DRUG REPURPOSING FOR COVID-19 THERAPY

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DRUG REPURPOSING FOR COVID-19 THERAPY

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EDITORIAL
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Editorial: Drug Repurposing for COVID-19 Therapy

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Editorial on the Research Topic

Drug Repurposing for COVID-19 Therapy

The rapid emergence in December 2019 of cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in China rapidly expanded to multiple countries leading to a pandemic situation in March 2020 and dramatic changes worldwide. COVID-19 immediately had major health consequences due to its severity, mainly in the population at risk, and to the lack of effective treatment to ameliorate the prognosis of the disease. Indeed, SARS-CoV-2 infection causes respiratory symptoms that range from mild forms to more serious ones, causing pneumonia, and multi–organ damage. Moreover, the sudden appearance and rapid propagation of COVID-19 produced an unexpected socio-economic crisis and major efforts have been devoted by multiple professionals to try to minimize the burden generated by this disease.

From the beginning of the pandemic, the scientific community made enormous efforts in order to rapidly develop vaccines that prevent the propagation of the SARS-CoV-2 infection. These research efforts result into an unprecedented success by reaching to the development of several efficient and secure vaccines in a time record in the history of vaccine development. Standard adenoviral approaches and novel mRNA strategies were used to successfully develop these novel vaccines and now there are still two enormous challenges opened for reaching an efficient vaccination campaign: the rapid distribution of these vaccines worldwide and the needs to raise awareness in the population about the safety and essential requirement of these vaccines to fight the COVID-19 pandemic.

Simultaneously to the vaccine development, multiple scientific groups concentrate their activities in an attempt to identify effective and safe pharmacological treatments against COVID-19. Indeed, both vaccines and pharmacological treatments are complementary to avoid the transmission of the viral infection and to prevent the severe consequences of the disease. In spite of the progress in the vaccination campaigns, pharmacological interventions are still needed to treat patients suffering the disease and to palliate the long-term consequences of the persistent forms of COVID-19. The efforts of the research were mainly devoted to the identification of compounds with anti-SARS-CoV-2 activity as well as drugs able to minimize the dramatic consequences of the exaggerated immune response leading to the most severe forms of the disease. However, due to the urgent need for a rapid development of pharmacological strategies, there was no time to start the long process required to develop novel compounds for such purposes. Therefore, the dominant research strategy was repurposing drugs for COVID-19 that were previously developed for other therapeutic purposes.

Research efforts of the scientific community were quickly translated in a large number of publications, including those devoted to the development of pharmacological approaches. The large majority of these publications met the rigorous criteria required for any prestigious scientific article.

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However, some few exceptions led to sound retractions that were largely commented and discussed by the general media, which emphasized once again the needs of the well-known rigorous peer review process in any scientific publication.

In order to collect the best evidence about drugs repurposed for COVID-19, we proposed and coordinated since May 2020 this Research Topic.

Several articles published in this Research Topic are devoted to antimalarial drugs that initially raised high expectancy due to their potential anti-SARS-CoV-2 activity. This initial interest was mainly focused on chloroquine and hydroxychloroquine, although the important risks associated to these treatments prompt overcome their potential benefits, as it is discussed and well-documented in several articles (Ren et al.; Kamat and Kumari; Manivannan et al.; Agarwal et al.; Younis et al.; Uckun et al.; Lozano-Cruz et al.). Antiretroviral drugs used for the Acquired Immunodeficiency Syndrome (AIDS) therapy, such as lopinavir and ritonavir, as well as antiviral drugs used for Ebola Viral Disease treatment, such as remdesivir, were also initially repurposed for COVID-19 therapy. However, the high expectancy for these drugs also promptly turned down (Gagliardini et al.; Li et al.), even if remdesivir is still one of the few drugs approved by regulatory authorities for treatment of patients with COVID-19. Other interesting approaches have also been proposed as novel potential therapeutic strategies with antiviral activity against SARS-CoV-2 including targeting the sigma one receptor with selective or non-selective ligands, such as the antipsychotic compounds (Stip et al.; Vela), modified ovalbumin (Liang et al.), methylene blue (Bojadzic et al.), *Bacillus* Calmette-Guérin vaccine (Patella et al.), vitamin D (Boulkrane et al.), vitamin C (Zhao et al.) and compounds that may inhibit the binding of the viral spike protein to ACE2 (Tsegay et al.).

It has been demonstrated that an exacerbated inflammatory and immunological response to SARS-CoV-2 induces the most severe cases of the disease. The excessive production of pro-inflammatory cytokines may lead to a cytokine storm syndrome that aggravates the respiratory distress. Several drugs have also been repurposed in order to mitigate the dramatic consequences of this cytokine storm syndrome. The efficient repurposing of a particularly potent glucocorticoid drug, dexamethasone, that has already well-demonstrated the efficacy for such a purpose is discussed in this Research Topic (Gozzo et al.). Several immunosuppressant and anti-rheumatic drugs (Rubsamen et al.; Soldevilla-Domenech et al.; Mary et al.; Cavalli et al.; Sarabia de Ardanaz et al.; Pala et al.), as well as modulators of estrogen receptor activity (Calderone et al.) and the statins (Vuorio et al.), have also been proposed as potential therapies for the severe COVID-19 cases associated to this cytokine storm. Due to the high prevalence of thromboembolic complications that often appear mainly in the severe forms of COVID-19, the use of anticoagulants including heparin has been proposed and the current evidence for addressing this novel approach is also discussed in this Research Topic (Gozzo et al.; Drago et al.).

Multiple other cellular and molecular pathways have also been suggested as additional possible targets for the repurposing of drugs for COVID-19 therapy, as discussed in other articles included in our topic (Hussman; Sarkar et al.; Zhang et al.; Blaess et al.; Al-Motawa et al.; Chen et al.; Khan et al.; Bezemer and Garssen; Sharma et al.; Zuo et al.; Xiong et al.; De Crescenzo et al.). The therapeutic perspectives in particular high risk populations, such as diabetic patients, have also been discussed in this topic (Sun et al.), as well as the new challenges open for the diagnosis and pharmacoepidemiological follow up of COVID-19 (Bianco et al.; Shoaib et al.; Powell et al.).

Finally, several articles highlighted how the repurposing process, as well as the approval of COVID-19 therapy in general, has represented an enormous regulatory challenge which forced the regulatory systems to rapidly adapt their rules to the pandemic (Gozzo et al.; Sultana et al.; Andrade et al.).

We believe that drug reuse has been an important attempt as an emergency strategy in a serious situation that could recur in the future. We cannot rule out that similar pandemics still threaten people as long as globalization affects all human activities. Therefore, we must consider the experience of drug reuse for COVID-19 as extremely helpful in enriching our experience in seeking therapeutic solutions when serious global health hazards occur.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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OPINION
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Selective Estrogen Receptor Modulators in COVID-19: A Possible Therapeutic Option?

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INTRODUCTION

Male and female genders exhibit significant differences in the outcome of infective diseases caused by several viral pathogens. Along with behavioral or social factors which can affect the exposure to infection and the availability of therapies, it is widely accepted that genetic and physiological factors can markedly influence sex-related differences in immune responses. In particular, receptors for gonadal hormones are expressed in many immune cell types and, consistently, sex-related differences in immune function are likely to be strongly influenced by circulating sex steroid hormones (Klein and Huber, 2010).

Concerning coronaviruses, epidemiological data from SARS epidemic (severe acute respiratory syndrome caused by SARS-CoV in 2002–2003) and MERS epidemic (Middle East respiratory syndrome, caused by MERS-CoV in 2012–2013) showed evident sex-dependent differences in disease outcome (Karlberg et al., 2004). Notably, such a sex-dependent difference is presently observed in the new SARS pandemic, broken out in 2019 and caused by SARS-CoV-2 (COVID-19). In particular, susceptibility to SARS-CoV-2 infection is almost similar in both genders, but higher severity and mortality are observed in male patients (Wenham et al., 2020).

ROLE OF THE "CYTOKINE STORM" IN COVID-19

The previous severe acute respiratory syndromes caused by SARS-CoV and MERS-CoV were often associated with rapid viral replication, huge infiltration of inflammatory cells, and excessive production of proinflammatory cytokines (cytokine storm syndrome), leading to lung injury and respiratory distress syndrome (Channappanavar and Perlman, 2017). Notably, accumulating evidence demonstrates that cytokine storm syndrome is involved also in the most severe cases of COVID-19 (Mehta et al., 2020). These patients rapidly develop respiratory distress syndrome, lung edema and failure (often associated with hepatic, myocardial, and renal injury, hemostasis alteration). Elevated levels of proinflammatory cytokines are observed in these patients. In particular, compared with non-intensive care patients, intensive care patients have higher levels of IL-2, IL-7, and TNF. Many cytokines detected in these patients belong to the Th17 type response

(as previously observed in MERS-CoV and SARS-CoV patients). The consequent IL-17-related pathway promotes broad pro-inflammatory effects by induction of specific cytokines, such as IL-1b, IL-6, TNF (responsible for systemic inflammatory symptoms), chemokines and matrix metalloproteinases (responsible for tissue damage and remodeling) (Wu and Yang, 2020). Moreover, pro-inflammatory cytokines, including IL-1b and IL-6, are directly induced by SARS-CoV-2 by interaction between viral components (probably nucleocapsid proteins) and toll like receptors of the host cells. Besides Th17 responses, patients diagnosed with COVID-19 showed marked rise of the Th1 subset (inflammatory cytokines IL-1β, IL-6, and IL-12) for more than 2 weeks after the infection onset (Russell et al., 2020).

In turn, IL-6 induced by SARS-CoV-2 in the lung seems to promote/amplify Th17 responses that may worsen the severe lung pathology in susceptible hosts (Hotez et al., 2020). In fact, IL-6 plays a crucial pathogenetic role in pulmonary injury induced by COVID-19. Accordingly, elevated levels of IL-6, produced by monocytes, lung interstitial fibroblasts, and alveolar macrophages, are observed in critical patients (Sun et al., 2020). Such a crucial role of IL-6 provided the rational basis for considering anti-IL-6 monoclonal antibodies (i.e. tocilizumab) as promising drugs for COVID-19 (Hotez et al., 2020).

ESTROGENS IN CYTOKINE REGULATION

The complex pathways of cytokine regulation may pave the way to new pharmacological approaches aimed at limiting IL-6 expression and cytokine storm. As reported above, COVID-19 outcomes show clear gender-related differences; notably, gonadal hormones deeply influence the immune response. Indeed, estrogen receptors (ERs) regulate the expression of IL-6 gene through inhibition of transcription factors NF-IL6 and NF-κB, and through disruption of NF-κB transactivation (Luo and Zheng, 2016). As well, estradiol (and probably progesterone) inhibits Th17 cell differentiation (Chen et al., 2015). ERα activation in immune cells reduces Th1 and Th17 responses and skews cytokine production towards a Th2 type, with enhanced antibody response.

ER modulation has been proposed in a murine experimental model of pulmonary inflammation as a useful pharmacological strategy. In particular, ERα are expressed in resident and infiltrated inflammatory cells of the lungs and activation of these receptors by estradiol markedly reduces the histological and biochemical markers of inflammation. Notably, these effects were observed in both male and female animals (Vegeto et al., 2010). Protective effects of ER mediators were also observed in murine models of pulmonary inflammation induced by influenza virus infection (Vermillion et al., 2018). Consistently, estradiol (Zhang and Liu, 2020) and other estrogen hormones (such as the horse estrogen equilin) has been presently reviewed as an alternative option for the treatment of COVID-19 (Suba, 2020).

SERMS AS POSSIBLE "ADJUVANT DRUGS" IN COVID-19

Noteworthy, the protective effects evoked by endogenous estrogens are also promoted by drugs belonging to the class of SERMs (selective estrogen receptor modulators) (Polari et al., 2018). These drugs exhibit a complex profile of mixed agonist/antagonist modulators of the ER subtypes and their effects on immune system and immune-mediated inflammatory responses have been described (Behjati and Frank, 2009). Indeed, many preclinical and clinical studies demonstrated that SERMs evoke significant anti-inflammatory responses and inhibit the expression of many proinflammatory cytokines, in different conditions of systemic or local inflammation (Suuronen et al., 2005; Nalbandian et al., 2005; Cerciat et al., 2010; Azizian et al., 2018).

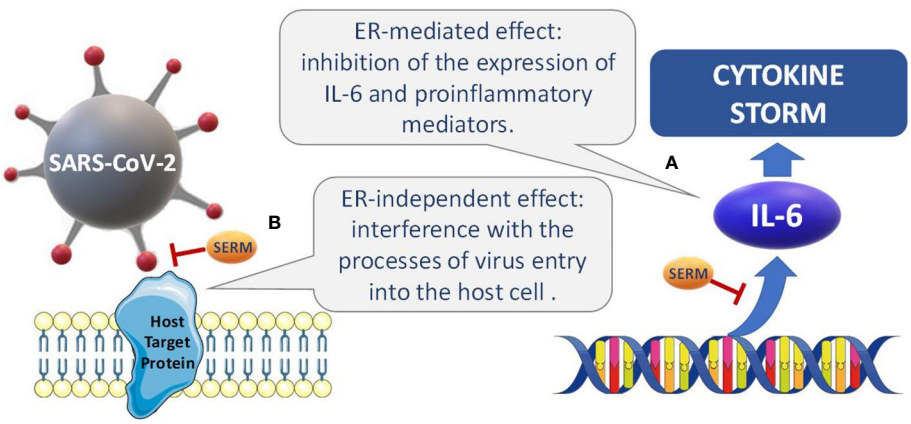

FIGURE 1 | Hypothesized mechanisms accounting for the potential effects of SERMs. **(A)** ERs regulate the expression of proinflammatory cytokines, such as IL-6, by inhibition of the transcription factors NF-IL6 and NF-κB, and disruption of NFκB transactivation. **(B)** in experimental studies on established cell lines, some SERMs have been reported to interfere with the processes of viral entry into the host cell and to inhibit different viral infections, including MERS-CoV, SARSCoV, and Ebola. Potential interactions with viral glycoproteins and with host proteins involved in the viral infection have been hypothesized.

Concerning coronavirus infections, a single preclinical study investigated the role of sex hormones in shaping gender-related vulnerability to SARS-CoV. In this study, male and female mice were infected with murine-adapted SARS-CoV (Channappanavar et al., 2017). Male mice were more vulnerable to SARS-CoV infection compared to female mice. Such a higher susceptibility of male mice to SARS-CoV was associated with higher viral titers, enhanced vascular leakage, and alveolar edema. These changes were also associated with elevated levels of inflammatory cytokines in lungs of male mice. Ovariectomy or treatment of female mice with an ER antagonist increased mortality, indicating a protective effect for ER signaling in mice infected with SARS-CoV. In contrast, treatment of female mice with SERMs (i.e. tamoxifen) led to increased levels of protection.

Moreover, beyond the effects of SERMs on ERs, these drugs seem to present useful ancillary properties. Besides their potential effects on proinflammatory cytokine expression (mediated by ERs), some SERMs seem to play broader roles in inhibiting viral replication by ER-independent mechanisms. Indeed, *in vitro* studies on established cell lines reported that some drugs of the SERM class interfere with processes of viral entry into the host cell and inhibit different viral infections, including MERS-CoV, SARSCoV, and Ebola virus. These effects may be due to potential interaction with viral glycoproteins and with host proteins involved in the viral infection (Zhou et al., 2020).

The hypothesized mechanisms of the potential effect of SERMs are summarized in **Figure 1**.

CONCLUSION

Taken together, these data suggest that ER modulation may be a suitable pharmacological approach for preventing/attenuating the cytokine storm and inflammation associated with COVID-19 and in particular the use of SERMs and/or "tissue selective estrogen complex" (TSEC, i.e. association of SERM and natural estrogen) may represent a promising pharmacological option. Such a therapeutic approach would be particularly useful for treatment of both male and female patients in early phase of the disease (with mild/moderate symptoms), in order to prevent or mitigate the possible evolution towards more serious and dangerous forms of the disease, due to the onset of the cytokine storm.

AUTHOR CONTRIBUTIONS

All the authors equally contributed to the manuscript writing.

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HYPOTHESIS AND THEORY
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Cellular and Molecular Pathways of COVID-19 and Potential Points of Therapeutic Intervention

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With the objective of linking early findings relating to the novel SARS-CoV-2 coronavirus with potentially informative findings from prior research literature and to promote investigation toward therapeutic response, a coherent cellular and molecular pathway is proposed for COVID-19. The pathway is consistent with a broad range of observed clinical features and biological markers and captures key mediators of pathophysiology. In this proposed pathway, membrane fusion and cytoplasmic entry of SARS-CoV-2 virus *via* ACE2 and TMPRSS2-expressing respiratory epithelial cells, including pulmonary type-II pneumocytes, provoke an initial immune response featuring inflammatory cytokine production coupled with a weak interferon response, particularly in IFN-λ–dependent epithelial defense. Differentiation of non-classic pathogenic T-cells and pro-inflammatory intermediate monocytes contributes to a skewed inflammatory profile, mediated by membrane-bound immune receptor subtypes (e.g., FcγRIIA) and downstream signaling pathways (e.g., NF-κB p65 and p38 MAPK), followed by chemotactic infiltration of monocyte-derived macrophages and neutrophils into lung tissue. Endothelial barrier degradation and capillary leakage contribute to alveolar cell damage. Inflammatory cytokine release, delayed neutrophil apoptosis, and NETosis contribute to pulmonary thrombosis and cytokine storm. These mechanisms are concordant with observed clinical markers in COVID-19, including high expression of inflammatory cytokines on the TNF-α/IL-6 axis, elevated neutrophil-to-lymphocyte ratio (NLR), diffuse alveolar damage *via* cell apoptosis in respiratory epithelia and vascular endothelia, elevated lactate dehydrogenase (LDH) and CRP, high production of neutrophil extracellular traps (NETs), depressed platelet count, and thrombosis. Although certain elements are likely to be revised as new findings emerge, the proposed pathway suggests multiple points of investigation for potential therapeutic interventions. Initial candidate interventions include prophylaxis to augment epithelial defense (e.g., AT1 receptor blockade, type III and type I interferons, melatonin, calcitriol, camostat, and lopinavir) and to reduce viral load (e.g., remdesivir, ivermectin, emetine, Abelson kinase inhibitors, dopamine D2 antagonists, and selective estrogen receptor modulators). Additional interventions focus on tempering inflammatory signaling and injury (e.g., dexamethasone, doxycycline, Ang1-7, estradiol, alpha blockers,

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and DHA/EPA, pasireotide), as well as inhibitors targeted toward molecular mediators of the maladaptive COVID-19 immune response (e.g., IL-6, TNF-α, IL-17, JAK, and CDK9).

Keywords: COVID-19, immunity, therapeutics, signal transduction, cytokines

INTRODUCTION

COVID-19 is a severe acute respiratory disease caused by the novel coronavirus SARS-CoV-2, which emerged in Wuhan, China in late 2019, quickly becoming a global pandemic, with over 10 million reported cases and 500,000 fatalities attributed to the disease through June 2020. Much of the response to the novel coronavirus has relied, by necessity, on a broad range of early reports relating to clinical features, biological markers, and candidate therapeutics. At the same time, many characteristics of the SARS-CoV-2 coronavirus and the acute respiratory distress produced by severe cases of COVID-19 infection mirror those observed in earlier coronavirus outbreaks, including SARS (severe acute respiratory syndrome, caused by SARS-CoV) and MERS (Middle-East respiratory syndrome, caused by MERS-CoV). Other conditions with informative overlap include ARDS (acute respiratory distress syndrome, resulting from pulmonary edema) and dengue hemorrhagic fever (DHF), which features severe and often fatal secondary immunopathology following dengue virus infection (Kurane, 2007) involving rapidly elevated cytokine expression, pulmonary edema, and acute respiratory failure.

The SARS-CoV-2 epidemic has emerged in the context of a rich existing literature detailing aspects of cellular and molecular pathways affected by prior CoV serotypes and related conditions. Much of the emerging literature specific to SARS-CoV-2 not only is strongly consistent with these findings but also features informative differences, particularly in lung tissue (e.g., weaker type III and type I interferon response, suppressed epithelial defense, and elevated pulmonary infectivity).

With the objective of linking early findings relating to the novel SARS-CoV-2 coronavirus with potentially informative findings from prior research literature and to promote investigation toward therapeutic response, a coherent cellular and molecular pathway is proposed for COVID-19. The pathway is consistent with a broad range of observed clinical features and biological markers and captures key mediators of pathophysiology.

In this proposed pathway, membrane fusion and cytoplasmic entry of SARS-CoV-2 virus *via* ACE2 and TMPRSS2-expressing respiratory epithelial cells, including pulmonary type-II pneumocytes, provokes an initial immune response featuring inflammatory cytokine production coupled with a weak interferon response, particularly in IFN-λ–dependent epithelial defense. Differentiation of non-classic pathogenic T-cells and pro-inflammatory intermediate monocytes contributes to a skewed inflammatory profile, mediated by membrane-bound immune receptor subtypes (e.g., FcγRIIA) and downstream signaling pathways (e.g., NF-κB p65 and p38 MAPK), followed by chemotactic infiltration of monocyte-derived macrophages and neutrophils into lung tissue. Endothelial barrier degradation and capillary leakage contribute to alveolar cell damage. Inflammatory cytokine release, delayed neutrophil apoptosis, and NETosis contribute to pulmonary thrombosis and cytokine storm. These mechanisms are concordant with observed clinical markers in COVID-19, including high expression of inflammatory cytokines on the TNF-α/IL-6 axis, elevated neutrophil-to-lymphocyte ratio (NLR), diffuse alveolar damage *via* cell apoptosis in respiratory epithelia and vascular endothelia, elevated lactate dehydrogenase (LDH) and C-reactive protein (CRP), high production of neutrophil extracellular traps (NETs), depressed platelet count, and thrombosis.

Although certain elements are likely to be revised as new findings emerge, the proposed pathway suggests multiple points of investigation for potential therapeutic interventions. These include prophylaxis to augment epithelial defense, reduce viral load, and temper inflammatory injury, as well as therapeutics targeted toward molecular mediators of the COVID-19 immune response.

CLINICAL FEATURES

Among patients with COVID-19 infection, cellular biomarkers in severe cases include elevated leukocyte and neutrophil counts, along with suppressed lymphocyte count, resulting in a significantly higher NLR ratio relative to non-severe cases (Huang C. et al., 2020; Qin et al., 2020). In a meta-analysis of nine studies including 1779 patients, 399 with severe disease, low platelet count was significantly associated with disease severity and mortality. Platelet count (thrombocytopenia) below the locally defined reference range is associated with a five-fold increase in the risk of severe disease (Lippi et al., 2020).

Molecular biomarkers of severe disease include elevated procalcitonin, serum ferritin, D-dimer, C-reactive protein (CRP), and inflammatory cytokines including IL-6, IL-2R, IL-7, IL-8/CXCL8, IP10, MCP-1/CCL2, MIP1A/CCL3, GM-CSF, and TNF-α, as well as IL-10 (Huang C. et al., 2020; Qin et al., 2020). However, the level of IL-10, a negative regulator of immune response, is reported to vary with COVID-19 severity and progression, with lower initial levels and subsequent decline associated with milder cases and possibly more successful viral clearance (Ouyang et al., 2020). Fast respiratory rate and elevated levels of lactate dehydrogenase (LDH), a marker of cell death, also predict severity (Huang H. et al., 2020).

Elevated inflammatory markers including IL-6, CRP, procalcitonin (PCT), and erythrocyte sedimentation rate (ESR) are observed in fatal cases (Zeng et al., 2020). Fatal acute lung injury is associated with T-lymphocyte dysregulation and cytokine-driven inflammation (Qin et al., 2020), with diffuse

pulmonary thrombosis and damage to endothelial cells (Poor et al., 2020).

In examination of postmortem tissue from all major organs of COVID-19 subjects, the primary finding is diffuse alveolar damage (DAD), featuring marked infection and viral burden in type II pneumocytes, along with pulmonary edema (Bradley et al., 2020; Carsana et al., 2020). CT examination is reported to have high diagnostic value, with multiple ground glass opacities being a prominent feature of disease progression (Li and Xia, 2020).

COVID-19 features infiltration of macrophages into lung tissue, with apoptosis of epithelial cells and pneumocytes. Infiltration of macrophages into alveolar cavities may be induced by MCP-1, with TGF-β1 and TNF-α contributing to proliferation and amplified cytokine production (He et al., 2006). Markers of infiltration include the neutrophil chemokine receptor CXCR2, along with monocyte chemotactic protein MCP-1/CCL2 and its receptor CCR2. Genes upregulated in severe and critically ill patients are enriched with members belonging to the NF-κB pathway (Hadjadj et al., 2020). Increased expression of TGF-beta in COVID-19 patients may promote fibroblast proliferation and contribute to pulmonary fibrosis (Xiong et al., 2020).

Several comorbid conditions are cited as risk-factors for progression and case fatality, including age, diabetes, vascular disease, cardiac dysfunction, hypertension, and cancer (Wu and McGoogan, 2020). Fever is the most common initial symptom, followed by cough, with maximum body temperature at admission, respiratory rate, CRP, and albumin significantly associated with progression in severity (Liu W. et al., 2020). Gastrointestinal symptoms are also reported but with lower frequency than in SARS or MERS (Ge et al., 2020).

The conditions associated with severe COVID-19 are not accurately described as "compromised immunity." Among 5700 hospitalized patients in the New York area with confirmed disease, the most frequent comorbidities reported were hypertension (56.6%), obesity (41.7%), diabetes (33.8%), and coronary artery disease (11.1%) (Richardson et al., 2020), all of which may be better described as conditions featuring predisposition to inflammation. Indeed, several key inflammatory cytokines associated with hypertension (TNF-α, MCP-1, and IL-6) (De Miguel et al., 2015) overlap those elevated in COVID-19.

ACE2-MEDIATED VIRAL ENTRY AND PRIMING OF INFLAMMATORY RESPONSE

Like the SARS coronavirus, the novel SARS-CoV-2 virus uses membrane-bound ACE2 to gain access to cells. ACE2 functions as an enzyme within the renin-angiotensin system (RAS), contributing to the regulation of blood pressure, fluid balance, and vasoconstriction. Angiotensin I (Ang I) generated by renin cleavage is converted by angiotensin-converting enzyme ACE to produce Ang II, which in turn activates AT1R receptors, contributing to increased blood pressure, vasoconstriction, oxidative stress, and pro-inflammatory signaling. The ACE2 enzyme has high affinity for Ang II, producing Ang(1-7). ACE2 thereby antagonizes the effects of Ang II and exerts a protective effect in conditions such as diabetes, hypertension, and cardiovascular disease (Cheng et al., 2020). Notably, elevated levels of Ang II are observed in ACE/ARB naïve COVID-19 cases, and high levels are associated with increased severity (Liu N. et al., 2020).

Initial genetic evidence of ACE2-mediated entry by SARS-CoV demonstrated that injection of spike protein in mice contributed to acute lung failure in mice and down-regulation of ACE2 expression. Inhibition of AT1R reduced lung pathology by blocking the effect of Ang II (Kuba et al., 2005). Notably, ACE2 is abundantly expressed on lung alveolar cells and enterocytes of the small intestine and is also present in vascular endothelia (Hamming et al., 2004), consistent with initial presentation of symptoms and sites of subsequent tissue damage.

SARS-CoV-2 viral entry is also dependent on priming of the viral S protein by the serine protease TMPRSS2, which may be partially blocked in some cell types by the serine protease inhibitor camostat mesilate. Full blockade was reported when camostat inhibition of TMPRSS2 was combined with an inhibitor of endosomal cysteine proteases cathepsin B/L (Hoffmann et al., 2020).

Despite exploitation of RAS by SARS-CoV-2, clinical evidence does not support the discontinuation of ACE-inhibitors or AT1R blockers (ARBs) as a strategy to limit infection, particularly as both types of inhibitors act to reduce the hypertensive and pro-inflammatory effects of Ang II. In SARS-CoV-2 infection, virus-induced ACE2 downregulation would be expected to lead to reduced production of Ang(1-7) and accumulation of Ang II, contributing to pulmonary edema and inflammation (Verdecchia et al., 2020).

Initial reports showed mixed evidence of clinical benefit of ACE inhibitors and AT1R blockers (ARBs) in COVID-19, with some showing insignificant effect (Peng et al., 2020; Richardson et al., 2020), as well as reports of protective effect among patients with pre-existing hypertension (Liu Y. et al., 2020; Yang et al., 2020). In a recent meta-analysis of five studies, the odds of death were reduced by a statistically significant 43% among 308 COVID-19 patients using ACE/ARB medications, compared with 1,172 patients not using ACE/ARB medications. A non-significant 19% reduction in the odds of hospitalization among users was also observed (Ghosal et al., 2020). In a separate, larger study of 610 cases and 48,667 high-coverage population–based controls, individuals with hypertension using ARBs were reported to have a 76% lower likelihood of developing COVID-19. However, a similar effect was not reported for ACE inhibitors (Yan et al., 2020).

Apoptosis of alveolar epithelial cells relies on autocrine generation of Ang II, while Ang(1-7) inhibits apoptosis through the Ang(1-7) receptor (Uhal et al., 2011). Exogenous delivery of Ang(1-7) is reported to reduce inflammation and improve lung function in ARDS models (Wosten-van Asperen et al., 2011). Recombinant ACE2 is also reported as a potentially

useful therapy in clinical studies of ARDS, producing a rapid decrease in plasma Ang II levels, as well as reduced IL-6 expression (Imai et al., 2007; Zhang and Baker, 2017).

PRO-INFLAMMATORY IMMUNE RESPONSE INITIATED BY TYPE-II ALVEOLAR PNEUMOCYTES

The innate pro-inflammatory response to SARS-CoV-2 infection in the lower respiratory tract may be most directly mediated by type-II alveolar pneumocytes, which highly express ACE2. Type-II pneumocytes act as epithelial immune cells and are capable of producing TNF-α, IL-6, IL-1β, MCP-1, and GM-CSF. Infected ACE2+ lung cells, but not uninfected cells, produce high levels of pro-inflammatory cytokines (Wong and Johnson, 2013). The age-related expression profile of ACE2 in uninfected human lung tissue is distinct from that in other ACE2-expressing tissues, showing a positive correlation with immune-cell and interferon-response marker genes in older individuals (>49 years) and a negative correlation in younger individuals (Li et al., 2020).

Local inflammatory cytokine expression in lung tissue of severe CoV infection may differ from that observed in circulating blood. SARS-CoV single-strand RNA is reported to provoke high production of pro-inflammatory TNF-α, IL-6, and IL-12 cytokines *via* activation of TLR7 and TLR8 (both highly expressed in lung tissue), amplifying the innate immune response (Li et al., 2013). Alveolar type-II cells are preferentially infected by SARS-CoV, resulting in the production of pro-inflammatory cytokines, with mRNA encoding IL-6 elevated approximately 10-fold in infected type-II cultures. In contrast, monocytes, monocyte-derived dendritic cells, and alveolar macrophages are not readily infected by SARS-CoV in culture and produce comparatively weak interferon and cytokine levels in response to viral exposure (Qian et al., 2013).

Likewise, the SARS-CoV-2 spike protein is a potent T-cell antigen, and direct activation of COVID-19 patient-derived peripheral blood mononuclear cells (PBMCs) by SARS-CoV-2 peptides in culture results primarily in production of T helper 1 (Th1)–related cytokines. However, IL-6 production is not observed in stimulated PBMCs. This finding suggests that direct antigen-specific T-cell activation may not induce production of IL-6 and that it may instead be mediated by innate immune cells (Weiskopf et al., 2020).

Based on intracellular cytokine staining, peripheral CD14+CD16+ monocytes are also implicated in the production of inflammatory cytokines in COVID-19 (Zhou et al., 2020). However, based on single-cell RNA sequencing of peripheral blood mononuclear cells (PBMCs) from seven COVID-19 cases and six healthy controls, peripheral monocytes and lymphocytes were not found to express substantial amounts of pro-inflammatory cytokines, suggesting that circulating leukocytes do not sufficiently account for COVID-19 cytokine storm (Wilk et al., 2020).

Such expression findings should be interpreted cautiously, as transcripts of many key immune genes demonstrate greater variation and transcription bursts than other genes (Gaublomme et al., 2015). Still, it appears likely that the cytokine storm observed in severe COVID-19 is mediated primarily by type II alveolar cells and local retention of blood cells that have migrated from peripheral circulation to infiltrate lung tissue.

INDUCTION OF NON-CLASSIC TH1 CELLS AND INTERMEDIATE CD14+CD16+ MONOCYTES

SARS-CoV-2 infection produces rapid activation of pro-inflammatory blood cell lineages. CD4+ Th1 lymphocytes co-expressing IFNγ and GM-CSF are reported almost exclusively in ICU patients with COVID-19, with relative absence of these cells in non-ICU patients and healthy controls. The percentage of CD14+CD16+ monocytes is also much greater in ICU patients with severe pulmonary complications. Pathogenic Th1 cells (GM-CSF+IFNγ+) are associated with increased proliferation of inflammatory CD14+CD16+ intermediate monocytes expressing both GM-CSF and IL-6. These contribute to the risk of inflammatory cytokine storm (Zhou et al., 2020).

Pathogenic GM-CSF+IFNγ+ Th1 cells have been described in inflammatory disease as "non-classic" Th1 cells (or "Th17/Th1" cells) and have been studied in conditions such as multiple sclerosis and juvenile rheumatoid arthritis. These CCR6+ Th17-derived cells have an intermediate gene expression profile between Th1 and Th17, with weaker suppression of Th17-associated genes *RORC2* and *IL-17A* than classic Th1 cells (Mazzoni et al., 2019). Th17 lymphocytes have an unstable phenotype and rapidly shift to a more aggressive non-classic Th1 phenotype in the presence of IL-12 and TNF-α. Inhibitors of TNF-α abrogate this transition (Cosmi et al., 2014). One of the earliest case reports of COVID-19 implicated an increased concentration of CCR6+ Th17 cells as a driver of severe respiratory damage (Xu et al., 2020). The potential therapeutic use of IL-17 inhibitors in COVID-19 has been proposed (Pacha et al., 2020).

The transcription factor Eomes, induced by the combined activity of IL-2 and IL-12, favors the induction of non-classic Th1 cells by selectively suppressing the expression of genes involved in Th17 differentiation. Knockdown of Eomes can be induced by tamoxifen (which also functions as a selective estrogen receptor modulator having tissue-dependent effects as a mixed agonist/antagonist) (Mazzoni et al., 2019). Non-classic Th1 cells are more pathogenic than Th17 cells (Kotake et al., 2017). The preferential induction of these cells is notable, as a comparison of gene expression between severe and non-severe COVID-19 patients reported that, in severe cases, the most significant biological function among differentially expressed genes (DEGs) having downregulated expression was the Th17 cell differentiation pathway (Ouyang et al., 2020).

Intermediate monocytes express the surface molecule CD14 and CD16, which encodes the FcγIII receptor. CD14+CD16+ intermediate monocytes produce high levels of pro-inflammatory TNF-α, coupled with low-to-absent levels of

anti-inflammatory IL-10 and have high antigen-presenting capacity. Elevated CD14+CD16+ cells are associated with increased ESR and C-reactive protein (CRP) levels (Ziegler-Heitbrock, 2007). Among monocytes, the highest expression of TNF-α receptor TNFR1 is observed in CD14+CD16+ cells (Hijdra et al., 2013). These monocytes can be mobilized under stress conditions, which may include, but are not dependent on, catecholamine release (Steppich et al., 2000).

Males are reported to have a significantly higher risk of mortality and mechanical ventilation than females in COVID-19, both before and after age-matching (RR, 1.4; 95% CI, 1.2–1.7) (Singh et al., 2020). In this context, it is notable that CD14+ monocytes and monocyte-derived macrophages deprived of 17 beta-estradiol express higher levels of CD16, with significant increases in TNF-α, IL-1β, and IL-6 production due to the absence of estrogen (Kramer et al., 2004). Additional factors potentially affecting gender differences in COVID-19 include androgen-mediated transcription of TMPRSS2 and X-linked effects (Wambier and Goren, 2020), as ACE2, androgen receptor, and TLR7 loci are all situated on the X chromosome.

The effects of CD14+CD16+ monocytes in elevating cytokine production and NLR ratios have been studied in other conditions. CD14+CD16+ cells are the preferential targets of Zika virus infection, with amplified proliferation of these cells and a reduction in the percentage and number of classical CD14+CD16- monocytes (Michlmayr et al., 2017). In acute leukemia, CD14+CD16+ monocytes are positively correlated with neutrophil proliferation and negatively correlated with CD4+ lymphocyte count (Jiang et al., 2015). Rheumatoid arthritis is characterized by preferential activation of intermediate CD14+CD16+ monocytes, which contribute to pathogenesis through the production of inflammatory cytokines including TNF-α, IL-1β, and IL-6 (Rana et al., 2018). In patients with type-1 diabetes, CD14+CD16+ monocyte production of IL-1β and IL-6 similarly contribute to pro-inflammatory pathology (Hamouda et al., 2019).

SKEWED INFLAMMATORY CYTOKINE PRODUCTION MEDIATED BY FC AND TLR RECEPTORS

Several membrane-bound proteins may contribute to the skewed inflammatory response, elevated cytokine production, and depressed platelet count observed in severe COVID-19. Fc receptors are cell surface proteins that mediate the phagocytosis and cytotoxic destruction of antibody-bound pathogens. Toll-like receptors (TLRs) are pattern-recognition receptors that participate in the innate immune response to extracellular pathogens.

FcγRIIIA (CD16) expression by monocytes is essential for antibody-dependent cellular toxicity (ADCC), which makes antibody-bound targets, such as virus infected cells, vulnerable to TNF-α–mediated cell death (Yeap et al., 2016). Meanwhile, the monocyte surface molecule CD14 cooperates with TLR2 in response to viral infection, activating nuclear factor-κB (NF-κB)–dependent transcription of genes encoding inflammatory cytokines, which may be inhibited *via* blockade of TLR2-mediated signaling (Zhou et al., 2010). Expression of TLR2 in monocytes is upregulated by IL-6 (Pons et al., 2006). Activation of TLR2 by SARS-CoV spike protein induces the production of inflammatory cytokines, including IL-6, IL-8, and TNF-α (Wang et al., 2007).

In addition to NF-κB activation, CD14-positive monocytes in SARS-CoV patients show an increase in phosphorylated mitogen-activated protein kinase MAPK p38. Augmented p38 MAPK activation in CD14 cells is associated with elevated IL-8 levels (Lee C. H. et al., 2004). The p38 MAPK signaling pathway is also implicated in the death of SARS-CoV–infected cells (Mizutani, 2007).

Given the observed proliferation of CD14+CD16+ intermediate monocytes in COVID-19 patients with severe pulmonary distress, it is possible that differential activation of Fcγ receptor subtypes, particularly FcγRIIA (inflammatory) and FcγRIIB (inhibitory), may contribute to an imbalanced inflammatory response. SARS macaque models produce skewed inflammatory cytokine production (including chemoattractants IL-8 and MCP-1) and absence of wound-healing similar to that observed in fatal human cases. Blockade of FcγRIIA reduces these effects (Liu et al., 2019). TNF-α and IL-10 synergistically upregulate FcγRIIA expression, while TNF-α downregulates FcγRIIB expression (Liu et al., 2005). Accordingly, TNF-α inhibition has been suggested as a potential therapeutic in SARS-CoV (Tobinick, 2004). Interestingly, the inhibitory FcγRIIB subtype is selectively upregulated in dendritic cells from RA patients with quiescent disease (Wenink et al., 2009).

Blockade of FcR activation *via* IVIG has been suggested for severe pulmonary inflammation and lung injury in SARS-CoV-2 (Fu et al., 2020). The anti-inflammatory effect is associated with its ability to recruit surface expression of the inhibitory Fc receptor FcγRIIB (Samuelsson et al., 2001). Among potentially repurposed therapeutics, IVIG is not without dangers (renal failure, thrombosis), and effectiveness is not established in MERS (Mustafa et al., 2018). Alternatively, human polyclonal immunoglobulin G from bovines has been reported to inhibit MERS-CoV *in vivo* (Luke et al., 2016).

Because depressed platelet count and dysregulated immune function is observed in COVID-19, the mediating role of Fcγ receptors in immune thrombocytopenia (ITP) may also be informative. In ITP, loss of self-tolerance to platelet protein leads to destruction of platelets and precursor megakaryocytes by binding of platelets to Fc receptors on macrophages. The inhibitory FcγRIIB receptor subtype (FCGR2B) prevents consumption by macrophages. Exogenous soluble FcγRIIB competitively binds antibody-bound platelets (Luke et al., 2016) and prevents autoantibody production (Shih et al., 2014). In contrast, FcγRIIA (FCGR2A) significantly aggravates the severity of antibody-mediated thrombocytopenia (McKenzie et al., 1999). Blocking FcγRIIIA (CD16) has also been shown to reduce ITP in mouse models (Flaherty et al., 2012).

In addition to viral entry *via* ACE2, antibodies against coronavirus spike proteins (anti-spike-S-IgG) can induce antibody-dependent enhancement (ADE) of viral entry *via* type II Fcγreceptors. Such enhancement has been studied in SARS-CoV infection (Wang et al., 2014) and appears to be dependent on the activation of Fcγ receptor II. Among FcR subtypes, FcγRIIA (CD32A) appears to mediate infectivity most efficiently (Jaume et al., 2011). In MERS-CoV, neutralizing antibodies can bind to the spike protein and enable alternative entry into FcγRIIA expressing cells (Wan et al., 2020). Accordingly, care in the selection of antigens is essential in the design of vaccine and antibody-based therapeutic strategies in order to avoid the potential for ADE.

Risk-genotypes associated with severe inflammatory pathology may be informative in the context of COVID-19. The FcγRIIA-R/R131 (rs1801274) genotype induces variation in the FcγRIIA receptor, while the CD14-159CC (rs2569190) genotype induces variation in CD14-mediated pro-inflammatory cytokine induction. Both are risk-genotypes for severe SARS (Yuan et al., 2005; Yuan et al., 2007) as well as aberrant immune response in pneumonia (Yuan et al., 2005), myasthenia gravis (van der Pol et al., 2003; Aricha et al., 2011), and acute asthma (Martin et al., 2006; Zhou et al., 2019).

NEUTROPHIL INDUCTION AND LUNG INFILTRATION

Severe SARS-CoV-2 infection is characterized by high neutrophil infiltration into lung tissue. In a study of 222 COVID-19 patients, disease severity was associated with significantly higher levels of both anti-virus IgG (IgG) and NLR ratio. Severity rates for patients with $NLR^{high}IgG^{high}$, $NLR^{high}IgG^{low}$, $NLR^{low}IgG^{high}$, and $NLR^{low}IgG^{low}$ phenotypes were 72.3, 48.5, 33.3, and 15.6%, respectively ($p < 0.0001$). Recovery rates for severe patients with these phenotypes were 58.8, 68.8, 80.0, and 100%, respectively ($p = 0.0592$). Notably, high NLR patients expressed the highest levels of IL-2, IL-6, and IL-10, with fatalities observed only in these patients (Zhang et al., 2020b).

Neutrophils comprise the majority of infiltrating cells into tissues undergoing inflammation. Transcriptional analysis of genes induced by SARS-CoV-2 features a host response characterized by weak induction of type I and type III interferons, coupled with enrichment of genes associated with cell death, leukocyte activation, and chemokine recruitment, including IL-1A, MCP-1 (CCL2), and IL-8 (CXCL8) (Blanco-Melo et al., 2020). In ARDS, MCP-1 and IL-8 induce chemotaxis of pro-inflammatory neutrophils into the lungs, where they are retained in the capillary bed and migrate into the alveolar space, contributing to cytokine production, formation of microthrombi, and cell death. GM-CSF, IL-8, and IL-2 contribute to delayed apoptosis, prolonging the amplified inflammatory response. In animal models of neutrophil-driven lung injury, cyclin-dependent kinase (CDK) inhibitors are reported to reduce inflammation and improve resolution by inducing neutrophil apoptosis (Potey et al., 2019). CDK9 is specifically implicated in this process (Wang et al., 2012).

Neutrophils can target pathogens and create a physical barrier to their migration by releasing NETs comprised of mesh-like extracellular DNA. NETs are observed at high levels in COVID-19 patients. Patient sera induce healthy control neutrophils to undergo NETosis. However, NETs may contribute to cytokine release and progression to respiratory failure (Zuo et al., 2020) and contribute to thrombosis *via* platelet-neutrophil interaction (Laridan et al., 2017).

ADHESION AND TISSUE RETENTION OF INFLAMMATORY LEUKOCYTES

The pathological inflammatory response observed in COVID-19 may be mediated by adhesion of hyperactivated and aggressive T-cells, monocytes, and neutrophils retained from peripheral circulation by vascular endothelia. Endothelial barrier degradation, capillary leakage, and extravasation into inflamed tissue may then contribute to the DAD observed in severe cases.

Phenotypic profiling of circulating leukocytes in critical COVID-19 patients indicates high activation of S-protein specific T-cells producing inflammatory cytokines, coupled with depletion of CD4+ and CD8+ T-cells expressing the LFA-1 integrin subunit CD11a. Conversely, recovery from respiratory distress is accompanied by a reversal of CD11a+ cell depletion (Anft et al., 2020). Hyperactivated T-lymphocytes and inflammatory macrophages recruited by chemokine signaling to lung tissue exhibit strong interaction with epithelial cells, contributing to increased cell death and lung injury. Elevated markers of immune cell trafficking in COVID-19 include MCP-1 and LFA-1. As monocyte recruitment and epithelial damage can be induced by binding of MCP-1 to ligands CCR1 or CCR5, blockade of these ligands has been suggested as a potential therapeutic approach (Chua et al., 2020).

Adhesion of inflammatory CD14+CD16+ monocytes and neutrophils to vascular endothelia is mediated by interaction of LFA-1 with its ligand, intercellular adhesion molecule ICAM-1. Inflammatory cytokines IL-1 and TNF-α induce ICAM-1 expression on endothelial cells. Expression of ICAM-1 selectively enhances adhesion of inflammatory non-classical and intermediate CD16+ monocytes under flow, with no effect on CD16- monocytes (Regal-McDonald et al., 2019). Docosahexaenoic acid (DHA) is reported to inhibit TNF-α-induced ICAM-1 expression (Lin H. C. et al., 2019), with similar inhibition of ICAM-1 expression reported for eicosapentaenoic acid (EPA) in aortic endothelia (Huang et al., 2015).

ICAM-1 facilitates cytokine-induced adhesion of neutrophils to vascular endothelia (Tonnesen, 1989). Notably, upregulation of ICAM-1 expression and inflammatory leukocyte recruitment is observed in ARDS (Müller et al., 2002) and respiratory syncytial virus (RSV) disease (Arnold and König, 2005). Similar upregulation is observed in Ang II-induced macrophage infiltration and cardiovascular pathology, which is ameliorated by ICAM-1 blockade (Lin Q. Y. et al., 2019). Blockade of ICAM-1 is also reported to markedly reduce pulmonary barrier damage in ARDS (Svedova et al., 2017).

Extravasation of CD14+CD16+ intermediate monocytes is mediated by secretion of MMP-9, a protease that degrades extracellular matrix proteins, resulting in the release of matrix-bound VEGF-A and increased vascular membrane permeability (Sidibe et al., 2018), In COVID-19 patients with respiratory failure, a significant increase is observed in circulating MMP-9, strongly correlated with neutrophil count (Ueland et al., 2020).

COVID-19 respiratory failure thus features co-expression of inflammatory cytokines with regulators of leukocyte recruitment and vascular integrity. This suggests a mechanism by which inflammatory leukocytes may degrade the alveolar-capillary barrier, with resulting destruction of lung tissue. Notably, electron microscopy of post-mortem lung tissue reveals extensive opening of junctional complexes. Hyperalbuminemia in severe COVID-19 patients, consistent with vascular permeability and capillary leakage, is strongly associated with mortality (Wu M. A. et al., 2020).

The potential importance of this mechanism in COVID-19 pathology is underscored by transcriptional and proteomic profiling. In bronchial epithelial cells infected with SARS-CoV-2, DEGs are enriched for members of pathways related to NF-κB, TNF-α, and IL-17 signaling. Specific genes shared by these pathways include *MMP9*, *ICAM1*, *CSF3*, and *IL6* (Enes and Pir, 2020). A protein-protein interaction network of DEGs shared between COVID-19, MERS, SARS, H1N1, and Ebola identifies *ICAM1*, *VEGFA*, *MMP9*, *IL6*, *TNF*, *IL-8*, *IL1B*, *STAT1*, *TLR2*, *TLR1*, *IRF7*, and *CXCL1* as hub genes (Alsamman and Zayed, 2020). Proteomic profiling of blood samples from COVID-19 patients identifies ICAM-1 and FCGR3A (CD16) as the most significant proteins in the classification of short vs. extended disease course (Tang). Likewise, in post-mortem lung tissue, IL-6, TNF-α, ICAM-1, and CASP-1 (an activator of inflammatory response and cell death) show significantly higher tissue expression, compared with control and H1N1 samples (Nagashima et al., 2020).

Although SARS-CoV-2 infection in pediatric cases is generally associated with asymptomatic resolution, a perplexing minority of children present with Kawasaki disease (KD)–like features, alternatively described as multisystem inflammatory syndrome (MIS). These patients present with high inflammatory markers, early gastrointestinal symptoms, and acute myocarditis, with therapeutic immune globulin reportedly contributing to recovery (Toubiana et al., 2020; Belhadjer et al., 2020). These cases may potentially be understood in the context of the same mechanisms of inflammatory leukocyte infiltration implicated above.

Specifically, acute KD is associated with increased proliferation of CD14+CD16+ intermediate monocytes (Katayama et al., 2000), while diminished inflammation in response to plasma exchange therapy is associated with a significant reduction in the percentage of CD14+CD16+ intermediate monocytes, relative to total leukocytes (Koizumi et al., 2019). The acute phase of KD also features transient depletion of CD11a-expressing T-cells from peripheral blood (Furukawa et al., 1993). In cultured vascular endothelial cells, patient sera from acute phase KD induces significantly higher expression of ICAM-1 than quiescent sera, with TNF-α contributing to ICAM-1 expression (Inoue et al., 2001). In KD cases exhibiting coronary artery abnormalities, a high and unresponsive NLR ratio is associated with resistance to IVIG treatment (Cho et al., 2017). Thus, the KD-like symptoms observed in a subset of pediatric COVID-19 cases are broadly consistent with the inflammatory mechanisms described in the proposed pathway.

WEAK INTERFERON DEFENSE AND NEUTROPHIL-DRIVEN CYTOTOXICITY IN LUNG EPITHELIA

SARS-CoV-2 infection is associated with increased levels of pro-inflammatory cytokines (Chen et al., 2020; Zhang, Guo, et al., 2020), yet the immune response in lung tissue features a relatively impaired response of type I (α/β), II (γ), and III (λ) interferons (Chu et al., 2020), along with down-regulation of interferon-induced genes. This contrasts with the interferon response in SARS-CoV, where preferential infection of alveolar type-II cells results in a marked increase of IFN-β and IFN-λ (IL-29) production (Qian et al., 2013).

The suppressed IFN-λ response observed in COVID-19 may be a key factor mediating viral infectivity. In human lung tissues, SARS-CoV-2 demonstrates markedly higher infectivity and replication than that of SARS-CoV, generating 3.2 times the number of infectious virus particles within 48 hours of infection (Chu et al., 2020).

While IFN-α and IFN-β receptors are primarily expressed on peripheral blood cells, IFN-λ receptors have restricted expression, preferentially defending epithelial cells, including respiratory pneumocytes. IFN-λ expression thus provides an initial line of defense to restrict viral replication in the upper airways, suppress excessive inflammation of the lower airways, and maintain the integrity of cellular barriers to inflammatory injury (O'Brien et al., 2020; Broggi et al., 2020).

In Dengue infection, IFN-λ inhibits replication of the DENV-2 virus in a dose-dependent manner *in vitro* (Palma-Ocampo et al., 2015). The rs7247086 variant of *IFNL1* (the T allele) is reported to be protective against DHF, suggesting that *IFNL1* may play a role in the pathogenesis and elevated cytokine expression observed in this condition (Arayasongsak et al., 2020).

Notably, MERS-CoV encodes two accessory proteins, NS4a and NS4b that contribute to suppression or evasion of innate antiviral immune pathways. In particular, both deletion of NS4a and mutation of catalytic or nuclear localization sites of NS4b result in increased expression of IFN-λ1 (Comar et al., 2019). The weak interferon response observed in COVID-19 suggests that the possibility that one or more SARS-CoV-2 viral proteins may exert a similar effect in suppressing IFN-λ expression, weakening front-line innate immune defense against viral infectivity. Similarly, viral proteins of RSV, the most important respiratory virus among infants, antagonize IFN-mediated

epithelial protection. Exogenous IFN-λ1 confers prophylactic benefit against viral infection (Villenave et al., 2015).

A recent genome-wide association study examined 300,000 loci to identify genetic factors associated with ACE2 expression in the presence of RNA virus infection. The most significant association was identified in three SNPs within the IFN-λ region of chromosome 19, controlling expression of IFNL3 and IFNL4. In the presence of RNA virus infection, ACE2 expression shows a significant negative correlation with IFN pathway genes. One of these SNPs is located near a frameshift mutation that disables the production of IFN-λ4 (Ansari et al., 2020). As both ACE2 and receptors for IFN-λ are preferentially expressed on type II alveolar pneumocytes, their association may be relevant in COVID-19 pathology, as suppressed IFN-λ expression coupled with elevated ACE2 expression could simultaneously suppress epithelial defense while amplifying the viral load.

Weak induction of IFN-λ in COVID-19 may be an important amplifier of cytokine production by impairing the control of inflammatory neutrophil responses. In animal models of ARDS induced by influenza-A virus (IAV) infection, neutrophils comprise the majority of infiltrating cells and are the primary source of pro-inflammatory cytokines. Neutrophils also express high levels of the interferon-lambda receptor IFNLR1 in proximity to epithelial cells, allowing IFN-λ to mediate sustained local anti-viral defense without amplifying inflammation. Accordingly, exogenous administration of pegylated recombinant IFN-λ in IAV-induced ARDS suppresses viral replication and improves lung function (Galani et al., 2017). IFN-λ also suppresses the migration of neutrophils and their proclivity to NETosis, thereby enabling the suppression of thromboinflammation (Chrysanthopoulou et al., 2017).

Low levels of IFN-λ in COVID-19 also appear likely to skew immune response toward neutrophil proliferation and suppressed lymphocyte response, contributing to the thrombosis, pro-inflammatory cytokine production, and fatality observed among NLR^{high} patients. Exogenous IFN-λ may reduce these consequences. CD14+ monocytes quickly express the IFN-λ receptor IFNLR1 upon differentiation to macrophages. IFN-λ stimulates the cytotoxic and phagocytic capacity of macrophages, as well as the secretion of cytokines that mediate T and NK-cell migration and cytotoxicity (Read et al., 2019).

CYTOKINE STORM FEATURING HIGH EXPRESSION OF IL-6 AND TNF-α

Increased IL-6 is an early indicator of cytokine release syndrome in COVID-19 patients (Wang et al., 2020). IL-6 concentrations are increased 2.9-fold in patients with complicated COVID-19 vs. uncomplicated (Coomes and Haghbayan, 2020), and IL-6 levels are predictive of respiratory failure (Herold et al., 2020; Zhang et al., 2020a).

The SARS-CoV spike protein induces (TNF-α converting enzyme) TACE-dependent shedding of the extracellular ACE2 receptor domain, resulting in loss of ACE2 function and production of TNF-α. NL63-S, a common cold coronavirus serotype, also uses ACE2 for entry, but does not induce similar ACE2 shedding or TNF-α production (Haga et al., 2008). TACE antagonists have been suggested as an approach to inhibit TNF-α and attenuate disease severity in SARS-CoV (Tobinick, 2004).

Cytokine storm on the IL-6/TNF-α axis appears likely to be mediated by phosphorylation of the NF-κB subunit p65. In SARS-CoV infection, the viral spike protein induces activation of NF-κB *via* IkB-α degradation, resulting in production of IL-6 and TNF-α (Wang et al., 2007). The viral nucleocapsid protein of SARS-CoV can also bind the NF-κB regulatory element on the IL-6 promoter, and activity is highest when the p65 subunit is present (Zhang et al., 2007).

Regulatory elements in the ACE2 gene control the transcription of PIR (pirin), a negative regulator of NF-κB subunit RELA (p65). SARS-CoV-2 disruption of ACE2 is proposed to reduce PIR expression (Fadason et al., 2020). PIR is proposed to function as a reversible switch that enables NF-κB response to changes in redox levels (oxidative stress) in the cell nucleus (Liu et al., 2013). Repression of PIR ablates inhibition of IL-6 expression (Wu et al., 2017).

Inhibition of NF-κB activation has been suggested as a therapeutic strategy to increase survival in SARS-CoV infection (DeDiego et al., 2014). Inhibition of JAK signaling may block p65 phosphorylation and attenuate proinflammatory cascade (Yang et al., 2017). Tocilizumab, a well-tolerated blocker of the IL-6 receptor, may have potential to dampen cytokine release syndrome in COVID-19 (Zhang C. et al., 2020). Because catecholamines augment the production of IL-6 and other inflammatory cytokines, α-1 adrenergic receptor inhibition (e.g., prazosin) has also been suggested as a candidate that may provide prophylactic benefit against cytokine storm (Konig et al., 2020).

Use of low molecular weight heparin is reported to be associated with improvement in aberrant coagulation and a reduction of IL-6 levels (Shi et al., 2020), and is reported to increase survival in COVID-19 (Negri et al., 2020; Tang et al., 2020). However, elevated anti-heparin-PF4 antibodies have been observed in severe COVID-19 patients, even in the absence of heparin exposure, and may contribute to heparin-induced thrombocytopenia, *via* binding of antibody-heparin complexes to the platelet FcγRIIA receptor (Liu X. et al., 2020). For that reason, the use of alternative anticoagulants (other than coumadin, which may provoke thrombotic complications) may be indicated (Izak and Bussel, 2014).

DISCUSSION

The rapid case growth and high fatality rate of COVID-19 have posed an urgent global health challenge. Major uncertainties exist in ascertainment, and case reports are likely to exclude large numbers of subclinical or asymptomatic cases that may contribute to infectivity and confound containment efforts. Meanwhile, conditional on cases that have been reported and

confirmed, the global case fatality rate of the disease exceeds 4.8%, with the United States experiencing the highest number of fatalities (127,000) through June 2020 (ncov-CSSE, 2020).

Despite incomplete knowledge of the pathophysiology relating to the novel coronavirus SARS-CoV-2, the proliferation of initial reports and small-scale studies carry stronger information content than may be evident amid the "noise" of this emerging literature, when integrated in the context of prior research on other CoV serotypes, ARDS, and related inflammatory conditions. From a noise-reduction perspective, information content can often be amplified by extracting jointly correlated signals from what might otherwise be individually weak sensors. The tractable pathway presented here is reflective of that effort.

Part of this analysis, by necessity, includes findings from early reports and pre-published data that may be modified or contradicted by subsequent studies. Accordingly, some elements of this pathway may require revision as new findings emerge. **Figure 1** illustrates this pathway.

Among the benefits of a coherent biological pathway, consistent with the observed clinical course of SARS-CoV-2, is that it connotes multiple points of intervention for potential therapeutic candidates. Emphatically, the candidates described below are not prescriptive but are instead discussed here to provoke pathway-informed investigation.

Potential investigational therapeutics consistent with the proposed COVID-19 pathway are listed in **Table 1**. Specific candidates are indicated as examples and do not comprise an exhaustive list. These candidates are not prescriptive but are instead intended to provoke further research and pathway-informed investigation.

Initial interventions with potential benefit early in SARS-CoV-2 infection may include approaches focused on augmenting epithelial defense, reducing viral load, and modifying inflammatory signaling. Potential candidates include the use ACE inhibitors and AT1R blockers (ARBs) to reduce the hypertensive and pro-inflammatory effects of Ang II, exogenous Ang(1-7), recombinant ACE2, pegylated IFN-λ, early administration of IFN-I, and α-1 adrenergic receptor inhibition.

In a study of 77 COVID-19 patients, treatment with IFN-α2b significantly reduced the duration of detectable virus in the upper respiratory tract, and reduced the duration of elevated IL-6 and CRP levels (Zhou Q. et al., 2020). However, evidence from SARS

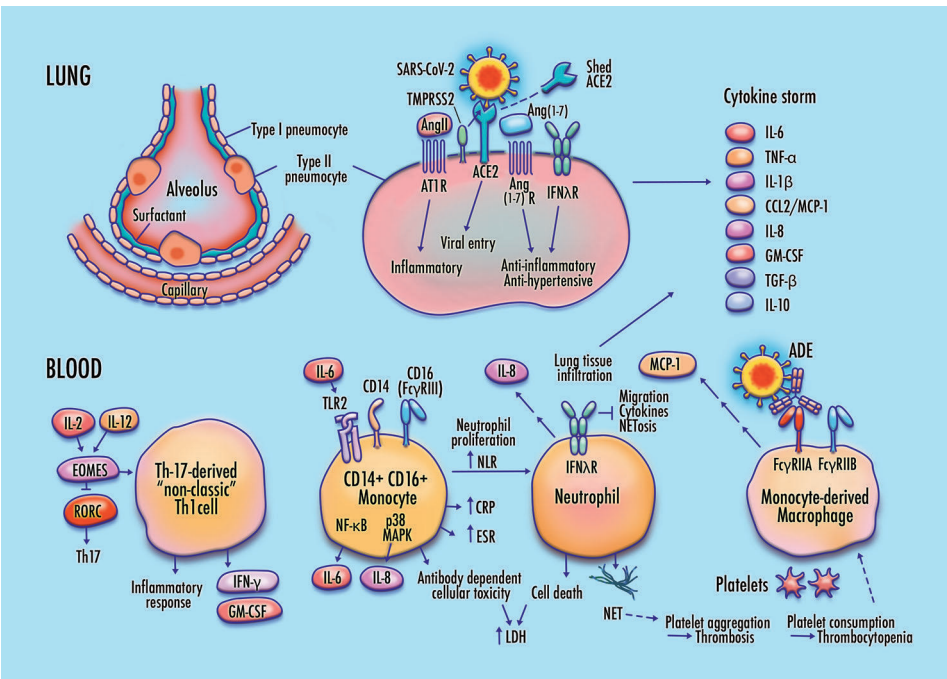

FIGURE 1 | Proposed features of cellular and molecular pathophysiology in COVID-19. Membrane fusion and cytoplasmic entry of SARS-CoV-2 virus *via* ACE2 and TMPRSS2-expressing respiratory epithelial cells, including pulmonary type-II pneumocytes, provokes an initial immune response featuring inflammatory cytokine production coupled with a weak interferon response, particularly in IFN-λ–dependent epithelial defense. Differentiation of non-classic pathogenic T-cells and pro-inflammatory intermediate monocytes contributes to a skewed inflammatory profile, mediated by membrane-bound immune receptor subtypes (e.g., FcγRIIA) and downstream signaling pathways (e.g., NF-κB p65 and p38 MAPK), followed by chemotactic infiltration of monocyte-derived macrophages and neutrophils into lung tissue. Endothelial barrier degradation and capillary leakage contribute to alveolar cell damage. Inflammatory cytokine release, delayed neutrophil apoptosis, and NETosis contribute to pulmonary thrombosis and cytokine storm. These mechanisms are concordant with observed clinical markers in COVID-19, including high expression of inflammatory cytokines on the TNF-α/IL-6 axis, elevated neutrophil-to-lymphocyte ratio (NLR), DAD *via* cell apoptosis in respiratory epithelia and vascular endothelia, elevated lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR), and CRP, high production of neutrophil extracellular traps (NETs), depressed platelet count, and thrombosis.

TABLE 1 | Potential investigational therapeutics consistent with proposed COVID-19 pathway.

Therapeutic candidate (not exhaustive)	Class	Potential mechanism and basis for investigation
Losartan, Irbesartan	Angiotensin II receptor AT1R blocker (ARB)	Blockade of pro-inflammatory, pro-hypertensive Ang II effects
Recombinant ACE2, Ang (1-7)	Exogenous RAS modulators	Restoration of anti-inflammatory, anti-hypertensive Ang(1-7) effect
Prazosin	Alpha-adrenergic blocker	Reduction of catecholamine-related amplification of cytokine response
Pasireotide	Somatostatin analogue	Reduction of cortisol-mediated NLR
Pegylated IFN-λ	Interferon-III	Augmented defense of respiratory epithelium, reduced cytokine production, NETosis and thrombosis
Calcitriol, Melatonin	Natural hormone supplement	Prophylaxis, reduced cytokine induction
Lopinavir, Camostat	Protease inhibitor	Disruption of viral entry
Remdesivir	Antiviral agent	Reduction of viral replication
Chlorpromazine, Trifluprom azine	Dopamine D2 receptor antagonist	Reduction of viral titer via disruption of clathrin-mediated endocytosis
Emetine, Ivermectin, Hydroxychloroquine	Anti-parasitic	Prophylactic reduction of viral titer
Imatinib, Dasatinib	Abelson (ABL) kinase inhibitor	Blockade of host-virus membrane fusion
Toremifene, Tamoxifen	Estrogen receptor modulator (tissue-dependent mixed agonist/antagonist)	Antiviral activity and inhibition of non-classic Th1 induction, potentially via receptor-independent mechanisms
Estradiol	Steroid hormone	Inhibition of CD16 and proliferation of inflammatory intermediate monocytes
DHA, EPA	n-3 polyunsaturated fatty acid	Reduced ICAM-1-mediated leukocyte adhesion and inflammatory response
Doxycycline	Tetracycline antibiotic	Antibiotic, anti-inflammatory effect on cytokine expression and MMP activity
Dexamethasone, Methylprednisolone	Glucocorticoid	Reduced inflammatory response
Sekukinumab, Broadalumab	IL-17 inhibitor	Reduced inflammatory response
Tocilizumab, Siltuximab	IL-6 inhibitor	Reduced inflammatory response
Etanercept	TNF inhibitor	Reduced inflammatory response
Tofactinib, Fedratinib	JAK inhibitor	Inhibition of NF-κB p65 signaling
Alvocidib	Cyclin-dependent kinase (CDK) inhibitor	Reduced inflammatory response and neutrophil-mediated cell death
FcγRIIB	Exogenous Fc receptor delivery	Reduced inflammatory response, potential inhibition of platelet consumption

and MERS cases suggests that while early delivery of IFN-I can reduce viral replication, later delivery may amplify risk by elevating pro-inflammatory response (Channappanavar et al., 2016; Channappanavar et al., 2019).

Among conservative, well-tolerated therapeutic candidates, melatonin exerts a protective effect on vascular endothelia by inhibiting NF-κB induced expression of MMP-9 (Qin et al., 2012). It is also reported to protect lung tissue from hypoxic stress by downregulating TNF, IL-6, and VEGF expression, with quercetin providing additional prophylactic effect (Al-Rasheed et al., 2017). Vitamin D attenuates TLR-mediated induction of inflammatory cytokines (Thota et al., 2013). This mechanism may be relevant in COVID-19 as low plasma levels of vitamin D are reported in SARS-CoV-2 infected individuals and significantly contribute to the risk of infection and hospitalization (Merzon et al., 2020). Calcitriol, the active form of vitamin D, is also reported to directly reduce the virus-induced cytopathic effect of SARS-CoV-2 infection in cultured human respiratory epithelial cells (Mok et al., 2020). The combination of melatonin and vitamin D has been proposed as a potentially synergistic intervention in COVID-19 (Martín Giménez et al., 2020).

Several classes of therapeutics may have benefit as potential viral entry inhibitors. In a screening of 290 compounds for antiviral activity against SARS-CoV and MERS-CoV, those promoting at least 50% viral inhibition in Vero E6 cells *in vitro* with little or no toxicity included selective estrogen receptor modulators (SERMs) (e.g., toremifene and tamoxifen), Abelson kinase (ABL) inhibitors (e.g., imatinib and dasatinib), dopamine D2 receptor antagonists (e.g., chlorpromazine and trifluprom azine), and antiparasitic agents (e.g., hydroxychloroquine and emetine) (Dyall et al., 2014). Research involving additional cell lines may be informative in this context, because while SARS-CoV-2 can be isolated from Vero E6 cells, cells engineered to express TMPRSS2 display a nearly 10-fold increase in SARS-CoV-2-infected cells than parental Vero E6 cells (Matsuyama et al., 2020).

SERMs such as toremifene are reported to potently inhibit Ebola virus, even without detectable expression of estrogen receptors, suggesting that SERMs may affect viral activity through an alternative pathway (Johansen et al., 2013). In CD14+ monocytes, SERMs are reported to reduce inflammatory signaling by downregulating TNF-α–stimulated NF-κB activation and to promote macrophage differentiation toward an M2 anti-inflammatory/repair phenotype (Polari et al., 2018). Toremifene was among two network-predicted therapeutics, along with the AT1R blocker irbesartan, with the strongest correlation between CoV-induced transcriptomes and drug-induced transcriptomes and having literature-based antiviral evidence (Zhou Y. et al., 2020).

ABL inhibitors are reported to have potent effect against SARS-CoV and MERS-CoV cell fusion, which is required for cytoplasmic delivery of the viral genome (Coleman et al., 2016). The D2 receptor antagonist chlorpromazine is reported to inhibit clathrin-mediated endocytosis in both SARS-CoV (Inoue et al., 2007) and MERS-CoV (Liang et al., 2018).

Several antiparasitic agents are recognized for exhibiting antimicrobial and anti-inflammatory properties, suggesting potential benefit against SARS-CoV-2 infection. For example,

ivermectin interferes with the nuclear import of proteins encoded by several RNA viruses and is reported to exert antiviral action against SARS-CoV-2 in Vero cells (Caly et al., 2020). Early evidence suggests that ivermectin treatment may be associated with reduced mortality risk in patients with COVID-19, particularly in those requiring oxygen support or mechanical ventilation (Rajter et al., 2020).

Hydroxychloroquine has been broadly used during the SARS-CoV-2 epidemic, with evidence of potential prophylactic effect (Colson et al., 2020) mediated by reduced viral replication (Keyaerts et al., 2004) and interference with ACE2 binding (Vincent et al., 2005). Chloroquine is also reported to reduce secretion of IFN-γ and IL-17 in activated Th1 and Th17 cells, respectively (Schmidt et al., 2017). However, evidence of therapeutic benefit for hospitalized patients has not been clearly established (Magagnoli et al., 2020; Shamshirian et al., 2020). In addition to potential risks of retinopathy and arrhythmia, combination therapy with azithromycin is reported to be associated with increased risk of heart failure and cardiovascular mortality (Lane et al., 2020).

A randomized, controlled trial of remdesivir including more than 1000 patients reported a reduction in average time to recovery to 11 days for the treatment group vs. 15 days for patients assigned to placebo. A small but insignificant reduction in the risk of fatality was also observed among treated patients (Ledford, 2020). In a screening of 16 therapeutic candidates specifically targeting SARS-CoV-2, the antiparasitic agent emetine was reported among four compounds achieving at least 50% in-vitro inhibition, along with remdesivir, lopinavir, and homorringtonine. Synergy between remdesivir and emetine was observed, enabling reduced dosages to achieve significant reduction in viral yield (Choy et al., 2020). In the context of SARS-CoV-2, adjuvant use of emetine may be of particular interest, given that emetine has a well-established role in enhancing interferon activity (Schellekens et al., 1975) and is reported to disrupt viral entry and replication (Yang et al., 2018). Considerations include pregnancy and cardiovascular risk.

The broad spectrum antibiotic doxycycline has been shown to exert anti-inflammatory effects by interfering with the expression of IL-6, IL-8, and TNF-α, reducing the recruitment of neutrophils and lymphocytes into inflamed tissue, and suppressing the activity of metalloproteinases (MMPs) (Di Caprio et al., 2015). Notably, doxycycline treatment was reported to reduce mortality by half in human patients with DHF, with survival associated with significant reductions in TNF and IL-6 levels (Fredeking et al., 2015). Administration of doxycycline also significantly decreases MMP-mediated capillary leakage and alveolar damage in virus-infected mice (Ng et al., 2012). These properties suggest potential therapeutic benefit of doxycycline across multiple fronts of COVID-19 immunopathology.

Corticosteroids are commonly used in the treatment of inflammatory conditions, but timing and duration of use are important considerations in the context of COVID-19. In SARS, early corticosteroid treatment (<7 days of illness) was associated with an increase in subsequent viral load (Lee N. et al., 2004). However, the use of steroids may be beneficial at the point of disease progression to acute respiratory distress and cytokine storm (Tomazini et al., 2020). Methylprednisolone use is reported to reduce the risk of death in patients with COVID-19 pneumonia that has progressed to ARDS (Wu C. et al., 2020). This result is consistent with clinical evidence in SARS, where pulse methylprednisolone was reported to be beneficial in a subset of patients with critical illness. Prolonged steroid administration without effective antimicrobial support is discouraged due to the risk of secondary infection (Tai, 2007).

In a randomized controlled trial comparing 2104 COVID-19 patients receiving dexamethasone and 4321 patients receiving standard-of-care, dexamethasone treatment reduced the risk of death by one-third in patients requiring invasive mechanical ventilation and by one-fifth in patients requiring oxygen without invasive ventilation. Dexamethasone did not reduce mortality risk in patients that had not progressed to the need for respiratory support at the time of randomization (Horby et al., 2020). However, in non-intubated patients with COVID-19 pneumonia, combination therapy including corticosteroids and tocilizumab is reported to increase survival (Mikulska et al., 2020).

Steroid use has been suggested as a possible factor contributing to the elevated NLR ratio observed in SARS patients. However high NLR is observed even in steroid-naïve patients, and elevated serum cortisol is reported to be correlated with the degree of neutrophilia and lymphopenia (Panesar et al., 2004). High adrenocorticotropic hormone (ACTH) production and induced cortisol release in response to SARS-CoV infection has been suggested to mimic the effect of corticosteroids in driving T-lymphocytes out of peripheral circulation (Panesar, 2003). The somatostatin analogue pasireotide may attenuate the skewed neutrophil/lymphocyte response observed in COVID-19.

Additional pathway-informed candidate therapeutics targeting molecular mediators of the COVID-19 hyperinflammatory response include biologics such as TNF-α inhibitors, IL-6 inhibitors, tamoxifen-mediated inhibition of Eomes, IL-17 inhibitors, CDK inhibition, exogenous delivery of soluble FcγRIIB, and JAK inhibitors. Among TNF inhibitors, etanercept was proposed as a potential first-line choice in SARS-CoV based on considerations of safety, short-half life, and limited immunogenicity (Tobinick, 2004). Early evidence relating to compassionate use of IL-6 inhibitors in SARS-CoV-2 (tocilizumab and siltuximab) appears promising, with unfavorable outcomes generally associated with treatment-resistant increases in IL-6. Well-designed clinical trials appear justified (Khan et al., 2020).

The high infectivity, rapid case growth, and severe outcomes of the SARS-CoV-2 epidemic have created an urgent global health crisis and a pressing need for therapeutic approaches to contain the number of fatalities. This epidemic has emerged in the context of a rich existing literature detailing aspects of cellular and

molecular pathways affected by prior CoV serotypes and related conditions. Much of the emerging literature specific to SARS-CoV-2 is strongly consistent with these findings, and also features informative differences, particularly in lung tissue (e.g., weaker interferon response, suppressed epithelial defense, and elevated pulmonary infectivity).

The resulting synthesis enables construction of a coherent biological pathway that suggests multiple points of investigation for potential therapeutic candidates. Given the high case fatality rate of COVID-19, such candidates may help to bridge an urgent gap. While results from ongoing randomized controlled clinical trials remain essential, critical patients may benefit in the interim from the estimation of preliminary odds ratios relating to repurposed therapeutics, based on outcomes of COVID-19 patients having existing exposure to pathway-relevant candidates.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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frontiers in Pharmacology

MINI REVIEW
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The Potential Role of Heparin in Patients With COVID-19: Beyond the Anticoagulant Effect. A Review

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is responsible of variable clinical manifestations, ranging from no symptoms to severe pneumonia with acute respiratory distress syndrome, septic shock, and multi-organ failure resulting in death. To date no specific antiviral drug have been approved for COVID-19, so the treatment of the disease is mainly focused on symptomatic treatment and supportive care. Moreover, there are no treatments of proven efficacy to reduce the progression of the disease from mild/moderate to severe/critical. An activation of the coagulation cascade leading to severe hypercoagulability has been detected in these patients, therefore early anticoagulation may reduce coagulopathy, microthrombus formation, and the risk of organ damages. The role of heparin in COVID-19 is supported by a lot of studies describing its pleiotropic activity but it must be proven in clinical trials. Several protocols have been designed to assess the risk-benefit profile of heparin (low-molecular-weight or unfractionated heparin) in hospitalized subjects. Although prophylactic doses may be adequate in most patients, it is important to wait the results of clinical trials in order to define the appropriate effective dose able to improve disease outcome.

Keywords: COVID-19, coagulopathy, heparin, pleiotropic activity, clinical trials

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INTRODUCTION

The clinical manifestations of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection range from asymptomatic infection to severe pneumonia with acute respiratory distress syndrome (ARDS), septic shock, and multi-organ failure resulting in death (Wang Y. et al., 2020).

A large Chinese epidemiological study showed that among 44,672 confirmed cases, 80.9% were mild, 13.8% severe, and 4.7% critical. The fatality rate for critical patients was 49%, higher in patients with comorbidities (cardiovascular disease 10.5%, diabetes 7.3%, chronic respiratory disease 6.5%, hypertension 6.0%, cancers 5.6%) than those without comorbidities (0.9%) (Wang Y. et al., 2020). Laboratory findings of Corona Virus Disease 19 (COVID-19) include lymphopenia with depletion of CD4 and CD8 lymphocytes, prolonged prothrombin time, elevated lactate dehydrogenase (LDH), D-Dimer, alanine transaminase, C-reactive protein (CRP), and creatinine kinase (Huang et al., 2020; Wang D. et al., 2020).

One of the most important mechanisms underlying the deterioration of disease is the cytokine storm (Shimabukuro-Vornhagen et al., 2018). This clinically severe phase is accompanied by high level of pro-inflammatory molecules, such as interferons α and β, and IL-6 (Mehta et al., 2020).

Severe disease is also complicated with coagulopathy and disseminated intravascular coagulation (DIC) has been reported in the majority of deaths (Tang et al., 2020a). Patients with progressive, severe COVID-19 infection with acute lung injury or ARDS have very high D-dimer and fibrinogen levels, related to a hypercoagulable state. Moreover, severe and critically ill COVID patients with prolonged immobilization are inherently at high risk of venous thromboembolism (VTE) and some patients who require mechanical ventilation may have acute pulmonary embolism (PE) or deep vein thrombosis (DVT), even without strong predisposing risk factors.

Thus, an early anticoagulation, which blocks uncontrolled blood clotting and reduce micro-thrombus formation, would lower the risk of major organ disfunction. Accordingly, even if the risk-benefit ratio has not been established, the World Health Organization (WHO) recommended in these patients thrombo-prophylaxis with either unfractionated or low molecular weight heparin (LMWH) (Driggin et al., 2020; WHO, 2020b; WHO, 2020a).

Aim of this work is to describe the link between inflammation, immune activation, and coagulopathy and the hypothetical pleiotropic role of heparin in COVID-19.

INFLAMMATION, SEPSIS, AND COAGULOPATHY

A variety of disorders (sepsis, systemic inflammatory conditions, trauma, malignant disease) lead to activation of the coagulation system, up to the most extreme form of DIC, and microvascular thrombosis is a frequent complication of critical illness conditions (Dhainaut et al., 2005; Ito, 2014).

Inflammation and coagulation are clearly linked by different molecular signals and their interactions play a major role in the pathophysiology of sepsis and DIC (Levi and Poll, 2015; Li and Ma, 2017).

Acute infections, including viral ones, induce a systemic inflammatory response and coagulation disruption (Subramaniam and Scharrer, 2018). The process is complex and multifactorial, involving cellular disruption and plasmatic elements of the hemostatic system and of the innate immune system to the pathogen (Gando et al., 2016). Thrombosis under certain circumstances plays a major physiological role in immune defense. The coagulation system and innate immunity (the so-called *immunothrombosis system*) play a beneficial role in early host defense against pathogens (Delvaeye and Conway, 2009; Fiusa et al., 2015), limiting microbial dissemination, protecting blood vessels, promoting recruitment and activation of leukocytes through fibrin, fibrinogen, and their degradation products, and stimulating cellular immune responses at the infection sites. Moreover, intravascular thrombi produce a distinct compartment where antimicrobial peptides can be concentrated and kept in contact with pathogens. However, aberrant or uncontrolled *immunothrombosis* may be harmful, determining an imbalance between pro-coagulant and anticoagulant mechanisms (Ito, 2014).

Multiple pathogenetic mechanisms have been identified in the coagulation cascade activation, and involving endothelial cells, von Willebrand factor, Toll-like receptor, and tissue-factor pathway (van Gorp et al., 1999; Ito, 2014). The effect is the deregulated thrombin generation, further worsened by the impairment of anticoagulant and fibrinolytic systems.

The pro-inflammatory mediators activate coagulation, which in turn promotes inflammatory activity (Opal, 2000; Russell, 2006; Hunt, 2014). In particular, inflammation promotes coagulation by leading to intravascular tissue factor expression, inducing the expression of leukocyte adhesion molecules on the endothelial cell, and down-regulating the fibrinolytic pathways by the up-regulation of plasminogen activator inhibitor-1 (PAI-1). On the other hand, thrombin stimulates inflammatory response in a self-propagating feedback loop.

The simultaneous impairment of pro-coagulant pathways and fibrinolytic systems as a result of systemic inflammation lead to platelet activation and fibrin deposition (Simmons and Pittet, 2015; Levi and van der Poll, 2017). It has been demonstrated that the most important mediators for orchestrating this imbalance during sepsis are cytokines (Levi et al., 1997), such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α), but also denatured DNA and cationic proteins, such as histones, released from damaged cells (McDonald et al., 2017)[21].

The final result of the uncontrolled activation of the coagulation system is multiple organ dysfunction (Iba and Levy, 2018; Li X. et al., 2020).

Moreover, it is relevant in the pathogenesis of specific organ damage, such as ARDS (MacLaren and Stringer, 2007; Frantzeskaki et al., 2017). The lung coagulopathy is related to a localized tissue factor-mediated thrombin generation, and depression of bronchoalveolar plasminogen activator-mediated fibrinolysis, mediated by the PAI-1 increase (Glas et al., 2013; Ozolina et al., 2016).

Thus, the involvement of the hemostatic system in severe COVID-19 is not surprising, being well documented that inflammation and sepsis are initiators of DIC (Voves et al., 2006). The most typical findings in patients with COVID-19 and coagulopathy are an increased D-dimer level, a modest decrease in platelet count, and a prolongation of the prothrombin time (Levi et al., 2020). The pattern is therefore different to that typically seen in sepsis, in which thrombocytopenia is more severe, and D-dimer not very high (Levi and Scully, 2018). In particular, markedly elevated D-dimer has been detected and associated with higher intensive care unit (ICU) admission and mortality, likely reflecting coagulation activation, cytokine storm development, and organ failure (Guan et al., 2020; Huang et al., 2020; Tang et al., 2020b; Zhou et al., 2020). Furthermore, post-mortem examinations show vascular thrombosis in small vessels of the lungs (Carsana et al., 2020; Menter et al., 2020; Wichmann et al., 2020), suggesting that the COVID-19 coagulopathy can include, besides a low-grade of DIC, a so-called "Pulmonary Intravascular Coagulopathy-PIC" (Belen-Apak and Sarialioglu,

2020; Fogarty et al., 2020; McGonagle et al., 2020), a localized pulmonary thrombotic micro-angiopathy determining organ damage (Levi et al., 2020).

It is believed that the coagulation cascade in COVID-2019 can be activated through the well-known mechanisms reported above, which lead to the deregulated thrombin generation both systemically and locally in the lungs, resulting in the deposition of fibrin with subsequent tissue damage and micro-angiopathy (Li T. et al., 2020). Moreover, SARS-CoV-2 would directly damage vascular endothelial cells through angiotensin-converting enzyme 2 (ACE2), which could represent the first injury triggering the abnormal coagulation in particular in the lung (Li H. et al., 2020). However, other studies showed that ACE2 pulmonary expression is restricted to type II pneumocytes, and is nearly absent in endothelial (McGonagle et al., 2020; Rivellese and Prediletto, 2020). In this context, the strict contact between type II pneumocytes and the pulmonary vascular network, and the severe local inflammatory reaction, is likely to drive the generalized pulmonary hypercoagulable state seen in patients with COVID-19 (Li H. et al., 2020; McGonagle et al., 2020; Rivellese and Prediletto, 2020). Nevertheless, the mechanisms contributing to coagulopathy in COVID-19 have to be comprehensively clarified yet.

TREATMENT STRATEGIES

To date, treatment of coagulopathy/DIC has been focused on the target of the primary associated pathology (Levi and Scully, 2018). This is limited in the case of COVID-19, due to the lack of approved antiviral drug treatment, so the management of patients is mainly focused on symptomatic and supportive care. Moreover, there are no treatments of proven efficacy to reduce the progression of the disease from mild/moderate to severe/critical, in particular counteracting the cytokine storm (Chen et al., 2020). However, reducing the release or activity of pro-inflammatory mediators can prevent or reverse the uncontrolled hyper-inflammation, thereby improving the condition of patients and a lot of drugs with this aim are under evaluation in clinical trials.

The use of anticoagulants for patients with severe COVID-19 has been recommended by expert consensus and by WHO (Driggin et al., 2020; WHO, 2020b).

The International Society of Thrombosis and Haemostasis (ISTH) introduced a new category identifying an earlier phase of sepsis-associated DIC, called "sepsis-induced coagulopathy" (SIC) (Iba et al., 2019). In this case or in patients with markedly elevated D-dimers, LMWH at prophylactic dose should be considered (Tang et al., 2020a).

The optimal thrombo-prophylactic regimen in patients with COVID-19 is unknown (Driggin et al., 2020). Given drug-drug interaction with direct oral anticoagulants and some anti-viral regimens, heparins, either unfractionated or low molecular weight, may be preferred.

Accurate patient assessment is necessary to balance the individual risk of thrombosis and bleeding. Therapeutic anticoagulation is not required unless another indication for therapeutic anticoagulation is documented (e.g. VTE, atrial fibrillation, or mechanical valve). Moreover, evidence of coagulopathy/DIC and especially elevated D-dimer levels observed even in early phase of PIC might be useful to guide therapeutic decision (Lillicrap, 2020).

Prophylactic dose LMWH is recommended for all hospitalized COVID-19 patients in the absence of contraindications.

However, standard prophylactic regimens may be insufficient in severe and critically ill patients with variable thromboembolic/bleeding risk, and monitoring of anti-Xa activity may be considered when LMWH is used in these patients (Duranteau et al., 2018).

In cases where there are no contraindications, empiric therapeutic anticoagulation has been proposed by the American Society of Hematology in the following cases (Ash, 2020):

- intubated patients who develop sudden clinical and laboratory findings highly consistent with PE;
- patients with physical findings consistent with thrombosis (superficial thrombophlebitis, peripheral ischemia or cyanosis, thrombosis of dialysis filters, tubing, or catheters);
- patients with respiratory failure, particularly when D-dimer and/or fibrinogen levels are very high, in whom PE or microvascular thrombosis is highly suspected and other causes are not identified (e.g., ARDS, fluid overload).

A normal level D-dimer level provides reasonable confidence that anticoagulation should continue at prophylactic doses.

However, the efficacy and safety of anticoagulation as well as the appropriate dose regimen able to improve disease outcome in patients with COVID-19 have yet to be defined in clinical trials.

PHARMACOLOGICAL PROPERTIES OF HEPARIN AND CLINICAL EVIDENCE IN COVID-19

Although primarily employed for its anticoagulant properties, it is known that heparin possesses anti-inflammatory, immunomodulatory, anti-viral, and anti-complement activity which may offer benefit beyond the anti-coagulation (Davidson et al., 2002; Hoppensteadt et al., 2008; Young, 2008; Ludwig, 2009; Li et al., 2012; Li et al., 2014; Li et al., 2015; Li and Ma, 2017; Thachil, 2020).

Heparin is a member of a family of polyanionic polysaccharides called glycosaminoglycans (Young, 2008). It remains one of the most important anticoagulant drugs in clinical practice, currently used for the prevention and treatment of venous thrombosis and PE, the management of arterial thrombosis in patients with acute myocardial infarction and in the prevention of re-thrombosis after thrombolysis, and the prevention of thrombosis in extracorporeal circuits and hemodialysis.

The mechanisms behind its pleiotropic effect are complex and not completely understood.

Its polyanionic nature allows to bind sites proteins such as antithrombin III, but also cytokines, chemokines, growth factors, adhesion molecules, cytotoxic peptides, tissue destructive enzymes, involved in inflammation (Day et al., 2004). Thus, the binding of acute phase and complement proteins may contribute to the anti-inflammatory activity of heparin (Weiler et al., 1992; Young et al., 1997).

Indeed, even if the binding of released cytokines may protect them from proteolytic degradation, heparin may alter the secondary and tertiary structure of cytokines and prevent the binding to their specific receptors (Balasubramanian and Ramanathan, 2000; Mummery and Rider, 2000; Jayanthi et al., 2017), thus, influencing their biological activity, limiting accumulation of inflammatory cells and activation and subsequent tissue damage. When given in pharmacological doses, exogenous heparin and heparinoids demonstrated to attenuate tissue damage, neutralizing a variety of mediators released from inflammatory cells (Elsayed and Becker, 2003).

In line with this assumption, a large number of studies have revealed that LMWH reduce the release and the biological activity of IL-6 and IL-8 (Qian et al., 2014; Shastri et al., 2015; Li et al., 2016; Liu et al., 2019).

In addition, heparin binding to P-selectin showed to inhibit leukocyte adhesion to endothelial cells, independently by its anticoagulant activity (Lever et al., 2000).

The dysfunction of endothelial cells and the reduction of glycocalyx are key characteristics of sepsis. Heparin, as a heparan sulphate (HS) analogue, may reconstitute the protective layer of proteoglycans to restore the natural vascular barrier (Nelson et al., 2008). The protective function on the endothelial tight junctions has been demonstrated in a model of lung damage induced by lipopolysaccharide, where heparin administration decreased edema and vascular leakage (Liu et al., 2019).

Moreover, the protective responses observed with heparin in experimental models of sepsis seem to be mediated by blocking the pro-inflammatory signaling pathways regulated by MAPK, NF-κB, and STAT3 (Iba and Levy, 2018; Li X. et al., 2020). It has been demonstrated that heparin is readily bound and internalized into the cytosolic compartment, where it can prevent the NF-κB translocation to the nucleus through the binding of the positively charged nuclear localization sequence (Letourneur et al., 1995; Akimoto et al., 1996; Dudas et al., 2000). Blocking of this transcriptional factor can reduce inflammatory gene activation and regulate the production of pro-inflammatory cytokines, chemokines, and adhesion molecules.

A novel immune-modulating mechanism of heparin related to blockage of circulating histones has been studied ***in vitro*** and in septic mouse models (Wildhagen et al., 2014). It is noteworthy that extracellular histones released from dead cells play important role in cellular damage and are robustly associated with endothelial dysfunction, organ dysfunction and even death during sepsis (Xu et al., 2009; Ekaney et al., 2014; Iba et al., 2015). Heparin demonstrated a strong affinity for extracellular histones and prevents their interaction with platelets, a potential mechanism contributing to the regulation of inflammation (Fuchs et al., 2011; Alhamdi et al., 2016).

Finally, the putative antiviral role of heparin has been studied in experimental models. Thanks to its polyanionic nature, heparin can bind to several proteins, such as cell surface glycoproteins and thus inhibit herpes simplex virus attachment (Shukla and Spear, 2001). Furthermore it has been demonstrated that in zika virus infection it prevents virus-induced cell death (Ghezzi et al., 2017).

Interestingly, ***in vitro*** and ***in vivo*** experimental studies have shown that human coronaviruses utilize heparin sulfate proteoglycans for attachment to target cells (Milewska et al., 2014), and interaction between the SARS-CoV-2 Spike S1 protein receptor binding domain (SARS-CoV-2 S1 RBD) and heparin has been recently showed, supporting the role of heparin in the therapeutic armamentarium against COVID-19 beyond the anticoagulant effect (Courtney Mycroft-West et al., 2020).

However, the exact benefit and safety of heparin as anti-inflammatory and antiviral agent in clinical setting are yet to be defined and conflicting results have been reported by previous clinical trials.

According to systematic reviews and meta-analyses regarding the use of heparin as a potential treatment for patients with sepsis, treatment with low doses of heparin is associated with significantly reduced 28-day mortality in sepsis (Liu et al., 2014; Wang et al., 2014; Zarychanski et al., 2015; Fan et al., 2016).

Another meta-analysis shows a reduction of the risk of 7-day and of 28-day mortality, and a significant improvement of PaO2/FiO2 ratio in patients with ARDS treated with high-dose LMWH (Li et al., 2018), demonstrating that treatment with heparin may be helpful in mitigating the pulmonary coagulopathy found in ARDS.

The existing evidence on the use of heparin to prevent or treat thrombotic complications in COVID-19 derives from retrospective and observational data.

Recently, a retrospective cohort study analyzed the relieving effect of LMWH in patients with COVID-19, to investigate the anti-inflammatory effects of heparin and the delay of disease progression (Shi C et al., 2020). Compared to the control group, patients treated with heparin had an improvement of hypercoagulability, a reduction of IL-6 and neutralization of its biological activity, and an increase in the percentage of lymphocytes. A large retrospective cohort showed lower mortality in COVID-19 patients treated with heparin, even after adjustment for age and gender (OR 95% CI 0.55, 0.37–0.82; $p = 0.003$), saturation of oxygen <90%, and temperature >37°C (OR 0.54, 0.36–0.82; $p = 0.003$), and use of concomitant medications (OR 0.42, 0.26–0.66; $p < 0.001$) (Ayerbe et al., 2020). Moreover, a recent observational study conducted in US found a reduced risk of mortality among patients (n = 786) hospitalized with COVID-19 who received anticoagulation (Paranjpe et al., 2020).

Randomized controlled trials are necessary to confirm these preliminary observations.

Ongoing Clinical Trials in COVID-19

As reported on the COVID-19 clinical trials registry (http://www.covid-trials.org, 2020) which collects all trials from International Clinical Trials Registry Platform (Chinese Clinical

Trial Registry, ClinicalTrials.gov, Clinical Research Information Service—Republic of Korea, EU Clinical Trials Register, ISRCTN, Iranian Registry of Clinical Trials, Japan Primary Registries Network, and German Clinical Trials Register), 16 clinical trials are ongoing (9/16 recruiting and 7/16 not-recruiting) to evaluate the effect of anticoagulation with heparin (low-molecular-weight—mainly enoxaparin—or unfractionated heparin) in hospitalized patients with COVID-19 (**Appendix 1**). More than 80% of these studies are open-label, randomized, two-arm trials, and at least 75% of protocols include a comparison between therapeutic anticoagulation (investigational arm) and thromboprophylaxis (control arm), in line with the uncertainty about the benefit/risk ratio of the two treatment strategies. As reported in **Appendix 2**, the primary outcome measures of heparin clinical trials are hard endpoints such as mortality or composite measure of clinical events and/or survival, as recommended by the WHO guidelines (WHO, 2020d).

Overall, almost 10,000 patients are expected to be enrolled. However, the completion of some studies (expected in the second half of 2020 and in 2021) would be difficult at least in European countries and China due to the reduction in the number of new cases and hospitalizations (WHO, 2020c).

CONCLUSION

Coagulation activation has been reported in COVID-19, determining pathological changes specifically involving the lung microvasculature, and an increased risk of DVT, PE, and DIC in severe phase. The use of anticoagulants, in particular heparin, is recommended by expert consensus for patients with severe COVID-19, although a final guidance cannot be implemented yet.

There are several ways in which probably heparin administration can benefit patients with COVID-19, beyond the anticoagulant effect.

Although prophylactic doses may be adequate in most patients, it would be important to administer therapeutic dosage based on the individual risk of coagulopathy and thrombosis. To assess the efficacy and safety in patients with COVID-19 in clinical trials is crucial in order to find the appropriate effective dose of LMWH/UFH and improve disease outcomes. Different well-designed clinical trials (randomized, controlled, with appropriate outcome measures, even if not-blinded) are ongoing. However, the completion of trials and the consequent definition of risk/benefit profile of drugs candidate for COVID-19 would be complicated by the reduced (albeit strongly awaited) spread of the virus.

AUTHOR CONTRIBUTIONS

LG wrote the first draft of the manuscript. PV and FD checked and revised the draft manuscript. All authors contributed to the article and approved the submitted version.

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APPENDIX 1 | Ongoing clinical trials with heparin in patients with COVID-19 (update May 28, 2020).

ID	Country	Treatment	Phase	Completion	Trial status	Design	Blinding	Arms	Patient setting	Size
2020-001709-21	France	Enoxaparin, tinzaparin, dalteparin, nadroparin	IV	NA	Recruiting	Randomized	Open-label	2	Hospital	550
2020-001823-15	France	Enoxaparin	IV	NA	Recruiting	Single-arm	Open-label	1	ICU	200
2020-001891-14	Spain	Enoxaparin	II	NA	Recruiting	Randomized	Open-label	2	Hospital	140
CHICTR2000030700	China	Enoxaparin	/	2020-Sep	Not recruiting	Randomized	Open-label	2	Hospital	60
CHICTR2000030701	China	Enoxaparin	/	2020-Sep	Not recruiting	Randomized	Open-label	2	Hospital	60
CHICTR2000030946	China	LMW heparin	IV	2020-Apr	Recruiting	Non-randomized	Unspecified	2	Hospital	120
NCT04344756	France	Tinzaparin, enoxaparin, dalteparin, unfractionated heparin	II	2020-Jul	Not recruiting	Randomized	Open-label	2	Hospital, ICU	808
NCT04345848	Switzerland	Enoxaparin Unfractionated heparin	III	2020-Nov	Recruiting	Randomized	Single	2	Hospital, ICU	200
NCT04354155*	United States	Enoxaparin	II	2022-Sept	Not recruiting	Single-arm	Open-label	1	Hospital	38
NCT04359277	United States	Enoxaparin Unfractionated heparin	III	2021-Apr	Recruiting	Randomized	Open-label	2	Hospital	1,000
NCT04360824	United States	Enoxaparin	IV	2021-Apr	Not recruiting	Randomized	Open-label	2	Hospital	170
NCT04362085	Canada	LMW heparin Unfractionated heparin	III	2020-Nov	Recruiting	Randomized	Open-label	2	Hospital	462
NCT04366960	Italy	Enoxaparin	III	2020-Aug	Recruiting	Randomized	Open-label	2	Hospital	2,712
NCT04367831	United States	Enoxaparin Unfractionated heparin	IV	2020-Nov	Recruiting	Randomized	Single	4	ICU	100
NCT04372589	Canada	Enoxaparin, tinzaparin, dalteparin, unfractionated heparin	/	2021-Jan	Not recruiting	Randomized	Open-label	2	Hospital	3,000
NCT04377997	United States	Enoxaparin Unfractionated heparin	II	2021-Jan	Not recruiting	Randomized	Open-label	2	Hospital, ICU	300

Four trials recently approved in Italy and not yet reported in the online registry are not included.
*NA, not available; ICU, intensive care unit. *Pediatric subjects.*

APPENDIX 2 | Main outcome measures of ongoing clinical trials with heparin in patients with COVID-19.

ID	Primary outcome measures
2020-001709-21	Onset of a symptomatic venous thromboembolic event, or symptomatic pulmonary embolism, or unexplained death when a pulmonary embolism cannot be excluded
2020-001823-15	Measurement of the anti-Xa activity of enoxaparin
2020-001891-14	Need for oxygen therapy escalation or invasive mechanical ventilation or mortality
CHICTR2000030700	Time to Virus Eradication
CHICTR2000030701	Time to Virus Eradication
CHICTR2000030946	Biochemical indicators
NCT04344756	• Survival without ventilation (NIV or mechanical ventilation) in patients not requiring ICU who need for oxygen but no NIV or high flow. • Ventilator free survival in patients with respiratory failure and requiring mechanical ventilation
NCT04345848	Composite outcome of arterial or venous thrombosis, disseminated intravascular coagulation, and all-cause mortality
NCT04354155	Safety of in-hospital thromboprophylaxis
NCT04359277	All-cause mortality, cardiac arrest, symptomatic deep venous thrombosis, pulmonary embolism, arterial thromboembolism, myocardial infarction, hemodynamic shock
NCT04360824	Mortality
NCT04362085	Composite outcome of ICU admission, non-invasive positive pressure ventilation, invasive mechanical ventilation, or all-cause death
NCT04366960	Incidence of venous thromboembolism
NCT04367831	Composite of being alive and without clinically-relevant venous or arterial thrombotic events at discharge from ICU
NCT04372589	Need for invasive mechanical ventilation or mechanical ventilation, and occurrence of death
NCT04377997	Composite efficacy endpoint of death, cardiac arrest, symptomatic deep venous thrombosis, pulmonary embolism, arterial thromboembolism, myocardial infarction, or hemodynamic shock

ICU, intensive care unit; NIV, non-invasive ventilation.

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Montelukast Drug May Improve COVID-19 Prognosis: A Review of Evidence

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With the lack of effective therapy, chemoprevention and vaccination, focusing on the immediate repurposing of existing drugs gives hope of curbing the pandemic. Interestingly, montelukast, a drug usually used in asthma, may be proposed as a potential adjuvant therapy in COVID-19. The aim of the present article was to review the properties of montelukast that could be beneficial in COVID-19. Ten experimentally supported properties were retrieved, either related to SARS-CoV-2 (antiviral properties, prevention of endotheliitis and of neurological disorders linked to SARS-CoV-2), and/or related to the host (improvement of atherogenic vascular inflammation, limitation of the ischemia/reperfusion phenomenon, improvement of respiratory symptoms), and/or related to serious COVID-19 outcomes (limitation of the cytokine storm, mitigation of acute respiratory distress syndrome), and/or related to tissue sequelae (antioxidant properties, anti-fibrosis effects). Based on gathered theoretical evidence, we argue that montelukast should be further tested to prevent and treat COVID-19 outcomes.

Keywords: coronavirus disease 2019, severe acute respiratory syndrome coronavirus 2, montelukast, lukasts, treatment, research

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INTRODUCTION

Coronaviruses are a large family of single-stranded RNA viruses, which infect animals and humans. Since December 2019, the coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; previously 2019-nCoV) is spreading worldwide. The virus is primarily spread between people during close contact, most often *via* small droplets produced by coughing, sneezing, and talking. COVID-19 is characterized by fever, cough, severe pneumonia, RNAaemia, combined with the incidence of ground-glass opacities, clot formation and endotheliitis, and a variety of clinical signs including fatigue, cardiac and neurological outcomes (Ahn et al., 2020). Of note, while the majority of cases result in only mild symptoms, some progress to acute respiratory distress syndrome (ARDS) possibly precipitated by significant increase in blood levels of cytokines and chemokines. This "cytokine storm", reportedly due to angiotensin-converting enzyme-2 (ACE2) downregulation by SARS-CoV-2 (Bourgonje et al., 2020), triggers a proinflammatory environment which is strongly associated with severe tissue damages, contributing to ARDS and fatal outcomes of COVID-19 patients (Kimura et al., 2013).

As of June 2020, the COVID-19 pandemic has affected millions of people in 196 countries and left hundreds of thousands dead. With the lack of effective therapy, chemoprevention and vaccination, focusing on the immediate repurposing of existing drugs gives hope of curbing the pandemic. Interestingly, a recent *in silico* exploration identified montelukast (MK), from the Leukasts family (LKs; i.e. cysteinyl leukotriene receptors antagonists), among the top-scoring clinically-oriented drugs likely to inhibit SARS-CoV-2 main protease (Huynh et al., 2020). One retrospective study consistently found that older asthmatic outpatients receiving MK had fewer episodes of confirmed COVID-19 than those not using MK (Bozek and Winterstein, 2020). The aim of this article was to review the properties of LKs, especially of MK, that could be beneficial in COVID-19 and would deserve further dedicated studies.

MONTELUKAST

MK works as a cysteinyl leukotriene (cysLT) receptor antagonist. Leukotrienes are inflammatory mediators produced by the immune system. They promote bronchoconstriction, inflammation, microvascular permeability, and mucus secretion in asthma and chronic obstructive pulmonary disease. Consequently, use of high-dose MK as an anti-inflammatory agent is effective in acute asthma (Wu et al., 2003). MK is mainly used as a complementary therapy in adults in addition to inhaled corticosteroids. The use of MK is also known to decrease the frequency and severity of wheezing after an upper respiratory tract infection caused by adenovirus, influenza, metapneumovirus or coronavirus (Brodlie et al., 2015). Common side effects include diarrhea, nausea, vomiting, mild rashes, asymptomatic elevations in liver enzymes and fever. In 2019 and 2020, concerns for neuropsychiatric reactions were added to the label in the UK and US where the most frequently suspected were nightmares, depression, insomnia, aggression, anxiety and abnormal behavior (Glockler-Lauf et al., 2019).

Apart from MK, LKs also include Zafirlukast (ZK) and pranlukast (PK). These three compounds may have properties of potential interest to treat COVID-19, the main ones of which are illustrated in **Figure 1** and described hereafter.

PROPERTIES OF MK RELATED TO SARS-COV-2

Antiviral Properties

Several antiviral properties of MK, potentially useful in COVID-19, have been described *in vitro* and *in vivo*, based on distinct mechanisms depending on the virus under investigation. For *Influenzae A* virus, an inhibition of the expression of the viral genome was observed with MK (Landeras-Bueno et al., 2016). For *flaviviridae*, in particular Zika virus, an irreversible and early inactivation of the virus was reported, probably due to some damage to the lipid membrane (Chen et al., 2020). Three distinct mechanisms were proposed to support the beneficial impact of MK on Zika virus: i) a direct antiviral action, ii) an antagonization of the cytokine storm, and iii) an inhibition of the vertical transmission by a MK-related neuroprotective effect on the brain of fetus. For the hepatitis C virus, MK induced a dose-dependent decrease in the levels of RNAs expressed, indicating an inhibition of viral replication (Ruiz et al., 2020). MK also attenuated the initial responses to respiratory syncytial virus (RSV) infection in neonate and adult mice, and reduced the consequences of RSV reinfection in mice initially infected as neonates (Han et al., 2010; Kloepfer et al., 2011). Finally, in humans, Morita et al. (2017) have reported a decrease of almost 50% in the number of colds in younger boys aged 1 to 5.

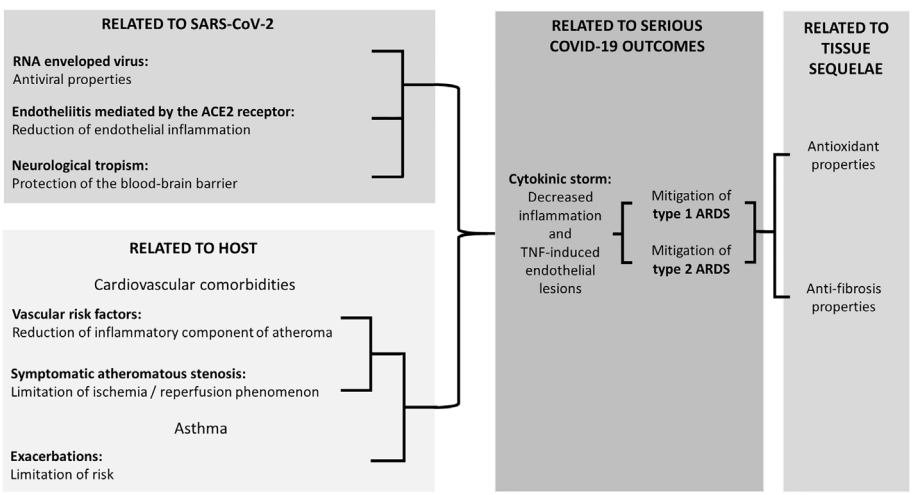

FIGURE 1 | Experimentally supported properties of Cyst LT1 receptor antagonists potentially beneficial in COVID-19.

Endotheliitis Induced by SARS-CoV-2 Infection

SARS-CoV-2 interacts with the ACE2 receptors to infect the host (Bourgonje et al., 2020). This process is thought to promote the development of endotheliitis (Varga et al., 2020), a condition that may be responsible for the multiplicity of clinical signs observed in COVID-19. MK antagonizes the inflammatory cascade induced by angiotensin II in vascular smooth muscle cells (Mueller et al., 2010) and could therefore constitute a specific treatment for the inflammation induced by this condition (Fidan and Aydoğdu, 2020).

Neurological Disorders Induced by SARS-CoV-2 Infection

Central nervous system disorders affect ca. 36.4% of patients with COVID-19 (Mao et al., 2020), mainly involving anosmia, dysgeusia, and headache. More serious manifestations such as seizures, delirium, encephalitis, and stroke have also been reported (Mao et al., 2020). LK limits the damage induced on the blood-brain barrier and has shown anti-convulsant properties in an experimental animal model of epilepsy (Lenz et al., 2014). Such protection of the blood-brain barrier could limit the occurrence and severity of brain damage (Zhou L. et al., 2019). It was also reported that MK improves fiber re-organization and long-term functional recovery after brain ischemia, enhancing recruitment and maturation of oligodendrocyte precursor cells (Gelosa et al., 2019). Additionally, a 6-week treatment with MK reduced neuroinflammation and elevated hippocampal neurogenesis through inhibition of the GPR17 receptor in younger and older rats (Marschallinger et al., 2015), with potential benefits for the prevention of manifestations such as delirium.

PROPERTIES OF MK RELATED THE HOST

Atherogenic Vascular Inflammation

It has been proposed that some severe complications of COVID-19 are mainly related to the host (Zhang et al., 2020). They are influenced by the age, gender and comorbidities, notably linked to preexisting inflammatory vascular and respiratory conditions. The cysLT are precisely strongly involved in the inflammatory phase of the atheromatous process although they are not used in this indication thus far. Antagonization of cysLT receptors greatly attenuates arterial spasm on human coronary arteries with atherosclerotic lesions, but it has no effect on healthy coronary arteries (Allen et al., 1993). A systematic review of the anti-atheromatous properties of MK in twenty-six animal and two human studies concluded that all studies supported the efficacy of LKs and MK on the atheromatous process (Hoxha et al., 2018). LKs could therefore reduce COVID-19 mortality in atheromatous patients, conferring a protection that would be (theoretically) proportional to the extent and severity of the atheromatous lesions (Almerie and Kerrigan, 2020).

Ischemia/Reperfusion

The ischemia/reperfusion phenomenon results in downstream vascular lesions following reperfusion. In patients with severe atheromatous disease, tissue hypoxia and hypoperfusion increase the risk of developing new endothelial lesions and ruptured atheroma plaque, inducing thrombosis and emboli. This may explain in part why COVID-19 is associated with an increased risk of arterial and venous thromboembolism, which affects approximately 30% of SARS-CoV-2-infected patients hospitalized in intensive care units (Klok et al., 2020). MK alleviates the ischemia/reperfusion phenomenon in animal models of intestinal anastomosis (Sayin et al., 2020), in skeletal muscles (Bilgiç et al., 2018), in the spinal cord (Korkmaz et al., 2015), and even following ovarian (Oral et al., 2011) or testicular torsion/distortion (Sılay et al., 2014). A coronary stent coated with MK particles is being developed (Zamani et al., 2016).

Asthma, Hyper-Reactivity Bronchitis, and Post-Infectious Cough

Asthma, for which LKs are usually prescribed, is a frequent and serious condition affecting 7%–8% of the population, though it is still under-diagnosed and under-treated (Deschildre, 2014). MK is effective against cough when it is an asthmatic equivalent, regardless of the functional respiratory parameters (Miwa et al., 2018). In contrast, MK has not shown any efficacy in chronic post-infectious cough (Wang et al., 2014), even though there was a high level of subjective improvement in the placebo group in this study (Wang et al., 2014). It would be of interest to examine MK on the mild symptomatic forms of COVID-19 respiratory damage (bronchospasms, cough, and chest pain).

PROPERTIES OF MK RELATED TO COVID-19 SERIOUS OUTCOMES

Cytokine Storm

The cytokine storm, corresponding to an unopposed generation of both pro-inflammatory and anti-inflammatory cytokines by the innate immune system, is responsible for most of the serious pulmonary complications of COVID-19 (Russell et al., 2020). The antagonist action of ZK on CystLT1 receptor protects the endothelium from inflammatory lesions induced by TNF-α (Zhou X. et al., 2019). By increasing IFN-γ production and inhibiting the expression of cytokines such as IL-1β, IL-6, and IL-8, the inflammatory chain-reaction could be better controlled (Han et al., 2010). Clinically, MK is used to reduce drug-related cytokine reactions induced by daratumumab (Chari et al., 2018) and rituximab (Kotchetkov et al., 2020). In this indication, MK is associated with a marked decrease in frequency and intensity of cytokine reactions and this action seems to be strengthened by the addition of an anti-H1, namely rupatadine (Kotchetkov et al., 2020).

Acute Respiratory Distress Syndrome

SARS-CoV-2-infected patients classically show mild symptoms that may gradually progress to more severe manifestations such as lethal ARDS. The type 1 ARDS is secondary to a direct alveolar inflammatory reaction, whereas the type 2 ARDS is secondary to systemic damage and occurs in the context of multi-visceral failure. To date, there is no effective chemotherapeutic treatment

for ARDS. The cornerstone of this condition remains the mechanical ventilation (Fan et al., 2018).

Regarding the type 1 ARDS, LK showed significant benefit on models induced by inhalation of irritant product like chlorine (Hamamoto et al., 2017) or pro-inflammatory lipids (Aquino-Junior et al., 2019), with a decrease in the intensity of the induced cytokine cascade and a lesser activation of neutrophils in the bronchoalveolar fluid. A similar effect was also reported in an animal model of malignant flu (Cardani et al., 2017).

Regarding the type 2 ARDS in an animal model of lung lesions induced by hepatic ischemia (Yeh et al., 2015) or hemorrhagic shock (Al-Amran et al., 2013), administration of LK resulted in a pulmonary reduction of neutrophil infiltration, lung inflammation, oxidative stress, and extent of lesions, along with a significant decrease in TNF-α and IL-6 cytokines in the pulmonary parenchyma and bronchoalveolar lavage.

PROPERTIES OF MK RELATED TO TISSUE SEQUELAE

Antioxidant Properties

An overproduction of reactive oxygen species (ROS) is crucial for viral replication and the subsequent virus-associated disease (Khomich et al., 2018). Experimental animal models of ARDS have shown enhanced ROS levels and disturbance of antioxidant defense during SARS-CoV infection (van den Brand et al., 2014). In cells infected with SARS-CoV, there was a greater amount of activated (phosphorylated) forms of all mitogen-activated protein kinase (MAPK) members (Khomich et al., 2018); i.e. a family of serine/threonine that are activated in response to environmental stresses including oxidative stress, DNA damage, carcinogenic stimuli and viral infections. Clinically, Shao et al. (2006) observed an upregulation of mitochondrial genes and genes responding to oxidative stress in peripheral blood mononuclear cells of convalescent SARS-CoV patients. Some of these genes, including PRDX1, FTH1 and FOS, are sensitive to oxidative stress and showed a remarkable elevation. These results support a role for oxidative stress during COVID-19. Importantly, protective effects of MK are not limited to inflammatory and microbial infectious attacks, but also include protection against chemotoxicity (bleomycin, cysplatin, doxorubicin, statin, paracetamol) (Hareedy et al., 2019) and radiotoxicity (Hormati et al., 2020) in animal experiments, which demonstrates some antioxidant properties resulting in increased mitochondrial mass and functionality, together with increased intracellular cyclic adenosine 3, 5′-monophosphate (cAMP) level and activation of the Krebs cycle (Wang et al., 2019).

Anti-Fibrosis Properties

Using MK may limit the residual extent of COVID-19 sequelae of pulmonary fibrosis, as for scar formation after lung surgery (Peng et al., 2017). MK regulates the extracellular remodeling matrix and inhibits the formation of fibrosis (Debelleix et al., 2018). This anti-fibrotic potential has been confirmed in an animal model of pulmonary fibrosis induced by bleomycin (Topaloğlu et al., 2018). Similarly, a recent meta-analysis confirmed in women that MK decreases the risk of retractile fibrosis after the placement of a silicone implant in breast reconstruction surgery (Wang et al., 2020).

CONCLUSIONS

Although quantity is not quality, these 10 effects of MK may constitute as many synergistic and potentiating therapeutic possibilities in COVID-19. MK is a commonly used drug that does not require any prior cardiological or biological examination; it can be prescribed for pregnant women and frail older adults, and it shows a "comfortable" therapeutic range. Moreover, it could be all the more effective for patients with comorbidities such as diabetes, sleep apnea, smoking, obesity, or symptomatic atherosclerotic lesions. We support the conduct of clinical trials testing the effect of MK in COVID-19 patients from a variety of populations, while keeping in mind its adverse effects. Finally, it should also be emphasized that a potential massive use of MK in COVID-19 would risk depriving asthma patients of their treatment, which should also be anticipated.

AUTHOR CONTRIBUTIONS

CA has full access to all of the data in the study, takes responsibility for the data, the analyses and interpretation and has the right to publish any and all data, separate and apart from the attitudes of the sponsors. All authors contributed to the article and approved the submitted version. Study concept and design: JB, J-MS, and CA. Drafting of the manuscript: JB and CA. Critical revision of the manuscript for important intellectual content: J-MS. Study supervision: CA.

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REVIEW
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Potential Therapeutic Options for COVID-19: Current Status, Challenges, and Future Perspectives

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The COVID-19 pandemic represents an unprecedented challenge for the researchers to offer safe, tolerable, and effective treatment strategies for its causative agent known as SARS-CoV-2. With the rapid evolution of the pandemic, even the off-label use of existing drugs has been restricted by limited availability. Several old antivirals, antimalarial, and biological drugs are being reconsidered as possible therapies. The effectiveness of the controversial treatment options for COVID-19 such as nonsteroidal antiinflammatory drugs, angiotensin 2 conversion enzyme inhibitors and selective angiotensin receptor blockers was also discussed. A systemic search in the PubMed, Science Direct, LitCovid, Chinese Clinical Trial Registry, and ClinicalTrials.gov data bases was conducted using the keywords "coronavirus drug therapy," passive immunotherapy for COVID-19', "convalescent plasma therapy," (CPT) "drugs for COVID-19 treatment," "SARS-CoV-2," "COVID-19," "2019-nCoV," "coronavirus immunology," "microbiology," "virology," and individual drug names. Systematic reviews, case presentations and very recent clinical guidelines were included. This narrative review summarizes the available information on possible therapies for COVID-19, providing recent data to health professionals.

Keywords: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), pandemic, COVID-19 proposed therapy, convalescent plasma, therapeutic challenges

INTRODUCTION

The contemporary century has witnessed the outbreak of several corona viral intimidations that cause a spotlight on public health, education, economy, and travels and respond to the threat of a global pandemic. The ongoing viral infection is caused by SARS-CoV-2, which establishes a novel coronavirus disease 2019 (COVID-19). Analogous (79.6% similar) to SARS-CoV, the SARS-CoV-2 is one of the members of a relatively largest family of the RNA viruses and contains four important structural proteins, such as the surface spike (S) glycoprotein, membrane (M) protein, small

envelope (E) glycoprotein, and the nucleocapsid (N) protein that help for its development completely (**Figure 1A**) (Schoeman and Fielding, 2019; Risitano et al., 2020). The positive-sense, single-stranded RNA genome of SARS-CoV-2 contains a cap at 5' end and polyadenylated (A) sequence at 3' end, serves as mRNA for replicase polyprotein translation (**Figure 1B**) (Wu et al., 2020).

From the beginning of the outbreak of SARS-CoV-2, it spreads immediately in most of the countries around the world and causes severe human diseases or death (Goumenou et al., 2020). The lack of effective drug therapy, and along with the high morbidity and mortality rates and its pandemic highlights the need for novel drug discovery for the treatment of COVID-19 (Tsatsakis et al., 2020).

Several national and international institutions and research groups are working collaboratively on a diversity of preemptive and beneficial interventions.

Cheap and widely available, dexamethasone is a steroid commonly used to treat allergic reactions, but also rheumatoid arthritis and asthma (Mititelu et al., 2020). British researchers who researched an effective treatment for COVID-19 reported that dexamethasone reduced deaths by a third among the most severely ill patients compared to regular treatment (Horby et al., 2020). It is currently conducting an analysis of the results obtained from the RECOVERY study arm regarding the use of dexamethasone-containing drugs in the treatment of hospitalized patients with COVID-19 infection. This component of the study looked at the effects of adding dexamethasone to regular therapeutic measures taken in adults who are being given invasive ventilation, those who are being given oxygen, or those who are not being given extra oxygen (Horby et al., 2020).

In the RECOVERY study, deaths occurred within 28 days of starting dexamethasone treatment. According to the preliminary results, in comparison with the routine measures, the administration of dexamethasone obtained the following: i) reduction by approximately 35% of the mortality rate in patients with invasive mechanical ventilation; ii) reduction by about 20% of the mortality rate in patients who were given oxygen without invasive ventilation; iii) nonreduction of the mortality rate in patients without oxygen therapy (Horby et al., 2020). As a result, in the UK, doctors have announced that patients will start receiving the first drug that has been shown to reduce COVID-19-associated death. While researchers believe that dexamethasone could save the lives of one in eight ventilator-connected patients, it has been shown to have few clinical benefits in less severe cases (Lu et al., 2020).

At least two major studies in the United States have shown that the antiviral drug remdesivir can reduce hospitalization period for patients with COVID-19. The results of these studies showed that remdesivir injections - originally intended as a treatment for Ebola - accelerated the patient's recovery compared to placebo (Beigel et al., 2020; Goldman et al., 2020).

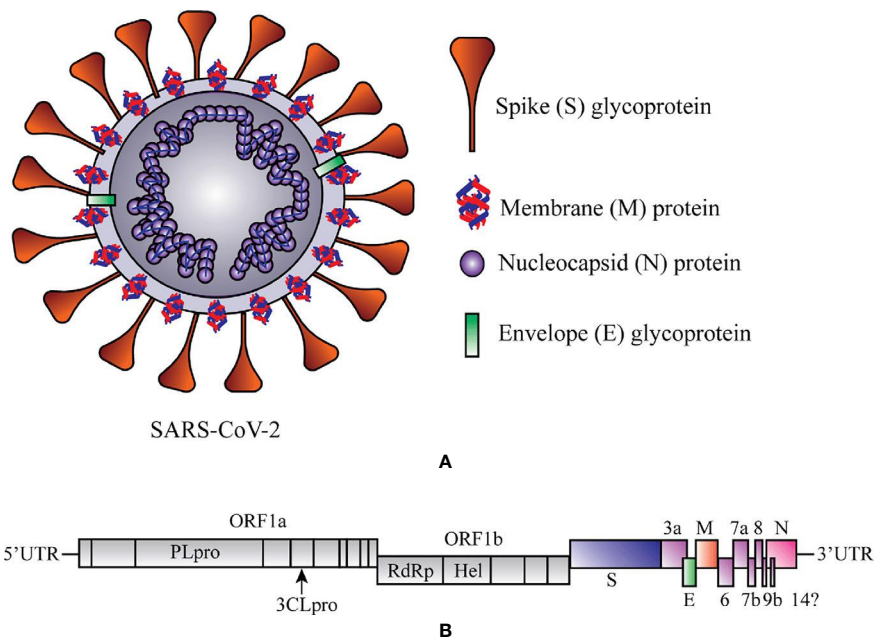

FIGURE 1 | Schematic representation of structure and RNA genome of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). **(A)** Structure of SAR-CoV-2. **(B)** RNA genome sequence of SARS-CoV-2. $3CL^{PRO}$, 3-Chymotrypsin-like protease; Hel, helicase; ORF1a/b, Open reading frame 1a/b; PL^{PRO}, Papain-like protease; RdRp, RNA-dependent RNA polymerase. At 5′ end 67% viral genome contains two open reading frames (ORF1a and ORF1b) that encode two significant replicase genes (*rep1a* and *rep1b*), and which helps to express large replicase polyprotein 1a/ab (pp1a and pp1ab) (Islam et al., 2020). These polyproteins produce nonstructural proteins (e.g., RNA-dependent RNA polymerase (RdRp) and helicase) after the cleavage with the help of two enzymes, papain-*like* cysteine protease (PL^{PRO}) and 3-chymotrypsin-*like* serine protease ($3CL^{PRO}$) (Zumla et al., 2016). At 3′ end 33% viral genome encodes the structural proteins (e.g., S, M, E, and N), which are required for the attachment of virus particle and entry of the viral genome into the host cell (Peiris et al., 2004).

Therefore, the US has authorized the emergency use of Remdesivir, an initiative followed by the European Union and several Asian nations, including Japan and South Korea (Gilead.Com, 2020).

China has completed clinical research on Favipiravir, an antiviral drug that has been shown to be clinically effective against the disease caused by the new coronavirus. Favipiravir, a flu medicine approved for clinical use in Japan in 2014, did not show any obvious side effects in the clinical trial (Heng et al., 2020).

More than 80 patients participated in the clinical trial, 35 of these patients received treatment with Favipiravir, and 45 were included in a control group. The results showed that patients treated with Favipiravir had negative results in testing for this virus in a shorter time compared to patients in the control group (Clinicaltrials.Gov, 2020a). Another randomized clinical trial also suggested that the therapeutic effect of Favipiravir was much better than that seen in the control group (Clinicaltrials.Gov, 2020bm). So, Favipiravir has been recommended by Chinese physicians and should be included in the diagnosis and treatment plan for COVID-19.

Prospective opportunities being reconnoitered include vaccine development, monoclonal antibodies (mAbs), interferon-based therapies, CPT, small-molecular drug therapies, and cell-based therapies (Li and De Clercq, 2020). (Calina et al., 2020).

In this comprehensive narrative review, we have sketched a current scenario on the most recent or ongoing clinical trials along with the remaining challenges and future perspectives of COVID-19 therapies.

METHODOLOGY

Since there is little information about these drug candidates in the peer-reviewed literature, aimed at this review, we also collected data from the publicly available websites and electronic and print media. In order to obtain all registered therapeutic and preventative interventions under clinical investigation, a systemic search (up to 10th June 2020) in the PubMed, Science Direct, LitCovid, Chinese Clinical Trial Registry, and ClinicalTrials.gov databases was conducted using the keywords "coronavirus drug therapy," "passive immunotherapy for COVID-19," "CPT," "drugs for COVID-19 treatment," "SARS-CoV-2," "COVID-19," "2019-nCoV," "coronavirus immunology," "microbiology," "virology," and individual drug names (**Table 1**). No language, country or study design restrictions were imposed. All information was evaluated in the knowledge about the treatment candidates, characteristics, dose/conc. (route of admin.), study systems, mechanism of action, and the stage of development of the COVID-19 therapies. The inclusion and exclusion criteria are given below.

Inclusion Criteria

1. Studies on current COVID-19 drug therapy performed in SARS-CoV-2 infected patients;
2. Studies that exploited single and/or multiple animals;
3. Registered clinical trials on the proposed, repurposed or experimental candidates for the COVID-19 treatment that are recorded in online registries such as ClinicalTrials.gov and the International Clinical Trials Registry Platform (ICTRP) of the WHO;
4. Therapeutic candidates with beneficial consideration after clinical trials;
5. Therapeutic candidates exhibited auspicious effectiveness in contrast to COVID-19;
6. Studies with or without proposing mechanism of actions of the therapeutic candidates in COVID-19;
7. The most recent or ongoing clinical trial(s) on the individual treatment candidate.

Exclusion Criteria

1. Data duplication, titles or abstracts not meeting the inclusion criteria;
2. Studies on antiviral drug candidates other than SARS-CoV-2;
3. Reports on treatment candidates that encode membrane (M), envelope (E), nucleocapsid (N) and spike (S) protein of genomic RNA other than SARS-CoV-2;
4. Active clinical trials were identified other than the ClinicalTrials.gov and the Chinese Clinical Trial Registry;
5. Previous clinical trial(s) on the individual candidate other than SARS-CoV-2 outbreak.

OLD AND NEW DRUGS POTENTIALLY PURPOSED FOR COVID-19 TREATMENT

From December 2019, several clinical trials (including those not yet recruiting, recruiting, active, or completed) of the proposed or repurposed drugs in several countries around the world are continuously proceeding to deliver real-world clinical data for the COVID-19 challenges. We selected a total of 72 most current or ongoing clinical trials of the COVID-19 drug candidates with their mechanism of actions after refining through inclusion and exclusion criteria that might be helpful to screen, therefore, considered as starting points to discover and develop antiviral drug candidates for COVID-19 (**Table 1** and **Figure 2**).

Table 1 shows i) antiviral drugs (nonspecific), ii) antiviral drugs (broad-spectrum), iii) antiviral drugs (antiretrovirals), iv) antimalarial drugs, v) antibiotics and antiparasitics, vi) nonspecific antiinflammatory and immunosuppressive drugs, vii) kinase inhibitors, viii) monoclonal antibodies, ix) hormonal preparations, x) cardiovascular drugs, and xi) blood and blood-forming organs.

Antiviral Drugs (Nonspecific)

Immunoglobulin (Ig)

It is an inhibitor of viral fragment crystallizable (Fc) receptor activation, which prevents antibody-dependent enhancement of infection and provides boosting effects of endogenous

TABLE 1 | Potential Drugs against COVID-19 and their mechanisms of action.

Treatment candidates	Characteristics	Dose/route of administration	Study systems	Mechanism of action	Stage of development (Registry/Status)	References
Antiviral drugs (nonspecific)						
Immunoglobulin	Inhibitor of viral Fc receptor activation	0.5g/kg/day (iv)	Patients (n=80)	↓ antibody-dependent enhancement of infection↑endogenous Nabs	Phase 2 and 3(NCT04261426/ Not yet recruiting)	(Cao et al., 2020; Clinicaltrials.Gov, 2020ah)
IFN-β1a	Cytokine signaling molecule	10 μg (iv)	Patients (n=7100)	↑cytoplasmic enzymes↓mRNA translation↓protein synthesis	Phase 4(NCT02735707/ Recruiting)	(Clinicaltrials.Gov, 2020bf)
IFN- β1b	Cytokine in the interferon family	0.25 mg (sc)	Patients (n=80)	↑cytoplasmic enzymes↓mRNA translation a↓protein synthesis.	Phase 2(NCT04276688/ Completed)	(Hung et al., 2020)
Interleukin-2	Cytokine signaling molecule	Low dose (IM)	Patients (n=20)	↑CD8+ T cells,↑CD4+ T,↑NK cell numbers.	Phase 1 (ChiCTR2000030167)	(Chictr.Org.Cn, 2020b)
CYNK-001	Cryopreserved allogeneic,off-the-shelf, placental-derived NK cell therapy	Multiple doses	Patients (n=86)	↑CD56+/CD3- NK cells	Phase 1 and 2(NCT04365101/ Recruiting)	(Clinicaltrials.Gov, 2020av)
Baloxavir Marboxil	Cap snatching inhibitor	80 mg once a day (orally)	Patients (n=10)	↓viral cap-dependent endonuclease.	Clinical trial(ChiCTR 000029544)	(Lou et al., 2020)
Antiviral drugs (broad spectrum)						
Favipiravir	RNA polymerase inhibitor	1,800 and 600 mg	Patients (n=100)	↓RNA-dependent RNA polymerase (RdRp)	Phase 3(NCT04336904/Active, not recruiting)	(Furuta et al., 2017; Clinicaltrials.Gov, 2020l)
Arbidol	Direct antiviral/host-targeting agent	200 mg,tid or 400 mg, tid (orally)	Patients (n=500)	↓ membrane haemagglutinin fusion.	Phase 4(NCT04246242/Not yet recruiting)	(Smartpatients.Com, 2020)
Remdesivir	Adenosine analog	200 and 100 mg (IV)	Patients (n=237)	↓ SARS-CoV-2 replication↓RNA polymerase	Phase 3(NCT04257656/ Terminated)	(Scavone et al., 2020; Wang et al., 2020b)
Galidesivir	Adenosineanalog	_	Patients (n=66)	↓viral RNA polymerase.	Phase 1(NCT03891420/ Recruiting)	(Clinicaltrials.Gov, 2020bp)
Antiviral drugs (antiretrovirals)						
ASC09	Protease inhibitor	100 mg or 300 mg (orally)	Patients (n=160)	prevention of proteolytic cleavage.	Not Applicable (NCT04261907/ Not yet recruiting)	(Clinicaltrials.Gov, 2020ak)
Azvudine	Nucleoside analog	1 mg (orally), 5 times daily	Patients (n=20)	↓reverse transcriptase →↓ replication of the virus.	Phase 3(ChiCTR2000029853)	(Chictr.Org.Cn, 2020e)
Danoprevir	Protease inhibitor	100 mg twice a day (orally)	Patients (n=11)	Danoprevir + ritonavir→↓transcription, ↓replication	Phase 4(NCT04291729/ Completed)	(Chen et al., 2020)
Darunavir and Cobicistat	Protease inhibitor	Single dose (orally)	Patients (n=30)	Darunavir + cobicistat→↓ Cyt P-450 CYP3A.	Phase 3(NCT04252274/ Recruiting)	(Clinicaltrials.Gov, 2020ab)
Lopinavir + Ritonavir	Protease inhibitor	100 and 400 mg (orally)	Patients (n=199)	↓metabolizing enzyme Cyt P450 3A by ritonavir↑ ½ life of lopinavir.	Clinical trial(ChiCTR2000029308)	(Cao et al., 2020; Dorward and Gbinigie, 2020)
Antimalarial drug						
Hydroxychloroquine	Antimalarial drug	200, 600, and 800 mg (orally)	Patients (n=3,000)	Hydroxychloroquine+Remdesivir →↓ viral replicationLopinavir/ritonavir + IF 1β →↓glycosylation of viral ACE-2.	Phase 3(NCT04308668/ Recruiting)	(Clinicaltrials.Gov, 2020bb)
Antibiotics and antiparasitics						
Carrimycin	A polyether antibiotic	_	Patients (n=520)	Acts against Gram-positive bacteria, mycoplasma; fungi, and yeasts.	Phase 4(NCT04286503/Not yet recruiting)	(Clinicaltrials.Gov, 2020k)
Suramin sodium	Used to treat trypanosomiasis, onchocerciasis	_	Patients (n=20)	↓glycosylation of viral ACE-2 ↓quinone reductase 2.	Clinical trial(ChiCTR2000030029)	(Chictr.Org.Cn, 2020d)
Ivermectin	Used to treat parasitic infections	600 μg/kg (orally)	Patients (n=60)	↓replication of SARS-CoV-2.	Phase 2(NCT04374279/Not yet recruiting)	(Clinicaltrials.Gov, 2020bv)
Dihydroartemisinine + piperaquine	Inhibitor of viral Fc receptor activation.	Dihydroartemisinin + piperaquine (40 mg +320 mg) (orally)	Patients (n=40)	Interaction between its peroxide bridge and haem iron may underlie its antiviral action.	Phase 4(ChiCTR2000030082)	(Chictr.Org.Cn, 2020a)

(Continued)

TABLE 1 | Continued

Treatment candidates	Characteristics	Dose/route of administration	Study systems	Mechanism of action	Stage of development (Registry/Status)	References
Azithromycin	Macrolide antibiotic	500 mg (orally)	Patients (n=200)	It blocks internalization into host cells during the early phase of infection.	Phase 2(NCT04369365/ Recruiting)	(Tran et al., 2019; Clinicaltrials.Gov, 2020bh)
Doxycycline	Semi-synthetic tetracycline antibiotic	200 mg/day (orally)	Patients (n=330)	↓ replication SARS-CoV-2↓IL-6 levels	Phase 3(NCT04371952/Not yet recruiting)	(Sargiacomo et al., 2020; Clinicaltrials.Gov, 2020y)
Nonspecific antiinflammatory and immunosuppressive drugs						
Corticosteroids	Immunomodulatingantiinflammatory	1mg/kg/day (IV)	Patients (n=86)	↓immune system↓inflammation↓ proinflammatory cytokines	Not Applicable(NCT04273321/ Completed)	(Clinicaltrials.Gov, 2020aa)
Fingolimod	Immunosuppressant	0.5 mg once daily (orally)	Patients (n=30)	modulating S1P→sequesters lymphocytes in lymph nodes.	Phase 2(NCT04280588/ Recruiting)	(Clinicaltrials.Gov, 2020al)
Leflunomide	DMARD and Immunosuppressant	300 mg once daily (orally)	Patients (n=20)	↓ dihydro-orotate dehydrogenase↓tyrosine kinases↓ intracellular transcription factors	Phase 1(NCT04361214/ Recruiting)	(Clinicaltrials.Gov, 2020ar)
Thalidomide	Immunosuppressant and sedative drug	100 m (PO, QN)	Patients (n=100)	↓TNF-α↓ cell surface adhesion molecules involved in leukocyte migration.	Phase 2(NCT04273529/Not yet recruiting)	(Clinicaltrials.Gov, 2020ac)
Colchicine	Antiinflammatoy and antigout agents	0.5 mg (PO)	Patients (n=6,000)	↓microtubule assembly→↓inflammasome activation, ↓chemotaxis,↓leukotrienes, ↓cytokines,↓ phagocytosis.	Phase 3(NCT04322682/ Recruiting)	(Niel and Scherrmann, 2006; Clinicaltrials.Gov, 2020m)
Ibuprofen	NSAID	200 mg	Patients (n=230)	↓COX,↓prostaglandins	Phase 4(NCT04334629/ Recruiting)	(Cole and Frautschy, 2010; Clinicaltrials.Gov, 2020as)
Naproxen	NSAID	250 mg	Patients (n=584)	Inhibits the activity of cyclooxygenase enzymes.	Phase 3(NCT04325633/Not yet recruiting)	(Knights et al., 2010; Clinicaltrials.Gov, 2020ae)
Piclidenoson/CF101	Antiinflammatory drug	2 mg (orally)	Patients (n=40)	It binds to the Gi protein associated A3AR, ↓antiinflammatory effect↓IL-17, ↓ IL-23.	Phase 2(NCT04333472/Not yet recruiting)	(Clinicaltrials.Gov, 2020az)
Kinase inhibitors						
Jakotinib hydrochloride	JAK inhibitor	50 mg/bid(orally)	Patients (n=90)	↓AAK1↓JAK.	Phase 2(NCT04312594/Not yet recruiting)	(Zhang et al., 2020; Clinicaltrials.Gov, 2020ap)
Ruxolitinib	JAK inhibitor	5 mg (orally)	Patients (n=402)	↓protein tyrosine kinases↓JAK 1, ↓JAK 2↓inflammation↓cellular proliferation.	Phase 3(NCT04362137/ Recruiting)	(Stebbing et al., 2020; Clinicaltrials.Gov, 2020ax)
Baricitinib	JAK inhibitor	4 mg/day(orally)	Patients (n=200)	Affinity for AP2-associated protein AAK1↓SARS-CoV-2 endocytosis.	Phase 2 and 3(NCT04320277/ Not yet recruiting)	(Cantini et al., 2020; Clinicaltrials.Gov, 2020f)
Tofacitinib	JAK inhibitor	10 mg twice a day	Patients (n=50)	↓ JAKs↓phosphorylation, ↓activation of STATs	Phase 2(NCT04332042/Not yet recruiting)	(Clinicaltrials.Gov, 2020bs)
Imatinib	Kinase inhibitor	800 mg/day (orally)	Patients (n=99)	↓tumor growth of bcr-abl transfected murine myeloid cells as well as bcr-abl positive leukemia lines.	Phase 2(NCT04357613/Not yet recruiting)	(Moen et al., 2007; Clinicaltrials.Gov, 2020am)
Monoclonal antibodies						
Tozumab +adamumab	TNF-α inhibitor	_	Patients (n=60)	↓TNF-α↓ IL-6, ↓ IL-10	Phase 4(ChiCTR2000030580)	(Chictr.Org.Cn, 2020c)
Ravulizumab/ ALXN1210	Component 5 (C5) inhibitor	Weight-based doses (IV)	Patients (n=270)	↓C5	Phase 3(NCT04369469/Not yet recruiting)	(Clinicaltrials.Gov, 2020ad)
Leronlimab/PA14/ PRO-140	CCR5 antagonist	700 mg (SC)	Patients (n=390)	A humanized IgG4 and monoclonal antibody (mAb) to CCR5 →↓coronavirus entry, ↓viral infection of CD4 T-cells, ↓CCR5	Phase 2(NCT04347239/ Recruiting)	(Clinicaltrials.Gov, 2020bo)
TJ003234	Anti-GM-CSF monoclonal antibody	3 and 6 mg/kg (IV)	Patients (n=144)	↑mAbs against GM-CSF.	Phase 1 and 2(NCT04341116/ Recruiting)	(Clinicaltrials.Gov, 2020bl)
Nivolumab/Obtivo®	IgG4 monoclonal antibody	3 mg/kg (IV)	Patients (n=92)	It binds to the PD-L1 receptor and blocks its interaction with PD-L1 and PD-L2.	Phase 2(NCT04343144/Not yet recruiting)	(Wolchok et al., 2013; Clinicaltrials.Gov, 2020bu)

(Continued)

TABLE 1 | Continued

Treatment candidates	Characteristics	Dose/route of administration	Study systems	Mechanism of action	Stage of development (Registry/Status)	References
Meplazumab	Humanized mAb	10 mg (IV)	Patients (n=20)	It binds to IL-5 and prevents it from binding to its receptor.	Phase 1 and 2(NCT04275245/ Recruiting)	(Bian et al., 2020)
Eculizumab	Recombinant humanized mAb	1,200 or 900 mg (IV)	Patients (n=120)	↓ C5 cleavage	Phase 2(NCT04346797/ Recruiting)	(Clinicaltrials.Gov, 2020w)
Clazakizumab	Anti-IL- 6 monoclonal	25 mg (IV)	Patients (n=60)	↑IgG1 which binds to IL-6 and prevents its interaction and signaling *via* IL-6R.	Phase 2(NCT04348500/ Recruiting)	(Clinicaltrials.Gov, 2020i)
Avdoralimab/ IPH5401	Anti-C5aR antibody	Multiple doses (IV)	Patients (n=108)	blocks C5aR,↓inflammatory response in the lungs.	Phase 2(NCT04371367/ Recruiting)	(Clinicaltrials.Gov, 2020e)
Lenzilumab	IgG1 kappa	IV infusion	Patients (n=238)	It targets CSF2/GM-CSF.	Phase 3(NCT04351152/ Recruiting)	(Clinicaltrials.Gov, 2020ay)
LY3127804	A selective mAb	IV administration	Patients (n=200)	It acts against Angiopoietin 2 (Ang2).	Phase 2(NCT04342897/ Recruiting)	(Clinicaltrials.Gov, 2020bj)
IFX-1	Antiinflammatories and Monoclonal antibody	Single dose/multiple doses (IV)	Patients (n=130)	↓C5a↓ Inflammation mediator modulators	Phase 2 and 3(NCT04333420/ Recruiting)	(Clinicaltrials.Gov, 2020aw)
Gimsilumab/KIN-1901	Fully mAb	High dose on Day 1 & low dose on Day 8	Patients (n=270)	↓ GM-CSF.	Phase 2(NCT04351243/ Recruiting)	(Clinicaltrials.Gov, 2020bn)
Actemra®/ Tocilizumab	IL-6 inhibitor	800 mg (IV)	Patients (n=100)	↓ IL-6interrupts the process of CRS.	Phase 2(NCT04335071/ Recruiting)	(Le et al., 2018; Clinicaltrials.Gov, 2020br)
Tocilizumab	IL-6 inhibitor (FDA granted)	800 mg (IV)	Patients (n=400)	↓ ILinterrupts the process of CRS.	Phase 2(NCT04317092/ Recruiting)	(Fortes et al., 1973)
Kevzara®/ Sarilumab	IL-6 inhibitor	200 and 400 mg	Patients (n=276)	↓immune response↓IL-6	Phase 2 and 3(NCT04315298/ Recruiting)	(Arrytown and Paris, 2020)
Bevacizumab	Anti-VEGF monoclonal IgG1 antibody	7.5 mg/kg in 100 ml saline	Patients (n=130)	↓ viral proliferation,↓migration,↑IgG1	Phase 2(NCT04344782/Not yet recruiting)	(Kazazi-Hyseni et al., 2010; Clinicaltrials.Gov, 2020bt)
Hormonal preparations						
Aviptadil	Analog of VIP	50–150 pmol/kg/h (IV)	Patients (n=120)	↓NMDA-induced caspase-3 activation in the lung,↓IL6, ↓ TNFα	Phase 2(NCT04311697/ Recruiting)	(Clinicaltrials.Gov, 2020ao)
Progesterone	Steroid hormone	100 mg (SC)	Patients (n=40)	↓inflammation↑repair of the respiratory epithelium.	Phase 1(NCT04365127/ Recruiting)	(Hall and Klein, 2017; Clinicaltrials.Gov, 2020bc)
Sildenafil	PDE5 blocker	0.1 g/day (orally)	Patients (n=10)	↓cGMP/competitive binding at the phosphodiesterase binding site.	Phase 3(NCT04304313/ Recruiting)	(Rogosnitzky et al., 2020; Clinicaltrials.Gov, 2020ba)
Triiodothyronine	Thyroid hormone	6 ml (IV)	Patients (n=60)	↓p38 MAPK activation↑tissue repair↑Akt activation	Phase 2(NCT04348513/Not yet recruiting)	(Pantos et al., 2020; Clinicaltrials.Gov, 2020bw)
Estradiol patch	Nuclear hormone	100 mg/day applied on the skin	Patients (n=110)	It interacts with a target cell receptor (Erα or Erβ) within the cytoplasm of the cell.	Phase 2(NCT04359329/ Recruiting)	(Clinicaltrials.Gov, 2020aj)
Cardiovascular drugs						
Losartan	ACE2 receptor inhibitor	50 mg (orally)	Patients (n=200)	↓ vasoconstrictor and aldosterone-secreting effects of angiotensin II↓ binding of angiotensin II to the AT1 receptor.	Phase 2(NCT04312009/ Recruiting)	(Tignanelli et al., 2020; Clinicaltrials.Gov, 2020au)
Valsartan	AT1R blockers (ARBs)	80 or 160 mg (orally)	Patients (n=651)	↓AT1R↑ACE2.	Phase 4(NCT04335786/ Recruiting)	(Clinicaltrials.Gov, 2020bx)
Ramipril	ACE inhibitor	2.5 mg (orally)	Patients (n=560)	Inhibition of the renin-angiotensin system.	Phase 2(NCT04366050/Not yet recruiting)	(Clinicaltrials.Gov, 2020bd)
APN01	rhACE2	Single dose/multiple dose (IV)	Patients (n=200)	It mimics ACE2 - which is used by the virus to enter cells - acting as a decoy that binds to the virus and renders it inactive.	Phase 2 (NCT04335136/ Recruiting)	(Taylor, 2020; Clinicaltrials.Gov, 2020bg)

(Continued)

TABLE 1 | Continued

Treatment candidates	Characteristics	Dose/route of administration	Study systems	Mechanism of action	Stage of development (Registry/Status)	References
Spironolactone	Antagonist of aldosterone	2 × 100 mg (orally)	Patients (n=60)	It acts through binding at the aldosterone-dependent sodium-potassium exchange site in the distal convoluted renal tubule.	Phase 4(NCT04345887/Not yet recruiting)	(Clinicaltrials.Gov, 2020bi)
Blood and blood forming organs						
Nafamostat Mesilate	Synthetic serine protease inhibitor and TMPRSS2-inhibitor.	IV administration	Patients (n=256)	↓thrombin,↓ factorXa, ↓ factor XIIa),↓kallikrein–kinin system,↓complement system, ↓pancreatic proteases.	Phase 2 and 3(NCT04352400/ Not yet recruiting)	(Bittmann et al., 2020; Clinicaltrials.Gov, 2020ai)
Camostat Mesilate	TMPRSS2-inhibitor.	200 mg (orally)	Patients (n=114)	↓ viral replication	Phase 2(NCT04353284/Not yet recruiting)	(Clinicaltrials.Gov, 2020g)
Vitamins or vitamin supplements						
Vitamin C	Antioxidants	50 mg/kg (IV)	Patients (n=20)	↓inflammatory process↓development of respiratory failure requiring intubation.	Phase 1 and 2(NCT04357782/ Recruiting)	(Clinicaltrials.Gov, 2020c)
		12g (IV)	Patients (n=140)	↓neutrophils accumulation in lung	Phase 2(NCT04264533/ Recruiting)	(Clinicaltrials.Gov, 2020by)
		50 and 100 mg/kg (IV)	Patients (n=200)	↓ inflammatory process↓development of respiratory failure requiring intubation.	Phase 2(NCT04395768/Not yet recruiting)	(Clinicaltrials.Gov, 2020an)
Vitamin D	Immune modulator	50,000 and 400,000 IU (orally)	Patients (n=260)	↓ RAS↓lung damage	Phase 3(NCT04344041/ Recruiting)	(Clinicaltrials.Gov, 2020x)
		50,000 IU once weekly(orally)	Patients (n=1,080)	↓ CAC.	Phase 2(NCT04363840/Not yet recruiting)	(Clinicaltrials.Gov, 2020aq)
		100.000 UI (orally)	Patients (n=1,265)	↓lung damage↓RAS	Phase 2(NCT04411446/Not yet recruiting)	(Clinicaltrials.Gov, 2020h)
Vitamin D3	Immuno-modulatory	100,000 IU(orally)	Patients (n=200)	↓ lung injury	Phase 2(NCT04400890/Not yet recruiting)	(Clinicaltrials.Gov, 2020be)

AP2, Adaptor protein-2; AAK1, Adaptor-associated kinase-1; ACE, Angiotensin converting enzyme; A3AR, A3 adenosine receptor; CRAC, Calcium release-activated calcium; CCR5, C-C chemokine receptor type 5; CSF2, colony stimulating factor 2; CRS, Cytokine release syndrome; C5, Complement 5; CAC, COVID-19-associated coagulopathy; DMARD, Disease-modifying antirheumatic drug; GM-CSF, Granulocyte-macrophage colony-stimulating factor; H-IG, Hyperimmune globulin; IV, Intravenous; IL-6, Interleukin-6; IgG4, Immunoglobulin G4; IM, Intramuscular; JAK, Janus-associated kinase; mAb, Monoclonal antibody; Nabs, Neutralizing antibodies; NRT, Nucleoside reverse transcriptase; NSAID, Nonsteroidal antiinflammatory drug; PO, Per oral; PDE, Phosphodiesterase enzyme; rhACE2, Recombinant human angiotensin-converting enzyme 2; RAS, Renin-angiotensin system; SC, Subcutaneous; SK2, Sphingosine kinase-2; S1P, Sphingosine 1-phosphate; TNF-α, Tumor necrosis factor alpha; TID, Three times a day; tPA, Tissue plasminogen activator; VEGF, Vascular endothelial growth factor; VIP, Vasoactive intestinal polypeptide.

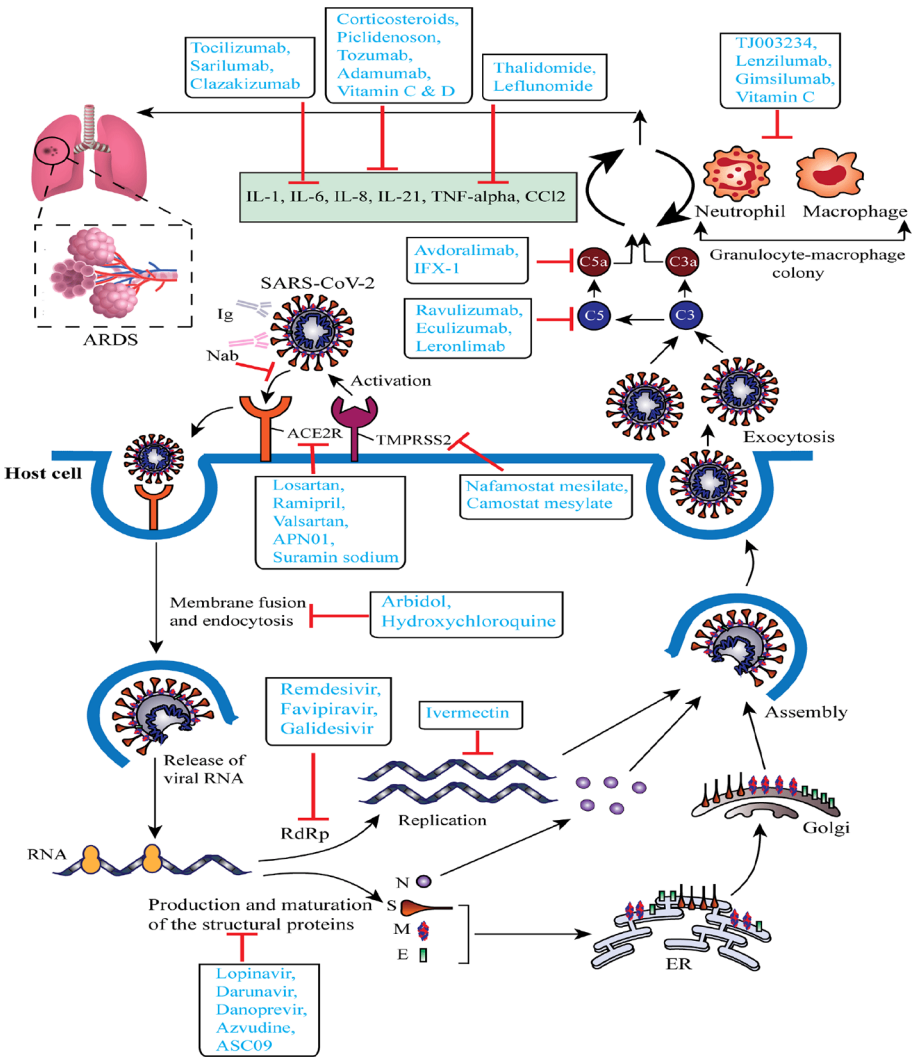

FIGURE 2 | Schematic representation of virus-based treatment responses by targeting the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) replication cycle and SARS-CoV-2 associated acute respiratory distress syndrome. The proposed targets of most important candidates are noted. ACE2R, angiotensin-converting enzyme 2 receptor; ARDS, acute respiratory distress syndrome; ER, endoplasmic reticulum; E, envelope protein; Ig, immunoglobulin; M, membrane protein; N, nucleocapsid protein; Nab, neutralizing antibody; RdRp, RNA-dependent RNA polymerase; S, spike glycoprotein; TMPRSS2, type 2 transmembrane serine protease.

neutralizing antibodies (Nabs) (Cao W. et al., 2020). Intravenous Ig is used to investigate to improve the treatment outcome of SARS-CoV-2 infection over the global pandemic with its capacity of proving passive immunity and antiinflammatory, and immunomodulatory effects. For this purpose, in phase 2/3 clinical trial (NCT04261426), 80 participants are treated with IV Ig at 0.5 g/kg/day dose for 5 days to understand the safety and efficacy of it in COVID-19 (Clinicaltrials.Gov, 2020ah).

Interferon (IFN)-β1a and -β1b

Interferon (IFN)-β1a is a cytokine signaling molecule used in the treatment of several chronic viral infections (e.g., HBV, HCV) that activates cytoplasmic enzymes, thereby, prevents mRNA translation and protein synthesis (Hensley et al., 2004; Clerico et al., 2007; Docea et al., 2016; Kamal et al., 2017). Recently, a research team of MJM Bontenprovides an adaptable research platform for the evaluation of treatment efficacy of IFN-β1a against the ongoing global pandemic in phase 4 clinical trial (NCT02735707) applying 10 μg intravenous (IV) dose once daily for 6 days in COVID-19 patients (n = 7,100) (Clinicaltrials.Gov, 2020bf). On the other hand, IFN-β1b, a cytokine used in the treatment of multiple sclerosis, is studied on 80 infected patients in phase 2 clinical trial (NCT04276688) with 0.25 mg subcutaneous (SC) dose for 3 days to evaluate the reduction of mortality rate (Hung et al., 2020). The combined therapy (lopinavir/ritonavir, ribavirin, and IFN-β1b) was found to

suppress the viral load and reduce the mortality rate in the infected patients compared with the lopinavir/ritonavir (Clinicaltrials.Gov, 2020at).

Interleukin (IL)-2

It is another cytokine signaling molecule used in the immunotherapy treatment, especially in cancer (e.g., melanoma) (Rosenberg et al., 1994) and in the prevention of viral infection (e.g., HIV) (Kovacs et al., 1996; Boda et al., 2018). IL-2 is lymphocytotrophic hormone that is recognized and characterized as a fundamental for the generation and regulation of the immune response (Smith, 1988). It is a T lymphocyte product that stimulates T cells for the progression of the cell cycle *via* a finite number of interactions with its specific membrane receptors (Smith, 1988). A controlled phase 1 intervention (ChiCTR2000030167) increases the production of CD4+ T, CD8+ T and NK cell numbers in 20 infected patients at a low dose intramuscularly (IM) (Chictr.Org.Cn, 2020b).

CYNK-001

According to Celularity, the investigational new drug (IND) has been cleared by the authority of US Food and Drug Administration (FDA) for the use of CYNK-001 as an experimental allogeneic shelf cell (e.g., NK cell) therapy derived from the human placental CD34+ cells to treat COVID-19 patients (Celularity, 2020). Recently, a world-leading company "Celularity Incorporated" has experimented with the efficacy and safety of CYNK-001 (NCT04365101) on 86 participants, suggesting it has enriched for CD56+/CD3-NK cells (Clinicaltrials.Gov, 2020av).

Baloxavir Marboxil (S-033188)

Previously, baloxavir marboxil (S-033188) is used as a first-in-class antiviral prodrug that is converted as to its active form (baloxavir acid) through hydrolysis (Koshimichi et al., 2018) and in turn acts as a selective inhibitor of the cap-dependent endonuclease (Hayden et al., 2018) and the neuraminidase (NA) inhibitors (NAI) (O'hanlon and Shaw, 2019), which is specially approved for influenza. In a recent investigation (ChiCTR 2000029544), it is reported that the baloxavir marboxil selectively inhibits cap-dependent endonuclease of SARS-CoV-2 in 10 infected patients with 80 mg (once a day) oral dose (Lou et al., 2020).

Antiviral Drugs (Broad-Spectrum, Inhibitors of RNA-Dependent RNA Polymerase)

Remdesivir (GS-5734)

Remdesivir (GS-5734), an approved HIV reverse transcriptase inhibitor, is a monophosphoramidate prodrug of an adenosine C-nucleoside with a similar chemical structure to the tenofovir alafenamide that consequently demonstrates as an active energetic C-adenosine nucleoside triphosphate analog and prevents RdRp as a broad-spectrum antiviral drug of several RNA viruses including as Coronaviridae and Flaviviridae (Agostini et al., 2018; Gordon et al., 2020; Ko et al., 2020). The first clinical use of remdesivir was for the treatment of Ebola. Based on the current pandemic, a report has recently been demonstrated an adaptive, randomized, placebo-controlled, double blind phase 3 (NCT04257656) clinical trial to evaluate the efficacy and safety of this drug (200 mg on day 1 and 100 mg once daily for 9 days) combined with the supportive care in the hospitalized 237 COVID-19 patients (Scavone et al., 2020; Wang et al., 2020b).

But this initial studies with remdesivir showed no benefit as underpowered (Davies et al., 2020; Wang et al., 2020a), this changed with the NIH study called COVID-19 Adaptive Treatment Trial (ACTT 3) in which the safety and efficacy of a treatment regimen consisting of remdesivir plus the interferon beta-1a immunomodulator in patients with coronavirus 2019 (COVID-19) will be evaluated (National Institutes of Health, 2020). Remdesivir has recently been granted a conditional marketing authorization in the European Union countries by the European Commission (Agency, 2020). A very recent study showed that this antiviral, originally developed against Ebola hemorrhagic fever, slightly reduces the recovery time of patients hospitalized with Covid-19 (15 to 11 days, on average). In contrast, this drug has not been shown to reduce mortality. The European Medicines Agency (EMA) has recommended the authorization of remdesivir (Velkury, Gilead Company) for patients infected with the new coronavirus, at EU level, by "conditional placing on the market." The EMA has recommended the use of remdesivir in adults and adolescents over 12 years of age who have pneumonia and need oxygen supplementation in critically ill patients (Agency, 2020).

The FDA has also authorized the use of remdesivir in infection with the new SARS-CoV-2 coronavirus, through the Special Emergency Use Authorization (EUA). This approval allows doctors to administer remdesivir to patients with suspected or confirmed infection, severe form (have blood oxygen saturation SpO2 ≤ 94%, require oxygen therapy, mechanical ventilation or extracorporeal membrane-to-arterial oxygenation/ECMO), even outside of clinical trials. However, EUA is not a complete approval, as further studies are needed to confirm the effectiveness of this treatment. Urgent approval follows the publication of encouraging results from two studies involving remdesivir:

i. Adaptive COVID-19 Treatment Trial (ACTT), organized by the US National Institute of Allergic and Infectious Diseases (NIAID): Phase III, randomized, placebo-controlled; 1,063 patients included; patients treated with remdesivir showed clinical improvement after a 31% shorter period; the study group had a median recovery time of 11 days, compared to 15 days in the control group; the study group had a mortality of 8%, compared to 11.6% in the control group (Health, 2020b).
ii. The SIMPLE study, organized by Gilead (the company producing remdesivir, veklury):

Phase III, without control group - patients receive a remdesivir treatment for 5 or 10 days;

Clinical improvement was similar in the two groups; half of the patients showed an improvement in the disease in the first 10 days, in the case of 5 days of treatment, and in the first 11 days (10 days of treatment); after 14 days, 60% of patients receiving remdesivir for 5 days were discharged, and 52.3% of those receiving 10 days were discharged (Gilead Sciences, 2020).

Favipiravir

Favipiravir (previously known as T-705 and Avigan) is a selective inhibitor of nonnucleoside RNA polymerase, which was developed and approved to treat influenza in Japan whereas it is already popular as a prodrug of purine nucleotide that is converted as to an active form namely favipiravir-ribofuranosyl-5′-triphosphate (RTP) by phosphoribosylation through the cellular enzymes (Furuta et al., 2013; Furuta et al., 2017). In response to the current global pandemic, with the help of a sponsor (Giuliano Rizzardini), a study (NCT04336904) on this drug is ongoing on 100 adult COVID-19 patients with 1800 mg/BID for day 1 and 600 mg/TID for day 2 and after that for a maximum of 14 days to evaluate the safety and efficacy of it combined with adequate supportive care (Clinicaltrials.Gov, 2020l).

Arbidol or Umifenovir

It is a selective broad-spectrum antiviral drug, which is initially licensed in Russia and China as a small indole-derivative molecule for the treatment of enveloped and nonenveloped virus infections (commonly influenza) through inhibiting the membrane haemagglutinin fusion (Blaising et al., 2014). Recently, a phase 4 clinical trial (NCT04246242) of arbidol is performed by the Xiangya Hospital of Central South University for determining the treatment efficacy and safety of it against the COVID-19 by applying the adaptable oral doses (e.g., 200 or 400 mg, TID) on 500 participants (Smartpatients.Com, 2020).

Galidesivir (BCX4430)

Galidesivir is another adenosine analog that demonstrates broad-spectrum antiviral activity against several types of viruses (e.g., togaviruses, filoviruses, arenaviruses, paramyxoviruses, orthomyxovirus bunyaviruses, CoVs, picornavirus, flaviviruses) and initially developed for the treatment of hepatitis C virus (HCV) (Westover et al., 2018). The first in-patient phase 1 clinical trial, is a randomized, placebo-controlled, and double-blind study to assess the safety, efficacy, pharmacokinetics, and tolerability of IV administration of galidesivir vs. placebo in hospitalized patients (n = 66) with either Group A (Yellow Fever) or Group B (COVID-19) (Clinicaltrials.Gov, 2020bp).

Antiviral Drugs (Antiretrovirals, Protease Inhibitors)

Lopinavir/Ritonavir

Lopinavir/ritonavir, a US FDA approved co-formulated antiretroviral therapy for treating HIV protease, established selective *in vitro* antiviral activity against $3CL^{PRO}$ and PL^{PRO} proteases of the SARS-CoV-2 (Chu et al., 2004; De Wilde et al., 2014). Based on liver cytochrome P450 inhibition, the simultaneous use of ritonavir may upsurge the plasma half-life of lopinavir (Barragan and Podzamczer, 2008). More recently, it has been cited that an improved clinical outcome of patients (n = 199) with SARS-CoV-2 is appeared to be associated with a randomized open-label trial (ChiCTR2000029308) of orally administered lopinavir/ritonavir (100 and 400 mg) vs. standard care (Cao B. et al., 2020; Dorward and Gbinigie, 2020).

Azvudine

It is an experimental nucleoside analog that may inhibit the reverse enzyme transcriptase for viral transcription and show the potential against COVID-19 (Wang et al., 2014). Nucleoside analogs (e.g., azvudine, remdesivir, galidesivir) are the adenine or guanine derivatives that prevent the viral RNA synthesis and inhibit RdRp by encoding viral replication of several RNA viruses, including hCoVs (De Clercq, 2019). A phase 3 clinical trial (ChiCTR2000029853) on azvudine is ongoing at the People's Hospital of Guangshan County to determine its better effectiveness against COVID-19 (Chictr.Org.Cn, 2020e).

Danoprevir

It is an orally available hepatitis C virus (HCV)NS3/4A protease inhibitor and recently approved for treating noncirrhotic genotype 1b chronic hepatitis C in China (Moucari et al., 2010). Danoprevir (100 mg/tablet) combined with ritonavir (100 mg/tablet) is currently in phase 4 clinical trial (NCT04291729) evaluating its safety and efficacy in COVID-19 patients (n = 11) and has shown the potential prevention against SARS-CoV-2 transcription and replication (Chen H. et al., 2020).

Darunavir

It is a US FDA approved nonpeptidic protease inhibitor (PI) for the treatment of HIV-1 infections, which is generally applied as a part of antiretroviral therapy (ART) together with a low boosting dose of ritonavir (Mckeage et al., 2009). The darunavir boosted with ritonavir (low dose) is swiftly absorbed and reaches peak plasma concentrations within 2.5–4 h (Rittweger and Arastéh, 2007). Darunavir is comprehensively and nearly absolutely metabolized by the hepatic cytochrome P450 (CYP) 3A4 enzymes (Rittweger and Arastéh, 2007). Patients with SARS-CoV-2 are being recruited in a randomized phase 3 clinical trial (NCT04252274) to evaluate the safety and efficacy of this drug and cobicistat (a potent human cytochrome P-450 3A (CYP3A) enzyme inhibitors used in the treatment of HIV/acquired immune deficiency syndrome (AIDS) infections) (Clinicaltrials.Gov, 2020ab).

TMC-310911(ASC09)

Structurally comparable to the darunavir, the TMC-310911 is a potent protease inhibitor that has been initially demonstrated for treating human immunodeficiency virus (HIV)-1 infections due to its proteolytic cleavage protection (Mina et al., 2020). Nowadays, several multinational companies are trying to develop the antiviral activity of this drug as a discerning agent against SARS-CoV-2 in combination with other HIV therapies, including ritonavir and lopinavir (Mina et al., 2020). Ascletis Pharmaceuticals Co., Ltd. is one of these multinational companies that provides information about an open-label trial (NCT04261907) of ASC09/ritonavir (300 mg/100 mg tablet) and lopinavir/ritonavir (200 mg/50 mg tablet), which are

experimented on 160 participants to evaluate and compare their effectiveness in COVID-19 (Clinicaltrials.Gov, 2020ak).

Antimalarial Drugs

The antimalarial drugs (e.g., hydroxychloroquine, chloroquine, quinacrine) are considered for a long time as effective therapies against malaria, and are also believed to have selective antiinflammatory effects against chronic inflammatory diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis), have antiviral effects against different types of RNA viruses (e.g., dengue, chikungunya, HIV, SARS-CoVs, MERS-CoV), and also have immunomodulatory effects by inhibiting autophagy and lysosomal activity in host cells *via* cytokine signaling (Canadian Hydroxychloroquine Study Group, 1991; Rogoveanu et al., 2018; Vijayvargiya et al., 2020). A randomized and placebo-controlled phase 3 clinical study (NCT04308668) is being recruited for the treatment of COVID-19 patients (n = 3000) to evaluate the effectiveness of postexposure prophylaxis and preemptive therapy with hydroxychloroquine (200 mg tablet; 800 mg once, followed in 6 to 8 h by 600 mg, then 600 mg once daily for 4 days) (Clinicaltrials.Gov, 2020bb).

The World Health Organization recently announced that it is discontinuing clinical trials with hydroxychloroquine and the Lopinavir-Ritonavir combination due to failure to reduce mortality in patients infected with the novel coronavirus (WHO, 2020). Preliminary results of the SOLIDARITY study showed that hydroxychloroquine and the Lopinavir-Ritonavir combination reduced little or no mortality in hospitalized COVID-19 patients compared to the therapeutic standard (WHO, 2020). Also, according to RECOVERY, the first major clinical trial conducted by Oxford University in the UK, has been stopped because the delivered results, they showed that hydroxychloroquine has no beneficial effect on COVID-19 (Torjesen, 2020).

Antibiotics and Antiparasitics

Carrimycin

On June 24, 2019, an interesting antibiotic (carrimycin) with a trade name of 'Bite' is originally developed for the treatment of upper respiratory infections approved by the country's National Medical Products Administration in China (Trialsitenews, 2020).

A randomized (1:1), multicenter, open-controlled phase 3 clinical trial (NCT04286503) on 520 COVID-19 patients with carrimycin (experimental group) and lopinavir/ritonavir or arbidol or chloroquine phosphate (active comparator group) was launched by Beijing Youan Hospital to analysis the safety and efficacy of carrimycinin COVID-19 (Clinicaltrials.Gov, 2020k).

Suramin Sodium

Since the 1920s, suramin sodium (polysulfonated naphthylurea) has significantly been used to treat trypanosomiasis and onchocerciasis in humans and has also been seen as a potent inhibitor of reverse transcriptase enzyme of various types of retroviruses including HIV/AIDS, and various autocrine growth factors including tumor growth factor-beta (TGF-β), insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), essential fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Hemady et al., 1996). It has also been used as an effective inhibitor of (Na^+-K^+)-activated ATPase and some hydrolytic and oxidative enzymes (Fortes et al., 1973). In retort to the ongoing pandemic, the hospitalized patients (n = 20) with proven SARS-CoV-2 infections at the First Affiliated Hospital of Zhejiang University School of Medicine are recruited to treat with it to evaluate its safety and efficacy in COVID-19 (Chictr.Org.Cn, 2020d).

Ivermectin

A macrocyclic lactone originally derived from an actinomycete (*Streptomyces avermitilis)* approved as a broad-spectrum antiparasitic and anthelmintic agent, is a 22,23-dihydro derivative of avermectin B1 with almost a similar structure to its naturally occurring precursor (abamectin) that is significantly used for the treatment of river blindness (onchocerciasis) and ectoparasitic disease, and also used against different types of nematode and arthropod parasites (Campbell et al., 1983; Campbell, 1985; Meinking et al., 1995). A randomized phase 2 (NCT04374279) clinical study has been applied in 60 severe COVID-19 patients to treat with standard care or standard care plus bicalutamide (150 mg once daily for 7 days) or ivermectin (600 μg/kg once daily for 3 days) (Clinicaltrials.Gov, 2020bv).

Dihydroartemisinine/Piperaquine

It is a fixed-dose combination antimalarial that contains 40 mg of dihydroartemisinin (potent and short-acting) and a 320 mg of partner drug, namely piperaquine (less-potent and long-acting) generally recommended by the World Health Organization (WHO) to treat uncomplicated malaria caused by *Plasmodium falciparum* (Amaratunga et al., 2016). On the other hand, the antiviral activity of dihydroartemisinin/piperaquine may underlie through interaction between its peroxide bridge and haem iron. Lately, a phase 4 clinical trial (ChiCTR2000030082) sponsored by the First Affiliated Hospital of Nanchang University is aimed to assess the anti-COVID-19 activity of this combination medicine on 40 COVID-19 patients (Chictr.Org.Cn, 2020a).

Azithromycin

The broad-spectrum antibiotic azithromycin is an orally administered acid-stableazalide antibacterial drug, which is an erythromycin derivative with a similar range of antimicrobial activity and developed pharmacokinetic physiognomies comparative to erythromycin (Peters et al., 1992; Zlatian et al., 2018). It is noted that the action of this drug is expanded significantly with a wide range of Gram-positive organisms, particularly *Haemophilus Influenza*-related with respiratory tract infections (Dunn and Barradell, 1996; Călina et al., 2017; Ungureanu et al., 2017). Most lately, azithromycin (500 mg) oral tablet has experimented as a prophylactic treatment following a randomized, single-blinded, placebo-controlled phase 2 trial (NCT04369365) in cancer patients (n = 200) undergoing antineoplastic therapy during the COVID-19 pandemic (Tran et al., 2019; Mohammad et al., 2020; Clinicaltrials.Gov, 2020bh).

Doxycycline

Doxycycline is a second-generation tetracycline that rapidly absorbed into the systemic circulation, distributed throughout the organism due to its function of lipophilicity, and eliminated through feces and urine (Saivin and Houin, 1988; Calina et al., 2016; Blejan et al., 2020). Xa study reports that doxycycline acts as a potent inhibitor of dengue viral replication and diminishesserum IL-6 levels at the time of viral infection (Sargiacomo et al., 2020). Patients (n = 330) with severe COVID-19 are recruited in a randomized, prospective, multicenter, double-blind phase 3 clinical study (NCT04371952) to evaluate the efficacy of doxycycline (200 mg/day) vs. a placebo (lactose 380 mg/capsule) (Clinicaltrials.Gov, 2020y).

NonSpecific AntiInflammatory and Immunosuppressive Drugs

Corticosteroids

Glucocorticosteroid hormones corticosteroids are repeatedly used to treat acute respiratory distress syndrome (ARDS) and severe lung injury due to their capacity of diminishing inflammatory and fibrotic phenomena and defeating deposition of collagen (Claman, 1972). There also have some controversial for the therapeutic efficiency of corticosteroids despite the popularity of their administering. To study an anti-ARDS efficacy against COVID-19, 86 COVID-19 patients were treated in a randomized, prospective, and placebo-controlled fashion with methylprednisolone, 1 mg/kg/day (IV) for 7 days, or placebo (Clinicaltrials.Gov, 2020aa).

Fingolimod

A first-in-class orally administered compound fingolimod (FTY720) is a frequent immunology modulator of sphingosine-1-phosphate–a receptor that has exposed clinical efficacy and expansion on imaging in a nonrandomized phase 2 intervention (NCT04280588) against 30COVID-19 participants (Clinicaltrials.Gov, 2020al). It is initially used in multiple sclerosis thanks to its function of sequestering lymphocytes in lymph nodes (Chun and Hartung, 2010).

Leflunomide

FDA approved immunomodulatory prodrug leflunomide to treat rheumatoid arthritis as a disease-modifying antirheumatic drug that is rapidly converted to its active metabolite (A771726) after oral administration (Fox, 1998; Prakash and Jarvis, 1999). The immunosuppressant leflunomide causes inhibition of dihydro-orotate dehydrogenase and tyrosine kinases and degradation of intracellular transcription factors (Rozman, 2002). In order to find out the tolerability of this drug with a high dose (300 mg once daily), the University of Chicago recruited a single-center tolerability phase 1 clinical trial (NCT04361214) with leflunomide in the ambulatory patients (n = 20) with mild COVID-19 (Clinicaltrials.Gov, 2020ar).

Thalidomide

Firstly, the CIBA pharmaceutical company manufactured thalidomide in 1954 thanks to the prescribed drug as a sedative, antiemetic, and tranquillizer for the morning sickness (Franks et al., 2004). Then it is profoundly marketed and endorsed throughout the world due to having its multi-purposes functions such as antiangiogenesis, antifibrotic, immune regulation effects, and antiinflammatory (Shannon et al., 2008). It inhibits excess production of tumor necrosis factor-alpha (TNF-α) and suppresses the leukocyte migration. A research group of the First Affiliated Hospital of Wenzhou Medical University randomly allocated 100 patients to receive thalidomide (100 mg, orally for 14 days) or placebo at the same dose of thalidomide in a first prospective, multi-center, placebo-controlled, double-blind phase 2 intervention (NCT04273529) to evaluate the safety and efficacy of this drug in COVID-19 (Clinicaltrials.Gov, 2020ac).

Colchicine

It is an alkaloid derivative derived from a plant source, namely *Colchicum autumnale* (Liliaceae) (Terkeltaub, 2009). Colchicine is standing with a long history for its application in inflammatory diseases, including familial Mediterranean fever, and severe gout and Behçet's disease (Niel and Scherrmann, 2006). In retort to the COVID-19, a research team of the Montreal Heart Institute assigned 6,000 COVID-19 patients to be given either colchicine or placebo (1:1 allocation ratio) for 30 days in a randomized, multi-center, double-blind, placebo, parallel controlled phase 3 clinical study to examine the reduction of mortality rate and lung difficulties associated with COVID-19 (Clinicaltrials.Gov, 2020m).

Ibuprofen

It is a nonsteroidal antiinflammatory drug (NSAID) involved in the class of 2 aryl propionic acid (2-APA) that was first announced in England in 1967 (Davies, 1998). It inhibits the production of prostaglandins by decreasing the activity of the enzyme cyclooxygenase (Cole and Frautschy, 2010; Rogoveanu et al., 2018). Ibuprofen is also familiar for the advanced treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, gout, and Bartter's syndrome (Kantor, 1979; Mititelu et al., 2020; Salehi et al., 2020). After registration (NCT04334629), the King's College London initiated a phase 4 clinical trial (multicenter, randomized, controlled trial) of this drug at a daily dose of 200 mg in 230 patients to examine the reduction in the austerity and advancement of lung difficulties associated with COVID-19 (Clinicaltrials.Gov, 2020as).

Naproxen

Stereochemically naproxen is a potent nitric oxide-releasing NSAID that is usually administered orally or rectally for the treatment of severe rheumatic disease and several nonrheumatic circumstances (Todd and Clissold, 1990). A study has been reported that a nitroxybutyl ester derivative of naproxen shows the less ulcerogenic in the gastrointestinal tract (GIT) than its mother NSAID (Davies et al., 1997). A randomized phase 3 clinical trial (NCT04325633) is established at Assistance Publique - Hôpitaux de Paris in hospitalized patients (n=584) with severe COVID-19 to treat with the standard of care plus naproxen(250 mg BID) and lansoprazole (30 mg daily) in order to determine the effectiveness of this drug in COVID-19 (Knights et al., 2010; Clinicaltrials.Gov, 2020ae).

Piclidenoson (CF101)

According to the report from the website of Can-Fite BioPharma, piclidenoson, commonly known as IB-MECA (methyl 1-[N6-(3-iodobenzyl)-adenin-9-yl]-b-D-ribofuronamide) is an active antiinflammatory agent that has been experimented in different types of experimental models. It acts after binding to the G protein associated A3AR, which induces a robust antiinflammatory effect by inhibiting IL-17 and -23.Patients (n = 40) with COVID-19 are assigned in an open-label, randomized, control phase 2 clinical trial (NCT04333472) to receive either piclidenoson (2 mg Q12H orally) with standard care as an experimental arm or standard care alone as a control arm (1:1 allocation ratio) on empty stomach of patients to evaluate the safety and efficacy against COVID-19 (Clinicaltrials.Gov, 2020az).

Kinase Inhibitors

Jakotinib Hydrochloride

Jakotinib hydrochloride, an AP2-associated protein kinase 1 (AAK1) inhibitor as well as a Janus kinase (JAK) inhibitor, was recommended a conceivable candidate, making an allowance for its high rate of persistent virological response in COVID-19 patients (Zhang et al., 2020). To determine the antiviral and antiinfective activity of this drug (50 mg/BID, orally), a randomized phase 2 clinical intervention (NCT04312594) sponsored by Suzhou Zelgen Biopharmaceuticals Co., Ltd is assigned in 90 COVID-19 patients with idiopathic pulmonary fibrosis (Clinicaltrials.Gov, 2020ap).

Ruxolitinib

Ruxolitinib, formally known as INC424 or INCB18424, is a US FDA approved orally bioavailable JAK1/2 inhibitor usually used in the treatment of myelofibrosis as an effective and discerning inhibitor (Harrison et al., 2012; Stebbing et al., 2020). Ruxolitinib, a more auspicious repurposed antiviral agent to examine its safety and efficacy against randomized patients (n = 402) with COVID-19 a multicenter, double-blind, controlled, phase 3 clinical intervention (NCT04362137) has been recruited to treat with either ruxolitinib at a dose of 5 mg/BID plus standard of carein 2:1 allocation ratio or oral matching-image placebo plus standard of care for 14 days (Clinicaltrials.Gov, 2020ax).

Baricitinib

Baricitinib, orally bioavailable, is another potent and selective inhibitor of AAK1 and JAK1/2 (Richardson et al., 2020). It is a more auspicious repurposed antiviral agent with a unique mechanism of action targetingAAK1 and JAK1/2, and reducing SARS-CoV-2 endocytosis through binding to the cyclin g-associated kinase (GAK) (Cantini et al., 2020). Treatment with this drug is accompanied with a high rate of continuous virological response in patients (n = 200) with mild to moderate SARS-CoV-2 infection in the response-guided nonrandomized, prospective, open-label, 2-week, phase 2 and 3 interventions (NCT04320277) conducted in the Fabrizio Cantini, Hospital of Prato (Clinicaltrials.Gov, 2020f).

Tofacitinib

Tofacitinib, a persuasive oral inhibitor of the JAK1/2/3 (family: kinases), can alleviate alveolar inflammation thorough blocking interleukins signal such as IL-2, -4, -6, -7, -9, -15, and -21, which is generally approved as an immunomodulator and disease-modifying therapeutic agent for rheumatoid arthritis (Fleischmann et al., 2012; Sandborn et al., 2012). To examine the primary outcome of this drug in COVID-19, a single group assignment, prospective cohort, phase 2 study (NCT04332042) is being arranged by the Armando Gabrielli, Università Politecnica delle Marche to treat SARS-CoV-2 related interstitial pneumonia in patients (n = 50) with it at a dose of 10 mg/BID for 14 days (Clinicaltrials.Gov, 2020bs).

Imatinib

Imatinib, an approved agent for chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor (GIST), can potentially inhibit the fusion protein Bcr-Abl and platelet-derived growth factor receptors (e.g., PDGFRα and PDGFRβ) (Peng et al., 2005; Moen et al., 2007). To study the antiviral effect of this drug, a research team of the Versailles Hospital randomly assigned 99 patients with nonsevere COVID-19 in phase 2, randomized, open-label, parallel clinical trial (NCT04357613), in a 1:1 ratio, to receive imatinib (800 mg/day) or standard therapy (Clinicaltrials.Gov, 2020am).

Monoclonal Antibodies

Tozumab/Adamumab

Tozumab is used as an immunotherapy for the treatment of bilateral lung lesions, whereas adamumab is used in rheumatoid arthritis (Ying et al., 2020). A combination therapy (tozumab combined with adamumab) is applied in severe and critical COVID-19 patients (n = 60) having pneumonia in phase 4, randomized, single-center, prospective, controlled parallel trial (ChiCTR2000030580) to evaluate its safety and efficacy in COVID-19 (Chictr.Org.Cn, 2020c).

Ravulizumab (Ultomiris or ALXN1210)

Ravulizumab (also called Ultomiris and ALXN1210), a humanized monoclonal antibody, is firstly manufactured by the Alexion Pharmaceuticals as a new inhibitor of complement C5 for paroxysmal nocturnal haemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) treatment (Mckeage, 2019). It was first approved intravenous drug for paroxysmal nocturnal hemoglobinuria (PNH) in USA in December 2018, which is developed from eculizumab to have a considerably higher terminal half-life (Röth et al., 2018). To determine the safety and efficacy of its in COVID-19,270 patients with severe pneumonia are randomly assigned to receive weight-based doses of ravulizumab (intravenously on Days 1, 5, 10, and 15) with the best supportive care by applying phase 3 open-label, randomized, controlled study (NCT04369469) (Clinicaltrials.Gov, 2020ad).

Leronlimab

FDA approved CCR5 (G protein-coupled receptor) antagonist, leronlimab (also called PA14 and PRO-140) is a humanized IgG4 and a monoclonal antibody (mAb) to CCR5 that significantly prevents CoV entry and inhibits viral infection of CD4 T-cells by blocking the CCR5 (Clinicaltrials.Gov, 2020bo). The unique mechanism of binding to CCR5, leronlimab may improve the

activities of DDR-based treatments for different types of cancer (e.g., prostate, pancreatic, breast, colon, and melanoma), permitting the reduction in dose of standard chemotherapy (Pestell et al., 2020). Researchers in CytoDyn, Inc. designed a randomized, double blind, adaptive, placebo controlled phase 2b/3 clinical trial (NCT04347239) to assess the efficacy, safety, and tolerability of subcutaneous leronlimab (weekly doses of 700 mg) in 390patients with severe COVID-19 (Clinicaltrials.Gov, 2020bo).

TJ003234

TJ003234, also known as TJM2 and TJ-003234RAR101, is an antigranulocyte macrophage–colony-stimulating factor (anti-GM-CSF) monoclonal antibody that produces a high level of mAbs against GM-CSF (Clinicaltrials.Gov, 2020bk). It is first discovered by a dynamic and global biotech company I-Mab Biopharma Co. Ltd. Again, this company arranged a multi-center, randomized, double-blind, placebo-controlled phase 1b/2 clinical trial (NCT04341116) to assess the safety and efficacy of its in COVID-19 (Clinicaltrials.Gov, 2020bl). In this study, 144 patients are assigned and divided into three groups to receive IV TJ003234 (3 mg/kg for 1st group and 6 mg/kg for 2nd group) and placebo (3rd group).

Nivolumab

Nivolumab (Optivo®), a human IgG4 monoclonal antibody, is a programmed death 1 (PD-1)immune checkpoint inhibitor (ICI) that directly binds to the PD-1 ligand 1 (PD-L1) receptor and blocks it's interaction capacity with the PD-L1 and -L2 (Wolchok et al., 2013). It is an approved drug, reversing T-cell anergy and boosting immune responses in several cancers, including metastatic melanoma, skin and lung cancer, and virus-associated tumors (CheckMate 358) and against the viral infections, including HIV (Le Garff et al., 2017; Topalian et al., 2017). To assess the safety, efficacy, and tolerability of intravenous nivolumab, a total of 92 patients with severe SARS-CoV-2 disease are randomly assigned in a randomized, multi-center, 2 parallel arms, open-label phase 2 clinical study and allocated in a 1:1 ratio for which they receive either nivolumab at a dose of 3 mg/kg on day 1 or standard care (Clinicaltrials.Gov, 2020bu).

Meplazumab

It is a humanized mAb that acts against host-cell-expressed CD147. Meplazumab blocks the infection of SARS-CoV-2 through binding with the S protein of SARS-CoV-2 (Bian et al., 2020). In a recent study, 20 COVID-19 patients with pneumonia are assigned in a single center, single-arm, open-label phase 2 clinical trial to receive meplazumab at an IV dose of 10 mg at 1st, 2nd, and 5th day to evaluate the therapeutic safety, efficacy, and tolerability of this drug in COVID-19 (Bian et al., 2020).

Eculizumab

Eculizumab (Soliris), another approved humanized mAb for the inhibition of intravascular hemolysis of PNH, is a potent terminal complement inhibitor that directly binds to the C5 complement protein and inhibits the cleavage of C5a and C5b-9 (Legendre et al., 2013). In order to evaluate the safety and efficacy of this drug in COVID-19, the researchers of the Assistance Publique - Hôpitaux de Paris conducted a cohort multiple randomized controlled phase 2 trial (NCT04346797) where 120 patients with moderate or severe pneumonia were allocated to receive either eculizumab at a dose of 1,200 mg on days 1st, 4th, 8th then 1,200 mg or 900 mg on day 12th or best standard of care (Clinicaltrials.Gov, 2020w).

Clazakizumab

Clazakizumab is an anti-IL-6 monoclonal, which is a hereditarily engineered high affinity humanized monoclonal antibody (IgG1) that directly binds to IL-6 and averts its interaction and signaling through IL-6R (Eskandary et al., 2019). To determine the sustainable rate of virological response of this drug in COVID-19, patients with COVID-19 with signs of pulmonary involvement are randomly conducted in a phase 2, randomized, placebo-controlled intervention (NCT04348500) clazakizumab at the dose of 25 mg in 50 cc NS has been given by IV infusion x 1 dose and placebo at the dose of 50 cc NS given by IV infusion x 1 dose (Clinicaltrials.Gov, 2020i).

Avdoralimab

Avdoralimab (IPH5401), an immunoglobulin G1-kappa, is a selective anti-C5aR antibody that potentially reduces the inflammatory response in the lungs (World Health Organization, 2019). In response to current viral infection, 108 COVID-19 patients with severe pneumonia are included in a randomized, double-blind, placebo-controlled phase 2 clinical study (NCT04371367) to receive IV avdoralimab and placebo to improve the proportion of infected patients (Clinicaltrials.Gov, 2020e).

Lenzilumab

The humaneered recombinant antihuman granulocyte-macrophage colony- stimulating factor (anti-hGM-CSF) antibody lenzilumabis an IgG1 kappa monoclonal antibody that targets human GM-CSF to treat chronic myelomonocytic leukemia (Patnaik et al., 2019). To appraise the supportable rate of safety, efficacy and tolerability of virological response of it in COVID-19, a phase 3 randomized, double-blind, placebo-controlled intervention (NCT04351152) involving 238 patients with pneumonia are randomized in a 1:1 ratio of this drug plus standard care vs. standard care (Clinicaltrials.Gov, 2020ay).

LY3127804

LY3127804, a selective mAb firstly developed by an American pharmaceutical company Eli Lilly and Company, is engineered high affinity humanized monoclonal antibody (IgG4 isotype) that discerningly targets to angiopoietin-2 (Ang-2) and counteracts phospho-Tie2, tumor growth and metastasis (Chintharlapalli et al., 2016; Pestana et al., 2018). Eli Lilly and Company initiates a randomized, double-blind, placebo-controlled, phase 2 intervention (NCT04342897) of LY3127804 in April 13, 2020, to evaluate the effectiveness of LY3127804 against ongoing viral infection. In this study, 200 hospitalized

patients with COVID-19 pneumonia have been receipted IV LY3127804 or placebo (Clinicaltrials.Gov, 2020bj).

IFX-1

It is applied as a first-in-class monoclonal antibody that serves as a C5a antagonist, which is being developed for the advanced treatment in COVID-19 patients by a German biopharmaceutical firm InflaRx in collaboration with Beijing (Clinicaltrialsarena.Com, 2020a). IFX-1 is one of the currently under development drugs generally used for the treatment as a skin disorder therapy, antiviral, antiinfective, antiinflammatory, and vascular disorder therapy. For the better advancement of this drug against COVID-19, the InflaRx has been assigned 130 COVID-19 patients with severe pneumonia in a two-arm (arm A: best supportive care plus IFX-1; arm B: best supportive care alone), randomized, open-label, pragmatic, adaptive, phase 2/3 clinical trial (NCT04333420) (Clinicaltrials.Gov, 2020aw).

Gimsilumab

A fully mAb gimsilumab that is developed by a pharmaceutical company Roivant Sciences Ltd. as a selective inhibitor of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Clinicaltrialsarena.Com, 2020b). In case of COVID-19, the Roivant Sciences Ltd. has been included 270COVID-19 participants having ARDS and lung complication secondary in a phase 2, adaptive, randomized, double-blind, placebo-controlled, multi-center study (NCT04351243) to check its safety and efficacy. In this study, subjects receive either gimsilumab at a higher dose on day 1 and a low dose on day 8 or saline solution as placebo on day 1 and day 8 (Clinicaltrials.Gov, 2020bn).

Tocilizumab

A humanized mAb tocilizumab (Actemra®) is an FDA approved IL-6 inhibitor that selectively inhibits IL-6-mediated proinflammatory signaling by blocking both soluble and membrane-expressed IL-6 receptors (Schiff et al., 2011) and also interrupts the process of cytokine release syndrome (CRS) (Le et al., 2018). Additionally, it has already been approved for the treatment of several types of arthritis, including rheumatoid, polyarticular juvenile idiopathic, systematic juvenile idiopathic and polyarticular juvenile idiopathic arthritis (Oldfield et al., 2009; Le et al., 2018). In response to the ongoing pandemic COVID-19, the University Hospital Inselspital, Berneinitiates is conducting a randomized, multicenter, double-blind, placebo-controlled phase 2 clinical trial(NCT04335071) in collaboration with the Roche Pharma to check its safety and efficacy at a dose of 8 mg/kg body weight, with a maximum single dose 800 mg patients (n=100) with severe pneumonia compared to a placebo group (Clinicaltrials.Gov, 2020br).

Sarilumab

The first fully human mAb sarilumab (Kevzara®, REGN88, and SAR153191) developed by jointly Sanofi and Regeneron Pharmaceuticals is an inhibitor of IL-6Rα that is firstly approved for the treatment of rheumatoid arthritis (Sieper et al., 2015). Sarilumab has a potent ability to bind directly to the soluble and membrane-bound IL-6R with the selective affinity, in that way preventing IL-6–mediated *cis* and *trans-signal*ing, thus may inhibit overactive inflammatory immune response associated with COVID-19 by inhibiting IL-6-mediated signaling (Genovese et al., 2015). Based on its IL-6 inhibitory capacity, the Regeneron Pharmaceuticals in collaboration with Sanofi starts have been started an adaptive phase 2/3, randomized, double-blind, placebo-controlled study (NCT04315298) to check out its clinical safety and efficacy in comparison to the control arm (Arrytown and Paris, 2020). For this, 2500 COVID-19 hospitalized patients with severe and critical phase are randomized to IV placebo or sarilumab at a single dose.

Bevacizumab

It is an anti-VEGF monoclonal IgG1 antibody that inhibits viral proliferation, migration, and survival by producing high levels of IgG1 antibody (Kazazi-Hyseni et al., 2010). In a randomized, open-label, controlled phase 2 clinical trial (NCT04344782), a most extensive hospital system in Europe (Assistance Publique - Hôpitaux de Paris) randomly assigns 130 patients with COVID-19 infection to receive either bevacizumab (7.5 mg/kg in 100 ml saline) as the experimental arm or standard of care as the control arm to examine safety and efficacy of this drug in COVID-19 (Clinicaltrials.Gov, 2020bt).

Hormonal Preparations and Related Drugs

Aviptadil

Aviptadil, an injectable formulation of the vasoactive intestinal peptide (VIP), is usually used in PAH (Al-Saikhan et al., 2015). It has also been awarded by FDA Orphan Drug Designation for the ARDS treatment and admitted to the FDA CoronaVirus Technology Accelerator Program. Some nonclinical studies reported that the aviptadil selectively prevents N-methyl-D-aspartate (NMDA)-induced caspase-3 activation in lung and constrains the production of IL-6 and TNF-α (Clinicaltrials.Gov, 2020ao). In 20 year history, aviptadil shows the safety and efficacy for sarcoid, pulmonary fibrosis, bronchospasm, and erectile dysfunction in phase 2 trials and ARDS in phase 1 trial. For further assuring the safety and efficacy of this drug against ARDS, the multi-national company NeuroRx, Inc. initiates a randomized, placebo-controlled, multicenter phase 2 clinical trial (NCT04311697) in hospitalized patients (n = 120) with COVID-19 associated ARDS (Clinicaltrials.Gov, 2020ao). In this study, patients are assigned randomly to receive an IV infusion of aviptadil (50–150 pmol/kg/h over 12 h) plus maximal intensive care or standard saline infusion plus maximal intensive care.

Progesterone

The steroid hormone progesterone has traditionally been considered as the mammalian pregnancy hormone, which also reduces inflammation and promotes repair of the respiratory epithelium (Lydon et al., 1995; Hall and Klein, 2017). To evaluate safety and efficacy of progesterone against SARS-CoV-2, a phase 1 randomized, single center, controlled trial (NCT04365127) is

conducted in which 40 patients (men) with COVID-19 who are 18 years of age or older receive either subcutaneous (SC) progesterone (100 mg/BID) plus standard care or standard care alone (Clinicaltrials.Gov, 2020bc).

Sildenafil

Sildenafil is an orally administered phosphodiesterase type 5 (PDE5) inhibitor that permits corpus cavernosum smooth muscle to relax and potentiating erections during sexual stimulation (Langtry and Markham, 1999; Georgiadis et al., 2020; Iordache et al., 2020a). It is also reported that the sildenafil can inhibit the breakdown of cyclic guanosine monophosphate (cGMP) through binding at the phosphodiesterase binding site (Iordache et al., 2020b; Rogosnitzky et al., 2020). A pilot study of sildenafil is designed in phase 3 clinical trial (NCT04304313) to check its citrate form tablet's safety, efficacy, and tolerability at a dose of 0.1g/day for 14 days in 10 COVID-19 patients (Clinicaltrials.Gov, 2020ba).

Triiodothyronine (T3)

It is a thyroid hormone that usually impedes the activation of p38 mitogen-activated protein kinase (MAPK) and promotes tissue repair through controlled protein kinase B (Akt) activation (Pantos et al., 2020). To evaluate its anti-COVID-19 activity, a phase 2, parallel, prospective, randomized, double-blind, placebo-controlled trial (NCT04348513) is designed to explore the probable effect of IVT3 solution (0.8 g/kg within 1 h and then followed by 0.113 g/kg/h for 48 h) in critically ill patients admitted in the intensive care unit (ICU) due to COVID-19 (Clinicaltrials.Gov, 2020bw).

Estradiol Patch

It is a nuclear hormone that interacts with a target cell receptor (Erα or Erβ) within the cytoplasm of the cell. The *in vivo* and *in vitro* studies have been demonstrated that estrogen acts in different types of viral infections and wound repair processes (Clinicaltrials.Gov, 2020aj). Thus, the it can be used in viral infections in the lung. To reduce the severity of SARS-CoV-19 disease, COVID-19 positive and probable COVID-19 positive patients (n = 110) are randomly assigned to receive estradiol patchat the dose of 100 μg/day for 7 days on the skin (Clinicaltrials.Gov, 2020aj).

Cardiovascular Drugs

Losartan

Losartan, a selective, orally available ACE inhibitor, which was developed to treat heart failure that acts through blocking the vasoconstrictor and aldosterone-secreting effects of angiotensin II *via* inhibiting the binding of it to the angiotensin II type 1 receptor (AT1R) (Tsatsakis et al., 2019; Tignanelli et al., 2020). Around 14% dose of losartan is converted to its 10 to 40 fold more active metabolite E3174 after an oral administration of it with its 6 to 9 h estimated terminal half-life. A research group led by the University of Minnesota has recently initiated a randomized, placebo-controlled, multi-center, double-blinded, phase 2 study (NCT04312009) for COVID-19 treatment (Clinicaltrials.Gov, 2020au). In this study, investigators assigned 200COVID-19 patients in a 1:1 ratio to get this drug at an oral dose of 50 mg/day or placebo for 7 days or hospital discharge (Clinicaltrials.Gov, 2020au).

Valsartan

A highly selective angiotensin II (Ang II) type 1 (AT_1) receptor blockers that potentially increases pulmonary vascular permeability through blocking AT1R activation and down-regulating the activity ofACE2 (Markham and Goa, 1997). It is evident that the COVID-19 is a high burden of morbidity and mortality because of the development of ARDS. The renin-angiotensin-system (RAS) is also related to developing ARDS and in the meantime, ACE2 is one of the enzymes involved in the RAS cascade (Trifirò et al., 2020). According to this perspective, some scientists of Radboud University initiate a double-blind, placebo-controlled 1:1 randomized phase 4 intervention (NCT04335786) in a total of 651 COVID-19 patients to treat them with valsartan in a dosage titrated to blood pressure up to a maximum of 160 mg/BID or placebo (80 or 160 mg) for 14 days or hospital discharge (Clinicaltrials.Gov, 2020bx).

Ramipril

Oral capsule ramipril is suggested as another RAS blocker that averts diabetes in people with hypertension or cardiovascular disease (Bosch et al., 2006). Exhibiting comparable pharmacodynamic responses to captopril and enalapril, ramipril is also considered as a long-acting ACE inhibitor (Todd and Benfield, 1990). It is a prodrug that is converted to its pharmacologically active metabolite ramiprilat after absorption through hydrolysis with a long estimated terminal half-life (Todd and Benfield, 1990). In response to ongoing infectious disease, the University of California, San Diego in collaboration with Pfizer is planning to initiate a randomized, double-blind, placebo-controlled, phase 2 trial (NCT04366050) to treat 560 COVID-19 patients with ramipril (2.5 mg/day) or placebo for 14 days (Clinicaltrials.Gov, 2020bd).

APN01

It is a recombinant humanACE2 (rhACE2), which is currently used for COVID-19 patients due to its ability to block viral entry and decreasing viral replication in the host cells (Taylor, 2020). APN01 is being tried to develop by the Apeiron Biologics for advanced treatment of COVId-19. For this reason, recently, a randomized, double-blind, phase 2 trial (NCT04335136) of APN01 is assigned in 200 COVID-19 participants (Clinicaltrials.Gov, 2020bg).

Spironolactone

It is an antagonist of aldosterone often used to treat patients with low renin essential hypertension, primary aldosteronism, hypokalemia, and diuretic (Loriaux et al., 1976). It acts as a competitive aldosterone antagonist through binding at the aldosterone-dependent Na^{1+}/K^{1+} exchange site in the distal convoluted renal tubule. A phase 4 clinical study (NCT04345887) is designed by Istanbul University-Cerrahpasa to assess the effects of spironolactone on oxygenation in COVID-19 ARDS patients (Clinicaltrials.Gov, 2020bi).

Agents Acting on Blood and Blood-Forming Organs

Nafamostat Mesilate

It is a proven serine protease inhibitor that is initially approved in Japan for the treatment of disseminated intravascular coagulation, acute pancreatitis, and anticoagulation in extracorporeal circulation (Tu et al., 2020). It inhibits different types of enzymatic systems, including coagulation and fibrinolytic systems (e.g., thrombin, Xa, XIIa), complement system, and kallikrein-kinin system (Bittmann et al., 2020). It has also been recognized that the nafamostat mesilate is an inhibitor of MERS-CoV S glycoprotein mediated viral membrane fusion through the inhibition of transmembrane protease, serine 2 (TMPRSS2) activity (Yamamoto et al., 2016). Based on previous experiments, a randomized, double-blind, placebo-controlled parallel-group, phase 2/3 trial (NCT04352400) of nafamostat mesilate is designed to evaluate its efficacy against 256 COVID-19 patients (Clinicaltrials.Gov, 2020ai).

Camostat Mesilate

It is another synthetic serine protease inhibitor that is initially used to treat dystrophic epidermolysis, chronic pancreatitis, and oral squamous cell carcinoma (Tu et al., 2020). It was first manufactured by the Nichi-Iko Pharmaceutical Co., Ltd. in combination with Ono Pharmaceutical, Japan (Ohkoshi and Oka, 1984). It has experimented that the camostat mesilate showed the inhibition effect of SARS-COV-2 replication in the *in vitro* study. Based on this previous preclinical study, a phase 2 clinical study is designed with 114 COVID-19 patients to treat them either with camostat mesylate at a dose of 200mg/TID or with placebo/TID for 7 days (Clinicaltrials.Gov, 2020g).

Vitamins

Vitamin C

Vitamin C (also known as L-ascorbic acid, ascorbic acid, and ascor, sodium ascorbate), a six-carbon lactone, is popular for its antioxidant properties that plays an essential role in reducing the inflammatory process, preventing from respiratory failure, deterring common cold, inhibiting the neutrophils accumulation in the lung, and also modulating the immune system (May and Harrison, 2013; Wilson, 2013; Carr and Maggini, 2017; Salehi et al., 2019b; Sharifi-Rad M. et al., 2020). In addition, some previous studies have been highlighted that the higher dose of IV vitamin C may be beneficial for the patients with acute lung injury, ARDS, and sepsis (Salehi et al., 2019a; Clinicaltrials.Gov, 2020c). It has also been reported that the deficiency of this vitamin may increase the risk and severity of influenza infections (Clinicaltrials.Gov, 2020by). Based on the previous reports, some clinical trials on this vitamin have been registered in order to evaluate its effectiveness in COVID-19.

A research group led by the Hunter Holmes Mcguire Veteran Affairs Medical Center has registered a nonrandomized, open-label, parallel, phase 1/2 clinical trial (NCT04357782) to evaluate the safety, tolerability, and efficacy of the IV vitamin C against SARS-CoV-2 infection and decreased oxygenation (Clinicaltrials.Gov, 2020c). In response to this study, 20 hospitalized patients (age: 18-99 years) are designed to receive this vitamin at a dose of 50 mg/kg given 6 hourly for 4 days (16 total doses) (Clinicaltrials.Gov, 2020c). Another research team of Zhongnan Hospital is actively functioning on vitamin C to evaluate its therapeutic efficacy against the severe SARS-CoV-2 infected pneumonia patients (n = 140) of 18 years and older-aged humans (Clinicaltrials.Gov, 2020by). This study is assigned in a randomized, placebo-controlled, phase 2 clinical trial (NCT04264533) to treat the patients with either vitamin C (12 g/BID for 7 days) plus sterile water (50 ml) or sterile water alone (50 ml/BID for 7 days) (Clinicaltrials.Gov, 2020by). In addition, vitamin C is also introduced in another randomized, multi-center phase 2 (NCT04395768) intervention in 200 COVID-19 patients (18 years and older), which is led by the National Institute of Integrative Medicine, Australia (Clinicaltrials.Gov, 2020an). In this study, the recommended dose of vitamin C is 50 mg/kg 6 hourly on day 1 followed by 100 mg/kg 6 hourly for 7 days (Clinicaltrials.Gov, 2020an).

Vitamin D

Vitamin D may provide the boosting and priming effects against the viral replication caused by several microbial peptides including cathelicidins and defensins (Grant et al., 2020), dysregulation of the renin-angiotensin system, and cytokine storm in the host (Clinicaltrials.Gov, 2020x) through modulating the innate and adaptive immune system (Aranow, 2011). According to the various pre-clinical studies, it is found that the SARS-CoV-2 replication in the host cell leads to severe ARDS by leading to a cytokine storm (Clinicaltrials.Gov, 2020x). Meanwhile, various studies (*in vivo* and *in vitro*) on vitamin D have been established that clearly highlight the activity of vitamin D against ARDS and COVID-19-associated coagulopathy (Clinicaltrials.Gov, 2020aq; Clinicaltrials.Gov, 2020x). Based on this critical information on vitamin D, some research groups are continuously working on clinical trials of vitamin D to evaluate its response against COVID-19 patients.

Recently, the University Hospital, Angers has registered a multicenter, randomized, phase 3 clinical trial (NCT04344041) to check out the efficacy of vitamin D for COVID-19 patients (Clinicaltrials.Gov, 2020x). In this instance, 260 patients with life-threatening COVID-19 are randomly allocated to get a high dose of oral vitamin D3 (400,000 IU) or a standard dose of vitamin D3 (50,000 IU) (Clinicaltrials.Gov, 2020x). Another institution Louisiana State University Health Sciences Center in New Orleans has also decided to initiate a multi-center, prospective, randomized, phase 2 intervention (NCT04363840) on SARS-CoV-2 infected patients (n = 1,080) to appraise the efficiency of the vitamin D (50,000 IU, once weekly for 2 weeks) in combination with aspirin (81 mg, once daily for 14 days) against the growing global health crisis (Clinicaltrials.Gov, 2020aq).

At the current crisis of COVID-19 pandemic, the investigators from the Hospital de Alta Complejidad en Red El Cruce Florencio Varela, Buenos Aires, Argentina design a phase 4, randomized, placebo-controlled clinical trial (NCT04411446) of vitamin D in 1,265 hospitalized COVID-19 patients to identify the outcome of vitamin D at a dose of 100.000 UI (total five capsules) compared with placebo at the similar dosage of vitamin D (Clinicaltrials.Gov, 2020h). Besides, to determine the therapeutic efficiency of vitamin D3, a

randomized, double-blind, placebo-controlled, proof-of-concept, phase 2 intervention (NCT04400890) has been registered. In response to this study, 200 participants are randomly apportioned in 2 arms (100 for active comparator and 100 for placebo comparator) to treat them with either resveratrol plus vitamin D3 (100,000 IU on day 1) or placebo with vitamin D3 (100,000 IU on day 1) for 15 days (Clinicaltrials.Gov, 2020be).

CONVALESCENT PLASMA THERAPY

Passive immunotherapy is one of the effective therapeutic approaches in the endemic or pandemic infectious disease, which is still used in ongoing pandemic expending polyclonal antibodies, rather as a hyperimmune preparation from the convalescent patient's sera who have already recovered from the infection (Dodd, 2012). Convalescent sera or immunoglobulin obtained from the donor is very effective in SARS-CoV-2 infected patients by emerging immediate immune responses in the host system will be possible to neutralize the viral particles in the host (**Figure 3**) (Rojas et al., 2020).

Passive immunotherapy has been used as a reliable treatment option for many infectious outbreaks, including the 2003 SARS-CoV-1 epidemic, 2009-2010 H1N1 influenza virus pandemic, 2012 MERS-CoV epidemic, and 2014 Ebola virus epidemic (Chen L. et al., 2020). Based on the previous experiences of CPT in viral infections, the clinical trials of convalescent plasma (CP) in different countries have been assigned to evaluate the safety, efficacy, and immunogenicity of passive immunotherapy for the treatment of COVID-19. As of now on 9th June 2020, approximately 40 clinical studies on the CP have been registered with the clinicaltrial.gov for SARS-Cov-19 infection. Based upon inclusion and exclusion criteria, we select a total of 15 clinical trials that are registered from May 2020 to Jun 2020 (**Table 2**).

A research team led by the Hospital for Sick Children in Canada registered a multi-centered, open-label, randomized controlled phase 2 clinical trial (NCT04377568) to evaluate the efficacy and safety of COVID-19 CP (C19-CP) for the treatment of COVID-19 in hospitalized children (Clinicaltrials.Gov, 2020ag). In this study, 100 hospitalized children (age up to 18 years) are randomized (1:2 ratio) to receive either C19-CP at the dose of 10 ml/kg plus standard care or standard care. Another research team of the University Hospital, Basel, Switzerland is energetically functioning on a pathogen-inactivated CP addition to best supportive care and antiviral therapy on experimental worsening in participants (n = 15) of 18 years and older age with COVID-19 (Clinicaltrials.Gov, 2020d).

An open-label, nonrandomized, controlled, phase 1/2 clinical trial (NCT04390178) is being carried out by the Joakim Dillner to evaluate the efficacy, safety, and tolerability of plasma collected from the donors who have recovered from the SARS-Cov-19 infection. In response to this study, 10 participants with varying degrees of COVID-19 illness are assigned nonrandomly to receive 180–200 ml of CP (Clinicaltrials.Gov, 2020o). Another nonrandomized, phase 1/2 clinical trial (NCT04384497) is designed by Joakim Dillner with 50 participants (age: 18 years and older) to treat them with CP (200 ml, up to a maximum of 7 CP infusions) for further investigation (Clinicaltrials.Gov, 2020t).

The Thomas Jefferson University registered an open-label, phase 2 clinical intervention (NCT04389710) with 100 SARS-

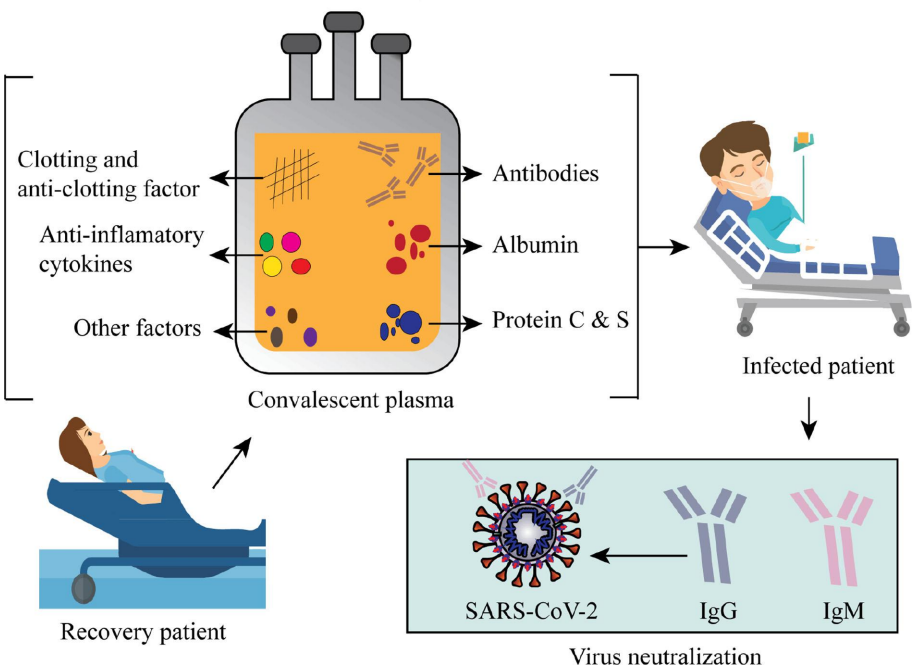

FIGURE 3 | Schematic represents convalescent plasma components and the viral neutralizing activity of convalescent plasma.

TABLE 2 | Convalescent plasma therapy in clinical studies.

Registry number	Sponsor	No. of patients (Age)	Dose/conc.	Phase (Status)	References
NCT04377568	The Hospital for Sick Children	100(up to 18 years)	10 ml/kg	2(Not yet recruiting)	(Clinicaltrials.Gov, 2020ag)
NCT04389944	University Hospital, Basel, Switzerland	15(18 years and older)	200 ml	Not applicable (Recruiting)	(Clinicaltrials.Gov, 2020d)
NCT04390178	Joakim Dillner	10(18–80 years)	180–200 ml	1 and 2(Active, not recruiting)	(Clinicaltrials.Gov, 2020o)
NCT04384497	Joakim Dillner	50(18 years and older)	200 ml	1 and 2(Recruiting)	(Clinicaltrials.Gov, 2020t)
NCT04389710	Thomas Jefferson University	100(18 years and older)	200–600 ml	2(Recruiting)	(Clinicaltrials.Gov, 2020q)
NCT04407208	Biofarma	10(18 years and older)	Three times of each 100 ml	1(Recruiting)	(Clinicaltrials.Gov, 2020u)
NCT04372979	Direction Centrale du Service de Santé des Armées	80(18–80 years)	Two units of units of each 200–230 ml	3(Not yet recruiting)	(Clinicaltrials.Gov, 2020af)
NCT04380935	Indonesia University	60(18 years and older)	Not given	2 and 3(Not yet recruiting)	(Clinicaltrials.Gov, 2020z)
NCT04403477	Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh	20(16 years and older)	200 and 400 ml	2(Recruiting)	(Clinicaltrials.Gov, 2020v)
NCT04408209	National and Kapodistrian University of Athens	60(18 years and older)	Not given	Not applicable (Recruiting)	(Clinicaltrials.Gov, 2020r)
NCT04383535	Hospital Italiano de Buenos Aires	333(18 years and older)	5–10 ml/kg/h	Not applicable(Not yet recruiting)	(Clinicaltrials.Gov, 2020n)
NCT04391101	Hospital San Vicente Fundación	231(18 years and older)	400 and 500 ml	3(Not yet recruiting)	(Clinicaltrials.Gov, 2020s)
NCT04395170	Lifefactors Zona Franca, SAS	75(18 years and older)	200–250 ml	2 and 3(Not yet recruiting)	(Clinicaltrials.Gov, 2020p)
NCT04374149	Prisma Health-Upstate	20(12–80 years)	Not given	2(Not yet recruiting)	(Clinicaltrials.Gov, 2020bq)
NCT04383548	Assiut University	100(21–50 years)	Not given	Not applicable(Not yet recruiting)	(Clinicaltrials.Gov, 2020j)

CoV-2 infected participants who have severe or life-threatening COVID-19. The participants typically receive 1–2 units (200–600 ml) of ABO compatible donor's CP administrating at a rate of 100–250 ml/h, which has the anti-SARS-CoV-2 antibody (Clinicaltrials.Gov, 2020q).

Most recently, a pilot study led by the Biofarma has enrolled 10 participants(age: 18 years and older) with severe COVID-19 at Gatot Soebroto Central Army Presidential Hospital Jakarta Pusat, Indonesia has undergone with the CP administrating at the 3 times of each 100 ml on day 0, 3, and 6, which has the minimum titer (1:80) of anti-SARS-CoV-2 antibody (Clinicaltrials.Gov, 2020u). On the other hand, a phase 3 clinical study involved in PlasCoSSA (randomized, controlled, triple-blinded, parallel study) is registered to evaluate the efficacy of the transfusion of SARS-CoV-2 CP as an early treatment of the COVID-19 (Clinicaltrials.Gov, 2020af). In this instance, 80 participants of 18-80 years aged are randomly conducted to receive an amotosalen inactivated IV injection of 2 units SARS-CoV-2 CPof each 200–230 ml (Clinicaltrials.Gov, 2020af).

Recently, the Indonesia University has registered for initiating a phase 2/3, randomized, open-label, controlled clinical study (NCT04380935) in the Referral Hospitals in Indonesia to evaluate the effectiveness and safety of CPT in COVID-19 patients with ARDS (Clinicaltrials.Gov, 2020z). In response to this study, the research group of Indonesia University is planning to assign 60 patients randomly to get either CP plus standard care or standard care (Clinicaltrials.Gov, 2020z).

The Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh has recently registered a phase 2, randomized, three-arm clinical trial with 20 participants testing positive for SARS-CoV-2 (Clinicaltrials.Gov, 2020v). Of interest, the apheretic CP is collected from donors who have recovered from COVID-19, which has the antibody titre >1:320. In this study, the intervention model is designed as the three arms (arm-A, B, and C) in which the participants are conducted to receive standard supportive treatment alone as the arm-A, standard supportive treatment plus 200 ml apheretic CP as the arm-B, and standard supportive treatment plus 400 ml apheretic CP as the arm-C to evaluate effectiveness, safety, and efficacy of the dose-depended CPT (Clinicaltrials.Gov, 2020v).

In addition, to determine the therapeutic efficacy of the CP, the titer of neutralizing anti-SARS-CoV-2 antibodies (IgG) obtained from the CP of the fully recovered patients from COVID-19 is administered on days 1–7, 14, 21, 28, and 35 from the start of treatment in 60 patients (age: 18 years and older) with severe SARS-Cov-19 infection (Clinicaltrials.Gov, 2020r). Another multi-center randomized, double-blind, placebo-controlled clinical trial (NCT04383535)has been also registered by the Hospital Italiano de Buenos Aires to evaluate the effect of CP vs. placebo (Clinicaltrials.Gov, 2020n). For this study, 333 patients with severe COVID-19 are conducted in a 2:1 ratio, to administer CP (222 patients) or placebo (111 patients). On the other hand, a phase 3 clinical study (NCT04391101) with 231 participants in a 2:1 ratio (CP:standard management), registered from Hospital San Vicente Fundación is running to evaluate the safety, efficacy, and tolerability of CP (400–500 ml) (Clinicaltrials.Gov, 2020s).

At the current crisis of the nCoV-19 pandemic, the investigators from Lifefactors Zona Franca, SAS designs a randomized, multicenter, phase 2/3 clinical trial (NCT04395170) of CP in 75

hospitalized patients with COVID-19 to assess the efficacy of CP at a dose of 200–250 ml on days 1 and 3 of the intervention compared to the intravenous anti-COVID-19 human immunoglobulin at a dose of immunoglobulin 10% IgG solution on days 1 and 3 of treatment (Clinicaltrials.Gov, 2020p).

Therapeutic plasma exchange (TPE) is an important intervention that helps instantly and scientifically to remove pathogenic antibodies and toxic candidates by using centrifugal separation of plasma or plasma membrane filtration. Sometimes, TPE in combination with tocilizumab and steroids has been used efficaciously for the treatment of severe 2, 3, 4 CRS following CAR-T treatment. To evaluate the efficacy of TPE, the Prisma Health-Upstate has registered a pilot study, where 20 patients are enrolled in a nonrandomized, open-label phase 2 clinical trial receive either TPE alone and or in combination with ruxolitinib (Clinicaltrials.Gov, 2020bq). In another study, the hyper immunoglobulins containing anti-SARS-CoV-2 immunoglobulin is being investigated in order to assess its efficacy as a passive immunization as well as treatment of early disease before the development of lower respiratory tract disease (e.g., pneumonia) (Clinicaltrials.Gov, 2020j).

DISCUSSION: CHALLENGES AND CLINICAL PERSPECTIVES ON COVID-19 PHARMACOTHERAPY

It is the greatest challengeable for the rapid identification of effective therapy developmental technologies and interventions for the COVID-19 associated paramount global public health crisis.

Compared with other viral infection, SARS-CoV-2 causes high proinflammatory disease state associated with COVID-19 through inducing lower levels of IFN–I and –III expression with a moderate reaction of IFN-stimulated genes (ISGs) and raising chemokine expression (**Figure 4**) (Blanco-Melo et al., 2020).

Mild forms of COVID-19 can be treated at home if the infection is not very symptomatic and the person can be

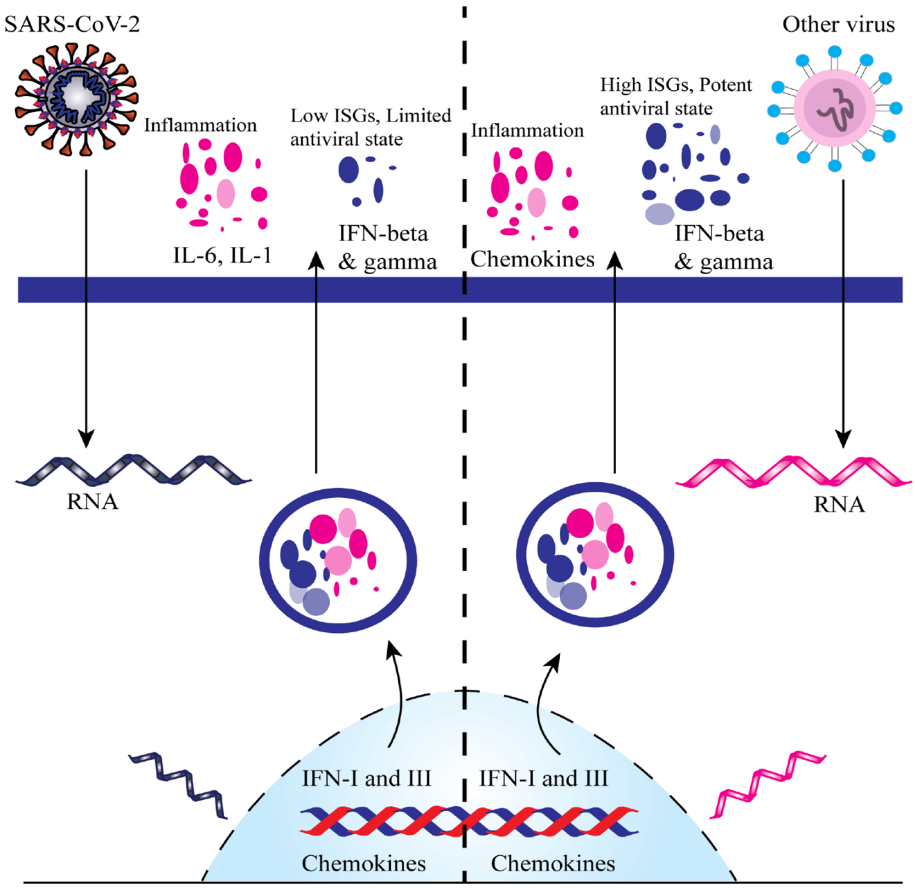

FIGURE 4 | A comparative scheme regarding the imbalanced host response of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection versus other common respiratory virus infections. ISGs, IFN-stimulated genes.

properly isolated (Godman, 2020). Patient care in these cases focuses on preventing transmission to others and monitoring the clinical condition to detect damage that could lead to hospitalization (Gao et al., 2020). Patient care in these cases is purely symptomatic (antipyretic), and preventive - the use of a mask in contact with other people, surface disinfection, hand hygiene, isolation of other people (Gao et al., 2020).

In other more severe clinical forms of COVID-19, patient care consists of the following aspects (Wiersinga et al., 2020):

i. Initially, symptomatic treatment is used - antipyretics to control fever (Jamerson and Haryadi, 2020)
ii. In case of hypoxia - oxygen therapy to maintain saturation> 94% - in patients who have signs of aggravation (apnea or severe dyspnea, central cyanosis, shock, coma, convulsions) - airway management, oxygen therapy minimum 5 L/min up to 10–15 L/min per mask; after stabilization SpO2 (blood oxygen saturation levels)> 90% is maintained; in some cases noninvasive ventilation is recommended (Dondorp et al., 2020).
iii. Co-infection treatment - even in case of suspicion of COVID-19, empirical antibiotic therapy is administered, especially in case of sepsis (1 h after the identification of sepsis), based on the clinical diagnosis (Chang and Chan, 2020).
iv. In case of ARDS (acute respiratory distress syndrome) - mechanical ventilation; with extreme care during the intubation maneuver which has a high risk of contamination; in severe ARDS it is recommended to use the sitting position; hydroelectrolytic rebalancing
v. Septic shock - hydroelectrolytic rebalancing antibiotic in the first hour, installation of central venous/arterial catheter (Fan et al., 2020).
vi. Prevention of complications is essential - maneuvers specific to each type of complication, especially in the case of those determined by prolonged immobilization and parenteral nutrition(Phua et al., 2020);
vii. The next supportive medicines will not be administered (due to the fact that they can aggravate the patient's condition) - hypotonic crystalloids, corticosteroids (no benefits have been proven so far, but many side effects and increased mortality due to secondary infections and side effects, to be used only if there are comorbidities that would may require such therapy) (Singh et al., 2020).

The reason for using corticosteroids in COVID-19 therapy is based on their ability to reduce the host's inflammatory responses in the lungs, inflammatory responses that could cause acute lung damage and acute respiratory distress syndrome. However, this benefit may be outweighed by their side effects, including delayed viral clearance and increased risk of secondary infection. Although direct evidence for the use of corticosteroids in COVID-19 is limited, analyzes of results in other viral pneumonias are relevant (Health, 2020a).

Observational studies in patients with SARS and MERS did not report any association between corticosteroid use and increased survival (Arabi et al., 2018), but showed an association of their use with delayed viral clearance in the respiratory tract and blood and the high frequency of complications, including hyperglycemia and psychosis (Keller et al., 2020).

In addition, a 2019 meta-analysis of 10 observational studies with 6,548 patients with influenza pneumonia found that corticosteroid therapy was associated with an increased risk of mortality (risk ratio [RR], 1.75 [95% CI, 1.3–2.4]; P <0.001) and a twice as high risk of secondary infections (RR, 1.98 [95% CI, 1.0–3.8]; P = 0.04)) (Ni et al., 2019).

Although the effectiveness of corticosteroids in acute respiratory distress syndrome and septic shock remains generally controversial, Russell and colleagues have argued that they are more effective in bacterial infections than in viral ones. A recent retrospective study of 201 COVID-19 patients in China found that for those who developed acute respiratory distress syndrome, methylprednisolone treatment was associated with a lower risk of death (23/50 [46%] with steroids vs. 21/34 [62%] without; HR, 0.38 [95% CI, 0.20–0.72]) (Russell et al., 2020).

Therefore, the potential adverse reactions and lack of proven benefits for corticosteroids in COVID-19 are arguments against their routine use in patients with COVID-19, unless there is a concomitant convincing indication, such as chronic exacerbation of obstructive disease or refractory shock.

Very recently, according to preliminary results from the RECOVERY study, dexamethasone, a common steroidal antiinflammatory drug, could reduce the death rate by one-third among patients severely affected by the new coronavirus. Dexamethasone is a glucocorticoid used since the 1960s in the treatment of many inflammatory conditions, but also in oncology (Bucolo et al., 2018).

Launched in March 2020, RECOVERY (Randomized Evaluation of COVid-19 Therapy) is one of the largest studies exploring potential treatments for COVID-19, and has included approximately 11,500 patients from over 175 hospitals NHS (National Health Service) in the United Kingdom. The results were surprising: one of the arms of the study, in which 2,104 patients were treated with six milligrams of dexamethasone per day (orally or intravenously) for ten days, was compared with 4,321 patients who received treatment considered be the current standard for SARS-CoV-2 infection (RECOVERY Collaborative Group, 2020).

In patients receiving standard treatment, mortality at 28 days was 41% in patients who required invasive ventilation, 25% in those who required only oxygen, and was lower (13%) in those who did not have need any intervention. It has been found that the use of dexamethasone reduces mortality by one third in ventilated patients - ratio 0.65; 95% confidence interval (0.48–0.88); p = 0.0003 - and one-fifth in patients with additional oxygen requirements - 0.80 (0.67–0.96); p = 0.0021, no significant benefit was observed in patients who did not require respiratory support - 1.22 (0.86 to 1.75); p = 0.14. These results demonstrate that if patients with COVID-19 requiring additional oxygen or invasive ventilation are given dexamethasone, it could save lives at extremely low cost. However, the WHO warns that the use of dexamethasone

should only be used in severe cases of SARS-CoV-2 infection, these being the situations in which benefits were noticed significant (Villar et al., 2020).

Thus, dexamethasone should become the new standard in the treatment of COVID-19, especially in severe cases and the WHO will soon update the therapeutic protocol guidelines for COVID-19 (WHO, 2020).

In the case of drug development, the shorter time period insisted health providers to focus on identifying existing drugs or drug candidates intended for other indications that may have efficacy against COVID-19 and put them into accelerated clinical trials. Further dose assessments can be combined into an extended phase 3 trial using a combination of clinical, viral load decline and immune response as endpoints. This kind of accelerated procedure will place an extensive load on controlling agencies that only the pandemic itself can rationalize. To solve this, WHO launched the harmonized "Solidarity Trial" in different countries to rapidly assess in thousands of COVID-19 patients to evaluate the effectiveness of current antiviral and antiinflammatory agents not yet evaluated specifically for COVID-19 (Cheng et al., 2020). Similarly, The US National Institute of Allergy and Infectious Diseases (NIAID) commenced an adaptive design for international phase 3 trial called "ACTT" to include up to 800 hospitalized COVID-19 persons at 100 places in numerous countries (Clinicaltrials.Gov, 2020b).

However, the more international arrangement would necessitate maintaining the high degree of regulatory coordination and normalization of clinical operations across many diverse settings. Another aspect of regarding drug development in COVID-19 is repurposed drugs, an accepted drug for the treatment of different ailments or medical conditions than that for which it was initially developed. Conversely, COVID-19 is a novel disease, the repurposed drugs will not totally effective, and extensive research will be needed to optimize them (Shi et al., 2020).

Like the other two therapies, CPT was a promising treatment for serious COVID-19, though it has hidden risks such as aggravating hyperimmune attacks. Moreover, this therapy is more effective in the earlier stage of disease and researches on SARS confirmed it. Therefore, the ideal timing of administering CPon COVID-19 patient needs to be cautiously measured (Zhao and He, 2020). Another challenging factor of CPT is SARS-CoV-2 neutralizing antibody titer. An investigation on SARS verified that the precise IgG began to upsurge about week 3 later of the onset, and reached at a high level at week twelve (Li et al., 2003). In addition, another study on influenza advocated that CPT with a neutralizing antibody titer level of 1:160 and more reduced mortality. Thus, CP from donors who have recovered at week 12 after onset with a neutralizing antibody titer level of not less than 1:160 is estimated to be more effective (Hung et al., 2011). Besides, the most common adverse reaction of CPT are transfusion-related problems, including fever, anaphylactic shocks, transfusion-related acute lung injury, circulatory overload and hemolysis (Maclennan and Barbara, 2006). Considering all these challenges, healthcare providers may use CPT for hospitalized patients to reduce morbidity and mortality.

Herd immunity (HdI) is the indirect protection from infection conferred to susceptible individuals when a sufficiently large proportion of immune individuals exist in a population. The time to reach HdI of a community depends on the reproduction number (R_0). It means the average number of people that a single infected person with the virus can infect those aren't already immune. The higher the R_0, the more people need to be resistant to reach HdI (Randolph and Barreiro, 2020). According to the scientific reports, the R_0 for COVID-19 is within 2 to 6 (Sanche et al., 2020). This means that one infected person can infect two to six other persons. It also means 17 to 50% of the population would need to be resistant before HdI kicks in and the infection rates start to go down. However, a single pathogen may have multiple R_0 values depending on the characteristics and transmission dynamics of the population. Therefore, the HdI threshold value ($1 - 1/R_0$) may vary between populations (Anderson and May, 1985).

The communicability of an infectious disease depends on many factors, such as population density and age structure, cultural behaviors, underlying comorbidity rates, differences in contact rates across demographic groups, which may affect the HdI threshold (Sharifi-Rad J. et al., 2020). The effective reproduction number (R_e or R_t) is also important to understand the population-level immunity. It is the average number of secondary cases generated by a single index case over an infectious period in a partially immune population. Thus, the goal of vaccination programs is to bring the value of R_e below 1 will be possible only when the HdI threshold exceeded. The pathogen spread cannot be maintained, therefore, a decline in the number of infected individuals will be seen within the population (Randolph and Barreiro, 2020).

The challenges of HdI in case of COVID-19 are: (i) less effectiveness, periodic outbreaks can still occur, (ii) unevenly distributed within a population, clusters of susceptible hosts that frequently contact one another may remain, (iii) the proportion of immunized individuals surpasses the HdI threshold, susceptible individuals will be found in the risk zone for local outbreaks, (iv) nonrelevant infection fatality rate (IFR) and case fatality rate (CFR). Still there is no straightforward, ethical path to reach the goal with HdI in case of COVID-19, due to the societal consequences of achieving it are devastating. A nonuniform COVID-19 case fatality rate (CFR) has been reported across age groups, with the vast majority of deaths occurring among individuals 60 years old or greater. Sex- and ethnicity-specific CFRs suggest that genetic, environmental, and social determinants may affect in susceptibility to COVID-19 and the severity of SARS-CoV-19 infections.

Sodium chloride (NaCl) also called 'table salt' as coating material on the fiber surface of the filtration unit of surgical mask effectively deactivated a number influenza virus species, suggesting a new strategy in the protective measures to avoid primary/secondary infection and transmission of many viruses, including SARS-CoV-19 (Quan et al., 2017). On the other hand, the natural adsorbents, including clay, charcoal, and clay minerals showed 99.99% adsorption of CoVs (Robson, 2020). Some minerals that may act against CoVs are selenium (Ma et al.,

2019), copper (Rupp et al., 2017), iron (Jayaweera et al., 2019), chromium (Terpiłowska and Siwicki, 2017), potassium (Punch et al., 2018), zinc (Te Velthuis et al., 2010), and so on. Moreover, medicinal plants or their derivatives are also evident to act against hCoVs (Kim et al., 2010; Aanouz et al., 2020; Islam et al., 2020).

LIMITATIONS

The main limiting aspects of this comprehensive review emerge from the studies that have been performed on these drugs that are still only experimental. Many of the studies analyzed included a relatively small number of patients and as a result data that are not statistically significant. That is why many old drugs with a well-known mechanism of action are re-proposed for the treatment of COVID-19. As no vaccine against COVID_19 has been approved yet, vaccine types have not been included in this paper. In addition, this review did not consider the cases of special patients such as the pediatric population and pregnant women, as they are excluded from clinical trials for ethical reasons.

The strength of this review is providing of the recent data with regard to the management of the COVID-19, within the environment in which information is rapidly changing and being made available; and it will be beneficial to health professionals.

CONCLUSION

The ongoing SARS-CoV-2 associated COVID-19 pandemic is continuously emerging worldwide and signifying the greatest spotlight on public health, education, travels, and economic conditions in the current world. The swiftness and dimensions of emerging therapeutic interventions hurled to explore potential treatments for COVID-19 highlight both the necessity and competence to produce superior evidence even at the time of a pandemic. Still, there is no single specific therapy that may give effective responses toward COVID-19. We believe, this paper will be able to provide sufficient information regarding the current treatment strategies and future directions for the pandemic SARS-CoV-2 infection.

Drug treatment is individualized according to the patient's symptoms. The patient receives adequate care to relieve and treat symptoms. Patients suffering from serious illnesses and complications (such as pneumonia, severe respiratory problems, diabetes, cancer, cardiovascular disease) receive optimal care to support vital functions. Throughout this period, since the beginning of the pandemic, drugs already used in other diseases have been used, the safety of which has already been tested on humans. However, there is a drug that has already been tested for the treatment of people infected with Ebola and MERS, Remdesivir and seems to be an option for a treatment that is available globally.

In spite of the pandemic condition, we cannot forsake the prerequisite for well-designed clinical trials. Therefore, the current situation highlights the urgency for adhering to clinical pharmacology and model-informed drug development to optimize COVID-19 therapies, designing adaptive solidarity trials to decrease therapeutic dilemmas in clinical trial settings, as well as implementing the right patient, right drug, right dosage, and right timing approach to maximize trial success. Finally, adaptive designs for COVID-19 will lead to the development of more vigorous infectious disease research infrastructure and funding to help mitigate future pandemics.

AUTHOR CONTRIBUTIONS

CS, MMo, MTI: conceptualization. MMa, AOD, and MTI: validation investigation. DC, AOD, JS-R, and MMa: resources. MMo, AOD, MMa, AM, DC, and JS-R: data curation. MMa, DC, and JS-R: review and editing. All authors: writing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Use of Enoxaparin to Counteract COVID-19 Infection and Reduce Thromboembolic Venous Complications: A Review of the Current Evidence

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The impact of the COVID-19 pandemic has been dramatic worldwide, with China, Italy, and now US at its epicenter. Researchers and clinicians are studying and testing different approaches in the attempt to prevent the infection and minimize its severity. Major efforts are focused on optimizing mechanical ventilation, antiviral, and supportive treatment; however, the role of heparin and low molecular weight (LMW) heparin in this setting has been largely overlooked. This review summarizes the available evidence about the role of heparan sulfate as a key entry mechanism for SARS-CoV-2; the efficacy of heparin and LMW heparin in counteracting its entry into the cell, the recent experimental findings obtained in *in vitro* studies using the LMW heparin enoxaparin Inhixa®, the role of heparin and LMW heparin in modulating the cytokine storm, and the evidence for the use of LMW heparin in the prevention and treatment of the thromboembolic complications of COVID-19. The available evidence suggests that LMW heparin appears as a promising tool in the treatment of COVID-19. Whether its systematic use is associated with a reduction in complications and ultimately mortality of these patients is being tested in several studies starting worldwide.

Keywords: enoxaparin, coronavirus, COVID-19, thromboembolism, induced thrombosis inflammation

INTRODUCTION

The spread of new coronavirus (SARS-CoV-2) has been recently declared a pandemic by the World Health Organization (WHO). Its dramatic impact is straining healthcare resources at their limit worldwide, first in China, then in western countries, with UK, Italy, and more recently US being the countries with the largest number of deaths to date. Researchers and clinicians are frantically studying and testing different approaches in the attempts to prevent the infection, minimize the severity, and prevent its complications (ICOTREG Group, 2020).

Four steps appear fundamental in the clinical outcome of COVID-19 infected patients: First, the cell infection by the virus; second, the so-called cytokine storm, i.e., the inflammatory response triggered by the infection; third, the pulmonary infiltration leading to a significant reduction in oxygen saturation; and lastly, the thromboembolic complications of the inflammatory response, contributing to rapid deterioration of the clinical status and death. Moreover, data are emerging indicating that diffuse bilateral pulmonary inflammation observed in COVID-19 is associated with a novel pulmonary-specific vasculopathy, which has been termed pulmonary intravascular coagulopathy as distinct to disseminated intravascular coagulation (Fogarty et al., 2020).

Mechanical ventilation and respiratory assistance remain the cornerstone treatment for patients with severe respiratory distress leading to death, especially among the elderly. On top of that, three main approaches can be envisioned to minimize the clinical consequences of COVID-19 infection: (a) prevention of virus entry into the cell and/or its replication, (b) modulation of the cytokine storm by anti-immune agents, and (c) prevention of the thromboembolic complications.

Clinical evidence about the efficacy of currently used pharmacological treatments remains scanty. At present, protocols developed in specialized centers have included the use of chloroquine and hydroxychloroquine with negative results, anti-virals such as lopinavir/ritonavir with negative results, and remdesivir, the latter with promising results and anti-inflammatory agents such as tocilizumab and desametasone with promising results. However, the search for innovative treatment approaches remains crucial for optimizing patient treatment. One overlooked research area is the attempt to inhibit the entry of SARS-CoV-2 into the cell, the very first step leading to the vicious circle described above.

This review will focus on: (a) the experimental evidence about the role of heparan sulfate as a key entry mechanism for SARS-CoV-2, (b) the efficacy of heparin and low molecular weight (LMW) heparin in counteracting its entry into the cell, (c) the recent experimental findings obtained in in-vitro studies using the LMW heparin enoxaparin (Inhixa®), (d) the role of heparin and LMW heparin in modulating the cytokine storm, and (e) the evidence for the use of LMW heparin in the prevention and treatment of the thromboembolic complications of COVID-19.

The available, albeit preliminary, evidence suggests that heparin in general and Inhixa® in particular appear as a promising additional tool in the treatment of COVID-19.

HEPARAN SULFATE AS AN ENTRY MECHANISM FOR SARS-COV-2

Virus tropism not only depends on its interaction with entry receptor but is also modulated by other factors, like attachment receptors, protease availability, and the activity of pathways responsible for internalization and trafficking of virus particles (Wickramasinghe et al., 2011; Promkuntod et al., 2013).

Many pathogens take advantage of the glycosaminoglycans heparan sulfate as a means to adhere and gain access to cells. Several years ago, the critical role of heparan sulfate has been clearly documented by de Haan et al. (2005). These authors have shown that murine hepatitis virus, a member of the betacoronavirus subfamily, acquires the ability to infect human cells by successive culture in infected cells thanks to the mutation, which confers the virus the ability to attach to heparan sulfate proteoglycan. Later studies confirmed that human coronavirus NL63 take advantage of heparin sulfate to attach to target cells through a structural M protein (Milewska et al., 2014; Milewska et al., 2018).

Recently, Mycroft-West C. et al. evaluated the interaction between the SARS-CoV-2 Spike S1 protein receptor binding domain (SARS-CoV-2 S1 RBD) and heparin and were able to show an interaction between the recombinant surface receptor binding domain and the polysaccharide, thus indicating the strong potential of repurposing heparin as an antiviral agent.

EFFICACY OF HEPARIN IN COUNTERACTING THE ENTRY OF SARS-COV-2

Heparan sulfate (HS) and heparin share similar structural characteristics, both of them are polysaccharides formed by repeated disaccharide covalently linked by uronic acid and acetylglucosamine with variable chain length and number of sulfate groups (average heparin disaccharide contains approximatively 2.7 sulfate groups, whereas heparan sulfate >1 sulfate group per disaccharide unit). In higher organisms, they can be found primarily on the cell surface or in the extracellular matrix, attached to a protein core. Heparin is a highly acidic polymer and its biological effects depend on both specific and nonspecific ionic interactions. The anticoagulant activity is related to the presence of a specific pentasaccharide sequence present in approximately 20–30% of commercially available heparin. The specific pentasaccharide sequence binds and potentiates the effect of antithrombin a naturally occurring anticoagulant, which can inhibit several serine proteases of the coagulation system, primarily FIIa (thrombin) and FXa. More recently, a heparin octasaccharidic sequence obtained by chemoenzymatic synthesis, in which glucuronic acid is replaced with sulfated iduronic acid, was shown to similarly bind to and activate antithrombin, thus paving the way for the development of heparin-like drugs that be obtained by a chemo-enzymatic approach (Elli et al., 2020).

However, heparin chains can have non anticoagulant effects by binding "nonspecifically" but also specifically to more than 100 proteins (Young, 2008). Significant clinical and basic science literature shows that heparin also possesses anti-inflammatory effects as it can modulate the function and activity of mediators of the immune response, acute phase and complement proteins, and growth factors. The activity of several proteins acting as mediators of inflammation, including CD11b/CD18, eosinophil cationic protein, IL-8, neutrophil elastase, major basic protein, P- and L-selectin, platelet growth factor 4, and stromal-derived factor 1a is modulated by heparin (Hao et al., 2019;

Hippensteel et al., 2020). A direct interaction of heparin with vascular endothelial cells (ECs), reducing recruitment of the innate immune system and inhibiting neutrophil activation, has also been shown. The anti-inflammatory effects of heparin and its constituent heparan sulfate glycosaminoglycan fragments are attributable to two general mechanisms: (i) inflammation dampening through interaction with proinflammatory mediators and (ii) prevention of the adhesion and infiltration of inflammatory cells to the diseased area (Hao et al., 2019; Hippensteel et al., 2020).

However, heparin utilization as anti-inflammatory agent has been hindered by the fear of bleeding, but the pleiotropic effects of heparin and its related compounds may have greater therapeutic potential than compounds directed against a single target due to the existing connection between inflammation, atherogenesis, thrombogenesis, and cell proliferation.

A potential role of heparin in counteracting the interaction of virus with host cell has been already documented. It competes with the herpes simplex virus for host cell surface glycoproteins to limit infection (Shukla and Spear, 2001) and it prevents cell death of human neural progenitor cells induced by Zika virus (Ghezzi et al., 2017).

The possibility that the infection by a SARS-CoV strain can be inhibited by heparin was demonstrated in an experiment conducted on the sputum specimen of an Italian patient infected by SARS (Vicenzi et al., 2004). The authors documented that the virus firstly binds to the abundant HS in the extracellular matrix, increasing its density on the cell surface, and promoting the recognition to its ACE2 receptor. Heparin (100 μg/mL) added 30 min before infection of Vero cells with SARS-CoV reduced the formation of plaques by 50%.

More recent data indicated that the human coronavirus NL63 similar to SARS-CoV-2 S1 RBD undergoes conformational change upon heparin binding, and this decreases the adhesion and hence the interaction with the ACE receptors. Since the interaction with heparan sulfate acts to facilitate ACE receptors binding by virus, it is also possible to block virus cell entry by modulating ACE 2 receptors, and recently Hoffmann et al. (2020) have shown that SARS-CoV-2 cell entry is blocked by camostate mesylate, a protease inhibitor acting on ACE2 and TMPRSS2.

Mycroft-West C. J. et al. showed that the addition of heparin to Vero cells at concentration spanning therapeutic use can inhibit SARS-Cov2 invasion between 44 and 80%. Heparin and low molecular weight heparin both bind to the Spike (S1) protein receptor binding domain, inducing conformational change. A hexasaccharide is required for conformational change. These findings are implied in the process of repurposing heparin a first line therapeutic agent as an antiviral agent and tailor made GAG based antiviral agent.

Yang et al. (2020) also showed by native mass spectrometry that both short (pentasaccharide) and relatively long (eicosasaccharide) heparin oligomers form 1:1 complexes with S1 protein receptor binding domain, supporting the existence of a single binding site. This association induces a conformational change with an important reduction of the ability to associate with ACE2. Heparin destabilizing effect is greater with the longer chains because of the electrostatic repulsion between the low-pI ACE2, and the heparin segments are not accommodated on the receptor binding domain surface.

Spike protein binding and infection by SARS-CoV-2 virus is potently blocked by unfractionated heparin, non-anticoagulant heparin, treatment with heparin lyases, and purified lung heparan sulfate (Clausen et al., 2020).

Thus, the available evidence indicates that heparan sulfate has a central role in the adhesion of the virus to the cell surface and that heparin leads to a conformational change of the SARS-CoV-2 surface protein and therefore limits its interaction with the ACE2 receptor, thus inhibiting SARS-CoV-2 infection (Kim et al., 2020). Heparan sulfate manipulation or the inhibition of viral adhesion by exogenous heparin can constitute new therapeutic opportunities (Kim et al., 2020).

ROLE OF ENOXAPARIN IN MODULATING THE CYTOKINE STORM

There is strong evidence indicating that a cytokine storm occurs during the evolution of SARS-CoV-2 infection. The development of cytokine storm leads ultimately to the necrosis of epithelial cells, increased permeability of vascular cells, and abnormal cellular and humoral immunity, eventually resulting in acute lung injury, acute respiratory distress syndrome (ARDS), and death (Arabi et al., 2017).

Evidence obtained in Chinese patients points to IL-6 release as a main trigger (Wan et al., ; Chen et al., 2020). In the study by Wan et al. on 123 patients, increased levels of IL-6 were observed in 76.2% of the patients with severe disease (16 of 21) compared with 30.4% of the patients with mild disease (31 of 102). Similar results were obtained in the 29 patients studied by Chen et al. In both studies, other cytokines including IL-1β, IL-8, IL-10, TNF-α, and hs-CRP were not significantly different in patients with mild vs. severe disease.

Several studies documented a role of heparin in modulating IL-6 release based on the initial observation of the heparin-binding properties of IL-6 (Mummery and Rider, 2000). For example, *in vitro* experiments demonstrated that the production of IL-6 and IL-8 induced by LPS is inhibited by heparin in human EC (Li et al., 2015) and by the non-anticoagulant fraction of enoxaparin in trypsin-treated pulmonary epithelial cells (Shastri et al., 2015).

Studies *in vivo* models indicated that the production of IL-6 and TNFα from alveolar macrophages induced by LPS can be attenuated by nebulized heparin (Chimenti et al., 2017).

Clinical data on the effect of enoxaparin on IL-6 level have been already documented several years ago (Zenáhlíková et al., 2010). However, very recent evidence suggests that LMW heparin has the potential to relieve inflammation in COVID-19 patients: in a retrospective cohort study, Shi et al. demonstrated that the use of LMW heparin was associated with a higher percentage of lymphocytes and, most importantly, a significantly lower level

of IL-6, suggesting a key role of LMW heparin in modulating inflammatory response (Shi et al., 2020).

Moreover, despite the mechanism underlying COVID-19 pulmonary vasculopathy is still unclear; the expression on both type II pneumocytes and vascular EC within the lungs of the ACE2 receptor exploited by COVID-19 supports the possibility of direct pulmonary EC infection, activation, and/or damage (Varga et al., 2020). Furthermore, the cytokine storm associated with COVID-19 infection will have major impacts upon thrombin generation and fibrin deposition within the lung (Zhou et al., 2020).

ENOXAPARIN AND VENOUS THROMBOEMBOLIC (VTE) COMPLICATIONS IN COVID-19 INFECTION

WHO's attention has been drawn to the vascular complications that accompany COVID-19 infection when developing severe acute respiratory syndrome (SARS). In a specific section, the interim guidance recently released (WHO, 2020) recommends thromboprophylaxis with either unfractionated or low molecular weight heparin (LMWH), since, as discussed earlier in this review, acute infections are strong prothrombotic stimuli and these patients are at increased risk of venous thromboembolism (VTE). Abnormal coagulation has been reported in a multicentre retrospective study in Chinese patients hospitalized with severe disease (Tang et al., 2020) in whom elevated D-dimer >1 gr/L was associated with in-hospital death, even after multivariate adjustment for other variables. In another study (Deng et al., 2020), non-survivors had significant higher levels of D-dimer, and 71% met the clinical criteria for disseminated intravascular dissemination (DIC).

Severe and critically ill COVID patients with prolonged immobilization are inherently at high risk of VTE, and pulmonary embolism (PE) should also be considered in those with clinical deterioration with hypoxia and hemodynamic instability. However, the optimal thromboprophylaxis regimen in hospitalized patients with COVID-related illness is unknown (Driggin et al., 2020). Standard LMWH prophylaxis may be insufficient, especially in the ICU patients who are characterized by a dynamic day-to-day variation both of thromboembolic and bleeding risk. Monitoring of anti-Xa activity may be considered when LMWH is used in these patients (Duranteau et al., 2018), and yet, failure rates with standard pharmacological prophylaxis with LMWH or UFH may not be negligible (5–15%) (Boddi, 2017). Current studies will clarify the ideal regimen in the COVID-19 clinical setting. This is even more important in light of the very recent observation of a high incidence (31%) of thrombotic complications in ICU patients with COVID-19 infections (Klok et al., 2020). The authors reinforced the recommendation to "strictly apply pharmacological thrombosis prophylaxis in all COVID-19 patients admitted to the ICU, and to increase the prophylaxis towards high-prophylactic doses, even in the absence of randomized evidence".

TABLE 1 | Potential effects of enoxaparin in the COVID-19 infection setting.

- Prevention of infection by decreasing virus cell entry and hence viral load
- Reduction of IL-6 release associated with cytokine storm
- Prevention of activation of coagulation cascade
- Prevention of venous thromboembolism
- Prevention and treatment of thrombosis of small and middle size vessels leading to lung failure

So far only data regarding observational retrospective studies of either LMWH or UFH for COVID-19 related illness are available, with mixed results (Hasan et al., 2020).

There are least 14 ongoing randomized clinical trials registered in ClinicalTrials.Gov, and they are all open label comparing standard prophylactic LMWH or UFH doses vs. intermediate therapeutic LMWH doses in patients hospitalized for SARS-CoV-2 in either general wards or intensive care units (Marietta et al., 2020).

The results of these studies are awaited to draw firmer conclusions on the role of heparin in SARS-CoV-2 related illness.

CLINICAL IMPLICATIONS AND CONCLUSIONS

The experimental and clinical evidence summarized in this review suggests a strong rationale for testing the use of enoxaparin in patients with COVID-19 infection. **Table 1** summarizes the potential beneficial effects. Whether a systematic use of this treatment is associated with a reduction in complications and ultimately mortality of these patients will be defined when the results of several studies starting worldwide will be available. Although randomized clinical trials remain the ideal setting to evaluate safety and efficacy of novel treatments, the threat posed by COVID-19 requires that clinicians are able to collect data in real-world setting.

In that respect, the fact that LWM heparin is already recommended as a preventive measure of venous thromboembolism allows clinicians to collect clinical data in real-world and help answering this crucial question for the optimal management of COVID-19 patients.

AUTHOR CONTRIBUTIONS

Conception/design: FD and AS. Collection and/or assembly of data: LG, LL, AS, and BC. Original draft preparation: FD, LG, LL, AS, and BC. Review and editing: LG, LL, and AS. All authors contributed to the article and approved the submitted version.

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frontiers in Pharmacology

BRIEF RESEARCH REPORT
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Anti-IL-6 Versus Anti-IL-6R Blocking Antibodies to Treat Acute Ebola Infection in BALB/c Mice: Potential Implications for Treating Cytokine Release Syndrome

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Cytokine release syndrome (CRS) is known to be a factor in morbidity and mortality associated with acute viral infections including those caused by filoviruses and coronaviruses. IL-6 has been implicated as a cytokine negatively associated with survival after filovirus and coronavirus infection. However, IL-6 has also been shown to be an important mediator of innate immunity and important for the host response to an acute viral infection. Clinical studies are now being conducted by various researchers to evaluate the possible role of IL-6 blockers to improve outcomes in critically ill patients with CRS. Most of these studies involve the use of anti-IL-6R monoclonal antibodies (α-IL-6R mAbs). We present data showing that direct neutralization of IL-6 with an α-IL-6 mAb in a BALB/c Ebolavirus (EBOV) challenge model produced a statistically significant improvement in outcome compared with controls when administered within the first 24 h of challenge and repeated every 72 h. A similar effect was seen in mice treated with the same dose of α-IL-6R mAb when the treatment was delayed 48 h post-challenge. These data suggest that direct neutralization of IL-6, early during the course of infection, may provide additional clinical benefits to IL-6 receptor blockade alone during treatment of patients with virus-induced CRS.

Keywords: Ebola (EBOV), COVID-19, SARS-CoV-2, Anti-IL-6, Anti-IL-6R, cytokine release syndrome

INTRODUCTION

Under normal circumstances, interleukin-6 (IL-6) is secreted transiently by myeloid cells as part of the innate immune response to injury or infections. However, unregulated synthesis and secretion of IL-6 has contributed to a host of pathological effects such as rheumatoid arthritis. (Swaak et al., 1988) Furthermore, IL-6 induces differentiation of B cells and promotes CD4+ T cell survival during

antigen activation and inhibits TGF-beta differentiation, providing a crucial link between innate and acquired immune responses (Korn et al., 2008; Dienz and Rincon, 2009). These actions place IL-6 in a central role in mediating and amplifying cytokine release syndrome (CRS), commonly associated with Ebola virus disease (EVD) infections. (Wauquier et al., 2010). CRS is known to be a factor in morbidity and mortality associated with acute viral infections including those caused by filoviruses and coronaviruses. For example, non-survivors of the West African EBOV epidemics exhibited significantly elevated levels of the overall inflammatory response cytokines and monokines compared to survivors (Ruibal et al., 2016). It is thought that prolonged exposure to elevated inflammatory cytokine levels is toxic to T cells and results in their apoptotic and necrotic cell death (Younan et al., 2018). Both lymphopenia and elevated serum Il-6 levels are found in Ebola virus infection and are known to be inversely correlated with survival in patients post-infection (Wauquier et al., 2010)and in mouse models of Ebola infection (Herst et al., 2020). However, IL-6 has also been shown to be an important mediator of innate immunity and important for the host recovery from acute viral infection (Yang et al., 2017). Elevated IL-6 levels are also observed in SARS-CoV-2 infections, severe influenza, rhinovirus, RSV infection, as well as in similar respiratory infections (Hayden et al., 1998; Tang et al., 2016; Kerrin et al., 2017; Conti et al., 2020). Originally developed for the treatment of arthritis, α-IL-6R mAbs have been used to treat CRS as a complication of cancer therapy using adaptive T-cell therapies. (Lee et al., 2014; Tanaka et al., 2016; Ascierto et al., 2020). Warnings admonishing the use of IL-6 blockers in the context of acute infection are present in the package inserts for tocilizumab (Genentech, 2014), sarilumab (Sanofi, 2017) and siltuximab (EUSA, 2015). Early mixed results of CRS treatment with IL-6 blockers (Herper, 2020; ClinicalTrialsGenetech, 2020; ClinicalTrialsEUSA, 2020; Taylor, 2020; Saha et al., 2020), and our own observations of the role of IL-6 in morbidity and mortality associated with Ebola virus infection (Herst et al., 2020), led us to evaluate the clinical effects of treatment with not only antibody directed against the IL-6 receptor, but also with mAb directed to IL-6 itself. We report here on the observed differences between treatments with α-IL-6R mAbs and α-IL-6 mAbs in a mouse model of EBOV infection and comment on how IL-6 blockade may be relevant to the management and therapy for patients with Ebola infection as well as patients infected with SARS-CoV-2.

METHODS

Virus Strain

For *in-vivo* experiments, a well-characterized mouse-adapted Ebola virus (maEBOV) stock (Bray et al., 1998; Lane et al., 2019) (Ebola virus M. musculus/COD/1976/Mayinga-CDC-808012), derived from the 1976 Zaire ebolavirus isolate Yambuku-Mayinga (Genebank accession NC002549), was used for all studies. All work involving infectious maEBOV was performed in a biosafety level (BSL) 4 laboratory, registered with the Centers for Disease Control and the Prevention Select Agent Program for the possession and use of biological select agents.

Animal Studies

Animal studies were conducted at the University of Texas Medical Branch (UTMB), Galveston, TX in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animal research. UTMB is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and has an approved OLAW Assurance. BALB/c mice (Envigo; n = 146) were challenged with 100 plaque forming units (PFU) of maEBOV *via* intraperitoneal (i.p.) injection as described previously (Hodge et al., 2016; Comer et al., 2019). Experimental groups of 10 mice each were administered rat anti-mouse-IL-6 IgG1 monoclonal antibody (BioXCell, BE0046, Lebanon, NH, RRID AB1107709) or rat anti-mouse-IL-6R IgG2 monoclonal antibody (BioXCell, BE0047, RRID AB1107588) at a dose of 100 μg in sterile saline *via* intravenous (i.v.) administration *via* an indwelling central venous catheter, or 400 μg *via* i.p. injection at 24, 48, or 72 h post-challenge. Antibody dosing was based on amounts previously reported to neutralize IL-6 and IL-6R in mice (Barber et al., 2014; Liang et al., 2015). Antibody dosing was performed once for the i.v. group or continued at 72-h intervals for the i.p. groups resulting in a total of four doses over the 14-day study period as summarized in **Figure 1** and **Tables S2–S5** (**Supplemental Materials**). Control mice (n=36) were challenge with maEBOV in parallel, but were treated with antibody vehicle alone. Serum IL-6 measurements were performed in control rodents at necropsy as previously described (Herst et al., 2020).

In Vivo Clinical Observations and Scoring

Following maEBOV challenge, mice were examined daily and scored for alterations in clinical appearance and health as previously described(Lane et al., 2019). Briefly, mice were assigned a score of 1 = Healthy; score 2 = Lethargic and/or ruffled fur (triggers a second observation); score 3 = Ruffled fur, lethargic and hunched posture, orbital tightening (triggers a third observation); score 4 = Ruffled fur, lethargic, hunched posture, orbital tightening, reluctance to move when stimulated, paralysis or greater than 20% weight loss (requires immediate euthanasia) and no score = deceased (**Table S1**, **Supplemental Materials**).

Statistical Methods

Descriptive and comparative statistics including arithmetic means, standard errors of the mean (SEM), Survival Kaplan-Meier plots and Log-rank (Mantel-Cox) testing, D'Agostino & Pearson test for normality, Area-Under-The-Curve and *Z* Statistics were calculated using R with data from GraphPad Prism files. The clinical composite score data used to calculate the AUC measures were normally distributed. The significance of comparisons (*p* values) of AUC data was calculated using the *Z* statistic. *p* values <.05 were considered statistically significant.

FIGURE 1 | Dosing Schedule for α-IL-6 and α-IL-6R mAbs used in this study.

RESULTS

Following maEBOV challenge, mice were dosed i.v. at 24, 48, or 72 h post-challenge with a single dose of α-IL-6R mAb, a single i.p. dose of α-IL-6R mAb 24 h after maEBOV challenge, or an initial i.p. dose of α-IL-6 or xtalpha-IL-6R mAb, followed by additional i.p. doses at 72-h intervals for a total of four doses. Mice were observed for up to 14 days as summarized in **Figure 1**. The average serum IL-6 concentration at necropsy for mice (n=5) challenged with maEBOV was 1092 ± 505 pg/ml, a concentration similar to that reported in a previous publication for mice challenged with 10 PFU of maEBOV (Chan et al., 2019). In mice not challenged with maEBOV the average serum IL-6 was 31 ± 11 pg/ml. The survival and average clinical score for mice receiving a single i.v. dose of α-IL-6R mAb is shown in **Figure S1A, B** (**Supplemental Materials**). Little to no effects on survival or clinical score were observed following maEBOV challenge and a single i.v. dose of α-IL-6R mAb.

The survival patterns for i.v. mAb treated and untreated groups following maEBOV challenge were statistically different and most untreated mice succumbed to maEBOV infection by day seven (**Figure S1**, **Supplementary Materials**). Because neither survival score alone or average clinical score represented the overall possible clinical benefits of mAb treatment, a secondary composite outcome measure was calculated from the quotient of mouse survival and the average clinical score for each day, similar to that previously reported (Kaempf et al., 2019). We then summed these scores across the last 12 days of observation to create an AUC Survival/Clinical Score (see **Figure S1C**, **Supplemental Materials**). The *Z* statistic and significance level for this metric was calculated for each experimental condition. We found a minor clinical benefit ($p < 0.01$) when mice were given one 100 μg dose of α-IL-6R mAb *via* central venous catheter at 72 h after maEBOV challenge, relative to vehicle alone, using the experimental design described in **Table S2** (**Supplementary Materials**).

Since the maEBOV challenge was administered intraperitoneally and murine peritoneal macrophages represent a significant depot of cells (Cassado et al., 2015) able to produce IL-6 (Vanoni et al., 2017) following toll-like receptor activation, we next compared the activities of α-IL-6 and α-IL-6R mAbs administered intraperitoneally following maEBOV challenge (**Figures 2–5**). We observed significant differences in the AUC Survival/Clinical Score when α-IL-6R mAb was administered 48 h post-maEBOV challenge and then repeated three times at 72-h intervals. The most significant beneficial effect on the AUC Survival/Clinical Score (**Figure 5**) was seen when α-IL-6 mAb was administered beginning at 24 h post-maEBOV challenge, and then repeated three times at 72-h intervals.

DISCUSSION

While EVD is classified as a viral haemorrhagic fever, there are many similarities between EVD and COVID-19, the disease caused by infection with SARS-CoV-2 that can present as an

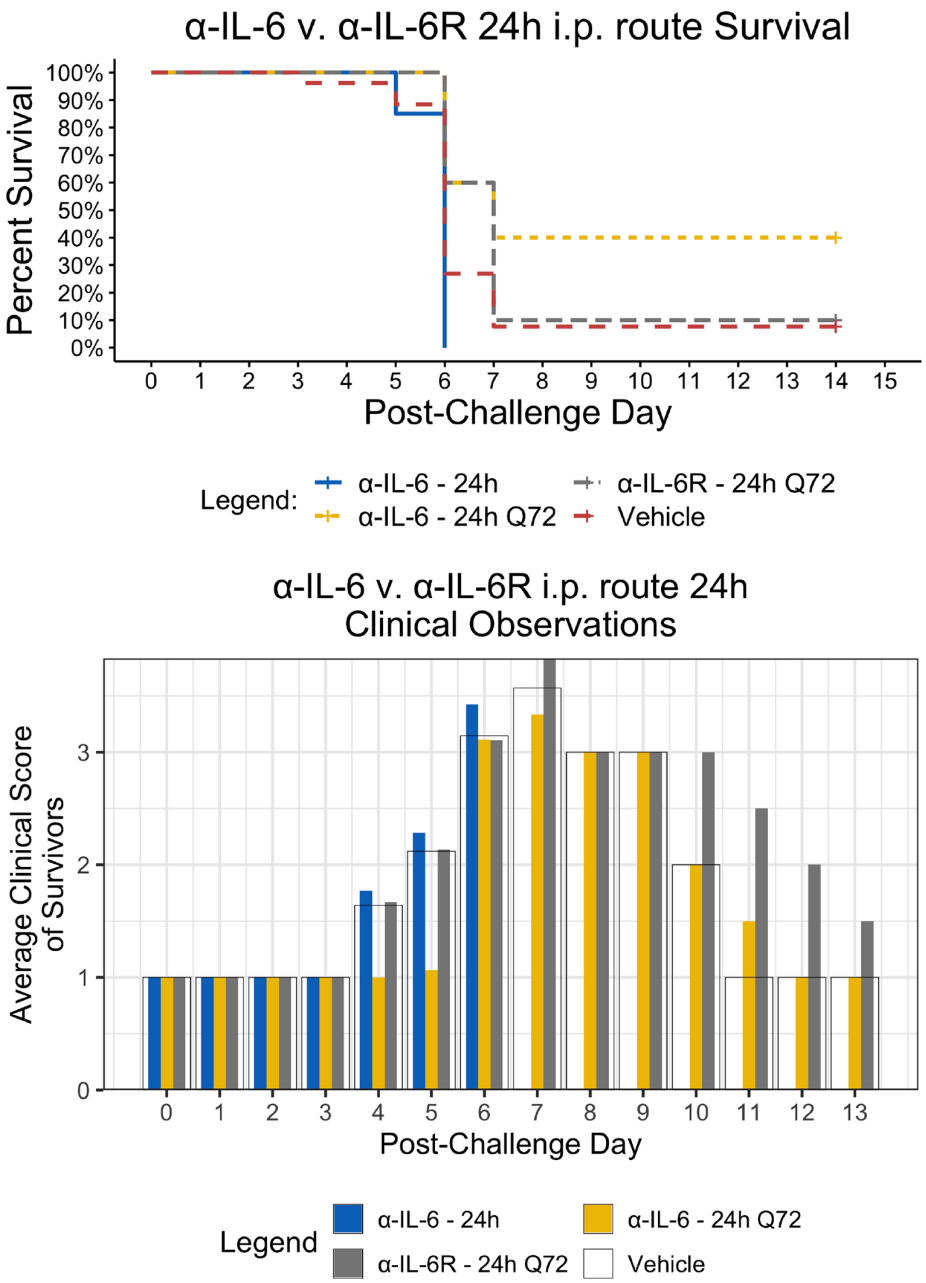

FIGURE 2 | Kaplan-Meier Survival Plots and Average clinical scores for a single or multiple i.p. doses of α-IL-6 or α-IL-6R administered 24 h after maEBOV challenge and followed by repeat dosing every 72 h for a total of four doses. The survival curves were significantly different by Log-rank (Mantel-Cox) testing ($P < 0.05$). SEM of the average clinicals scores were < 10% of the mean.

acute respiratory distress syndrome (ARDS) (Zhou et al., 2020; Chen et al., 2020; Huang et al., 2020a; Lescure et al., 2020). Like EVD, elevated IL-6 was found to be significantly correlated with death in COVID-19 patients (Ruan et al., 2020), suggesting that patients with clinically severe SARS-CoV-2 infection might also have a CRS syndrome (Huang et al., 2020b). Both EVD and COVID-19 (Younan et al., 2019; Tan et al., 2020) are associated with lymphopenia. Since the severity of SARS-CoV-1 infection has been shown to be associated with increased serum concentrations of IL-6, clinical scientists have proposed non-corticosteroid based immunosuppression by using IL-6 blockade as a means to treat hyper inflammation observed in certain

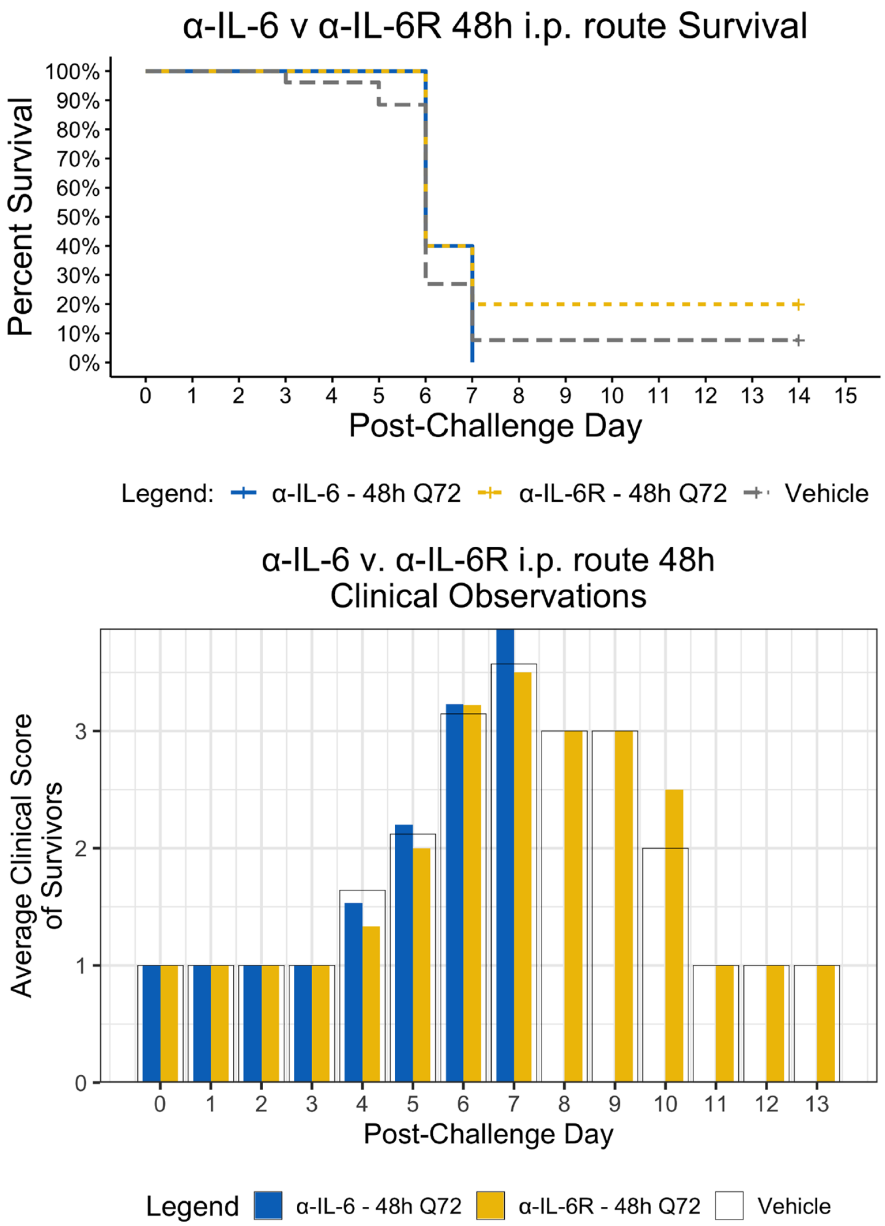

FIGURE 3 | Kaplan-Meier Survival Plots and Average clinical scores for multiple i.p. doses of α-IL-6 or α-IL-6R administered 48 h after maEBOV challenge and followed by repeat dosing every 72 h for a total of four doses. The survival curves were significantly different by Log-rank (Mantel-Cox) testing ($P < 0.05$). SEM of the average clinical scores were < 10% of the mean.

patients with SARS-CoV-2 infections (Wong et al., 2004; Mehta et al., 2020a). The potential value of using IL-6 blockade to treat COVID-19 patients was discussed early during the 2020 SARS-CoV-2 outbreak (Liu et al., 2020; Mehta et al., 2020b). Indeed, a recent (5/24/2020) search of ClinicalTrials.gov revealed at least 62 clinical trials examining the efficacy and safety of α-IL-6R mAbs and α-IL-6 mAbs for management of patients with COVID-19; 45 studies for tocilizumab (α-IL-6R mAbs), 14 for sarilumab (α-IL-6R mAbs) and 3 for siltuximab (α-IL-6 mAbs). Most of the studies involve the use of α-IL-6R mAbs and have shown promising results (summarized in **Tables 1**, **2**), but there is clear need for improvement.

Using a mouse model of Ebola infection, we found clinical benefit when mice were administered multiple i.p. doses of α-IL-6R mAb 48 h after maEBOV challenge. At both earlier (24 h) and later (72 h) time points of initiation of administration of α-IL-6R

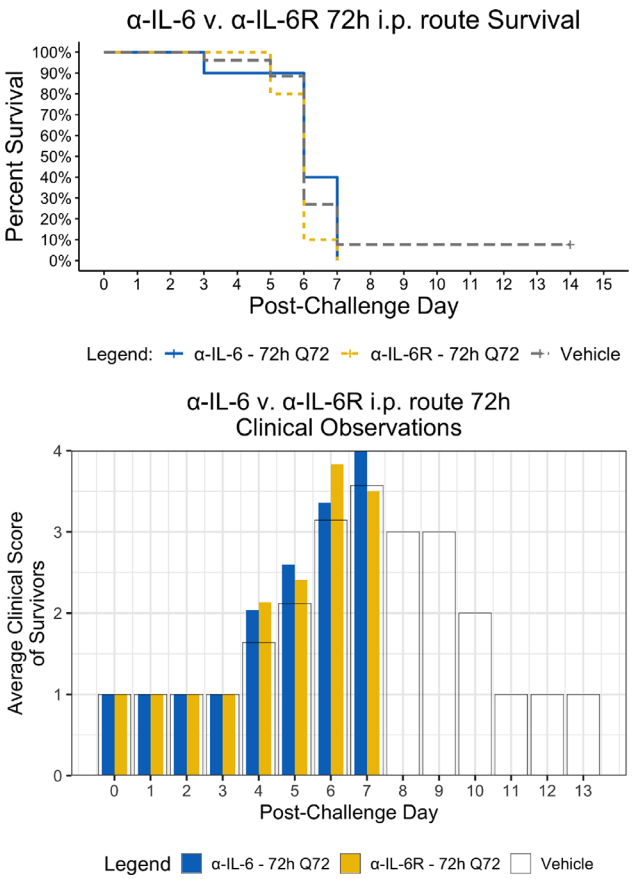

FIGURE 4 | Kaplan-Meier Survival Plots and Average clinical scores for multiple i.p. doses of α-IL-6 or α-IL-6R administered 72 h after maEBOV challenge and followed by repeat dosing every 72 h for a total of four doses. The survival curves were significantly different by Log-rank (Mantel-Cox) testing ($P < 0.05$). SEM of the average clinical scores were < 10% of the mean.

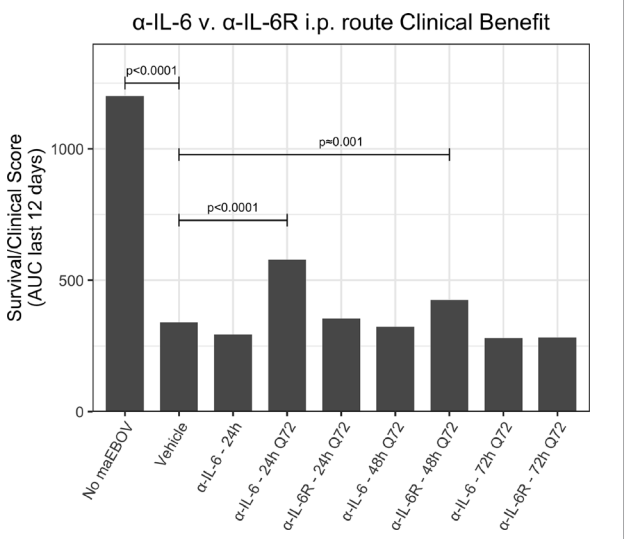

FIGURE 5 | A clinical benefit metric was calculated as an area under curve for survival/clinical scores for 120 mice receiving a single or multiple i.p. doses of α-IL-6 or α-IL-6R mAb following maEBOV challenge on day 0. The given p values are determined from the Z statistic calculated for each experimental condition.

mAb, we observed little to no effects on the clinical benefit score. Similarly, we found clinical benefit when α-IL-6 mAb was administered beginning at 24 h post-maEBOV challenge, and then repeated three times at 72-h intervals, but no benefit was observed if α-IL-6 mAb was initiated at 48 or 72 h post challenge. These data suggest that α-IL-6 mAb therapy may also have clinical benefits similar to α-IL-6R mAb particularly when given early during the course of maEBOV infection.

Previous experiments in the murine EBOV system (Herst et al., 2020) suggest that some degree of activation of innate immunity and IL 6 release benefits survival post maEBOV challenge. It may be the case that the observed clinical benefits of α-IL-6 mAbs are associated with incomplete blockade of the Il-6 response particularly later than 24 post challenge. Overall our data suggest that human clinical trials evaluating the benefits of α-IL-6 mAbs *versus* α-IL-6R mAbs titversus combined early α-IL-6 mAb and later α-IL-6R mAb is warranted to evaluate the potential of IL-6 pathway blockade in the during Ebola or SARS-CoV-2 infection.

Although antibody blood levels were not obtained during the mouse studies described here, we present a pharmacokinetic model based on literature values (Medesan et al., 1998; EUSA, 2015; Sanofi, 2017) shown in **Table S5** in **Supplemental Materials**. Simulated PK curves for each of the three experiments described is shown in **Figure 6**. Dosing α-IL-6 mAb at 24 h after challenge produced a clinical benefit, whereas dosing α-IL-6R beginning at the same time point did not. The shorter terminal half-life of α-IL-6 mAb ($T_{1/2}$ = 57h) *versus* α-IL-6R mAb ($T_{1/2}$ = 223h), possibly due to isotype specific differences in glycosylation (Cobb, 2019) may help explain why giving α-IL-6 mAb early after infection provided the most observed clinical benefit. As can be seen from the simulated PK profile in **Figure 6C**, repeated dosing every 72 h, beginning 24 h after challenge, is predicted to maintain blood levels peaking at about 200 *μg/ml*. This is in contrast to blood levels predicted after similar dosing of α-IL-6R where the blood levels continue to increase over the study period. These differences seen in the simulated PK profiles may have allowed α-IL-6 mAb to partially block IL-6, allowing innate immunity to develop, while still providing sufficient blockade to reduce the deleterious clinical effects of IL-6 as the study progressed. In addition, it may be that the stoichiometry of α-IL-6 blockade *versus* α-IL-6R may favor achieving partial blockade early during the evolution of CRS given that the amount of IL-6 present may exceed the number of IL-6 receptors. It is also possible that IL-6 may act on other sites not blocked by α-IL-6R mAb, and that this may yield a potential

TABLE 1 | Summary of recent literature on use of α IL-6R mAb for treatment of SARS-CoV-2 infection.

Patient Population	Design, Number of Patients, and Primary Outcomes	Treatment/Dose	Conclusions and Reference
RT-PCR confirmed Sars Cov-2 pneumonia, SpO2<93% in room air or mechanical ventilation	PROSPECTIVE TWO ARMS: Standard of Care (n=365) and Standard of Care plus Tocilizumab (n=179) OUTCOME: Survival	Tocilizumab (α-IL-6R) i.v. 8mg/Kg in two infusions 12h apart not exceeding 800mg total	Significantly improved survival associated with use of Tocilizumab(p<0.001) Guaraldi et al., 2020
RT-PCR confirmed Sars Cov-2 pneumonia, SpO2<93% in room air or mechanical ventilation	PROSPECTIVE SINGLE ARM: Severe Disease versus Non-Severe Disease (n=239) OUTCOME: Clinical parameters and historical survival	Tocilizumab (α-IL-6R) i.v. 8mg/Kg not exceeding 800mg total	Tocilizumab-treated patients with severe disease had survival similar to that of Tocilizumab-treated patients with nonsevere disease. Price et al., 2020
RT-PCR confirmed Sars Cov-2 pneumonia, SpO2<93% in room air, ICU admission with or without mechanical ventilation	PROSPECTIVE TWO ARMS: Standard of Care (n=420)and Standard of Care plus Tocilizumab (n=210) OUTCOME : Survival	Tocilizumab (α-IL-6R) i.v. one or two doses of 400mg	Patients receiving Tocilizumab had significantly decreased hospital-related mortality (p<0.004) Biran et al., 2020
Clinical Diagnosis of COVID-19	RETROSPECTIVE SINGLE ARM: Pre- and Post-Tocilizumab outcome (n=15) OUTCOME: Clinical parameter: CRP level	Tocilizumab (α-IL-6R) i.v. 80-600mg once or multi 80-160mg doses	Reduced C-Reative protein levels relative to pretreatment levels Luo et al., 2020
RT-PCR confirmed Sars Cov-2 pneumonia, Sp02<90% in room air	PROSPECTIVE SINGLE ARM: Pre- and Post-Tocilizumab (n=100) OUTCOME: Clinical parameters: BCRSS respiratory score	Tocilizumab (α-IL-6R) i.v. 8mg/Kg in two doses 12h apart. Discretionary third dose	Improvement of clinical symptoms and reduced BCRSS scores associated with treatment with Tocilizumab. Toniati et al., 2020
RT-PCR snd X-ray confirmed Sars Cov-2 pneumonia, Sp02<90% in room air	RETROSPECTIVE CASE-CONTROL STUDY: Standard of Care (n=25) and Standard of Care plus Tocilizumab (n=20) OUTCOME: Survival	Tocilizumab (α-IL-6R) i.v. once or twice	Significantly Improved survival associated with adminstration of Tocilizumab (p<0.002). Klopfenstein et al., 2020
RT-PCR confirmed Sars Cov-2 pneumonia, SpO2<93% in room air requiring mechanical ventilation	PROSPECTIVE TWO ARMS: Standard of Care (n=76) and Standard of Care plus Tocilizumab (n=78) OUTCOME: Survival	Tocilizumab or Sarilumab (α-IL-6R) i.v. 8mg/Kg not exceeding 800mg total	Improved survival associated with administration of Tocilizumab deduced from 45% reduction in hazard of death [hazard ratio 0.55 (95% CI 0.33, 0.90)]. Somers et al., 2020

advantage of using α-IL-6 mAb to treat CRS brought about by a viral infection.

It may be possible to develop a controlled release formulation of α-IL-6 mAb to obtain a clinically beneficial effect from the administration of α-IL-6 mAb, α-IL-6R mAb, or a combination of both, after a single injection early during the course of SARS-CoV-2 infection. For example, **Figure 6**, bottom-right panel, shows various predicted controlled release PK profiles of α-IL-6 mAb that could be achieved by using delivery systems producing different first order rates of delivery from an injection depot of 20 mg/kg. Correlation of these release profiles with the AUC Survival/Clinical score described here in pre-clinical models could lead to the development of a single dose treatment mitigating the effects of CRS on the host.

CONCLUDING REMARKS

Although the previous reports of use of IL-6 blockers to treat CRS have shown mixed results, recent clinical data for α-IL-6 and α-IL-6R mAbs have shown early promise in human trials for treatment of severe influenza and corona virus infections (Gritti et al., 2020; Xu et al., 2020). Pre-clinical studies

TABLE 2 | Summary of recent literature on use of α IL-6R mAb for treatment of SARS-CoV-2 infection.

Patient Population	Design, Number of Patients, and Primary Outcomes	Treatment/Dose	Conclusions and Reference
RT-PCR confirmed Sars Cov-2 pneumonia, SpO2<92% in room air	PROSPECTIVE SINGLE ARM: Pre- and Post-Tocilizumab (n=63) OUTCOME: Clinical parameters (CRP levels and ratio PaO2/FiO2)	Tocilizumab (α-IL-6R) i.v. 8mg/Kg not exceeding 800mg total once or twice	Improvement in clinical parameters. Sciascia et al., 2020
RT-PCR and X-Ray confirmed Sars Cov-2 pneumonia, SpO2<93%	PROSPECTIVE TWO ARMS: Standard of Care (n=28) and Standard of Care plus Tocilizumab (n=28) OUTCOME: Survival	Tocilizumab (α-IL-6R) i.v. 400mg total	No significant Improvement in clinical parameters, but faster recovery in subset with less severe disease. Della-Torre et al., 2020
RT-PCR confirmed Sars Cov-2 pneumonia, SpO2<93% in room air or mechanical ventilation	PROSPECTIVE SINGLE ARM: Pre- and Post-Tocilizumab (n=15) OUTCOME: Clinical parameters	Sarilumab (α-IL-6R) s.c. 400mg one or two doses	Rapid improvement in clinical and biochemical outcomes responders (%66), but (33%) were non-responders. Montesarchio et al., 2020
RT-PCR confirmed Sars Cov-2 pneumonia. SpO2<92%	PROSPECTIVE SINGLE ARM with two subgroups (A (n=149): requiring FiO2<45% and B (n=106): requiring FiO2>45%) OUTCOME: Survival	Tocilizumab (α-IL-6R) i.v. 400mg or Sarilumab (α-IL-6R) i.v. 400mg given once or twice	Improved survival in patients with severe disease (subgroup A) as compared to the subgroup B suggests that anti-IL-6 R intervention should occur prior to the onset of critical illness for maximum benefit. Sinha et al., 2020

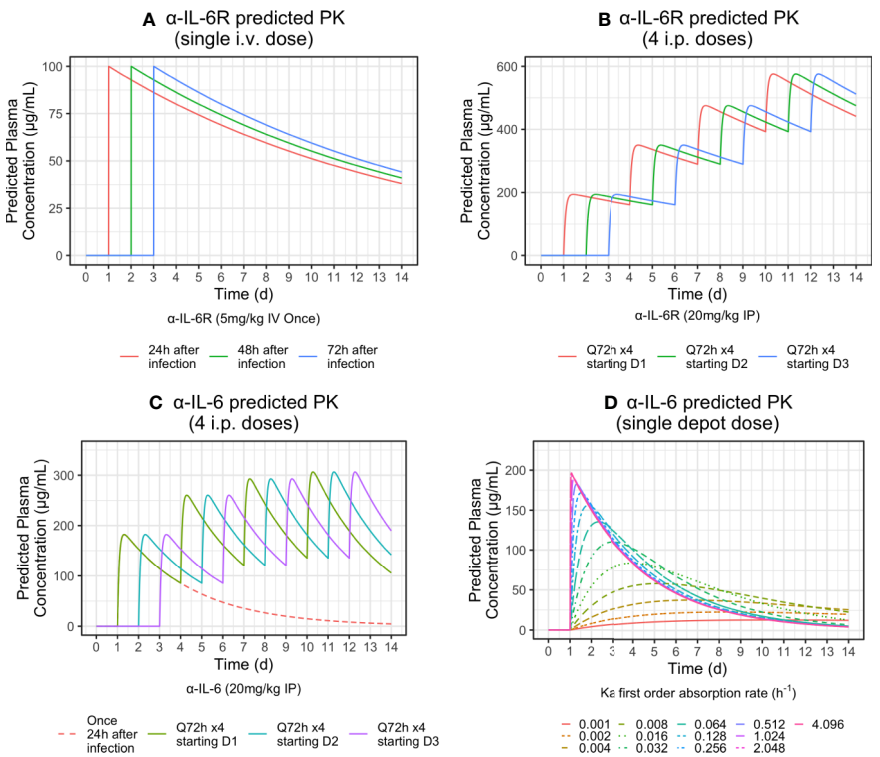

FIGURE 6 | Simulated PK profiles for i.v. and i.p. routes of administration based on literature PK parameters shown in **Table S5** in **Supplementary Materials** were determined. The top-left panel **(A)** models the i.v. delivery experiment. The top-right panel **(B)** and bottom-left panel **(C)** model i.p. delivery experiments one and two. For each of these simulations, mice were dosed a total of four times at 72-h intervals, beginning 24 h after challenge. The bottom-right panel **(D)** models release profiles for simulated controlled release scenarios with different absorption rates as indicated by the listed Ka parameters after a single depot injection of 20 mg/kg.

and various ongoing clinical trials evaluating the potential benefit of IL-6 blockers, for example, early α-IL-6 mAb and later α-IL-6R mAb, for the treatment of patients with CRS may provide clinical correlation with the results presented here.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by UTMB which operates under OLAW assurance number D16-00202(A3314-01).

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. CM and TB performed the study under BSL-4 conditions and generated the data presented here.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPINION
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The Regulatory Challenges for Drug Repurposing During the Covid-19 Pandemic: The Italian Experience

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INTRODUCTION

The search for safe and effective treatments for Covid-19 started early and focused in particular on drug repurposing of available molecules in the hope of finding valuable therapeutic options as quickly as possible.

As reported on *Covid19db*, a free online database of trials of medicinal products to prevent or treat Covid-19, the percentage of trials including repurposed drugs exceeds 60% of the total number of interventional drug-based trials (AnticancerFund; Pan Pantziarka et al., 2020).

The main advantages of drug repurposing over *de novo* medicine research are the faster and potentially cheaper development and the reduced risk of failure due to safety concerns (Bertolini et al., 2015; Pushpakom et al., 2019; Verbaanderd et al., 2019). Therefore, the regulatory authorities were rapidly overwhelmed by requests for clinical trials and compassionate use program approval.

One of the national regulatory agencies that has suffered the most because of the Covid-19 crisis has been the Italian one.

Moreover, in a situation of absolute emergency with hospitals full of critical patients, clinicians tried to save lives with off-label drugs used according to the available (although weak) evidence.

CLINICAL TRIALS APPROVAL: SUCCESSFUL ATTEMPT OF CENTRALIZATION

The Italian Medicines Agency (AIFA) is the Competent Authority for issuing the authorization of all clinical trials of medicinal products together with the local Ethics Committees (ECs) competent for the clinical sites for their formal approval (**Supplementary Appendix 1A**). As reported in a national registry (AIFA, 2019b), despite the progressive reduction established by a Decree (Decree-Law, 2013) 90 ECs are still working in Italy, which should be further reduced to 40 local ECs and three national ones (Petrini and Brusaferro, 2019) in order to optimize their performance and improve efficiency. This condition can indeed produce disparities in terms of opinions and/or timing for approval.

On an exceptional basis, during the Covid-19 crisis an emergency regulation for clinical trials and compassionate uses was issued with the Law Decree N. 18 on 17 March 2020 (Decree-Law, 2020). According to the aforementioned Decree, Covid-19 protocols have to be preliminarily evaluated and subsequently approved by the Technical Scientific Committee (CTS) of AIFA, by the AIFA Clinical Trial Office and by the EC of the National Institute for Infectious Diseases Lazzaro Spallanzani in Rome, as

single ECs, which express a nationwide opinion (**Supplementary Appendix 1B**). The rationale of the measure was to speed up the approval process thanks to a single national body (instead of the multitude of ECs usually involved based on territorial criteria) and to guarantee a high level of quality of the assessment thanks to the expertise of the institution on the treatment of infectious diseases. Moreover, the Decree established that both AIFA and Istituto Spallanzani are responsible for the activation of Covid-19 compassionate use programs (CUPs), which must be assessed and eventually authorized as a matter of urgency.

One month from these provisions, AIFA published a summary report on the work performed by the Commission and the national EC (AIFA, 2020d). In the reference period, 80 Covid-19 protocols and proposals had been assessed, and 16 positive opinions (20%) had been issued. The main reasons behind the rejections were concerns about the study design, the rationale, and a not adequately defined population.

If we look at the clinical trials approved by AIFA under normal conditions, in the last published report (AIFA, 2019a) we find 714 protocols assessed during 2018 (almost 60 per month), of which 666 had been approved (93.3%). Probably the highest percentage of positive opinion is related to the highest quality of submission under normal circumstances.

According to the latest update, about 40 clinical trials for Covid-19 have been approved in Italy out of a total of 156 protocols submitted (Popoli, 2020) (**Supplementary Appendix 2**), even in light of the difficulty of completion due to the reduction in the number of new cases and hospitalizations.

Despite the expected complexity of studies management due to the emergency, well-designed clinical trials were favored. Indeed, the great majority of approved studies were randomized–controlled trials (**Supplementary Appendix 3**), even if less than 25% were blinded. However, a critical point was undoubtedly the definition of the *"standard of care"* in the absence of authorized treatments. In order to guide clinical practice and trial design, AIFA published and periodically updated reports concerning drugs recommended according to national and international guidelines (AIFA, 2020a).

Other measures have been put in place to ensure the safe conduct of clinical trials and promote research on Covid-19.

Due to the exceptional restrictive measures introduced in order to fight the Covid-19 pandemic and in line with European directions (EMA, 2020b), AIFA provides indications regarding the management of all clinical trials that must be conducted with the highest protection of participants and maintaining adequate supervision even during emergencies (AIFA, 2020b). For instance, sponsors were invited to implement a risk-proportionate action plan, in view of the need to minimize contact between patients and the investigational team, and not to overload healthcare facilities. Moreover, carrying out procedures directly at the patient's home may be considered. On-site monitoring visits can be replaced by exceptional and alternative monitoring such as telephone calls or video-calls with the trial site staff, or can be postponed.

Finally, the Italian Ministry of Health recently authorized the financial support of 10 important research projects on Covid-19 (Ministry of Health, 2020).

OFF-LABEL USE FOR COVID-19 AND EMERGENCY APPROVAL

According to the European Medicines Agency (EMA), off-label use "relates to situations where the medicinal product is intentionally used for a medical purpose not in accordance with the authorized product information" (EU, 2017).

A major advantage of off-label use is the potential and rapid satisfaction of medical needs, especially in cases where no other options are available, even if it could increase the risk of inappropriate use and medical error due to the lack of a defined risk-benefit ratio (Bellis et al., 2014). Therefore, appropriateness in off-label drug prescriptions must be carefully assessed in order to ensure this use occurs only in the presence of data supporting a favorable risk/benefit profile.

Off-label prescribing is not currently regulated by European Union (EU) legislation, but some countries have adopted specific laws (**Supplementary Appendix 4**) (EU, 2017).

Italy has gained a lot of experience in off-label regulation and management as a result of the so-called "*Di Bella case*" (Di Bella, 2010). In order to limit off-label use, to guarantee patients' well-being and reduce unmotivated risks, the Italian Parliament issued Law 94 in 1998, which allows physicians to perform off-label prescriptions in individual and exceptional cases, provided that the following requirements are respected:

- the assumption of responsibility of the prescriber,
- an adequate informed consent of the patient,
- and the existence of scientific evidence of the efficacy and safety of the medicine derived from at least phase II clinical trials (Financial-Law, 2008).

Moreover, the Law establishes that the National Health System (NHS) does not cover the cost of treatment, which must be granted by patients themselves. In a hospital setting, prescribers must request the authorization for off-label treatment to the local Health Director, and costs are covered by the hospital budget.

The only case in which the Italian NHS can reimburse an off-label drug is its use under Law 648/1996 as reported in specific lists, updated based on new scientific evidence resulting from at least phase II clinical trials (Law 648, 1996).

The Covid-19 emergency obliged national authorities to consider the possibility to allow a systematic off-label use of some medicines notwithstanding the aforementioned rules (**Figure 1**). In particular, this happened for hydroxychloroquine/cloroquine, lopinavir/ritonavir, and darunavir/cobicistat, whose use in patients with Covid-19 was promptly and provisionally approved for reimbursement, despite the non-applicability of the Law 648/1996 (above all due to the lack of evidence from phase II clinical trials), in order to manage their uncontrolled off-label use, which was already spreading nationwide. This decision allowed the standardization of prescriptions giving official instructions on how to use these medicines, but also to carry out an appropriate surveillance because of the obligation for prescribers to promptly share data about treated patients.

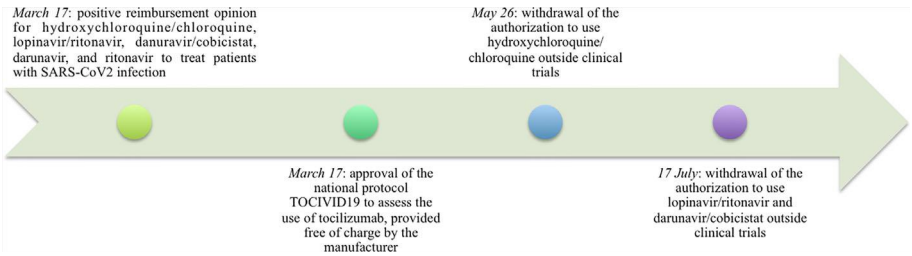

FIGURE 1 | Timeline of the main AIFA opinions on off-label drug use issued during the Covid-19 emergency.

Recently, AIFA published the Report on Medicines used during the Covid-19 epidemic showing a very high increase of hydroxychloroquine use compared to January 2019, a sign of clinicians hopes for this drug in the absence of available alternatives (AIFA, 2020e).

Subsequently, due to the lack of evidence and the possible risk of serious adverse events (Boulware et al., 2020; Cao et al., 2020; EMA, 2020a; FDA, 2020; Lother et al., 2020; Mehra et al., 2020a; Mehra et al., 2020b; WHO, 2020a; WHO, 2020b), AIFA revoked the authorization.

It is noteworthy that the next drug in terms of use following the anti-malarial is the antibiotic azithromycin, the use of which has never been officially authorized outside clinical trials (AIFA, 2020e; AIFA, 2020a). These findings deserve further analyses.

The case of tocilizumab is different, it has been provided free of charge by the Company since the beginning of March. In this case, in order to monitor all patients treated with the drug and to collect solid real-world data, AIFA together with Istituto Nazionale Tumori, IRCCS, Fondazione G. Pascale (Napoli) promoted a nationwide trial that involved hundreds of clinical centers and enrolled thousands of patients (AIFA, 2020c). The final results of the study are expected to be published in the near future.

CONCLUSION

The Covid-19 pandemic tested the regulatory authorities' ability to react to an emergency. In this context, Italy promptly implemented many measures (including centralization of clinical trials approval, simplification of the trial management obligation, financial support for research proposals, off-label use funding and governance) in order to simplify the practice of drug repurposing but also to maintain a strict control on drug access. Although some decisions were later withdrawn, the Italian regulatory authority was vigilant, efficient, and adaptable to face such a great challenge. Moreover, centralization has proven to be a successful choice, and a way forward in the future, albeit perfectible.

This success can be useful in order to start reviewing some old regulations and to further simplify some procedures, to make the system competitive and guarantee equal access to patients.

Finally, a dialogue among European member states and other authorities worldwide is desirable to set common criteria for proper off-label use management.

AUTHOR CONTRIBUTIONS

LG wrote the first draft of the manuscript. FD checked and revised the draft manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.588132/full#supplementary-material

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Vulnerabilities of the SARS-CoV-2 Virus to Proteotoxicity—Opportunity for Repurposed Chemotherapy of COVID-19 Infection

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The global pandemic of COVID-19 disease caused by infection with the SARS-CoV-2 coronavirus, has produced an urgent requirement and search for improved treatments while effective vaccines are developed. A strategy for improved drug therapy is to increase levels of endogenous reactive metabolites for selective toxicity to SARS-CoV-2 by preferential damage to the viral proteome. Key reactive metabolites producing major quantitative damage to the proteome in physiological systems are: reactive oxygen species (ROS) and the reactive glycating agent methylglyoxal (MG); cysteine residues and arginine residues are their most susceptible targets, respectively. From sequenced-based prediction of the SARS-CoV-2 proteome, we found 0.8-fold enrichment or depletion of cysteine residues in functional domains of the viral proteome; whereas there was a 4.6-fold enrichment of arginine residues, suggesting SARS-CoV-2 is resistant to oxidative agents and sensitive to MG. For arginine residues of the SARS-CoV-2 coronavirus predicted to be in functional domains, we examined which are activated toward modification by MG – residues with predicted or expected low pK_a by neighboring group in interactions. We found 25 such arginine residues, including 2 in the spike protein and 10 in the nucleoprotein. These sites were partially conserved in related *coronaviridae: SARS-CoV and MERS. Finally, we* identified drugs which increase cellular MG concentration to virucidal levels: antitumor drugs with historical antiviral activity, doxorubicin and paclitaxel. Our findings provide evidence of potential vulnerability of SARS-CoV-2 to inactivation by MG and a scientific rationale for repurposing of doxorubicin and paclitaxel for treatment of COVID-19 disease, providing efficacy and adequate therapeutic index may be established.

Keywords: COVID-19, coronavirus, methylglyoxal, glyoxalase, doxorubicin, paclitaxel, proteomics, bioinformatics

INTRODUCTION

A global pandemic of COVID-19 disease caused by infection with the SARS-CoV-2 coronavirus has developed from January 2020. It has produced a global public health emergency with currently (20th July 2020) over 14 million infections and *ca*. 600,000 deaths, with both rapidly increasing. New treatments are urgently required for COVID-19 disease until effective vaccines are developed. A rapid route to achieve this is repurposing of existing drugs with previously undisclosed activity against coronavirus infection.

As a strategy to identify drugs for repurposing, we sought to explore whether the SARS-CoV-2 may have vulnerabilities in the viral proteome to modification by endogenous reactive metabolites. Pharmacological increase of reactive metabolites will then produce a virucidal effect and therapeutic response for COVID-19 disease. Important reactive metabolites producing major quantitative modification of the proteome in physiological systems are: reactive oxygen species (ROS) and methylglyoxal (MG) (Winterbourn, 2008; Rabbani and Thornalley, 2015). Key to characterizing the vulnerability of the viral proteome to reactive metabolites, ROS and MG, is location of their susceptible amino acid residue targets in functional domains of viral proteins and activation of these residues toward reaction with reactive metabolites. ROS are formed by mitochondria through trace leakage of electron flux in oxidative phosphorylation, by oxidases and other sources. They are metabolized by antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxins (Murphy et al., 2011). The reactive dicarbonyl metabolite, MG, is formed mainly by trace level degradation of triosephosphate glycolytic intermediates, glyceraldehyde-3-phosphate and dihydroxyacetonephosphate, and is mainly metabolized by glutathione-dependent glyoxalase 1 (Glo1) of the glyoxalase pathway (Rabbani et al., 2016b) (**Figure 1A**). The most susceptible targets in proteins to modification by ROS are cysteine residues which are oxidized to cystine and cysteine sulfenic and sulfonic acids (Winterbourn, 2008). The most susceptible targets in proteins to modification by MG are arginine residues which are glycated to hydroimidazolone MG-H1 with loss of charge, all related electrostatic interactions and, typically, resistance to proteolytic cleavage close to the site of modification (Rabbani et al., 2016b) (**Figure 1B**).

Key to exploring if reactive metabolites of the host can be exploited to produce a virucidal response against SARS-CoV-2 is to identify proteomic vulnerabilities of the virus. Currently it is unknown if target amino acid residues of reactive metabolites are enriched in functional domains of the viral proteome, and if these targets amino acids are activated toward modification by reactive metabolites. It also unknown if there are investigational new drugs or current clinically approved drugs that increase reactive metabolites to virucidal levels in the cellular environment where SARS-CoV-2 undergo cell fusion and propagation. To address these gaps in knowledge, we initiated a series of studies using bioformatics tools, available proteomics data and a cell model used in SARS-CoV-2 virus propagation. Herein we predict the susceptibility of the SARS-CoV-2 virus to increased MG or "dicarbonyl stress" (Rabbani and Thornalley, 2015). This is based on enrichment of arginine residues in functional domains of the SARS-CoV-2 proteome and predicted activation of many of these arginine residues to modification by MG through neighboring group interactions. We also identified two clinical antitumor drugs that increase the cellular concentration of MG to virucidal levels and are candidates for consideration for repurposing for evaluation for treatment of COVID-19.

MATERIALS AND METHODS

Reagents and Chemicals

Doxorubicin, Paclitaxel, monoclonal anti-Glo1 antibody (rat), anti-Rat IgG (whole molecule)–Biotin conjugate, D-Lactic dehydrogenase were purchased from Sigma-Aldrich (Poole, Dorset, UK). Geneticin G-418 (potency rating – 700 μg) was purchased from Fisher Scientific (Loughborough, UK). S-p-bromobenzylglutathione cyclopenyl diester (BBGD) was prepared an purifeid in-house, as described (Thornalley et al., 1996). The HEK293 cell line was purchased from the American Tissue Culture Collection (ATCC, Virginia, USA).

Plasmids, pIRES2-GLO1-EGFP and pIRES-EGFP, PJT laboratory were prepared and purified in-house, as described (Ahmed et al., 2008).

Sequences of SARS CoV-2, SARS-CoV, and MERs and Human Host Proteins

Reference sequences of the 29 proteins of the SARS-CoV-2 proteome (**Table S1**) and sequences of analogous proteins of SARS-CoV and *Middle East Respiratory Syndrome* (MERS) *coronaviridae* were obtained from the NBCI reference sequence database (www.ncbi.nlm.nih.gov). Sequences of reviewed proteins of the human proteome, 18,821 – excluding fetal proteins, were obtained from the UniProtKB database (www.uniprot.org).

Receptor Binding Domain Analysis

Receptor binding domain (RBD) analysis is a protein primary sequenced based informatics method to deduce amino acid residues in functional domains of proteins—defined as sites of protein-protein, protein-nucleic acid, and protein-ligand or substrate interaction. It is applicable to any protein. The optimized protocol uses a window of five amino acid residues moved sequentially along the sequence of a protein, assuming a gyration angle between two consecutive residues in the sequence of 100°, to deduce sequential mean Eisenberg hydrophobicity and mean dipole moment for the central amino acid. Values cannot be deduced for the two amino acids at the N- and C-termini of proteins and they are therefore missing from the amino acid residue prevalence reports (Gallet et al., 2000). This approach had 80% accuracy when validated against a database of known interacting proteins (Gallet et al., 2000). We developed an R script to obtain mean hydrophobicity and hydrophobic moment for all UniProtKB proteins and SARS-CoV-2 proteins.

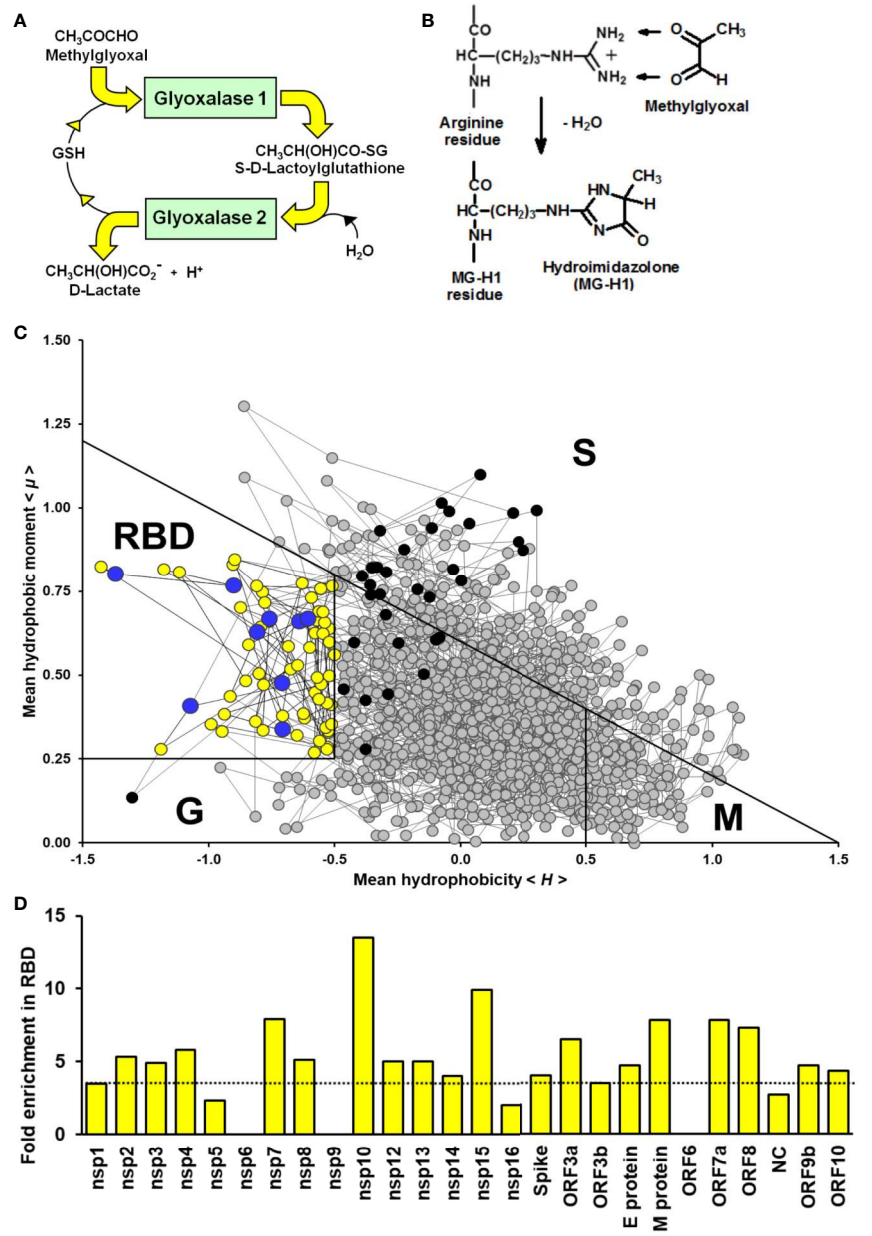

FIGURE 1 | Methylglyoxal—an endogenous arginine-modifying reactive metabolite and receptor binding domain analysis of functional arginines in the SARS-CoV-2 proteome. **(A)** Metabolism of methylglyoxal by the glyoxalase pathway (Rabbani et al., 2016b). **(B)** Modification of arginine residues by methylglyoxal (MG) to form hydroimidazolone, MG-H1. **(C)** Receptor binding domain (RBD) plot for SARS-CoV-2 Spike protein. Line-linked filled circles represent the primary sequence. The RBD is the area bound by the trapezium in the upper left-side region of the chart. Key: ● arginine residue in the RBD; ●, arginine residues outside the RBD; ●, other amino acid residues in the RBD; and ●, other amino acid residues outside the RBD. Other predicted domains: surface (S), globular (G), and membrane (M). **(D)** Arginine enrichment in individual proteins of SARS-CoV-2 proteins. Proteins not shown have no arginine residues (nsp11, ORF7b and ORF14). Dotted line - mean fold enrichment of the human host proteome, 3.5.

Prediction of Arginine Residues Activated for MG Modification

Arginine residues of proteins which are activated toward reaction with MG by decrease of pK_a of the guanidino side chain which facilitates formation of the MG-guanidino sidechain encounter (Rabbani and Thornalley, 2012; Rabbani et al., 2016a). Arginine residue sidechain pK_a is decreased by interaction with neighboring amino acid residues with positively charged sidechains. For an α-helix, interactions with lysine or arginine residues at positions -4, -3, +3 and +4 in the sequence with respect to the target arginine residue are expected to decrease the arginine target residue pK_a by side chain interaction along the side of the helix axis. Longer range

interactions occur between these and other types of secondary structure domains in the tertiary structures of proteins where arginine residue pK_a may be predicted from crystallographic data. We explored the peptide environments of arginine residues in predicted functional domains of the SARS-CoV-2 proteome, identifying arginine residues with neighboring interacting lysine and arginine residues, predicted secondary structure and predicted arginine residues target pK_a where crystallographic data are available. Information on predicted secondary structure was extracted from *in silico* predicted models: nsp1, YxJyvF; nsp3, 5hYU6g; M-protein, 9LzAZz (http://3dbionotes.cnb.csic.es/ws/covid19) (Waterhouse et al., 2018). Arginine side chain predicted pK_a values are given, deduced by DelPhiPKa program (Wang et al., 2016) using nucleoprotein (NC) crystal structure (pdb file 6VYO; Chang *et al.*, to be published) and AMBER forcefield (predictions were similar with CHARMM and PARSE forcefields). To identify similar arginines residues in SARS-CoV and MERS proteins, we used the Clustal Omega software on-line (Madeira et al., 2019).

Culture of HEK293 Cells *In Vitro*

The HEK293 cell line, seeding density 2×10^4 cells cm^{-2}, was cultured in Dulbecco's Modified Eagles Medium (DMEM) containing phenol red, L-glutamine and 4,500 mg/L glucose, supplemented with 10% Fetal Bovine Serum (FBS), 100 U penicillin and 0.1 mg/ml streptomycin. pIRES2-GLO1-EGFP plasmid (Glo1+ vector) and pIRES-EGFP plasmid (empty vector) were prepared as described (Ahmed et al., 2008). HEK293 cells were stably transfected with Glo1+ and empty vector using Lipofectamine 2000, according to the manufacturer's instructions (plasmid DNA: Lipofectamine 2000, 1:4). After 48 h, cells were sub-cultured, G-418 disulphate was added (2 mg/ml; 405 μg/mg potency) culture continued. Transfected colonies with GFP fluorescence were selected using a cloning disk (3.2 mm) and glass cylinder selector (8 mm, 150 μl) and cultured further with G-418 disulphate (1 mg/ml, 705 μg/mg potency) containing medium. Assessment of Glo1 activity and protein, as described (Arai et al., 2014; Xue et al., 2014), indicated a four- to fivefold increase in Glo1 activity and protein. HEK293 cells stably tranfected with empty and GLO1+ vectors were incubated with and without cell permeable Glo1 inhibitor, S-p-bromobenzylglutathione cyclopenyl diester (BBGD) (Thornalley et al., 1996), doxorubucin and paclitaxel at the concentrations indicated (diluted from 100 mM stock solution in DMSO) for 2 days and effect on cell growth assessed by viable cell number counts, using the Trypan blue exclusion method and median growth inhibitory concentrations GC_{50} deduced. Cellular MG concentration and flux of formation of D-lactate, a surrogate measure of flux of formation of MG, was assayed as described (Rabbani and Thornalley, 2014; Irshad et al., 2019).

Statistical Analysis

Datasets were checked for normality of distribution and parametric statistical tests for assessment of significance of difference between study groups applied: *Student's t-test* for two groups and one-way *ANOVA* for 3 or more study groups.

RESULTS

Enrichment of Arginine Residues in the Functional Domains in the SARS-CoV-2 Proteome

We acquired primary amino acid sequences of the 29 proteins of the SARS-CoV-2 proteome (**Table S1**) and also, for comparison, 18,821 reviewed protein sequences of human host proteins from the UniProt Knowledgebase (UniProtKB; www.uniprot.org) excluding fetal proteins. We found a similar prevalence of cysteine and arginine residues in the viral proteome: 3.14% and 3.63%, respectively (**Table 1**). We applied RBD analysis to identify functional domains of viral proteins and to thereby deduce the prevalence and enrichment of cysteine and arginine residues therein. The RBD analysis outcome is illustrated as a plot of mean hydrophobicity against mean dipole moment for the widow of 5 amino acid residues moved sequentially along the sequence of a protein. An example of the RBD analysis of the SARS-CoV-2 Spike protein is given in **Figure 1C**. Functional domains are located in a trapezium-shaped domain on the top-left side of the plot – regions of low mean hydrophobicity and high mean dipole moment of proximate groups of amino acid residues. This analysis showed that 4.8% of cysteine residues were in functional domains of the SARS-CoV-2 proteome whereas a much greater proportion of arginine residues, 30.7%, were in functional domains. The enrichment of arginine residues in functional domains was 4.9-fold—the highest of any amino acid, whereas there was a slight negative enrichment, 0.8-fold, or depletion of cysteine residues in functional domains. Other

TABLE 1 | Receptor binding domain of SARS-CoV-2 viral proteomes.

Amino acid	N		Prevalence		Proportion in RBD (%)	Fold Enrichment
	All	RBD	All	RBD		
Arg	358	110	3.63	17.7	30.7	4.9
Cys	310	15	3.14	2.4	4.8	0.8
Met	203	7	2.06	1.1	3.5	0.6
Tyr	448	23	4.54	3.7	5.1	0.8
Trp	113	2	1.15	0.3	1.8	0.3

*Receptor binding domain (RBD) analysis was applied to SARS-CoV-2 proteome (see **Table S1**) using a window of five amino acids and gyration angle between two consecutive residues in the sequence of 100° (Gallet et al., 2000). Complete amino acid profile is given in **Table S2**.*

amino acid residues susceptible to oxidative damage were also depleted in functional domains: met 0.6, tyr 0.8, and trp 0.3 (**Table 1**). The SARS-CoV-2 proteome is, therefore, resistant to oxidative inactivation but susceptible to functional inactivation by MG. For individual SARS-CoV-2 proteins, the majority had arginine residue enrichment in the functional domains greater than the mean of the human host proteome of *ca.* 3.5: range 2.3–13.5 (**Figure 1D**).

Arginine Residues Activated for MG Modification by Predicted Neighboring Group Interaction

We next sought to identify arginine residues in the predicted functional domains of SARS-CoV-2 proteins which are activated toward modification by MG based on potentially neighboring group side-chain interaction with arginine and lysine residues and, where crystallographic data are available, predicted target arginine residue pK_a. For example, in human serum albumin, neighboring group interactions with R186, R218 and R410 decrease the pK_a values of their sidechain guanidino groups to 12.5, 12.2, and 12.5 from the basal pK_a of 13.8 (Fitch et al., 2015). The reactivity with MG of these arginine residues increases by 20- to 40-fold through increase of the trace level conjugate base of the sidechain guanidino group (Fitch et al., 2015; Rabbani et al., 2016a). In low-level extent of modification of albumin by MG in experimental investigations *in vitro* and similar low-level extent of modification by MG of human serum albumin found similarly *in vivo*, MG was detected on these residues preferentially (Ahmed et al., 2005).

Applying RBD analysis and inspecting sequences for arginine or lysine residues at positions -4, -3, +3 and +4 with respect to the target arginine, we found the following number of arginine residues reactive toward MG modification and protein inactivation in predicted functional domains in SARS-CoV-2 proteins: nsp1, 2; nsp2, 3; nsp3, 3; nsp8, 1; nsp12, 1; nsp15, 2; spike protein, 2; M-protein, 1; NC, 10; and ORF10, 1. There were 25 functional arginines potentially activated for MG modification: 5 sites were in predicted α-helices and 2 in NC with predicted pK_a lowered by neighboring group interaction and thereby activated toward MG-modification (**Table 2**).

Uniquely for related coronaviridae, SARS-CoV-2 spike protein has an interacting arginine triad, $R_{682}RAR_{685}$, at the S1/S2 cleavage site (Andersen et al., 2020) with both R682 and R685 predicted sites susceptible to MG modification (**Figure 2A**). Modification of this triad by MG is expected to confer resistance to proteolytic cleavage by transmembrane serine proteases (TMPRSSs) and blocking cell fusion for virion entry into pulmonary alveolar epithelial and other cell target sites, uncoating and replication (Meng et al., 2020). Trapped in the extracellular environment, there is excepted to be an improved host immune response to the virus; *cf.* viral host immunity boosted by similar aldehyde-modifying agents (Herrera-Rodriguez et al., 2019).

SARS-CoV-2 nucleoprotein is highly susceptible to modification and functional inactivation by MG. Nucleoprotein binds the 3' end of the viral single strand RNA genome of SARS-CoV-2 and it is arginine-rich, as is typical of RNA-binding proteins (Tan and Frankel, 1995). By analogy with SARS-CoV, residues 42–187 are involved with RNA binding with R93 playing a critical role (Mcbride et al., 2014). It is predicted to have sites susceptible to MG modification. The crystal structure of a segment of nucleoprotein, residues 50–173, is available and this enabled prediction of pK_a values of arginine residues in this region. pK_a shifts from 13.8 of inactivated arginine (Fitch et al.,

TABLE 2 | SARS-Cov-2, SARS-Cov, and MERS proteins with putative activated arginine residues in functional domains.

	Activated arginine in RBD		
Protein	SARS-CoV-2	SARS-CoV	MERS
nsp1	R43 (LSEARQHLK, R73 (VFIKRSDAR), R124 (KVLLRKNGN)	R43, R73, R124	R124
nsp2	R64 (WYTERSEKS), R107 (TIQPRVEKK)	R64, R107	R64, R107
nsp3	R30 (ELDERIDKV), R586 (STIQRKYKG), R712 (EFLKRGDKS)	R30, R586, R712	
nsp8	R75 (YKQARSEDK)		
nsp12	R555 (KNRARTVAG)	R555	R555
nsp15	R61 (LWAKRNIKP), R138 (FRNARNGVL)	R138	
Spike protein	R682 (TNSPRRAR), R685 (PRRARSVAS)	R685	R685
Protein M-protein	SARS-Cov-2 R105 (RLFARTRSM)	SARS-Cov-2 R105	MERS R105
ORF8	R52 (RVGARKSAP)		
NC	R36 (RSGARSKQR), R40 (RSKQRRPQG), R41 (KQRRPQGL), R88 (IGYYRRATR; $pK_a \approx 12.7$), R89 (GYYRRATRR, $pK_a \approx 13.9$), R93 (RATRRIRGG; $pK_a \approx 12.6$), R95 (TRRIRGGDG; $pK_a \approx 12.2$); R185 (QASSRSSSR), R191 (SSRSRNSSR), R195 (RNSSRNSTP), R262 (PRQKRTATK)	R36, R40, R41, R88, R89, R93, R95, R185, R191, R195, R262	R36, R88, R89, R191, R195, R262
ORF10	R234 (RMNSRNYIA)		

Key: R, putative activated arginine, R and K, putative neighboring interacting residues decreasing pKa of the target R residue. , predicted α-helix from in silico predicted models: nsp1, YxJyvF; nsp3, 5hYU6g; M-protein, 9LzAZz (http://3dbionotes.cnb.csic.es/ws/covid19) (Waterhouse et al., 2018) and nsp15, crystal structure (pdb file 6VWW) (Kim et al., 2020). Arginine side chain predicted pK_a values are given, deduced by DelPhiPKa program (Wang et al., 2016) using nucleoprotein crystal structure (pdb file 6VYO; Chang et al., to be published) and AMBER forcefield (predictions were similar with CHARMM and PARSE forcefields). Sequence alignment of SARS-Cov-2, SARS-CoV and MERS proteins was performed using the Clustal Omega software on-line (Madeira et al., 2019).

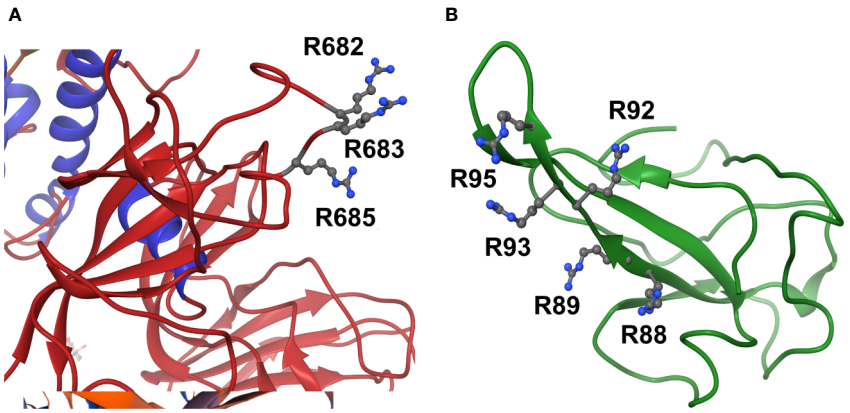

FIGURE 2 | Activation of functional arginine residues toward modification by methylglyoxal in the SARS-CoV-2 proteome. **(A)** Activated arginine residues triad of Spike protein, $R_{682}R_{683}AR_{685}$. **(B)** Activated arginine residue pentad of nucleoprotein, $R_{88}R_{89}ATR_{92}R_{93}IR_{95}$. Molecular graphics produced from Spike protein (pdb file 6VSB) (Wrapp et al., 2020) and nucleoprotein segment crystal structure (pdb file 6VYO; Chang et al., to be published) using Chimera 1.14 (Pettersen et al., 2004).

2015) for R93 and R95 indicated *ca.* 16 and 40-fold increased reactivity toward MG modification, compared to nonactivated target residue. These residues lie in a pentad of reactive arginine residues where MG modification at R93 and R95 is expected to inactive the nucleoprotein (**Figure 2B**). The SR-rich region of 182–196 is important for virus replication (Tylor et al., 2009) and is also a target for MG modification and inactivation at 3 sites: R185, R191 and R195. MG modification of the nucleoprotein, and also membrane protein, will block viral replication and virion assembly, respectively.

There are similar MG modification sites in functional domains of related *coronaviridae*. The proteome of severe acute respiratory syndrome coronavirus (SARS-CoV) had 23 similar MG modification sites to those of SARS-CoV-2, and MERS coronavirus proteome had 12 similar MG modification sites. In all of these *coronaviridae* there were multiple MG modification sites in functional domains of the nucleoprotein (**Table 2**). Given the high activation of multiple arginine residues in functional domains of the SARS-CoV-2 proteome, it is likely that pharmacological increase of endogenous MG concentration will produce modification at multiple susceptible and functional sites, producing protein inactivation and antiviral response.

Pharmacological Increase of Cellular MG to Virucidal Levels by Cell Permeable Glyoxalase 1 Inhibitor and Clinical Antitumor Drugs, Doxorubicin, and Paclitaxel

Antiviral activity of supraphysiological concentrations of MG was reported historically (De Bock et al., 1957). More recently, inhibition of cytopathic effect of strains of influenza B by MG was investigated. The most sensitive strain gave a median inhibitory concentration of 23 ± 7 μM MG (Charyasriwong et al., 2016). These studies assessed antiviral activity by cytopathic response – concentration of MG required to prevent 50% lysis of infected cells, using relatively high multiplicity of infection (MOI). Median effective concentrations for antiviral effects of pharmacological agents tend to be lower in physiologically relevant range of MOI than in pathogenic response assessment (Wang M. et al., 2020). These studies also used exogenous MG in cellular *in vitro* models where MG is rapidly metabolized by Glo1 and onward through the glyoxalase pathway to D-lactate (Rabbani et al., 2016b). The cellular concentration of MG is 1–4 μM and the plasma concentration 130–250 nM (Rabbani and Thornalley, 2014; Xue et al., 2016; Irshad et al., 2019). The optimum approach to achieve an antiviral effect is to increase cellular MG concentration by inhibition of Glo1. BBGD is a potent cell permeable Glo1 inhibitor prodrug. It delivers the Glo1 competitive inhibitor, S-p-bromobenzylglutathione (K_i = 160 nM), into cells and has established antitumor and antimalarial activity (Thornalley et al., 1994; Thornalley et al., 1996) (**Figure 3A**). We studied the effect of BBGD and clinically approved antitumor drugs on the cellular concentration of MG in human HEK293 cells – a cellular model employed for SARS-CoV-2 propagation (Chien et al., 2016). BBGD increased the endogenous concentration of cellular MG by 4-fold to *ca.* 20 μM – a level similar to that which inhibited viral cytopathic activity (Charyasriwong et al., 2016).

We have a longstanding interest in anticancer activity of Glo1 inhibitors and overexpression of Glo1 in multidrug resistant tumors (Thornalley et al., 1996; Rabbani et al., 2018). Hence, we have been studying the likely involvement of increased MG in the mechanism of action of clinical antitumor drugs. Interestingly, we found clinical antitumor agents, doxorubicin and paclitaxel (**Figure 3A**), also increased cellular MG by a similar extent (**Figure 3B**). Increased MG concentration induced by doxorubicin and paclitaxel is linked to increased glucose metabolism and related increased formation of MG as a byproduct of glycolysis. Indeed, flux of formation of D-lactate

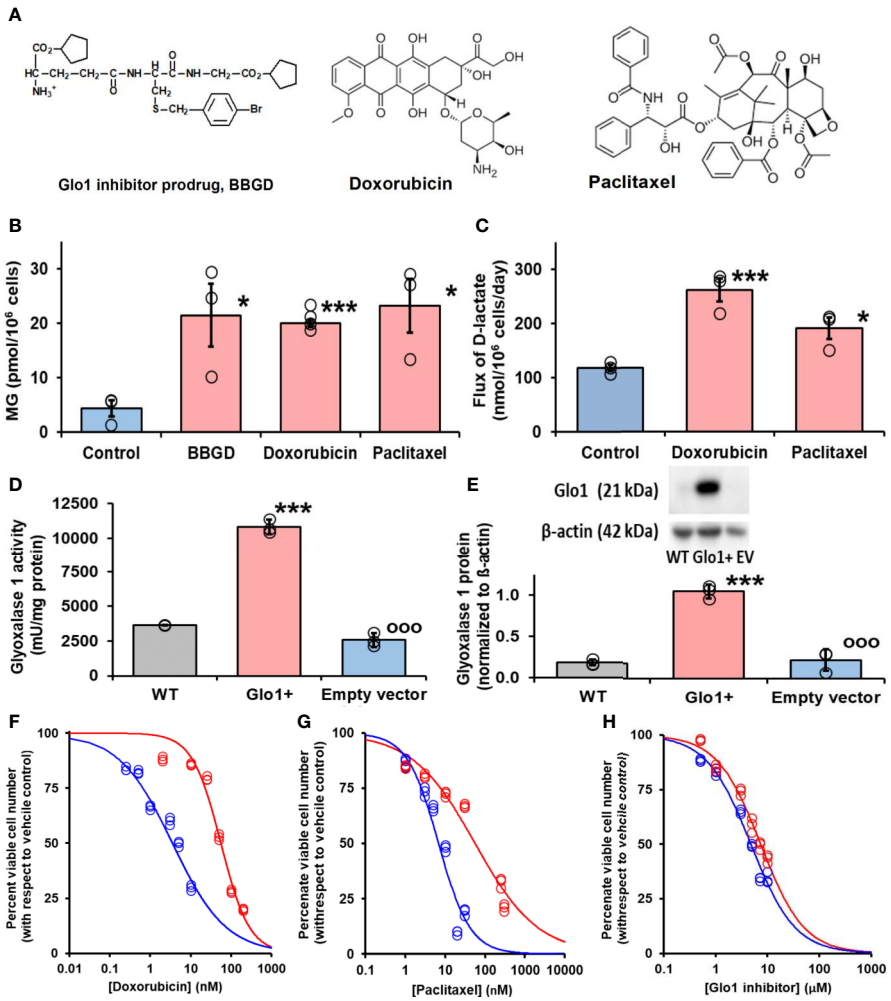

FIGURE 3 | Glyoxalase 1 inhibitor prodrug, doxorubicin and paclitaxel increase cellular concentration of methylglyoxal to virucidal levels. **(A)** Molecular structures of drugs. Glyoxalase 1 inhibitor prodrug, S-p-bromobenzylglutathione cyclopentyl diester (BBGD). Delivers competitive Glo1 inhibitor, S-p-bromobenzylglutathione, K_i = 160 nM, into cells (Allen et al., 1993; Thornalley et al., 1996). Doxorubicin – topoisomerase inhibitor.(Tewey et al., 1984) Paclitaxel – stabilizer of microtubule assembly (Schiff et al., 1979). **(B, C)** Increase in cellular methylglyoxal (MG) in HEK293 cells and flux of formation of D-lactate (surrogate for flux of MG), respectively, incubated *in vitro* with and without investigational agent and drugs indicated. For assay of MG, cells were incubated with and without treatment for 3 h and for flux of D-lactate incubated for 24 h. Data are mean ± SEM (n = 3 except n = 4 for MG estimation with Doxorubicin; individual data points are shown). Drug concentrations: BBGD, 7.4 μM; doxorubicin, 6.0 nM, paclitaxel, 21 nM. Significance: b. P<0.02 and c. P<0.01 (*one-way ANOVA*) and * and ***, P<0.05 and P<0.001 with respect to control (*t-test*). **(D, E)** Activity and protein of Glo1, respectively, in HEK293 cells: wild-type (WT) and cells stably transfected to overexpress Glo1 (Glo1+) and empty vector (EV). Glo1 activity and protein were increased four- to fivefold. This was maintained for > 10 passages. **(F–H)** Effect of Glo1 expression on anti-proliferative activity. Key: blue – empty vector, red – Glo1 overexpression. HEK293 cells were incubated with and without treatment for 48 h. Data (six drug concentrations in triplicate) were fitted by nonlinear regression to the dose-response equation, V = 100 x $GC_{50}^{n}/(GC_{50}^{n} + [Drug]^{n})$, solving for GC_{50} and, n (logistic regression coefficient). **(F)** Doxorubicin: empty vector, GC_{50} = 3.54 ± 0.28 nM, n = 0.71 ± 0.05; Glo1+, GC_{50} = 55.9 ± 3.4 nM, n = 1.24 ± 0.10 (16-fold resistance). **(G)** Paclitaxel: empty vector, GC_{50} = 6.8 ± 1.0 nM, n = 1.07 ± 0.17; and Glo1+, GC_{50} = 56.4 ± 7.2 nM, n = 0.55 ± 0.04 (8-fold resistance). **(H)** BBGD: GC_{50} = 4.78 ± 0.18 μM, n = 1.02 ± 0.05; and Glo1+, GC_{50} = 7.37 ± 0.30 μM, n = 1.04 ± 0.06 (twofold resistance).

—a surrogate indicator of flux of formation of MG—was increased by both drugs (**Figure 3C**).

Increase of cellular MG also likely contributes to the antiproliferative effect of BBGD (Rabbani et al., 2018). The involvement of MG in the antiproliferative activity of doxorubicin and paclitaxel is unknown. We explored this by determining the effect of overexpression of Glo1 on inhibition of HEK293 cell growth. Vector-derived stable transfectant HEK293 cell lines were prepared with Glo1 expression increased four- to fivefold and empty vector transfectant control (**Figures 3D, E**), imposing a four- to fivefold increased rate of MG metabolism in Glo1 overexpressing cells. When these transfectant cell lines were treated with growth inhibitory concentrations of drugs, there was an increase of median growth inhibitory concentration GC_{50}

value and resistance to inhibition of cell growth in HEK293 cells with stable overexpression of Glo1 (**Figures 3F–H**). The GC_{50} values were (mean ± SD; empty vector vs Glo1+): doxorubicin, 3.54 ± 0.28 nM vs 55.9 ± 3.4 nM (16-fold resistance); paclitaxel – 6.8 ± 1.0 nM vs 56.4 ± 7.2 nM (eightfold resistance). For treatment with BBGD there was an antiproliferative effect with limited change in GC_{50} value with Glo1 overexpression: 4.78 ± 0.18 μM vs 7.37 ± 0.30 μM (2-fold resistance). The limited effect on antiproliferative effect of BBGD is expected as the delivered Glo1 inhibitor also inhibits the overexpression factor, Glo1.

DISCUSSION

The enrichment of arginine residues in functional domains of the SARS-CoV-2 proteome provides important evidence to support an arginine-modifying agent strategy for inactivation of the virus and virucidal activity. Arginine residues are also enriched in the human host proteome but less so that in SARS-CoV-2; 3.6 versus 4.9. This characteristic of the human proteome was noted previously (Gallet et al., 2000) —now updated herein with UniProtKB current sequence information. In addition, in the SARS-CoV-2 proteome there is a high number of arginine residues activated by neighboring groups for reaction with MG. This particularly applies to the nucleoprotein and, uniquely for the SARS-CoV-2 coronavirus, to the furin cleavage site of the spike protein. For the 25 arginine residues identified with predicted reactivity toward MG modification in the SARS-CoV-2 proteome, there is supporting secondary structure and predicted low pK_a value evidence for seven of them. These arginine residue targets are in key proteins: nucleoprotein, M-protein, and Spike protein. A further important feature for susceptibility of viral proteins to MG modification is protein abundance: high abundance of a protein increases its susceptibility to reaction with MG. Previous earlier studies of the SARS virion suggested proteins of highest abundance were: nucleoprotein, M-protein, Spike protein and nsp3 (Neuman et al., 2008). Assuming a similar relative abundance of proteins in SARS-CoV-2, multiple arginine residues reactive toward modification and inactivation by MG were found in the 4 most abundant proteins of the SARS-CoV-2 proteome.

We predict the SARS-CoV-2 proteome is sensitive to modification by MG in functional sites. The proteome of human host alveolar cells is also likely to have increased modification by drug-induced increase of cellular MG. Protein domains sensitive to MG modification are chaperonin containing T-complex protein-1/T-ring complex protein-1 (CCT/TriC-1) chaperonins of protein (Irshad et al., 2019). Modification of these proteins is expected to be low but may delay folding of viral proteins and contribute to antiviral activity of drugs increasing the cellular concentration of MG.

We also explored use of proteomics data from previous studies where MG modification was detected at 411 in different sites in the cytosolic extract of human endothelial cells in culture (Irshad et al., 2019) in an attempt to identify a proteomic MG modification motif to map onto the SARS-CoV-2 proteome (data not shown). Although we could determine amino acid frequencies round the MG-modified arginine targets, there was a dropout of peptides (failure to detect) without lysine and arginine residues on the N-terminal side of the arginine target in mass spectrometric detection. In comparisons with unmodified arginine target peptide sequences, this led to an artifactual enrichment of lysine and arginine residues on the N-terminal side of the MG-modified arginine target. This peptide dropout was likely due to an additional missed cleavage by trypsin when the target arginines were modified by MG, making the related tryptic peptides difficult to detect in mass spectrometry analysis due to the loss of arginine residue charge and increased sequence length. This requires further investigation and likely studies with proteases other than trypsin in which MG modification changes tryptic peptide formation.

SARS-CoV-2 was rich with arginine residues in functional sites activated to MG modification. Other *coronaviridae*—SARS-CoV-2 and MERS—had similar MG modification sites in protein crucial for virion viability – particularly the nucleoprotein. This suggests that pharmacological agents increasing cellular concentration of MG, inducing dicarbonyl stress, may have virucidal activity against multiple *coronaviridae*.

The SARS-CoV-2 proteome was predicted to be relatively resistant to oxidative damage because oxidant-sensitive cysteine residues were negatively enriched, or depleted, in functional sites; enrichment ratio 0.8. A similarly depletion of methionine residues in functional sites was found, enrichment ratio 0.6 (**Table 1**), which are also susceptible to oxidative damage (Winterbourn, 2008). For induction of proteotoxicity, therefore, drugs which increase arginine-directed MG are predicted to be more effective than drugs which induce oxidative damage to proteins.

Doxorubicin and paclitaxel are clinical antiproliferative antitumor agents with mechanisms of action targeting inhibition of topoisomerase-II in DNA replication and stabilization of the interphase and microtubular network and mitotic spindle in mitosis, respectively (Schiff et al., 1979; Tewey et al., 1984). Herein, we show that increase in MG contributes to their mechanism of antiproliferative activity. Doxorubicin increases glucose metabolism by increasing expression of glucose transporter GLUT1 and hexokinase-2 (Demel et al., 2015). Paclitaxel stabilizes microtubules, decreasing free tubulin concentration; the latter increasing mitochondrial voltage-dependent anion channel (VDAC) activity and thereby *in situ* activity of hexokinase (Maldonado et al., 2010). These mechanisms are available in the lung epithelial cells primarily targeted by SARS-CoV-2 (Pezzulo et al., 2011; Lottes et al., 2014). Increased glucose metabolism produces a corresponding increase in the formation of MG – evidenced herein by increase in flux of formation of D-lactate; there may be disproportionately large increase in MG if expression of enzymes of onward metabolism of triosephosphates, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase, are not increased along with hexokinase activity and glycolysis becomes

dysregulated or unscheduled (Irshad et al., 2019; Rabbani and Thornalley, 2019).

BBGD has been evaluated previously in human cell cultures and tumor-bearing mice. It enters human cells in culture and hydrolyses to the Glo1 inhibitor, S-p-bromobenzyl-glutathione, and inhibits Glo1 with maximum cellular concentration of MG occurring after 3 h (Thornalley et al., 1996). *In vivo* studies were performed with BBGD and similar compounds in tumor bearing mice (Thornalley et al., 1996; Sakamoto et al., 2001). S-p-Bromobenzylglutathione is expected to eventually undergo excretion from cells and metabolism by the mercapturic acid pathway with urinary excretion of N-acetyl-S-p-bromobenzylcysteine. Common strains of laboratory mice have markedly higher plasma esterase activity than human subjects, so an esterase-deficient strain of mouse is required in experimental investigations to avoid esterase-dependent inactivation of BBGD before reaching target tissues (Kavarana et al., 1999).

We envisage increased cellular MG interacting with the virus replication cycle as follows. SARS-coronaviruses replicate in the cytoplasm of infected host cells. Their replication complexes are associated with a reticulovesicular network of modified endoplasmic reticulum (ER) that integrates convoluted membranes and interconnected double membrane vesicles (Knoops et al., 2008). Viral RNA released by host cell fusion with the incoming virion is translated to express viral proteins. Multiple copies of the nucleoprotein enclose and package the genomic RNA. Spike protein, M- and E-proteins are inserted into the membrane of the rough ER and transported from the ER-to-Golgi intermediate compartment to meet the nucleocapsid and assemble into particles by budding; M-protein playing a pivotal role, interacting with all viral assembly partners. Virions are transported through the constitutive secretory pathway out of the cell (De Haan and Rottier, 2005). Increasing cellular MG in virally-infected cells is expected to increase the modification of arginine residues of viral proteins – particularly nucleoprotein, spike protein and M-protein. Modification in functional sites of viral proteins, typically highly structured domains, converts cationic, hydrophilic arginine residues to uncharged hydrophobic MG-H1 residues. This produces protein misfolding, binding of misfolded proteins by heat shock proteins and ubiquitin ligases for degradation. Replication of SARS-CoV-2 is thereby slowed or terminated. Where viral proteins are modified by MG before folding, the change in hydrophobicity will likely impair correct folding and also direct the nascent polypeptide for ubiquitination and proteolysis. If some virions escape this proteotoxicity, MG modification on the spike protein may block or impair cell infectivity and thereby enhance viral immunogenicity; *cf.* β-propiolactone – an approach used in a SARS-CoV-2 vaccine in clinical evaluation (Gao et al., 2020). Indeed, further investigations may be merited to explore the use of MG modification to produce inactive virus for vaccine development studies. Most vaccines against SARS-CoV-2 in development contain whole or fragments of the spike protein (Jeyanathan et al., 2020).

Doxorubicin, paclitaxel and BBGD are expected to increase the cellular concentration of MG in human host tissues other than the lung. This may be advantageous as recent evidence suggests SARS-CoV-2 may directly infect endothelial cells of the kidney, heart and liver (Varga et al., 2020) and renal tubular epithelium and glomerular podocytes (Su et al., 2020). Increase of MG at these nonpulmonary sites may decrease viral load and decrease risk of vascular and renal complications of COVID-19. Relatively short-term treatment with drugs increasing cellular MG may be beneficial in patients with COVID-19. We acknowledge that, contrary to this, chronic increase of MG in clinical diabetes is rather associated with increased risk of vascular complications—including diabetic kidney disease (Rabbani et al., 2016b).

In the search for drugs to repurpose for COVID-19 disease, we suggest doxorubicin and paclitaxel be considered. These drugs have not been proposed hitherto although they have been evaluated for antiviral activity, particularly with respect to inhibition of viral helicase (Ash and Diekema, 1987; Bergamini et al., 1992; Borowski et al., 2002; Briguglio et al., 2011). Paclitaxel also suppressed inflammation in a murine model of bacterial pneumonia (Mirzapoiazova et al., 2007). However, a concern is the established adverse effects of these drugs found in cancer chemotherapy: bone marrow suppression (primarily neutropenia) and peripheral neuropathy for paclitaxel, and cumulative congestive heart failure for doxorubicin. Toxicity is related to dose and duration of treatment (Rowinsky, 1997; Barrett-Lee et al., 2009). Drug treatment of COVID-19 may be shorter than in cancer chemotherapy: for example, median hospitalization time of patients surviving severe symptoms of COVID-19 was 28 days (Wang L. et al., 2020) and a typical course of cancer chemotherapy with paclitaxel and doxorubicin is 6 months or longer (Rowinsky, 1997; Barrett-Lee et al., 2009). If high potency antiviral effect of these agents is found, low dose and short duration of treatment is expected to decrease risk of adverse effects.

The approach to drug repurposing for COVID-19 developed herein addresses the intrinsic vulnerability of SARS CoV-2 proteome to endogenous reactive metabolites, with respect to the human host, and identified drugs to exploit this. Other strategies for repurposing drugs are based on SARS CoV-2 protein interactions with human host proteins and drugs targeted to them, virion endosomal processing and viral protease inhibition (Chen et al., 2020; Gordon et al., 2020; Wang M. et al., 2020).

CONCLUSIONS

We provide evidence of vulnerability of SARS-CoV-2 to modification and inactivation by MG. We also reveal, for the first time, increase in cellular concentration of MG in the antiproliferative activity of doxorubicin and paclitaxel—thereby providing a mechanistic rationale for repurposing of these drugs against SARS-CoV-2 and treatment of COVID-19 disease. Doxorubicin and paclitaxel may have potential for application for treatment of COVID-19 and may now be considered for evaluation in SARS-CoV-2 live virus cultures and animal models.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All methods were carried out in accordance with relevant guidelines and regulations and all experimental protocols were approved by University of Warwick Genetic Modification & Biosafety Committee (Project no. 305).

AUTHOR CONTRIBUTIONS

MA-M accessed protein sequence information on the SARS-CoV-2, applied RBD analysis and produced the molecular graphics images. HA cultured HEK293 cells, prepared and propagated plasmids, prepared stable transfectant cell lines and performed metabolite and drug treatment studies. PW and AF collated and curated data on arginine sequence environments. MX provided technical guidance and support to HA and performed SARS-CoV2, SARS-CoV, and MERS sequence alignments. PJT assisted with MG analysis. NR and PJT acquired the funding, designed and supervised the studies, contributed to the data analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.585408/full#supplementary-material

SUPPLEMENTARY TABLE 1 | SARS-CoV-2 viral proteome. *Sequence from reference (Chan et al., 2020).

SUPPLEMENTARY TABLE 2 | Receptor binding domain of SARS-CoV-2 viral proteome. RBD analysis was applied to SARS-CoV-2 proteome (see **Table S1**) using a window of 5 amino acids and gyration angle between two consecutive residues in the sequence of 100° (Gallet et al., 2000).

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SYSTEMATIC REVIEW
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Assessment of Chloroquine and Hydroxychloroquine Safety Profiles: A Systematic Review and Meta-Analysis

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Background: Chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) have recently emerged as potential antiviral and immunomodulatory options for the treatment of 2019 coronavirus disease (COVID-19). To examine the safety profiles of these medications, we systematically evaluated the adverse events (AEs) of these medications from published randomized controlled trials (RCTs).

Methods: We systematically searched MEDLINE, the Cochrane library, the Cochrane Central Register of Controlled Trials (CENTRAL), and the ClinicalTrials.gov for all the RCTs comparing CQ or HCQ with placebo or other active agents, published before June 20, 2020. The random-effects or fixed-effects models were used to pool the risk estimates relative ratio (RR) with 95% confidence interval (CI) for the outcomes.

Results: The literature search yielded 23 and 19 studies for CQ and HCQ, respectively, that satisfied our inclusion criteria. Of these studies, we performed meta-analysis on 6 studies for CQ and 18 studies for HCQ. We did not limit our analysis to published records involving viral treatment alone; data also included the usage of either CQ or HCQ for the treatment of other diseases. The trials for the CQ consisted of a total of 2,137 participants (n = 1,077 CQ, n = 1,060 placebo), while the trials for HCQ involved 2,675 participants (n = 1,345 HCQ and n = 1,330 control). The overall mild and total AEs were significantly higher in CQ-treated non–COVID-19 patients, HCQ-treated non–COVID-19 patients, and HCQ-treated COVID-19 patients. The AEs were further categorized into four groups and analyses revealed that neurologic, gastrointestinal (GI), dermatologic, and sensory AEs were higher in participants taking CQ compared to placebo, while GI, dermatologic, sensory, and cardiovascular AEs were higher in HCQ-treated COVID-19 patients compared to control patients. Moreover, subgroup analysis suggested higher AEs with respect to dosage and duration in HCQ group. Data were acquired from studies with perceived low risk of bias, so plausible bias is unlikely to seriously affect the main findings of the current study.

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Conclusions: Taken together, we found that participants taking either CQ or HCQ exhibited more AEs than participants taking placebo or control. Precautionary measures should be taken when using these drugs to treat COVID-19. The meta-analysis was registered on OSF (https://osf.io/jm3d9).

Registration: The meta-analysis was registered on OSF (https://osf.io/jm3d9).

Keywords: chloroquine, hydroxychloroquine, safety profiles, meta-analysis, adverse events

INTRODUCTION

The 2019 coronavirus disease (COVID-19) is caused by the novel and highly infectious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Since its discovery in December of 2019 in Wuhan, it has now caused a global pandemic. As of June 20, 2020, there were 8,735,394 confirmed cases and 461,786 deaths from the disease, which brings the mortality to approximately 5.3%. Thus, significant efforts have been made to develop a vaccine for SARS-CoV-2. Although it is estimated that vaccine development will take at least 12–18 months (Amanat and Krammer, 2020), two medications—chloroquine (CQ) and hydroxychloroquine (HCQ)—have emerged as possible contenders to treat COVID-19.

Emerging evidence has suggested that these drugs are effective in treating SARS-CoV-2 *in vitro* (Vincent et al., 2005; Liu et al., 2020). Viral replication begins when the virus attaches and penetrates the host cell. In the case of SARS-CoV-2, it uses its surface unit (S1) of the S protein to attach to the angiotensin-converting enzyme 2 (ACE2) receptor, which facilitates viral entry (Hoffmann et al., 2020). When African green monkey kidney VeroE6 cells were pretreated for an hour with CQ or HCQ prior to four different multiplicities of infection by SARS-CoV-2, both drugs prevented viral entry as well as post-entry stages of SARS-CoV-2 infection (Liu et al., 2020). Inhibition of viral entry may be due to the interference of terminal glycosylation of the ACE2 receptor (Vincent et al., 2005). Additionally, CQ and HCQ can alkalinize the phagolysosome, which disrupts the pH-dependent steps of viral fusion and uncoating—processes that are absolutely essential for viral replication (Rolain et al., 2007).

Moreover, both CQ and HCQ have immunomodulatory properties (Schrezenmeier and Dörner, 2020) that may be beneficial in extreme, life-threatening COVID-19 cases. Indeed, there has been a recent surge in COVID-19 patients with severe hyper immune activity, known as the *cytokine storm syndrome* (Mehta et al., 2020). In this patient population, immunosuppression is likely to be beneficial, since the over-active immune response is paradoxically causing more harm than benefit to the patients. Therefore, CQ and HCQ have recently become appealing due to their antiviral and anti-inflammatory properties, which may help treat COVID-19, especially under dire circumstances.

Although the promising findings suggest that CQ and HCQ are great candidates, much concern exists regarding their mechanisms, effective dosing regimen, clinical efficacy, and adverse effects with respect to COVID-19. Indeed, our current knowledge on CQ and HCQ are derived from non–COVID-19 patients treated for diseases such as malaria, rheumatoid arthritis, and systemic lupus erythematosus. The rise in popularity of these drugs as potential medications to treat COVID-19 and the current desperate need for better therapeutics have fueled rapid and ongoing research and clinical trials (Cortegiani et al., 2020) to further elucidate their antiviral and anti-inflammatory properties, pharmacodynamics, and safety profiles with respect to COVID-19.

Currently, the safety profiles of these drugs for COVID-19 are not entirely known due to the lack of large clinical trials, as well as sparse randomized controlled trials (RCTs). Moreover, the drugs have a narrow therapeutic range, which presents another challenge when using these drugs (Frisk-Holmberg et al., 1983; Touret and de Lamballerie, 2020). We therefore designed a meta-analysis to assess CQ/HCQ AEs in non–COVID-19 and COVID-19 patients. We believe that despite the shortcomings, comprehensively evaluating the existing data on these drugs can provide powerful and valuable insights regarding their safety profiles, which will not only drive future clinical trials, but also help health professionals make informed decisions.

METHODS

The meta-analysis was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The PRISMA flow diagram was included in the **Supplementary Materials**.

LITERATURE SEARCH AND INCLUSION CRITERIA

A comprehensive search strategy was designed to retrieve relevant clinical data from published literature. Our objective was to identify all RCTs that compared the safety profiles of CQ or HCQ with placebo or other active agents. We searched MEDLINE, the Cochrane Library, the Cochrane Central Register of Controlled Trials (CENTRAL), and the ClinicalTrials.gov for all the RCTs comparing CQ or HCQ with placebo or other active agents, published before June 20, 2020. We also searched conferenced proceedings to acquire relevant papers. Medical subject headings (MeSH terms) and keywords such as "randomized controlled trial," "adverse effects," "tolerability,"

"toxicity," and "side effects" were used. This review was not restricted to studies conducted in the English language; it includes records from any countries that compared CQ or HCQ with placebo or other active agents, since there is a wealth of information in RCTs from many different countries.

Due to the lack of large clinical trials and small numbers of RCTs, we decided to include all the RCTs reporting adverse events (AEs) in patients with different disease conditions, including rheumatoid arthritis, systemic lupus erythematosus, infectious diseases such as HIV infection, and immune diseases such as Primary Sjögren's Syndrome. We included all RCTs in adult patients that compared CQ or HCQ with other active agents or placebo.

To be included in the analysis, the study had to fulfill the following criteria: (1) randomized trials which could be open-label, single-blind, double-blind, or parallel group studies; (2) use of CQ or HCQ as one of the interventions; (3) studies comparing CQ or HCQ with placebo or other active agents; and (4) available data on safety and tolerability data for CQ or HCQ.

Studies were excluded from meta-analysis if: (1) they presented data on children only; (2) they lacked placebo group; (3) study did not present safety and tolerability outcomes; (4) full text could not be sourced; (5) CQ or HCQ was used in combination with other drugs.

DATA COLLECTION AND OUTCOME MEASURES

Bibliographic details and abstracts of all citations retrieved by the literature search were downloaded to EndNote X9. All studies were screened and evaluated by two independent reviewers (LR and PT), which were then checked by a third reviewer (SY). Discrepancies were resolved by discussion in group conferences. Completed data were then thoroughly checked by two additional reviewers (WX and JO). Data including first author, year of publication, trial design, country where studies took place, purpose of treatment, trial duration, dosage regimen, outcomes and AEs were extracted using a standardized form and presented in table format. Safety evaluation included monitoring of AEs and vital signs. Withdrawals due to AEs were reported.

STUDY QUALITY ASSESSMENT AND RISK OF BIAS

Risk of bias in the individual studies included for meta-analysis was assessed using the Cochrane risk assessment tool (Higgins et al., 2011). The assessment was performed by two independent reviewers (WX and JO) and further checked by two additional reviewers (LR and PT). The completed information is provided in **Supplementary Table S1**.

STATISTICAL ANALYSIS

Comparison of safety and tolerability outcomes was made between interventions by pooling data from studies using a direct meta-analysis technique. All terminology used when analyzing data was in accordance with the Common Terminology of Clinical Adverse Events handbook. Outcomes were summarized as relative risk ratios. Random-effects model (Barili et al., 2018) was used to pool the risk estimates relative ratio (RR) with 95% confidence interval (CI) for the outcomes. If $I^2 \geq 40\%$, the heterogeneity is high. Although we did not alter this in our software output, but $I^2 < 0\%$ may be considered as $I^2 = 0\%$. We analyzed results from RCTs that had placebo controls. Subgroup analyses were performed to see the effects of different age, duration, and dosage on relative risk of total AEs. For the HCQ studies, subgroup analysis of different pathologies on relative risk on total AEs was also assessed. Random-effects meta-regression models were used to test whether the relative risk of total AEs was affected by the age, dosage, or trial duration. Comparisons with no events in either group were excluded. I^2 statistics was included in all the meta-analyses that were performed, which is a percentage of variance attributed to study heterogeneity. Heterogeneity tests were performed. Publication bias was conducted with restricted maximum likelihood method. Sensitivity analyses were conducted by leaving one study out, or by removing all studies with zero events. Analyses were performed using STATA 16 (Stata, College Station, TX, USA). Sensitivity analyses was performed with OpenMeta[Analyst] (CEBM, Brown University) or STATA 16.

RESULTS

Process of Identifying Eligible Clinical Trials

We identified records that involved either CQ (n = 2,577) or HCQ (n = 1,689). Of the published records we identified, we initially screened them through the titles and abstracts to examine if they were relevant to our objective of identifying safety profiles for CQ and HCQ. Therefore, 170 and 26 records were initially excluded for CQ and HCQ, respectively. Of the remaining ones (n = 70 for CQ and n = 84 for HCQ), we performed a more thorough review using the inclusion and exclusion criteria described in the methods. In total, 23 CQ and 19 HCQ studies satisfied our requirements. The literature search strategy used for each database was listed in the supplementary materials. Therefore, a total of 6 studies and 18 studies were used for data extraction for CQ and HCQ, respectively (**Figure 1**).

Characteristics of Trials, Patients, and Interventions

Table 1 describes the characteristics of the trials, patients, and interventions of CQ, while **Table 2** describes the same parameters for HCQ. The trials indicated with asterisks next to the primary author's last name were the trials used for our meta-analyses. As shown in the tables, we did not restrict our systematic review to just the United States. Additionally, investigators used CQ as treatment options for breast cancer (Amanat and Krammer, 2020), malaria (Beck et al., 2020), hepatitis (Vincent et al., 2005), viral infections (Rolain et al.,

2007), and lupus erythematosus (Amanat and Krammer, 2020). To conduct our meta-analysis for CQ, we used 6 double-blinded, placebo-controlled, randomized studies that used CQ for the treatment of breast cancer, autoimmune hepatitis, dengue fever, and influenza. Age of participants ranged from 22 to 57 years old. Dosing regimen ranged from approximately 107 mg/day to 1,000 mg/day. Of these studies, general findings reported in the studies noted that CQ did not have a significant effect when compared with placebo. However, of the studies that compared CQ with other medications, the authors noted that CQ was generally more effective.

Similarly, the 19 HCQ studies (**Table 2**) that we examined were conducted from a plethora of countries and used HCQ to treat a myriad of disorders, which included dermatologic disorders (Amanat and Krammer, 2020), rheumatoid arthritis (Rolain et al., 2007), HIV (Liu et al., 2020), Primary Sjögren's Syndrome (Liu et al., 2020), graft-versus host disease (Amanat and Krammer, 2020), diabetes (Liu et al., 2020), chronic spontaneous urticaria (Amanat and Krammer, 2020), dementia (Amanat and Krammer, 2020), kidney failure (Amanat and Krammer, 2020), cardiovascular disease (Amanat and Krammer, 2020), and COVID-19 (Rolain et al., 2007). To conduct our meta-analysis for HCQ, we used RCTs that were pilot studies (one specifically for COVID-19), 3 open-label, 1 single-blinded, and the rest double-blinded. These studies are shown with asterisks next to the primary author's last name in the table. For these particular records, age of participants ranged from 33 to 70 years. Dosage schedule ranged from 200 mg/day to 1,200 mg/day, with a mode of 400 mg/day, depending on the treated disorder. COVID-19 patients required a higher dosage (>400 mg/day), but a lower duration (<2 weeks) relative to other treated conditions. General outcomes from about a third of the studies revealed that HCQ had no significant effect, while the rest of the studies showed that it was effective for the disorders.

Mild, Severe, Total AEs, and Withdrawals Due to AEs From Trials Involving CQ and HCQ in Non–COVID-19 Patients

The CQ meta-analyses of mild, serious, total AEs, and withdrawals due to AEs were based on 6 comparisons between CQ and placebo (control), while the HCQ meta-analyses of mild, serious, total AEs, and withdrawals due to AEs were based on 16 comparisons between HCQ and placebo (control), as depicted in **Figure 2**. When assessing mild AE (**Figure 2A**), the overall relative risk (RR) of CQ compared with placebo was 2.17 (95% CI 1.36–3.45, $p < 0.01$), while the overall RR of HCQ compared with placebo was 1.35 (95% CI 1.13–1.61, $p < 0.01$). The RR for severe AEs (**Figure 2B**), however, was insignificant for both drug usage when compared with placebo. When assessing total AEs of either drug compared with placebo (**Figure 2C**), the combined RR for CQ was 2.30 (95% CI 1.39–3.79, $p < 0.01$), while for HCQ it was 1.34 (95% CI 1.13–1.60, $p < 0.01$). There was statistical evidence of overall heterogeneity between CQ trials with regard to total AEs ($I^2 = 59.51\%$). Withdrawals due to AEs was near significant with CQ compared with placebo. As evident in **Figure 2D**, the overall RR was 2.03 (95% CI 1.01–4.07, $p = 0.05$). There was no evidence of heterogeneity ($I^2 = 0\%$). Taken together, these data suggest that both drugs induced higher mild and total AEs as compared to control.

System Analyses From Trials With CQ and HCQ in Non–COVID-19 Patients

Based on the reported AEs, we divided our analyses to examine four groups: neurologic, gastrointestinal (GI), dermatologic, and ophthalmic AEs. Neurologic AEs reported by participants included headache, dizziness, neuropathy/seizure, or other central nervous system (CNS) related AEs; GI AEs included vomiting, nausea, abdominal pain, diarrhea, liver dysfunction, or non-specific GI AEs; dermatologic AEs included rash, itchiness, dryness; and sensory AEs included blurred vision, pain, or auditory problems. With the usage of CQ, there was a significant increase in all four groups of AEs (**Figure 3**). The overall RR was 2.73 (95% CI 2.12–3.51, $p < 0.01$) for neurologic AEs; 2.84 (95% CI 2.06–3.93, $p < 0.01$) for GI AEs; 1.88 (95% CI 1.10–3.23, $p < 0.05$) for dermatologic AEs; and 4.60 (95% CI 1.66–12.71, $p < 0.01$) for sensory AEs. No heterogeneity between the trials were observed. With the usage of HCQ, there was no significant increase in any of the groups that we examined. These data suggest that patients treated with CQ experienced more neurologic, dermatologic, ophthalmic, and GI AEs relative to placebo control, while patients treated with HCQ did not experience more of these AEs compared to control.

Further analyses on heterogeneity, as well as publication bias, can be seen in **Supplementary Figures S4–S7**. Study and quality assessment can be seen in **Supplementary Table S1**. Risk of bias was assessed using eight different categories with judgment of risk indicated as either positive (low risk) or negative (high risk). The majority of the studies used in this meta-analysis were deemed low risk by two independent reviewers. We therefore believe that plausible bias would unlikely affect the key findings of the current study.

Mild, Severe, Total AEs, and Withdrawals Due to AEs From COVID-19 Studies Involving HCQ

The HCQ meta-analyses of mild, serious, total AEs, and withdrawals due to AEs were based on five comparisons between HCQ and placebo (control) in COVID-19 studies, as depicted in **Figure 2**. When assessing mild AE (**Figure 4A**), the overall relative risk (RR) of HCQ compared with placebo was 3.25 (95% CI 1.59–6.64, $p < 0.01$). The RR for severe AEs (**Figure 4B**), however, was insignificant. When assessing total AEs of HCQ compared with placebo (**Figure 4C**), the combined RR was 2.79 (95% CI 1.49–5.25, $p < 0.01$). Withdrawals due to AEs was not significant. As in **Figure 4D**, the overall RR was 2.13 (95% CI 0.97–4.67, $p = 0.06$). There was no evidence of heterogeneity ($I^2 = 0\%$). Taken together, these data suggest that HCQ induced higher mild and total AEs as compared to control in patients with COVID-19.

Stratification of the AEs into distinct groups revealed that COVID-19 patients treated with HCQ exhibited increased dermatologic (overall RR 3.23, 95% CI 1.01–10.33, $p < 0.01$), GI

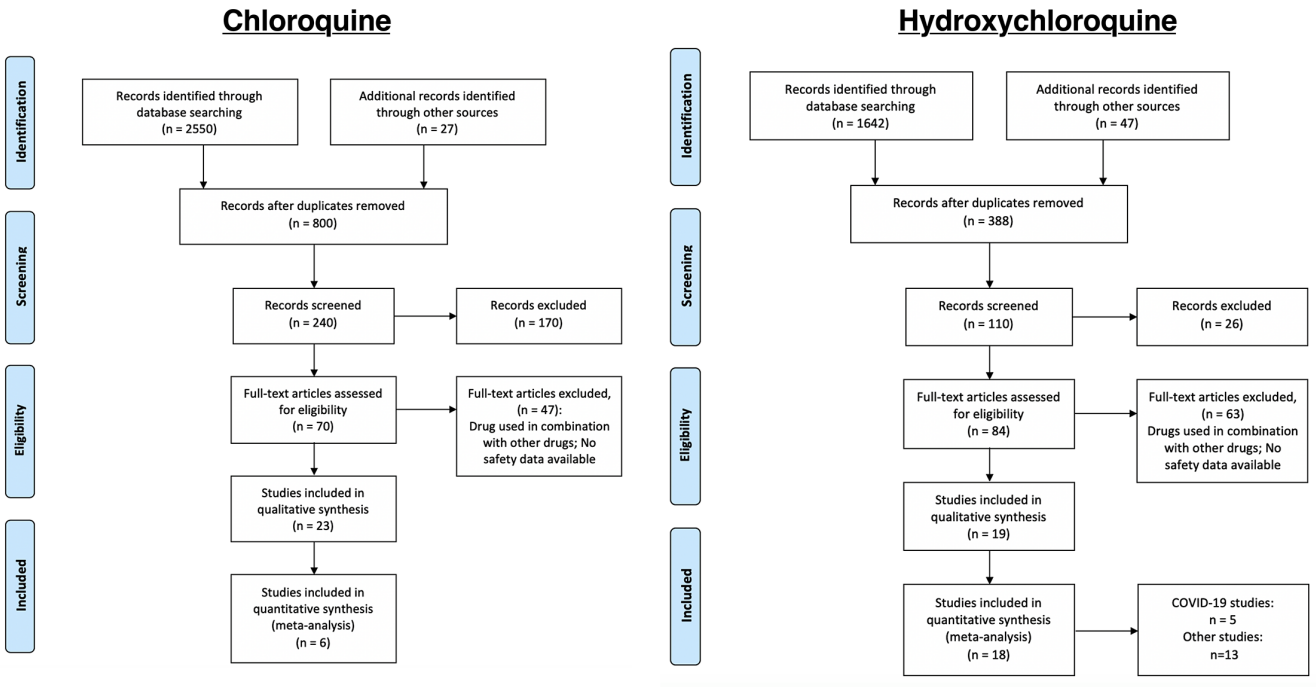

FIGURE 1 | Process of identifying eligible clinical trials. Records were identified through MEDLINE, CENTRAL, and ClinicalTrials.gov. We used the same process of study collection for both CQ and HCQ. We performed an initial screening, followed by a more stringent screening using our selection criteria. The studies that remained after all the exclusion were the ones used for data extraction. In total, we identified 23 and 17 studies for CQ and HCQ, respectively, which are described in, **Table 1** and **2**. Of those studies, 6 CQ and 16 HCQ records are controlled RCTs, so we used these studies for our data analysis.

(overall RR 5.69, 95% CI 2.42–13.35, $p < 0.01$), sensory (overall RR 4.70, 95% CI 1.09–20.20, $p < 0.01$), and cardiovascular (overall RR 4.98, 95% CI 1.65–15.03, $p < 0.01$) AEs relative to control patients. There was evidence of heterogeneity between trials with respect to GI AEs ($I^2 = 84.57\%$).

Stratification of All AEs

To fully appreciate the wealth of information from the RCTs from all the CQ/HCQ reports, we constructed a flow chart that contains information on the number of participants who experienced a certain AE, as well as the percentages. Four groups (CNS, GI, skin, and sensory) underwent meta-analyses (**Figure 4**), since they had robust records in the studies that we examined. In **Figure 5**, panels A and B show the charts for CQ and HCQ, respectively. The 6 CQ studies contained a total of 1,077 participants for CQ-treated group and it contained a total of 1,060 participants for placebo-treated. Of these participants, 435 (40.4%) and 270 (25.5%) AE were reported in the CQ and placebo group, respectively. The highest reported AEs for the CQ group occurred in the CNS, with about 18.7% of overall CQ participants reporting headache, dizziness, neuropathy, or other CNS-related AEs. In contrast, placebo group had higher records for respiratory distress, such as coughing, sore throat, or running nose.

The 18 HCQ studies contained 1,345 participants for HCQ-treated group and 1,330 participants for control group. Of these participants, 802 HCQ-treated participants and 807 control participants were part of the COVID-19 studies, while 543 HCQ-treated participants and 523 control participants were part of the non–COVID-19 studies. Total AEs reported for HCQ was 489 (36.4%), while total AEs reported for control was 228 (17.1%). GI AEs, such as diarrhea, nausea, liver damage, abdominal pain, and other non-specific GI AEs seemed to be the most dominant for both groups. Interestingly, cardiovascular AEs were reported in three of the studies (hypertension, acute coronary syndrome, and bradycardia) in non–COVID-19 patients that we examined. For COVID-19 studies, QT Prolongation was reported most frequently. Together, these stratified data provide ample information regarding the percentage of participants who experienced specific AEs.

Subgroup Meta-Analysis for CQ and HCQ With Respect to Age, Duration, Dosage, and Treated Disorder

Since we found a significant increase in total AEs when taking either drugs, we tested whether differences in age, duration, or dosage had any bearing on the results. We therefore performed subgroup meta-analysis. First, we examined age (**Figure 6A**). We divided the CQ trials into two groups: participants <30 years old and participants ≥30 years old. We stratified the HCQ trials into two groups: participants <50 years old and participants ≥50 years old. These ages were chosen to ensure that there was robust

TABLE 1 | Characteristics of CQ studies.

Study	Study Type	Country	Treated Disorder (n patients)	Trial Duration (weeks)	Dosage	Summary of Outcomes	Intervention (n of patients)	Age (mean or median)	Total n of AEs	Total n of serious AEs
*Arnaout et al. (2019)	Double-Blinded, Placebo-Controlled, Randomized, Window of Opportunity Trial	Canada	Breast Cancer (70)	2–6	500 mg/day CQ or Placebo for 2–6 weeks	No significant effects	CQ: 46 Control: 24	57.4 ± 9.7 55.7 ± 8.4	35 8	0 0
Divala et al. (2018)	Open-Label, Randomized, Single-Centered, Three-Armed	United States/ Malawi	Placental Malaria (900)	20–28 of gestation to birth	Days 1-2: 600 mg Day 3: 300 mg≥ 4 weeks later (CQ-IPTt) or 600 mg at enrollment, then 300 mg/week until delivery (prophylaxis)	CQ IPTp was not better than SP-IPTp	CQ: 600 SP-IPTp: 300	33.00 ± 12.11 33.95 ± 11.91	5 3	0 0
*Terrabuio et al. (2019)	Double-Blinded, Interventional, Parallel-Group, Placebo-Controlled, Randomized, Single-Centered	Brazil	Autoimmune Hepatitis (AIH) (61)	156.4	250 mg/day for 36 months	CQ safely reduced relapse risk of AIH; no subgroup with greater benefit from CQ use	CQ: 31 Control: 30	37.7 ± 16.1 39.1 ± 16.9	17 5	0 0
Abreha et al. (2017)	Randomized	United States/ Ethiopia	Vivax Malaria (398)	6	25 mg/kg over 3 days	Primaquine (PQ) + CQ or Artemether-Lumefantrine (AL) reduced vivax malaria recurrence 5 folds over 1 year	CQ: 206 AL or AL+PQ: 192	Median: 18 CQ+PQ: 17 AL: 18 AL+PQ: 18	165 165	0 0
Grigg et al. (2018)	Open-Label, Randomized, Two-Armed	Australia/ Malaysia	Uncomplicated *Plasmodium Knowlesi* Malaria (123)	6	25 mg/kg at enrollment, 6, 24, and 48 h	Artemether-Lumefantrine (AL) was effective at treating *knowlesi* malaria	CQ: 58 AL: 65	Median: 31 Median: 30	25 29	0 0
Valecha et al. (2016)	Multicentric, Open-Label, Phase III Study	India	Acute, Uncomplicated *Plasmodium Vivax* Malaria (317)	≥6	CQ: 4 doses (total 10 tablets of 250 mg each) for 3 days	FDC of arterolane maleate (AM) and PQP cures *vivax* marlaria	CQ: 158 AM+PQP: 137	33.7 ± 13.45 33.2 ± 11.81	135 127	0 4
Siqueira et al. (2017)	Open-Label, Non-Inferiority, Randomized	Brazil	*Vivax* Malaria (380)	6	25 mg/kg over 3 days	Artesunate-Amodiaquine (ASAQ) is more effective than CQ at preventing *P. vivax* infection	CQ: 189 ASAQ: 190	34.7 ± 15.9 35.7 ± 16.4	52 68	0 5
Peymani et al. (2016)	Triple-Blinded, Placebo-Controlled, Randomized, Pilot	Iran	Hepatitis C (10)	8	150 mg/day for 8 weeks	CQ was potentially safe for HCV non-responders	CQ: 6 Control: 13	49 50	0 0	7 0
Grigg et al. (2016)	Open-Label, Randomized	Australia/ Malaysia	Uncomplicated *Plasmodium Knowlesi* Malaria (252)	6	25 mg/kg at enrollment, 6, 24, and 48 h after treatment	Artesunate-Mefloquine (AM) was highly effective at treating *P. Knowlesi* Malaria	CQ: 125 AM: 127	Median: 32 Median: 33	316 302	0 2
Chopra et al. (2014)	Assessor-Blinded, Parallel Efficacy, Randomized, Two-Armed	India	Musculoskeletal Pain and Arthritis Following *Chikungunya* virus infection (70)	24	250 mg/day for 24 weeks	No significant improvement over meloxicam	CQ: 38 Meloxicam: 32	50.2 45.4	7 5	0 0

(Continued)

TABLE 1 | Continued

Study	Study Type	Country	Treated Disorder (n patients)	Trial Duration (weeks)	Dosage	Summary of Outcomes	Intervention (n of patients)	Age (mean or median)	Total n of AEs	Total n of serious AEs
*Borges et al. (2013)	Double-Blinded, Placebo-Controlled, Randomized	Brazil	Dengue (129)	3 days	1,000 mg/day for 3 days	CQ reduced pain; improved well-being of patients; but did not affect disease duration	CQ: 63 Control: 66	31.64 ± 11.74	2 0	0 0
*Paton et al. (2011)	Double-Blinded, Placebo-Controlled, Randomized	Singapore	Influenza (1,516)	12	Week 1: 500 mg/day Weeks 2–12: 500 mg/week	No significant effects	CQ: 757 Control: 759	23.6 23.5	341 249	3 5
Awab et al. (2010)	Open-Label, Perspective, Randomized	Afghanistan	*Vivax* Malaria (536)	8	25 mg/kg for 3 days	CQ was effective for *Vivax* Malaria treatment	CQ: 268 DP: 268	Mean: 11 Median: 12	15 2	0 0
*Tricou et al. (2010)	Double-Blinded, Placebo-Controlled, Randomized	Vietnam	Dengue (307)	3 days	Days 1–2: 600 mg Day 3: 300 mg	CQ did not reduce viraemia/NSI antigenaemia (AG) in dengue patients	CQ: 153 Control: 154	22 22	18 6	0 0
*De Lamballerie et al. (2008)	Double-Blinded, Placebo-Controlled, Randomized	France	Chikungunya Infection (54)	5 days	Days 1–3: 600 mg/day Days 4-5: 300 mg/day	No significant effect on acute Chikungunya infection	CQ: 27 Control: 27	Range: 18-65	7 0	0 0
Villegas et al. (2007)	Double-Blinded, Placebo-Controlled, Randomized	Thailand	*Vivax* Malaria in Pregnancy (1,000)	Weekly till delivery	500 mg/week	CQ was safe and effective as a prophylaxis against *P. Vivax* during pregnancy	CQ: 500 Control: 500	26.1 ± 6.4 25.4 ± 6.3	2 1	0 0
Laufer et al. (2006)	Randomized	United States/ Malawi	Uncomplicated *Plasmodium Falciparum* Malaria (210)	4	Days 0–1: 10 mg/kg Day 2: 5 mg/kg	CQ was effective in Malawi after 12 years	CQ: 80 Sulfadoxine-Pyrimethamine: 87	2.6 ± 2.2 2.9 ± 2.2	0 0	0 0
Dunne et al. (2005)	Double-Blinded, Randomized	India	*Plasmodium Vivax* Malaria (199)	4	Days 1–2: 600 mg Day 3: 300 mg	CQ was tolerated as well, but was more effective	CQ: 102 Azithromycin: 97	30.0 ± 11.8 31.7 ± 11.6	33 20	2 0
Mucenic et al. (2005)	Pilot Study	Brazil	Remission of Autoimmune Hepatitis (32)	≥52	250 mg/day for ≥12 months	CQ group had lower relapse frequency	CQ: 14 Control: 18	27.29 ± 15.23 26 ± 13.59	18 0	0 0
Bezerra et al. (2005)	Double-Blinded, Randomized	Brazil	Lupus Erythematosus (33)	26.1	250 mg/day for 6 months	Clofazimine (CFZ) equally as effective as CQ diphosphate (CDP)	CQ: 17 CFZ: 16	34.4 34	21 21	0 0
Llanos-Cuentas et al. (2001)	Open-Label, Randomized, Comparison	Peru	Acute *Plasmodium Falciparum* Malaria (29)	4	Day 1: 600 mg Days 2–3: 300 mg	Atovaquone/Proguanil (A/P) much more effective than CQ	CQ: 14 A/P: 15	Range: 12–65	29 26	0 1
Hatz et al. (1998)	Comparative, Open, Parallel Group, Randomized, Single-Centered	Switzerland/ Tanzania	Acute *Plasmodium Falciparum* Malaria (26)	4	Day 1: 10 mg/kg Days 2–4: 5 mg/kg	CGP-56697 highly effective against *P. Falciparum* in this part of Tanzania	CQ: 130 CGP-56697: 130	Median: 2 Median: 2	17 6	0 0
Kofi Ekue et al. (1983)	Double-Blinded, Randomized	Zambia	Symptomatic *Falciparum* Malaria (99)	6	Day 1: 900 mg Days 2–3: 300 mg	No significant differences between MQ and CQ	CQ: 49 MQ: 50	Range: 13-51	62 45	0 0

TABLE 2 | Characteristics of HCQ studies.

Study	Study Type	Country	Treated Disorder (n patients)	Trial Duration (weeks)	Dosage	Summary of Outcomes	Intervention (n of patients)	Age	Total n of AEs	Total n of serious AEs
*Boulware et al. (2020)	Randomized, double-blind, placebo-controlled trial	United States and Canada	COVID-19	1	800 mg once, then 600 mg 6 to 8 h later, then 600 mg daily	HCQ did not prevent illness compatible with COVID-19	HCQ: 349 Control: 351	41 40	140 59	0 0
*Jun et al. (2020)	Randomized Pilot Study	China	COVID-19 (30)	1	400 mg/day for 5 days	Prognosis of common COVID-19 patients is good	HCQ: 15 Control: 15	50.5 ± 3.8 46.7 ± 3.6	4 3	0 0
*Cavalcanti et al. (2020)	Multicenter, randomized, open-label, controlled trial	Brazil	COVID-19	1	400 mg twice daily for 7 days	HCQ did not improve clinical status compared with standard care	HCQ: 221 Control: 227	51.3 ± 14.5 49.9 ± 15.1	67 40	2 2
*Mitjà et al. (2020)	Multicenter, open label, randomized controlled trial	Spain	COVID-19	1	800 mg on day 1, 400mg daily for 6 days	No benefit was observed with HCQ beyond the usual care	HCQ: 169 Control: 184	41.6 41.7	121 16	8 12
*Tang et al. (2020)	Multicenter, open label, randomized controlled trial	China	COVID-19	2-3	1,200 mg/d for 3 days and then 800 mg/d	HCQ did not result in a significantly higher probability of negative conversion of virus than control	HCQ: 70 Control: 80	48.0 44.1	21 7	2 0
*Boonpiyathad et al. (2017)	Single-Blind, Placebo-Controlled, Randomized	Thailand	Anti-Histamine Refractory Chronic Spontaneous Urticaria (CSU) (55)	12	400 mg/day for 12 weeks	HCQ was effective as an adjunct treatment for CSU	HCQ: 46 Control: 24	33.00 ± 12.11 33.95 ± 11.91	5 3	0 0
*Wasko et al. (2015)	Double-Blinded, Parallel-Arm, Placebo-Controlled, Randomized	United States	Pre-Diabetes (32)	13 ± 1	400 mg/day for 13 ± 1 weeks	HCQ improved both ß-cell function and insulin sensitivity in non-diabetic patients	HCQ: 17 Control: 15	>18	3 3	0 0
*Gottenberg et al. (2014)	Double-Blinded, Parallel-Group, Placebo-Controlled	France	Primary Sjogren's Syndrome (120)	48	400 mg/day Placebo or HCQ for 24 weeks, then 400 mg/day HCQ for 24 weeks	No significant effects	HCQ: 56 Control: 64	56.3 ± 11.9 55.6 ± 13.9	5 7	5 7
*Solomon et al. (2014)	Blinded, Crossover, Randomized	United States	Rheumatoid Arthritis and Insulin Resistance (30)	16	6.5 mg/kg HCQ or placebo daily for 8 weeks, then crossover to other arm for 8 weeks	No significant change in insulin resistance; minor improvements to total LDL cholesterol	15 (HCQ → Placebo) 15 (Placebo → HCQ)	56 ± 11.4 56 ± 11.4	2 0	0 0
*Rotaru et al. (2014)	Randomized, Pilot, Triple Masking	United States	Kidney Failure, Chronic Cardiovascular Disease Arteriosclerosis (8)	25	200 mg/day for 10 days ± 4 days, then 200 mg twice daily for 6 months	Terminated (Lack of Funding)	HCQ: 7 Control: 1	18-65: 4 >65: 3 18–65: 1	2 0	0 0
*Paton et al. (2012)	Double-Blinded, Randomized, Placebo-Controlled	United Kingdom	HIV (83)	48	400 mg/day for 48 weeks	No significant effects	HCQ: 42 Control: 41	37.1 ± 7.7 38.3 ± 10.8	41 26	0 0
*Fong et al. (2007)	Double-Blinded, Placebo-Controlled, Randomized	United States	Chronic Graft-Versus-Host Disease (95)	55	121 days at 800 mg/day	No effects	HCQ: 46 Control: 49	48 46	1 1	0 0
*Gerstein et al. (2002)	Double-Blinded, Placebo-Controlled, Randomized	Canada	Type 2 Diabetes Mellitus (135)	78.2	300 mg first month, 450 mg s, and 600 mg third, daily	HCQ improved glycemic control in patients with poorly controlled type 2 diabetes	HCQ: 69 Control: 66	57.5 57.5	3 1	0 0
*Van Gool et al. (2001)	Double-Blinded, Parallel-Group, Multicenter	The Netherlands	Dementia in Early Alzheimer's Disease (168)	78.2	<65 kg: 200 mg/day >65 kg: 400 mg/day; 18 months	No significant effects	HCQ: 83 Control: 85	70.4 ± 8.3 70.7 ± 8.5	20 15	5 2

(Continued)

TABLE 2 | Continued

Study	Study Type	Country	Treated Disorder (n patients)	Trial Duration (weeks)	Dosage	Summary of Outcomes	Intervention (n of patients)	Age	Total n of AEs	Total n of serious AEs
*Sperber et al. (1995)	Double-Blinded, Placebo-Controlled, Randomized	United States	HIV-1 (40)	8	800 mg/day for 8 weeks	HIV-1 RNA declined significantly in the HCQ group over 8 weeks; increased in placebo group	HCQ: 19 Control: 19	39.1 ± 6.6 40.6 ± 12.5	0 0	0 0
*The HERA Study Group (1995) *Clark et al. (1993)	Double-Blinded, Placebo-Controlled, Randomized Double-Blinded, Placebo-Controlled, Randomized	Canada Mexico	Early Rheumatoid Arthritis (120) Early Rheumatoid Arthritis (126)	36 24	200 mg/day for 2 weeks. If no side effects, 400 mg/day 400 mg/day for 24 weeks	Improved pain and disability of recent arthritis HCQ effectively improved early rheumatoid arthritis	HCQ: 59 Control: 60 HCQ: 65 Control: 65	53 ± 13.5 53 ± 14.8 39 36	25 19 28 28	1 0 0 1
*Kruize et al. (1993)	Double-Blinded, Crossover, Placebo-Controlled	The Netherlands	Primary Sjogren's Syndrome (19)	52.2	400 mg/day for 12 months	No significant effects	10 (HCQ → Placebo) 9 (Placebo → HCQ)	52.8 ± 16.1 51 ± 15.8	0 0	1 0
Faarvang et al. (1993)	Double-Blinded, Multicenter, Parallel-Group, Placebo-Controlled, Randomized	Denmark	Rheumatoid Arthritis (91)	26.1	250 mg/day HCQ and 2g/day Placebo OR 250 mg/day + S for 6 months	HCQ and Sulphasalazine (S) had no improvement over HCQ alone	62 (HCQ + Placebo & HCQ + Sulphasalazine) 29 (Placebo + Sulphasalazine)	61 61	7 0	0 0

comparison, since the number of RCTs was very limited. We found that there was no group difference in either case, which suggests that age (younger vs. older) had no bearing on the total AEs experienced in participants.

Next, we assessed whether duration had any relevance to total AEs (**Figure 6B**). CQ trials were divided into two groups: <1 week and ≥1 week. Although there was no significant difference between the two groups for CQ, there was evidence of heterogeneity (I^2 = 55.79%) between the two groups. It is important to note that when these studies were separately analyzed, there was statistical significance for either group ($p < 0.05$). Upon close inspection of the HCQ trials, we noted that trials for non–COVID-19 patients generally had longer duration than trials for COVID-19 patients. We therefore divided HCQ trials into two groups: ≤2 weeks and >2 weeks. This division allowed us to test whether there is a difference between RR with respect to trial duration for COVID-19 patients (shorter duration) and non–COVID-19 patients (longer duration). We found that with this division, there was a significant difference between the two test groups ($p = 0.03$), with evidence of overall heterogeneity between the two groups (I^2 = 78.95%).

Furthermore, to determine if there were significant differences between a low versus a high dosage with respect to total AEs for either drug according to their respective median values. We stratified the dosages of the CQ studies into two groups: <500 mg/day and ≥500 mg/day (**Figure 6C**). This arbitrary grouping ensured that we included enough studies in each group for CQ, since the number of RCT for CQ is limited. There was no statistical group difference for CQ reports. For the HCQ studies, we used >400 mg/day and ≤400 mg/day, since this grouping divided the non–COVID-19 studies from the COVID-19 studies. As evident in our meta-analyses, there was significant difference between 2 subgroups for HCQ, in which the overall RR of total AEs was 1.72 (CI 95% 1.15–2.58, $p < 0.05$). Additionally, there was evidence of heterogeneity (I^2 = 82.97%) between the two groups. Taken together, this indicates that a high dosage of HCQ (>400 mg/day) could lead to a significant increase in total AEs compared to a lower dosage.

Finally, we stratified for indication of use in another subgroup analysis to assess whether the treated disorders impacted total AEs (**Supplementary Figure S1**). The overall RR was 1.74 (CI 95% 1.21–2.50, $p = 0.12$), which indicates that the underlying pathologies did not significantly impact total AEs in HCQ-treated patients. Upon closer inspection, the overall RR of total AEs was significant in COVID-19 patients taking HCQ; however, the other non–COVID-19 conditions did not exhibit this trend. The subgroup analysis was not conducted in CQ group due to the limited number of studies.

Taken together, there was no statistical evidence to suggest that age (younger vs. older) differentially affected the total AEs when using either drug. In contrast, there was statistical evidence to suggest that dosage and duration has a significant impact on total AEs in the HCQ-treated patients.

Meta-Regression Analyses for CQ and HCQ

Meta-regression analyses were performed to determine the relationship between RR and age, duration of trial, and

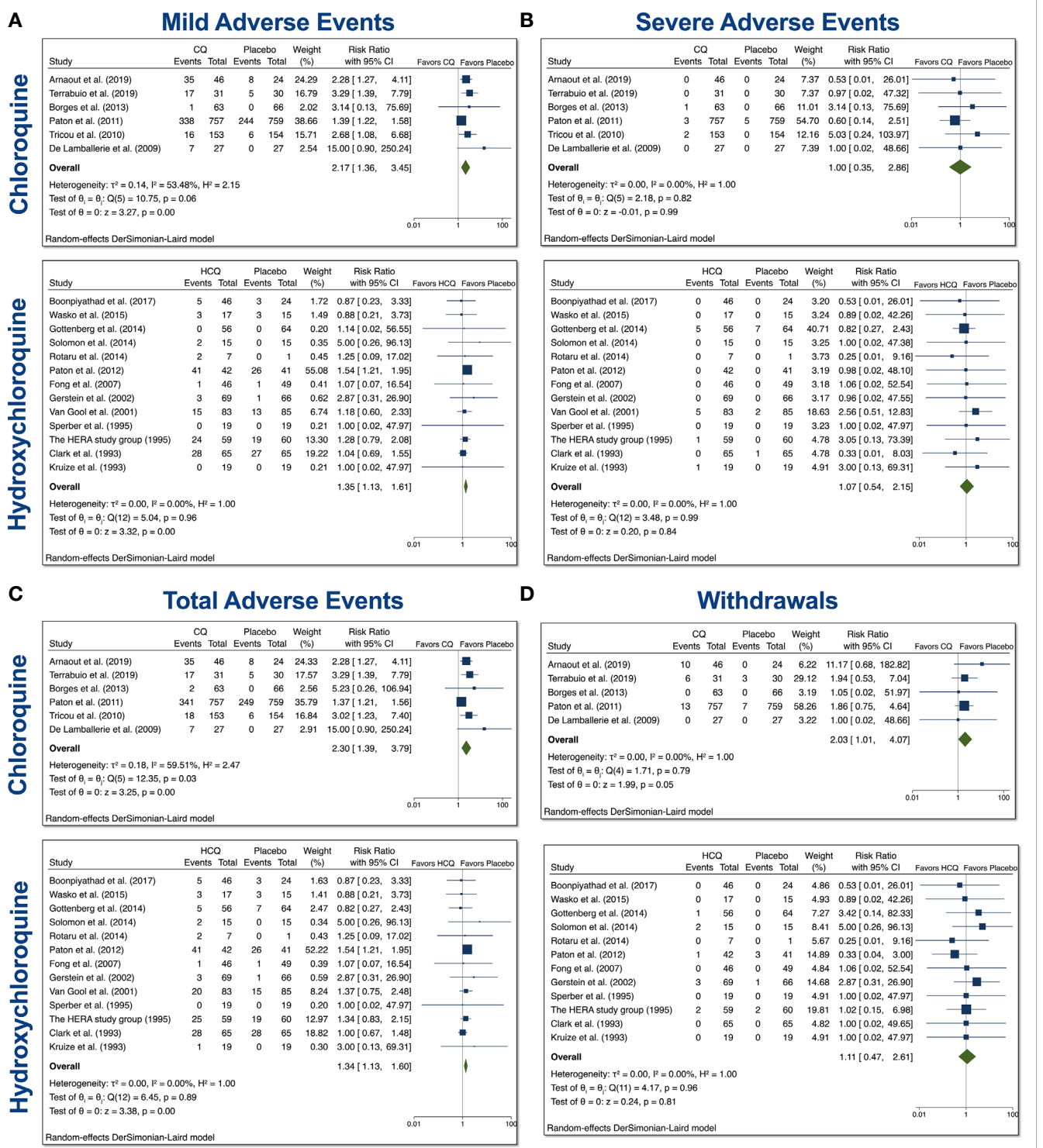

FIGURE 2 | Mild, severe, total AEs, and withdrawals due to AE from trials involving CQ and HCQ in non–COVID-19 patients. We performed 6 comparisons between CQ and placebo and 16 comparisons between HCQ and placebo, as evident in the forest plots. AEs were divided into **(A)** mild, **(B)** severe, and **(C)** total. **(D)** Additionally, we also examined withdrawals from trials due to AEs. Meta-analyses were performed. We tested heterogeneity between trials, as well as overall effect. Statistical data are displayed in the forest plots.

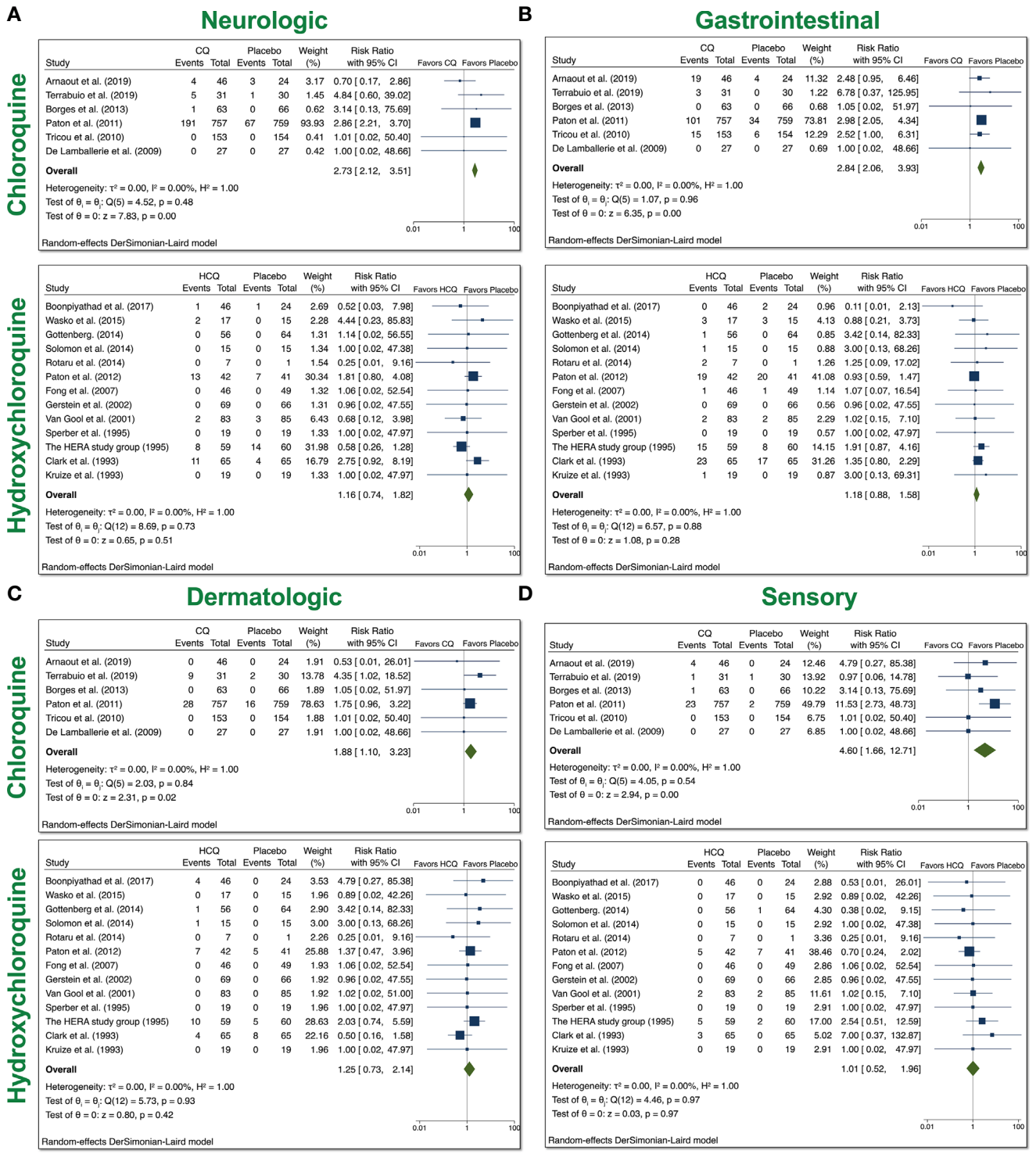

FIGURE 3 | System analyses from trials with CQ and HCQ in non–COVID-19 patients. We performed 6 comparisons between CQ and placebo and 16 comparisons between HCQ and placebo, as evident in the forest plots.. AEs were divided into four groups: **(A)** neurologic, **(B)** gastrointestinal (GI), **(C)** dermatologic, and **(D)** sensory AEs. Using meta-analyses, we tested heterogeneity between trials, as well as overall effect. Statistical data are displayed in the forest plots.

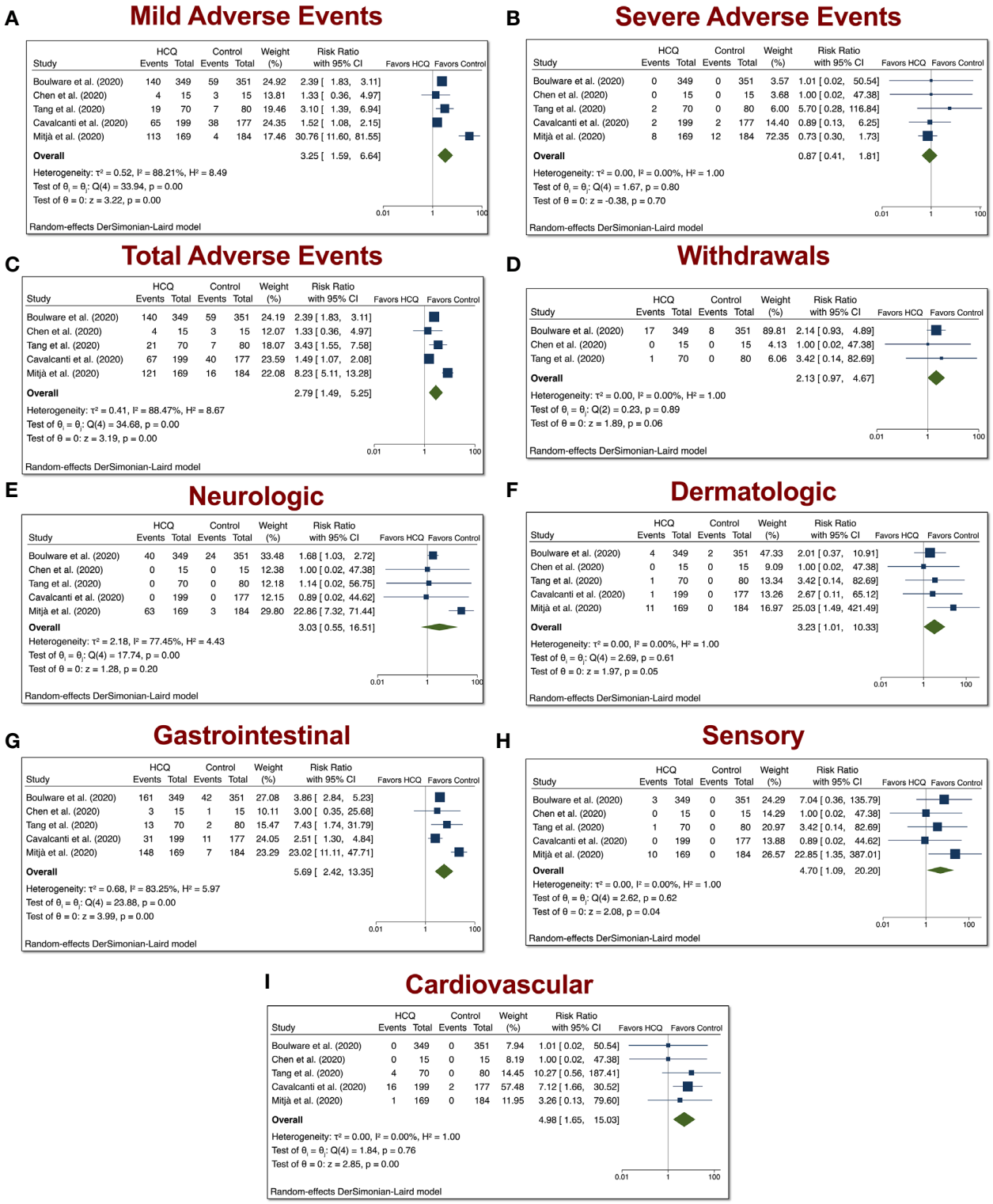

FIGURE 4 | Mild, severe, total AEs, and withdrawals due to AEs from COVID-19 studies involving HCQ. The HCQ meta-analyses of **(A)** mild, **(B)** severe, **(C)** total, **(D)** withdrawals due to AEs, **(E)** neurologic, **(F)** dermatologic, **(G)** gastrointestinal, **(H)** sensory, **(I)** and cardiovascular AEs were based on five comparisons between HCQ and control in COVID-19 studies.

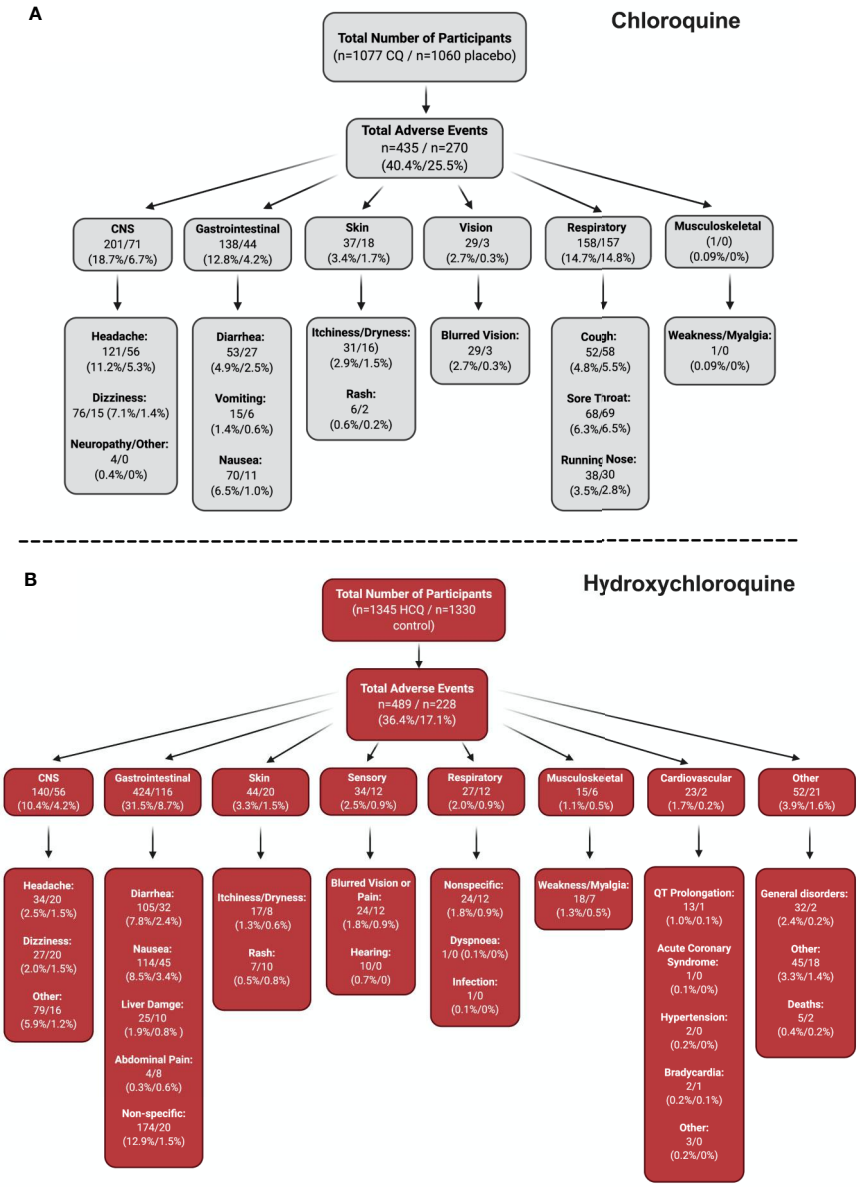

FIGURE 5 | Stratification of all AE. To fully appreciate the wealth of data regarding CQ and HCQ AE, we divided the AE into different categories. Panel **(A)** depicts the data for CQ, while panel **(B)** shows the data for HCQ. Both panels begin with the total number of participants in the studies (n = 6 CQ, n = 18 HCQ), which is then followed by the total number of AE. The AE were then divided into different systems, which is then broken down into specific AE. Figure was generated using BioRender.

dosage, as depicted in **Supplementary Figures S2, S3**. We examined if age of participants, duration of trial, or dosage has any effects on total AEs or withdrawals due to AEs. The size of the symbols indicates more weight toward a particular study. In all plots, the predicted regression lines and 95% confidence-interval lines are displayed. Regression of logarithm of RR of total AE with CQ and dosage revealed that dosage had an effect on total AEs. Age and duration of trial did not affect the total AEs for CQ.

DISCUSSION

The current pandemic with SARS-CoV-2 has relentlessly claimed thousands of lives and caused significant economic hardship. The urgent need for viable therapeutic options while vaccine development is in progress has resulted in the proposal of numerous antiviral medications (Beck et al., 2020). CQ and its derivative HCQ have been proposed as potential drugs to treat COVID-19. However, little is known regarding their safety profiles

due to the lack of RCTs. To address this urgent issue, we performed a systematic review and meta-analysis by pooling the existing published data of AEs for CQ and HCQ relative to control.

It is important to note that CQ/HCQ used for the treatment of chronic diseases generally had a longer duration regimen and lower dosage (**Table 2**). To take this into account, we separated the COVID-19 studies from the non–COVID-19 ones. We found that the usage of either drug increased the relative risk (RR) for mild and total AEs in non–COVID-19 patients (**Figure 2**). Further system analyses showed that overall participants in the CQ trials experienced more neurologic, GI, dermatologic, and sensory AEs (**Figure 3**). However, we did not observe a significant elevation in any of these AEs in HCQ-treated non–COVID-19 patients relative to control patients.

COVID-19 studies included five trials from patients treated with HCQ. We found a significant increase in mild and total AEs in HCQ-treated COVID-19 patients relative to control patients (**Figure 4**). Dermatologic, GI, sensory, and cardiovascular AEs were significantly elevated in COVID-19 patients treated with HCQ. Although cardiovascular AEs was not as common in the non–COVID-19 patients, it was more prevalent in the COVID-19 patients. This may be due to an increase in dosage given to COVID-19 patients.

Given the severity of cardiovascular AEs, it is critical to note that six studies reported cardiovascular AEs including hypertension, acute coronary syndrome, bradycardia, and QT prolongation (Gottenberg et al., 2014; Rotaru ea, 2014; Cavalcanti et al., 2020; Mitja et al., 2020; Tang et al., 2020). Although there were no cardiovascular AEs reported in the CQ studies that we analyzed, its cardiotoxicity has also been noted in a plethora of studies (Chatre et al., 2018). An excellent systematic review article by Chatre et al. documented cardiac complications that are attributed to CQ and HCQ (Chatre et al., 2018). In their review, they found that among other cardiovascular complications, conduction bundle or atrioventricular block were reported more frequently. Moreover, QT interval prolongation has been noted in numerous studies (Rey et al., 2003; Morgan et al., 2013; Chorin et al., 2020; van den Broek et al., 2020) and has also been found in studies involving COVID-19 patients (Cavalcanti et al., 2020). Severely prolonged QT interval can lead to lethal arrhythmias and sudden cardiac death. Therefore, the prevalence of these cardiovascular AEs warrants periodic electrocardiogram

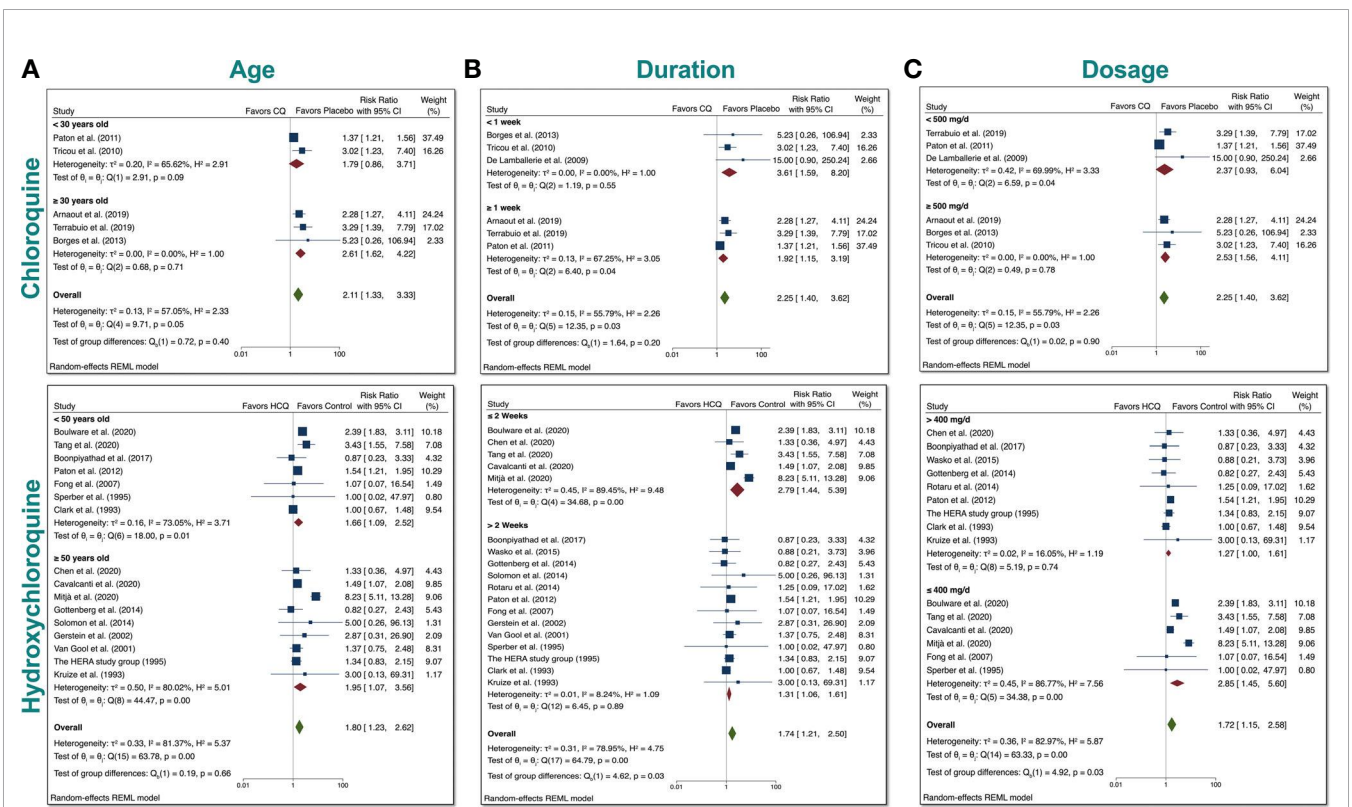

FIGURE 6 | Subgroup meta-analyses for CQ and HCQ with respect to age, duration, dosage. We stratified the dosages used in the studies for both CQ and HCQ into two subgroups. We then performed subgroup analysis for dosage and trial duration. **(A)** For age, we separated CQ trials into <30 years old and ≥30 years old, while we separated HCQ trials into <50 years old and ≥50 years old. **(B)** For drug duration, we divided CQ studies into <1 week and ≥1 week, while we divided HCQ studies into <6 months and ≥6 months. **(C)** And for dosage, we wanted to investigate if there was a difference in using <500 mg/day versus using ≥500 mg/day for CQ, and ≥400 mg/day versus <400 mg/day for HCQ. Statistical data are presented in the figures.

(ECG) monitoring when participants are undergoing these therapies, as cardiovascular AEs can be fatal.

Overall, participants who took CQ exhibited more AEs (40.4%) relative to control (25.5%, **Figure 5**). In the HCQ studies, 36.4% of total AEs were reported versus 17.1% for control. The high percentage of total AEs occurring with CQ participants is concerning, but consistent with the consensus that HCQ is a safer alternative to CQ (McChesney, 1983; Finbloom et al., 1985; Felson et al., 1990; Liu et al., 2020). When total AEs were stratified according to different organ systems, we found that CQ had more participants exhibiting CNS AEs (18.7%), while HCQ participants had more participants experiencing GI AEs (31.5%). It is worth noting that only 10.4% of HCQ participants exhibited CNS AEs. The extra hydroxyl group in HCQ may decrease the occurrence of CNS AEs. More mechanistic, controlled studies need to be performed to confirm this finding.

Furthermore, subgroup analyses (**Figure 6**) of CQ reports revealed no evidence in differences of RR of total AEs when studies were divided by age (younger vs. older), dosage (lower vs. higher) and duration (shorter vs. longer). When we performed meta-regression analyses (**Supplementary Figure S2**), there was a relationship between dosage and total AEs in the CQ group, which suggests that the subgroup meta-analyses for dosage would be more robust if more CQ RCTs existed. In contrast, subgroup analysis of HCQ reports suggested that lower duration (<2 weeks, **Figure 6B**) and higher dosage of HCQ (≥400 mg/day) could lead to more total AEs (**Figure 6C**). Indeed, the duration and dosage regimen of HCQ significantly differ for COVID-19 patients and non–COVID-19 patients. COVID-19 patients received higher dosage for a shorter duration, while non–COVID-19 patients received a lower dosage for a longer duration.

Given the long half-life of HCQ (Tett et al., 1989), it is plausible that the longer the duration of dosing regimen, or the higher the dosage, the more total AEs would be observed. Therefore, caution is recommended when taking higher dosage or longer duration of HCQ. Although we did not find a difference in total AEs when accounting for the different treated disorders (**Supplementary Figure S1**), this may be due to the limited number of studies for each disorder. However, upon closer inspection, there is evidence that COVID-19 patients experienced an overall RR of total AEs that was in favor of the control, while non–COVID-19 treated conditions such as rheumatoid arthritis and diabetes did not. Therefore, it is important to consider the underlying condition when examining the presented data, as this affects the dosing schedule and duration, which consequently impacts the occurrence and type of AEs.

Limitations

Here, we present a comprehensive analysis that reveals the increase in AEs associated with either CQ or HCQ. However, RCTs have several limitations when it comes to identifying adverse drug reactions or adverse events, including under-reporting, poor reporting, and lack of information on long-term outcomes. In addition, this systematic review and meta-analysis is limited due to the lack of large RCTs. For instance, although we did not observe an increase in severe AEs associated with taking either medication, there has been numerous records showing cardiovascular AEs. Moreover, due to the sparse RCTs, the analyses reported may be affected in a few instances according to the sensitivity analyses performed. These analyses took into account removing one study (**Supplementary Figures S8–S10**), or removal of all the studies that did not report any events (**Supplementary Figures S11–S13**). In this study, by including all the known RCTs in the meta-analysis, we were able to more confidently report our findings. Despite including all these studies, however, this meta-analysis would benefit significantly from larger RCTs, as this would provide better representations of both drugs' safety profiles. Indeed, several RCTs are currently ongoing that involve both medications, which would help drive future analyses.

CONCLUSIONS

Taken together, our data show that participants taking either CQ or HCQ experienced more mild and total AEs relative to placebo control. Precautionary measures should be taken when giving these medications for their therapeutic impact.

AUTHOR CONTRIBUTIONS

LR and PT designed the study. LR and PT screened and evaluated studies. LR performed statistical analyses. SY checked studies included. PT and SY checked statistical analyses. LR, WX, JO, and PT performed comprehensive characterization of studies. SY and NC provided expertise. LR, PT, and NC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.562777/full#supplementary-material

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Role of Endolysosomes in Severe Acute Respiratory Syndrome Coronavirus-2 Infection and Coronavirus Disease 2019 Pathogenesis: Implications for Potential Treatments

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Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is an enveloped, single-stranded RNA virus. Humans infected with SARS-CoV-2 develop a disease known as coronavirus disease 2019 (COVID-19) with symptoms and consequences including acute respiratory distress syndrome (ARDS), cardiovascular disorders, and death. SARS-CoV-2 appears to infect cells by first binding viral spike proteins with host protein angiotensin-converting enzyme 2 (ACE2) receptors; the virus is endocytosed following priming by transmembrane protease serine 2 (TMPRSS2). The process of virus entry into endosomes and its release from endolysosomes are key features of enveloped viruses. Thus, it is important to focus attention on the role of endolysosomes in SARS-CoV-2 infection. Indeed, coronaviruses are now known to hijack endocytic machinery to enter cells such that they can deliver their genome at replication sites without initiating host detection and immunological responses. Hence, endolysosomes might be good targets for developing therapeutic strategies against coronaviruses. Here, we focus attention on the involvement of endolysosomes in SARS-CoV-2 infection and COVID-19 pathogenesis. Further, we explore endolysosome-based therapeutic strategies to restrict SARS-CoV-2 infection and COVID-19 pathogenesis.

Keywords: endolysosome, endocytosis, two pore channel

INTRODUCTION

Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) that causes the pandemic disease known as coronavirus disease 2019 (COVID-19) (Contini et al., 2020; Gudbjartsson et al., 2020) is an enveloped virus that contains a large single-stranded RNA genome (Chan et al., 2020; Huang et al., 2020; Lu et al., 2020; Ren et al., 2020). SARS-CoV-2 belongs to the same beta-coronavirus family as does SARS-CoV that caused the SARS outbreak in China in 2002 (Cherry, 2004) and Middle East respiratory syndrome coronavirus (MERS-CoV) that caused the MERS outbreak in Saudi Arabia in 2012 (Zaki et al., 2012; Li and Du, 2019). Similar to other enveloped coronaviruses, SARS-CoV-2 enters host cells by endocytosis and uses host cell machinery for replication.

Spiked glycoproteins on the outer surface of coronaviruses are recognized by and bind to cell surface receptors such as angiotensin-converting enzyme 2 (ACE2) (Huang et al., 2006; Hoffmann et al., 2020b; Shang et al., 2020b) as well as possibly other co-receptors (Raj et al., 2013). Following binding, receptor-bound virus is endocytosed whereupon the viral genome is delivered into the cytoplasm; endocytosis mechanisms are pH-dependent and -independent (Dimitrov, 2004; White and Whittaker, 2016). Viruses that co-opt pH-independent mechanisms, an example of which is HIV-1, fuse with cell surface membranes and use endocytic pathways to achieve infection (White and Whittaker, 2016). Viruses that enter cells by pH-dependent mechanisms fuse with endosome membranes and use host factors associated with endosomes to enable viral entry into cells (Yang et al., 2004; White and Whittaker, 2016).

Coronaviruses use endolysosome-associated cathepsin B and L proteases under acidic conditions and are considered to be late penetrating viruses (late-entry kinetic mechanism) (Follis et al., 2006; Bosch et al., 2008; Millet and Whittaker, 2014; Coutard et al., 2020; Hoffmann et al., 2020a; Hoffmann et al., 2020b; Pranesh et al., 2020). Following entry, coronaviruses are released into the cytosol from endolysosomes or are targeted for degradation in lysosomes. In addition, some coronaviruses including SARS-CoV-2 can escape endolysosomes and replicate in autophagosome-like structures in the cytosol (Maier and Britton, 2012; Chen et al., 2014; Gassen et al., 2019; Gassen et al., 2020). Accordingly, it is important to focus attention on the role of endolysosomes in early stages of interactions between the virus and host cells as well as COVID-19 pathogenesis.

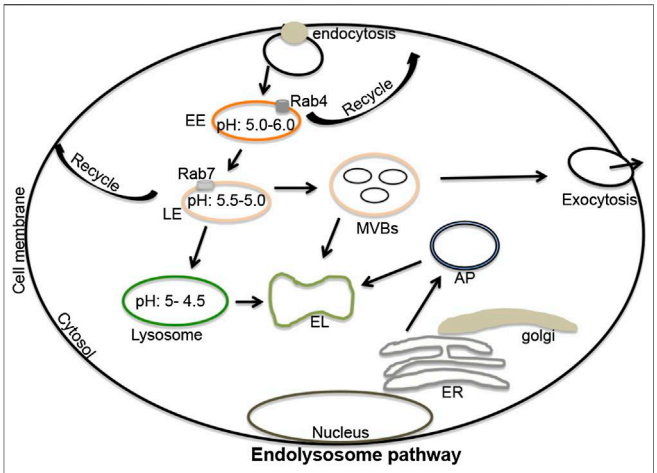

FIGURE 1 | The endolysosome pathway: Extracellular signaling molecules upon binding to cell surface receptors can be engulfed by endocytosis. These endocytosed vesicles can mature and differentiate into early endosomes (pH 5.5–6.0), late endosomes (pH 5.5–5.0), lysosomes (pH 5.0–4.5), and endolysosomes (a fusion process of lysosomes and late endosomes). Various marker substances can differentiate early from late endosomes including Rab4 (early endosomes), and Rab5 and Rab7 (late endosomes). Both early and late endosomes regulate recycling processes that return constituent molecules back to plasma membranes. Late endosomes can produce multi-vesicular bodies, which can fuse with lysosomes or can be released from cells in the form of extracellular vesicles (exosomes). Lysosomes regulate the degradation of extracellular materials in endolysosomes produced by fusions with late endosomes. Lysosomes can also fuse with autophagosomes to form autolysosomes; sites where extracellular and intracellular components are degraded. EL, endolysosomes; ER, endoplasmic reticulum; EE, early endosomes; LE, late-endosomes; MVBs, multi-vesicular bodies; AP, autophagosomes; Rab, ras-related protein 4, 5 and 7).

THE ACIDIC NATURE OF ENDOLYSOSOMES

Endosomes are formed from plasma membrane invaginations; a process known as endocytosis. These acidic organelles are categorized further as early, late and recycling endosomes; all with different compositions and hydrogen ion (H^+) content (Luzio et al., 2007; Huotari and Helenius, 2011; Gautreau et al., 2014). Rab4 and Rab5 are important components of early endosomes and function optimally at a pH range of 5.5–6.0. Early endosomes participate in signaling between the extracellular and intracellular environments (Pálfy et al., 2012; Villaseñor et al., 2016); they can recycle to plasma membranes thereby returning endocytosed constituents back to the cell surface (McCaffrey et al., 2001; Grant and Donaldson, 2009; Hsu and Prekeris, 2010). Alternatively, early endosomes can mature and transform into late endosomes (Bright et al., 2005; Luzio et al., 2007); these are differentiated from early endosomes by the expression of Rab7 and have an optimal pH range of 5.0–5.5 (Vanlandingham and Ceresa, 2009; Guerra and Bucci, 2016). Late endosomes can also recycle to plasma membranes (Guerra and Bucci, 2016), can produce multi-vesicular bodies from which extracellular vesicles (exosomes) originate, or can fuse with lysosomes (Piper and Luzio, 2001; Traub, 2010). The fusion of late endosomes with lysosomes generates endolysosomes under more acidic conditions ranging from pH 4.5–5.0 (**Figure 1**) (Mullock et al., 1998; Luzio et al., 2007; Luzio et al., 2010). The tight range of H^+ concentrations in these organelles controls enzymatic activities as well as fusions between autophagolysosomes and lysosomes, and lysosomes and endosomes; pH also affects autophagy and other important cellular processes (Luzio et al., 2007; Luzio et al., 2010; Nakamura and Yoshimori, 2017). Vacuolar-ATPase (v-ATPase) activity largely regulates the acidic nature of endolysosomes and does so by controlling the flux of cations and anions via hydrolysis of free ATP that drives protons against their electrochemical gradient into the lumen of endolysosomes (Mindell, 2012; Halcrow et al., 2019a; Khan et al., 2019a).

Endolysosomes are involved in a wide range of cellular processes including membrane trafficking, catabolism of extracellular and intracellular components, immune responses and antigen presentation, cell secretions, and cell life and death (Eskelinen and Saftig, 2009; Munz, 2012; Repnik et al., 2013; Bright et al., 2016; Truschel et al., 2018; Khan et al., 2019a; Afghah et al., 2020). These acidic organelles have also been implicated in various pathological conditions; structural and functional changes have been reported in various neurodegenerative disorders as well as in cancer (Repnik et al., 2013; Bright et al., 2016; Davis, 2018; Halcrow et al., 2019a; Khan et al.,

2019a). Because endolysosome pH regulates structural and functional features of endolysosomes, the involvement of v-ATPase in disease pathogenesis has received much attention and the v-ATPase complex has been targeted for therapeutic reasons. Indeed, inhibitors of v-ATPase and other strategies to keep endolysosomes from de-acidifying has shown benefit against diverse pathological conditions including different types of cancer (Whitton et al., 2018; Halcrow et al., 2019a; Halcrow et al., 2019b), neurological complications (Colacurcio and Nixon, 2016), and infectious diseases (Luzio et al., 2007).

CORONAVIRUS ENTRY INTO AND ESCAPE FROM ENDOLYSOSOMES:

Coronaviruses once endocytosed can avoid immune surveilence detection and degradation; thus enhancing infection (Hofmann and Pöhlmann, 2004; Belouzard et al., 2012; Shang et al., 2020a; Letko et al., 2020; Stower, 2020). SARS-CoV and MERS-CoV bind principally to dipeptidyl peptidase 4 while SARS-CoV-2 appears to bind mainly to ACE2; regardless, coronavirus spike proteins are activated by the host proteases TMPRSS2 or cathepsin B/L (Bosch et al., 2008; Shirato et al., 2013; Zhou et al., 2015; Hoffmann et al., 2020b; Pranesh et al., 2020). In addition, SARS-CoV-2 and MERS-CoV are activated by furin and this enhances viral entry especially in cells with lower expression levels of lysosomal cathepsin (Follis et al., 2006; Millet and Whittaker, 2014; Coutard et al., 2020; Hoffmann et al., 2020a).

Coronaviruses enter host cells by pH-dependent endocytosis (Yang et al., 2004; Burkard et al., 2014; Hoffmann et al., 2020b) and the acidic environment of endolysosomes is regulated not only by v-ATPase (Mindell, 2012), but also by Na^+/K^+-ATPase (Cain et al., 1989), mucolipin (TRPML1) channels (Li M. et al., 2017), big potassium channels (BK and MaxiK) (Khan et al., 2019b), Niemann-Pick type C (NPC1) (Wheeler et al., 2019a; Wheeler et al., 2019b; Höglinger et al., 2019; Lim et al., 2019), and two-pore channels (TPCs) (Marchant and Patel, 2015; Grimm et al., 2017; Khan et al., 2020). To date, TPCs and NPC1 have both been implicated in coronavirus infectivity.

TPCs are present in two forms; TPC1 and TPC2. TPC1s are mainly localized on early endosomes while TPC2s are mainly found on late endosomes/lysosomes (Brailoiu et al., 2009; Pitt et al., 2010; Zakon, 2012). Both subtypes of TPCs can help orchestrate interactions between endolysosomes and such viruses as Ebola (Sakurai et al., 2015), MERS-CoV (Gunaratne et al., 2018), and SARS-CoV-2 (Ou et al., 2020); TPCs regulate the trafficking of virus to late-endosomes/lysosomes following entry into cells. Not surprisingly then, TPC inhibitors can block entry of SARS-CoV-2 into cells and restrict the release of viral RNA into the cytosol (**Figure 2**) (Ou et al., 2020). TPCs are also involved in chloroquine-mediated endolysosome leakage and facilitated the release of HIV-1 Tat protein from endolysosomes thus enabling activation of HIV-1 LTR transactivation in the nucleus (Khan et al., 2020). Therefore, TPCs appear to promote virus entry and facilitate the release and transport of viral RNA to replication sites by inducing endolysosome permeability and depolarization.

NPC1 appears to also play a role in virus entry and infectivity. SARS-CoV enters into early endosomes, traffics to NPC1-positive late endosomes and lysosomes, and accesses highly active cathepsin L protease that triggers fusion mechanisms (**Figure 2**) (Shah et al., 2010; Zheng et al., 2018). MERS-CoV, Ebola, and SARS-CoV-2 use similar mechanisms to enter into host cells (Mingo et al., 2015; Zhou et al., 2016; Ballout et al., 2020).

AUTOPHAGY AND CORONAVIRUS REPLICATION

Autophagy is a process by which extracellular and intracellular macromolecules are engulfed in and degraded by autophagolysosomes; structures formed by fusion of lysosomes with autophagosomes (Eskelinen and Saftig, 2009; Kenney and Benarroch, 2015; Yim and Mizushima, 2020). Autophagy is regulated by diverse proteins including autophagy-related-genes (ATGs), *Beclin*, ubiquitin-binding protein (p62), 5′-adenosine monophosphate-activated protein kinase, serine/threonine kinase 1 (Akt), and S-phase kinase-associated protein 2 (Skp2) (He and Klionsky, 2009; Badadani, 2012).

The process of autophagy degrades invading viruses, enhances antigen processing and presentation, and induces adaptive immune responses (Lee and Kim, 2007; Delgado et al., 2009; Richetta and Faure, 2013; Choi et al., 2018a). For example, toll-like receptors are pattern recognition receptors that sense viral RNA and DNA in endolysosomes, induce type I-interferon responses, and following induction of autophagy antiviral immune responses are decreased and invading viruses are degraded (Lee and Kim, 2007; Dalpke and Helm, 2012; Choi et al., 2018a). Autophagy has antiviral effects independent of the degradation process; interferon-γ can suppress replication of norovirus (Hwang et al., 2012; Baldridge et al., 2016; Biering et al., 2017). Additionally, viruses can modulate, escape, and inhibit autophagy at multiple steps to survive and replicate in host cells (Pattingre et al., 2005; Kyei et al., 2009; Chaumorcel et al., 2012).

Autophagy plays a role in viral infections including those caused by coronaviruses (Prentice et al., 2004a; Killian, 2012; Maier and Britton, 2012). Mouse hepatitis virus (MHV) has been used as a model for coronavirus infections (Prentice et al., 2004a); the replication complex of MHV generates double-membrane vesicles (DMVs) resembling autophagosomes (Snijder et al., 2006; Clementz et al., 2008; Gadlage et al., 2010) within which the autophagy markers LC3 and ATG12 colocalize (Prentice et al., 2004a). MHV replication is impaired when the autophagy marker ATG5 is knocked down (Prentice et al., 2004a). Replication proteins of SARS-CoV colocalize with LC3 and autophagy appears to play an important role in SARS-CoV replication (Prentice et al., 2004b). In contrast, SARS-CoV and MHV replication was not impaired when ATG5 and ATG7 were knocked down (Zhao et al., 2007; Reggiori et al., 2010; Schneider et al., 2012). The MERS-CoV and SARS-CoV associated protein, membrane-associated papain-like proteases, suppressed autophagy flux by blocking the fusion of lysosomes and autophagosomes (Chen X. et al., 2014; Gassen et al., 2019).

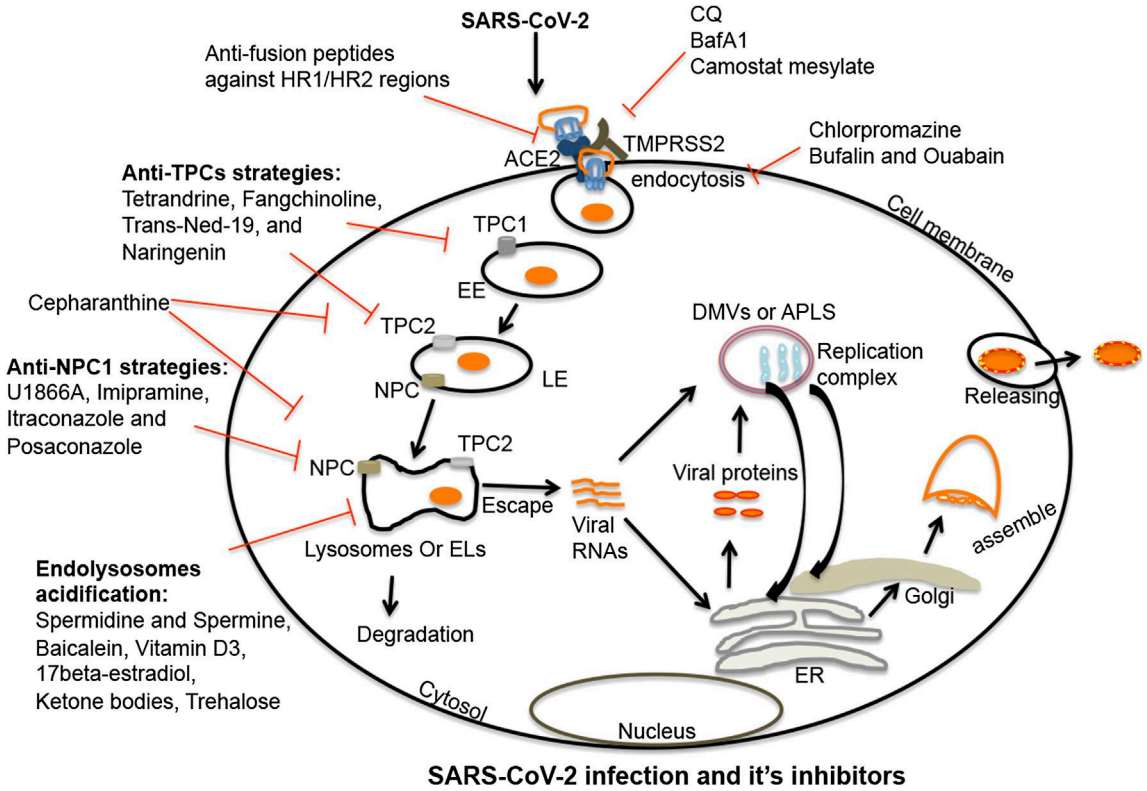

FIGURE 2 | Endolysosome-mediated therapeutic strategies against SARS-CoV-2: SARS-CoV-2 enters cells following interactions between viral spike proteins and cell surface ACE2 receptors. Once endocytosed, spike proteins in endosomes are primed in late endosomes/lysosomes by cathepsin enzymes (B/L); this enhances virus entry. Post-fusion, virus is either released from or degraded in endolysosomes. SARS-CoV-2 once released from endolysosomes, enters the cytosol where it produces a replication complex to generate viral genomic and sub-genomic RNA. Following replication, viral structural proteins get inserted into the ER and move to the ERGIS (endoplasmic reticulum–Golgi intermediate compartment) secretory pathway for virus assembly. Following assembly, virions are transported to vesicles and released from cells by exocytosis. Thus, various stages are targetable for intervention. The first target might be fusion between spike proteins and host ACE2 receptors. A second target might be de-acidification of endolysosomes and blocking the priming of spike proteins by deactivating serine proteases. A third target might be clathrin-mediated endocytosis. Fourth, TPC and NPC1 inhibitors could effectively inhibit the virus infection by de-acidifying endolysosomes and blocking the trafficking of cholesterol. Endolysosome acidification may also be a therapeutic target because of its capacity to block the escape of viral RNA to the cytosol and enhance the degradation of the virus in lysosomes. Shown in the figure are multiple compounds and drugs capable of targeting each of these important steps in the virus cycle. SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; TMPRSS2, transmembrane protease serine 2; ACE2, angiotensin-converting enzyme 2; EL, endolysosome; EE, early endosome; LE, late-endosome; TPC1/2, two-pore channel 1 and 2; NPC1, Niemann-Pick disease type C1; viral RNAs, viral ribonucleic acids; DMVs, double-membrane vesicles; APLS, autophagosome-like structures; ER, endoplasmic reticulum.

Similarly, SARS-CoV-2 suppresses autophagy by modulating multiple autophagy regulatory factors (Gassen et al., 2020), by blocking the degradation of viral factors, and by increasing the formation of DMVs to promote virus replication. Induction of autophagy reduced the replication and infectivity of MERS-CoV (Gassen et al., 2019; Carmona-Gutierrez et al., 2020) and SARS-CoV-2 (Maier and Britton, 2012; Gassen et al., 2020).

ENDOLYSOSOME-BASED THERAPEUTIC STRATEGIES TO INHIBIT SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 INFECTION

Because endolysosomes influence coronavirus infections, these organelles might be targeted against SARS-CoV-2 infection and COVID-19 pathogenesis. Given the urgency of need and the tremendous costs involved in developing new drugs, a good approach to therapeutic drug development is the repurposing of drugs known to accumulate in and affect the function of endolysosomes. The diprotic weak base drugs chloroquine (CQ) and hydroxychloroquine (HCQ), that de-acidify endolysosomes, have shown effectiveness in controlling SARS-CoV-2 infection in *in vitro* studies, however the effectiveness of CQ/HCQ against COVID-19 has not been established for COVID-19 patients (Liu et al., 2020; Wang et al., 2020; Yao et al., 2020). Endolysosome de-acidification can restrict replication of SARS-CoV-2 because acidic conditions are necessary for SARS-CoV-2 to enter into and be released from host cells. In the context of SARS-CoV-2 infection, CQ and HCQ have been used in combination with azithromycin (Andreani et al., 2020; Carlucci et al., 2020); a weak base antibiotic known to accumulate in endolysosomes (Kong

et al., 2017; Choi et al., 2018b; Andreani et al., 2020). Of course, CQ and HCQ have other pharmacological actions, but the involvement of endolysosome de-acidification in SARS-CoV-2 infection is supported by findings that other endolysosome de-acidification drugs; ammonium chloride, bafilomycin A1 and monensin all block coronavirus infections at the entry-level (Hoffmann et al., 2020b; Pranesh et al., 2020; Yang and Shen, 2020).

However, de-acidification may have other unintended consequences that might result in increased viral levels. Acidic conditions in endolysosomes are necessary for TLR-induced type-I interferon-mediated antiviral immune responses and antigen presentation (Dalpke and Helm, 2012; Munz, 2012; Choi et al., 2018a; Viret et al., 2020). Acidic endolysosomes are also important for autophagy, which is important for initiating innate immune responses and the degradation of viruses (Dalpke and Helm, 2012; Choi et al., 2018a). Accordingly, de-acidification of endolysosomes might hamper autophagy-mediated antiviral responses (Kužnik et al., 2011) by deactivating RNA sensors (Belizaire and Unanue, 2009; Kužnik et al., 2011; Kazi et al., 2013; Hussman, 2020; Offerhaus et al., 2020; Schrezenmeier and Dörner, 2020). Therefore, improving innate immune responses using synthetic RNAs, oligonucleotides, or small agonists of TLRs as well as type-I interferon treatment might improve clinical responses to CQ and HCQ (Dalpke and Helm, 2012; Freund et al., 2019; Hussman, 2020; Lee and Shin, 2020).

INHIBITION OF CORONAVIRUSES AT THE ENTRY LEVEL

The spike protein of SARS-CoV-2 is necessary for viral entry into cells governed by receptor-mediated endocytosis (Hofmann and Pöhlmann, 2004; De Clercq, 2006; Burkard et al., 2014; Burkard et al., 2014; Zheng et al., 2018; Jiang et al., 2020; Ou et al., 2020; Shang et al., 2020a; Tay et al., 2020). SARS-CoV-2 spike is a trimer with three receptor-binding domains (RBDs) of S1 heads on top of a trimeric S2 stalk (Gui et al., 2017; Shang et al., 2020a; Walls et al., 2020). Following proteolytic cleavage, the RBD of S1 conformationally switches from a laid-down position to a standing-up position in order to facilitate fusion with cell membranes (Hofmann and Pöhlmann, 2004; Gui et al., 2017; Yuan et al., 2017); the laid-down position has a significantly higher binding capacity (Walls et al., 2020; Wrapp et al., 2020) and escapes host immune surveillance. These features of the spike protein might make development of vaccines and antibody-based therapies more challenging (**Figure 2**) (Rossmann, 1989; Sui et al., 2014; VanBlargan et al., 2016; Gui et al., 2017; Chu et al., 2020; Xia et al., 2020). Never-the-less, huge efforts are on-going to develop vaccines and antibody-based therapies based on the structural and binding properties of RBDs (Jiang et al., 2005; Du et al., 2014; Shang et al., 2020b; Tai et al., 2020). Additional sites for intervention against viral infection include the spike S2 stalk that contains HR1 and HR2 hydrophobic regions; stable six-helix-bundle (6-HB) structures that fuse the virus with the host cell membrane (**Figure 2**) (Bosch et al., 2003; Bosch et al., 2004; Aydin et al., 2014; Wang et al., 2019). These mechanisms might represent sites for intervention against viral replication because targeting these hydrophobic regions has been shown to restrict infection of HIV-1 (Kong et al., 2016; Yuan et al., 2019), SARS-CoV-2, and other coronaviruses (Bosch et al., 2004; Xia et al., 2019a; Xia et al., 2019b; Wang et al., 2019; Xia et al., 2020).

Post-fusion with plasma membranes, many viruses enter cells by endocytosis and clathrin-mediated endocytosis (Inoue et al., 2007; Wang et al., 2008). Therefore it is not surprising that the anti-schizophrenia drug chlorpromazine (Ban, 2007) that inhibits clathrin-mediated endocytosis inhibits infection by the coronaviruses MHV (Pu and Zhang, 2008), MERS-CoV (Burkard et al., 2014), and SARS-CoV (Inoue et al., 2007; Wang et al., 2008). Similarly, Na^+/K^+-ATPase pump-based inhibitors bufalin and ouabain restricted MERS-CoV infection (Burkard et al., 2014; Burkard et al., 2015; Amarelle and Lecuona, 2018) by inhibiting clathrin-mediated endocytosis (Ko et al., 2020). An additional FDA approved drug that might find use against COVID-19 is camostat mesylate that is used for the treatment of pancreatitis (Ramsey et al., 2019); it inhibited serine proteases and restricted MERS-CoV, SARS-CoV, and SARS-CoV-2 infections by inhibiting TMPRSS2 activity (**Figure 2**) (Shirato et al., 2013; Bojkova et al., 2020; Hoffmann et al., 2020b). Also, the cathepsin L inhibitors Z-FY (t-Bu)-DMK, K11777, and teicoplanin blocked the entry of SARS-CoV and MERS-CoV (Huang et al., 2006; Adedeji et al., 2013; Zhou et al., 2016; Baron et al., 2020). Accordingly, the aboved named agents might find use against SARS-CoV-2 infection (**Figure 2**) and the pathogenesis of COVID-19.

EFFECTS OF ENDOLYSOSOME PH ON CORONAVIRUS INFECTION

The coronavirus spike protein is activated under acidic conditions by the endolysosome proteases TMPRSS2 and cathepsins B, L; conditions that promote fusion with host cell membranes and entrance into cells (Hoffmann et al., 2020b). Consistent with this, de-acidification of endolysosomes by CQ, bafilomycinA1, and ammonium chloride have all been shown to deactivate TMPRSS2 and cathepsin B, L as well as suppress coronavirus infection (**Figure 2**) (Simmons et al., 2004; Vincent et al., 2005; Wang et al., 2008; Shirato et al., 2013; Al-Bari, 2017; Gao et al., 2020; Hoffmann et al., 2020b). Although mentioned earlier, it is important to consider more specifically the involvement of endolysosome-resident ion channels and proteins that regulate endolysosome pH including TPCs, NPC1, and v-ATPase.

TPCs are calcium- and sodium-permeable channels that regulate cell membrane trafficking and endolysosome pH (Wang et al., 2012; Lagostena et al., 2017; Khan et al., 2020). Because of the involvement of TPCs in the regulation of endolysosome pH it is not surprising that TPC activation increased the entry and trafficking of SARS-CoV-2 (Ou et al., 2020), MERS-CoV (Gunaratne et al., 2018) and Ebola

(Sakurai et al., 2015) while the TPC inhibitors tetrandrine and Ned-19 significantly inhibited the entry and trafficking of viruses in host cells (**Figure 2**) (Ou et al., 2020). Moreover, apilimod and vaculin-1 restricted SARS-CoV-2 infection by reducing PIKfyve enzyme activity (Kang et al., 2020; Ou et al., 2020); PIKfyve is a regulator of PI(3,5)P2, an endogenous activator of TPCs (Dove et al., 2009; Kirsch et al., 2018). Further, the natural flavonoid naringenin inhibited TPCs (Tsai and Tsai, 2012; Pafumi et al., 2017; Benkerrou et al., 2019; Bai et al., 2020) and has antiviral activity against hepatitis C (HCV) (Nahmias et al., 2008), influenza A (Dong et al., 2014), Zika (Cataneo et al., 2019), and Dengue (Frabasile et al., 2017). Additionally, naringenin suppressed acute inflammation by inducing lysosome-mediated degradation of inflammatory cytokines (Jin et al., 2017) and ameliorated radiation-induced lung fibrosis (Zeng et al., 2018; Zhang et al., 2018). Thus, naringenin and other drugs targeting TPCs might be considered as possible therapeutic strategies against COVID-19 (**Figure 2**).

Niemann-Pick disease type C1 (NPC1) is an endolysosome-resident protein (Higgins et al., 1999) that regulates trafficking of late endosomes and lysosomes (Ko et al., 2001; Zhang et al., 2001; Ganley and Pfeffer, 2006; Sztolsztener et al., 2012), membrane trafficking of essential cellular factors such as cholesterol and sphingolipids (Chen et al., 2005; Infante et al., 2008; Kwon et al., 2009; Lange et al., 2012; Höglinger et al., 2019), and regulation of endolysosome pH and calcium (Elrick et al., 2012; Liu and Lieberman, 2019; Wheeler et al., 2019a). Impaired NPC1 is an underlying cause of Niemann-Pick disease; a lysosome storage disease (Lloyd-Evans et al., 2008; Schuchman and Desnick, 2017). NPC1 has been implicated in the infectivity of Ebola virus, MERS-CoV and SARS-CoV following late entry kinetics and access to cathepsin L in late endosomes and lysosomes (Shah et al., 2010; Mingo et al., 2015; Zhou et al., 2016; Zheng et al., 2018; Ballout et al., 2020;). Because SARS-CoV-2 also uses similar cell entry and cleavage mechanisms, NPC1 might become a target against SARS-CoV-2 infection; the desired effect being endolysosome de-acidification and accumulation of lipids in endolysosomes (Zheng et al., 2018; Wheeler et al., 2019a; Ballout et al., 2020; Pranesh et al., 2020; Sturley et al., 2020). Indeed, increased levels of 25-hydroxycholesterol (25-HC) restricted viral infection of *Filoviruses* (Liu et al., 2013), *Coronaviridae* (ZhangY. et al., 2019b), and *Flaviviridae* (Chen Y. et al., 2014; Li et al., 2017). Elevated levels of 25-HC and 7-ketocholesterol (7-KC) (Willard et al., 2018) in NPC compromised cells may restrict infection by SARS-CoV-2. Moreover, available NPC1 inhibitors U1866A and imipramine inhibited several enveloped viruses including MERS-CoV and SARS-CoV (Wrensch et al., 2014), Ebola (Herbert et al., 2015; Lu et al., 2015), HIV-1 (Tang et al., 2009), HCV (Elgner et al., 2016), influenza A (Eckert et al., 2014), and chikungunya (Wichit et al., 2017) by de-acidifying endolysosomes and increasing lipid accumulation (Lange et al., 2012) (**Figure 2**). Also, the anti-fungal drugs itraconazole and posaconazole are not only inhibitors of NPC but also have antiviral activity (Strating et al., 2015; Trinh et al., 2017; Meutiawati et al., 2018; Rhoden et al., 2018; Schloer et al., 2019; Takano et al., 2019a; Takano et al., 2019b). In addition, cepharanthine, an inhibitor of TPC2 and NPC1, has antiviral activity (**Figure 2**) (Zhang et al., 2005; Matsuda et al., 2014; Lyu et al., 2017; Bailly, 2019; Kim et al., 2019; Fan et al., 2020; Rogosnitzky et al., 2020). Thus, TPCs and NPC1 might both attract attention as possible targets to block SARS-CoV-2 infection and suppress COVID-19.

The v-ATPase pump is an ion channel that is crucial for regulating endolysosome pH (Mindell, 2012); higher or lower activity levels of v-ATPase significantly affects endolysosome functions (Colacurcio and Nixon, 2016; Halcrow et al., 2019a). CQ, Baf A1 and ammonium chloride all cause de-acidification and deactivation of proteases in endolysosomes as well as inhibit coronavirus infections (Vincent et al., 2005; Gao et al., 2020; Hoffmann et al., 2020b). The SARS-CoV $3CL^{pro}$ protease de-acidifies endolysosomes by direct interaction with the G1 subunit of v-ATPase (Lin et al., 2005) and blocks degradation of viral factors thereby enhancing virus replication. Endolysosome acidification may also restrict coronavirus infections by blocking the escape of viral RNA to the cytosol and promoting viral degradation in lysosomes (Carmona-Gutierrez et al., 2020; Gassen et al., 2020; Yang and Shen, 2020). A number of natural compounds acidify endolysosomes and might be tested for their ability to enhance coronavirus degradation; these include spermidine and spermine (Gassen et al., 2020), baicalein (Zhu et al., 2020), vitamin D3 (Hu et al., 2019; Daneshkhah et al., 2020), 17-beta-estradiol (Lipovka and Konhilas, 2014; Xiang et al., 2019; Khan, 2020; Suba, 2020), ketone bodies (Hui et al., 2012; Camberos-Luna et al., 2016), trehalose (Sharma et al., 2020), wogonin (Li et al., 2016), apigenin (Zhang X. et al., 2019), and butein (Ansari et al., 2018) (**Figure 2**).

CONCLUSION

The high fatality rate of COVID-19 especially among people with pre-existing co-morbities and rapidly increasing case numbers of SARS-CoV-2 infections has created a huge global need for effective therapeutic interventions against COVID-19. Because of the urgent need for therapeutics, re-purposing already approved pharmaceuticals might be the quickest available strategy. SARS-CoV-2 enters into endolysosomes where it can escape detection by immune surveillance and from there can traffic to the cytosol where it can propagate. Endolysosomes generally and endolysosome pH more specifically may represent important targets against SARS-CoV-2 replication and COVID-19 pathogenicity, and several compounds and drugs are available that may be repurposed for immediate testing. Reviewed above were several potential targets to block SARS-CoV-2 infection including endocytosis following binding of the spike protein with its receptor (ACE2), RNA replication and transcription, translation and proteolytic processing of viral proteins, virion assembly, and release from infected cells (Guy et al., 2020; Poduri et al., 2020); all targets involving the endolysosome

system. In considering approaches against SARS-CoV-2 infection and COVID-19 pathogenesis, the involvement of endolysosomes should be considered.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the manuscript.

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frontiers in Pharmacology

REVIEW
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Challenges for Drug Repurposing in the COVID-19 Pandemic Era

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The coronavirus disease (COVID-19) pandemic has affected an estimated 16 million persons and caused 0.6 million deaths worldwide by September 2020. The pandemic has led to a rush to repurpose existing drugs, although the underlying evidence base is of variable quality. The improving knowledge of the virology and clinical presentation of COVID-19 is leading to a broadening pool of potential pharmacological targets. The aim of this review is to describe regulatory and pharmacological aspects of drug repurposing and to identify drugs proposed for repurposing in COVID-19 based on registered clinical trials, discussing the evidence to support their use in the treatment of this disease. The challenges of the correct interpretation of existing pre-clinical/clinical evidence as well as the generation of new evidence concerning drug repurposing in COVID-19 will also be discussed.

Clinical Trial Registration:
https://clinicaltrials.gov, identifier NCT04321174, NCT04342663, NCT04280705, NCT04244591, NCT04359329, NCT04348695, NCT04304313, NCT043505931

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INTRODUCTION

To date, the global coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to more than 16 million infected patients and more than 600,000 deaths worldwide by September 2020 (European Centre for Disease Prevention and Control, 2020). To date, only remdesivir, an investigational antiviral compound, has received a conditional marketing authorization from the European Commission for the treatment of COVID-19 in adults and adolescents from 12 years of age with pneumonia who require supplemental oxygen (European Medicines Agency, 2020a). Remdesivir has also been approved in Japan (Gilead, 2020) and has received Emergency Use Authorization in the United States from the Food and Drug Administration (Food and Drug Administration, 2020a). No other treatments have been approved to date.

There is great interest in drug repurposing (also known as repositioning or rediscovery) to accelerate the identification of drugs that can cure or prevent COVID-19. The value of drug repurposing is to speed up the traditional process of drug discovery by identifying a novel clinical use for drugs that have already proven to be safe and effective in humans and are approved for other indications. This strategy can also reduce the costs required for the development of new drugs, with notable savings in preclinical phase I and II (Pushpakom et al., 2018). The rationale of drug

repurposing lies in the fact that the same molecular pathways may be involved in different diseases (Oprea et al., 2011). One of the key drivers for the repositioning of drugs is the serendipitous discovery of pharmacological activity on new targets, which would then suggest that a new possible indication of use (Pushpakom et al., 2018). Some classical examples of serendipity-based drug repurposing are thalidomide, originally developed for the treatment of morning sickness in pregnant women and now used in multiple myeloma (Jacobson, 2000), sildenafil, initially conceived for the treatment of angina and hypertension and today used for the treatment of erectile dysfunction (Ghofrani et al., 2006) and amantadine, an antiviral which was originally indicated for influenza and was then used to treat Parkinson's disease (Lee and Kim, 2016).

Repurposing has several implications in the drug regulatory setting as well as in the scientific setting, especially if it occurs during a public health emergency such as the COVID-19 pandemic. In this review, challenges for drug repurposing in the COVID-19 pandemic era will be described. In addition, drugs proposed as candidates for repurposing in COVID-19 will be identified and the rationale behind their use will be discussed.

DRUG REPURPOSING FROM A REGULATORY PERSPECTIVE

Repurposing officially falls into a number of categories, bearing in mind that physicians in most countries have the right to use drugs outside the existing approved label and frequently do. However, "off-label" use is often frowned upon by regulatory drug agencies and scientific societies, as the effectiveness and safety of drugs for off-label indications may not be established. In most cases, to have a new indication approved, substantial investment is required. A patent is essential for the sponsor of the development program to ensure return on investment with sales. Patents based on "product" or "composition-of-matter" not only give the patentee the right to exclude others from making and selling the drug for the same purpose as the patentee but also block the marketing of any new use that another party discovers. The patent process also runs alongside extensions of exclusivity built into some regulatory processes. In contrast, so-called "use" patents protect a selected therapeutic use. Repurposed drugs fall into four categories based on those described by the Discovering New Therapeutic Uses for Existing Molecules initiative of National Institute of Health (NIH) through the National Center for Advancing Translational Sciences (NCATS) (https://ncats.nih.gov/ntu): 1) Therapeutic assets (of any modality) with remaining patent life but never approved for human use; 2) Therapeutics with remaining patent life that are currently approved for one or more indication(s) but have potential use in others; 3) Therapeutic assets with no patent life that are not currently marketed because they were either never approved or were withdrawn; 4) Therapeutics with no patent life currently manufactured by generic companies, approved for certain indications, and available by prescription from healthcare providers. The first two can be developed by the patent holder or licensed to another company for development; the second two can be developed by establishing a "use" if it fits patentable criteria of being sufficiently novel, useful, and non-obvious. All types of products must meet all the normal benefit-risk assessment requirements of drug regulation based on the evidence of quality, safety and efficacy and with appropriate prescriber information, if they are to be distributed to the public. In addition, compliance with health technology assessment (HTA) to oversee pricing and reimbursement and the way the product is positioned in national guidance for standard of care is becoming increasingly important in all major countries and, to a greater or lesser extent, government controlled.

Regulation and HTA require one or more clinical trials for the new indication unless it is an exceptional situation. One such exceptional situation concerns extremely rare conditions, such as anthrax and Ebola, which can be registered on animal data and human safety data from healthy volunteers or other indications. An example of this is Anthrasil™, Anthrax Immune Globulin Intravenous (Human), approved by FDA in 2015 to treat patients with inhalational anthrax in combination with appropriate antibacterial drugs already registered under the animal rule. Another exceptional situation concerns a product currently used for the indication. This applies in Europe as "well-established use". When an active ingredient of a medicine has been used for more than 10 years its efficacy and safety may be considered well-established. In such cases, marketing authorization may be based on results from the scientific literature. There are also regulatory mechanisms for the active ingredient to be used at a different dose (hybrid applications) and an example of this is dexamethasone registered by EMA as Neofordex™ under well-established use in 2016 for multiple myeloma. Most products, however, will need more than a single clinical trial to establish the new indication for repurposing. If the indication is completely new, repurposed drugs normally start with preclinical pharmacology, including animal models and safety, aimed at defining dose and length of treatment, followed by a translational medicine and a clinical program, similarly to the approval pathway of a new drug.

Regulatory decisions are made on benefit-risk balance and in a rapidly evolving pandemic two issues come in to play, namely the speed of drug approval and the urgency of clinical needs. The speed of developing treatments to the point of approval is key to protect patients from the infection as quickly as possible. Drug development of new biologicals or chemicals, other than vaccines, takes a minimum of 2–3 years even if there is a candidate that has shown efficacy in animal trials and have scaled down clinical trials to 6–12 months. Pre-clinical activities of manufacturing to establish product quality and the necessary safety studies normally take at least 2 years before clinical trials can start. Repurposed drugs, however, could appear on the market after simply completing one or more clinical trials. As the medical need for a drug increases and more patients die from the disease, the benefit of the drug increases in the assessment of benefit-risk balance. For new biologicals or small-molecule drugs the risk

element is not yet established in clinical medicine and it takes time to accumulate enough patients for this purpose under the new indication, even if efficacy can be established quickly. A potential way is to accelerate the generation of evidence to separate safety trials from efficacy trials, as in medicines and vaccines registered under the animal rule (Food and Drug Administration, 2015; European Medicines Agency, 2018). In United States there is specific guidance for developing products where efficacy can only be established by the animal rule (FDA, 2015) but in Europe the guidance is included within a specific guidance for types of development such as the vaccine guideline (European Medicines Agency, 2018). The alternative is to use repurposed drugs where the human risk profile is known.

In situations such as the COVID-19 pandemic, regulatory agency decisions may have to accelerate their decision process, thus collaboration and communication between national regulatory and HTA agencies becomes essential. The aim should be to reach peak acceleration of review without compromising the benefit-risk balance assessment. Some changes in processes may be continued after the pandemic and it could be argued that they have demonstrated general improvement in process efficiency such as rolling review in EMA which has been planned to be extended in their strategy up to 2025 (European Medicines Agency, 2020b). Some compromises in process, however, such as monitoring and audit of clinical trial sites is unique to lockdown and is not planned to continue.

All drug regulatory agencies have committed to speeding up regulations and all are collaborating through the International Coalition of Medicines Regulatory Authorities (ICMRA) whose website contains valuable information on COVID-19 trial programmes for medicines and vaccines in development. Each individual agency has also issued guidance which not only speeds the time to obtain clinical trial approval but also makes scientific advice more rapidly accessible, dropping normal agency timelines and conducting video meetings. With regard to minimal evidence, all major agencies (Europe, United Kingdom, Japan and the United States) already had robust processes in place for accelerated approvals which require minimal evidence. This is illustrated by remdesivir, a repurposed drug that was originally developed for Ebola. Remdesivir received Japanese authorization under the Exceptional Approval Pathway, Emergency Use Authorization in the USA and in the United Kingdom access was granted under the Early Access to Medicine Scheme. Conditional approval for remdesivir by EMA for use to treat COVID-19 in adults and adolescents with pneumonia requiring supplemental oxygen was given under the new accelerated pathway under guidance issued in May 2020 (EMA, 2020). The main efficacy study assessed was NIAID-ACTT-1 (Beigel et al., 2020) involving 1,063 hospitalized patients with COVID-19 (120 with mild to moderate disease and 943 with severe disease) showed that Veklury™ (remdesivir) can speed up the recovery time in some patients, allowing them to spend less time in hospital or on treatment. The further clinical data required by EMA is published in their web site (European Medicines Agency, 2020c; European Medicines Agency, 2020d). In both United States and EU pediatric data planning is also required. It is worth noting that although remdesivir use was supported by *in vivo* studies due to its initial indication for Ebola virus, it was trialed in a Phase III clinical trial for Ebola,with that safety data being also used for the evaluation of the Emergency Use Approval in the United States (Mulangu et al., 2019).

The expedited drug development and regulatory decision-making processes may come at the cost of complete drug safety and effectiveness data. Indeed, the drug development process, which usually takes 12–15 years, is reduced to 12–18 months or less. Normally, conducting Phase III clinical trials is a lengthy process; reducing the time needed so drastically can only be achieved by conducting shorter, fewer or in extreme cases, no Phase III clinical trials. This can occur in a pandemic when the recruitment stage coincides with a period of low incidence of the disease, such as the trial of remdesivir by Wang et al., which was stopped early because there were not enough infected patients available in China to reach trial recruitment targets (Wang et al., 2020). Therefore, during a pandemic, drugs can be licensed with less information available than would normally be acceptable. In the case of COVID-19 it depends on the prevalence of the disease but repurposed products could be licensed on the basis of their benefit risk evaluations for other indications, biomarker data and limited phase III studies for the COVID-19 indication. Emergency Use Authorization (FDA), Conditional Marketing Authorization (EMA) or approval under the Early Access to Medicines Scheme (MHRA) for COVID-19 were based on very limited clinical data. Even the full Marketing Authorization are likely to be granted on the basis of limited clinical data compared to the conventional licensing requirements. Therefore, real-world studies to monitor the safety and effectiveness post-licensing are necessary during a pandemic. Regulatory decisions are likely to be based on limited Phase III data or in some circumstances only on the results of Phase II trials with a conditional license to conduct studies in the post-marketing phase. It is well known that there are gaps in available safety information at the time of licensing for all medicinal products. This is the nature of clinical development which focuses on efficacy, and is the reason for post-authorization risk management plans which aim to address any safety uncertainties during the postmarking phase. Such uncertainties are significantly higher for products that have gone through expedited development and licensing. Hence, robust post-authorization studies with early and regular interim reporting are not only highly necessary but, in some cases, may be a condition of the Marketing Authorization.

DRUGS CANDIDATES FOR REPURPOSING IN COVID-19 INFECTION

To identify drugs being seriously considered for repurposing, clinicaltrials.gov, a repository for clinical trials, was searched for all registered clinical trials concerning COVID-19 on July 2, 2020.

TABLE 1 | Overview of drugs proposed as potential inhibitors of one or more steps of SARS-CoV-2 lifecycle and undergoing experimental studies at 2nd July 2020– source: clinicaltrials.gov.

Pharmacological class	Drug	Proposed mechanism in the treatment of SARS-CoV-2 infection
Kinase inhibitors	Baricitinib	It could exert anti-viral effects by its affinity for AP2-associated protein AAK1, reducing SARS-CoV-2 endocytosis
	Imatinib	It accumulates in lysosomes resulting in some antiviral activities by lysosomal alkalization required for virus/cell fusion
Antibacterials	Doxycycline	It could reduce pro-inflammatory cytokines levels and chelate matrix metalloproteinases used for cell fusion and viral replication
Antidiabetic drugs	Dapagliflozin	During virus infection, serum lactate dehydrogenase level excessively rises. Dapagliflozin has been reported to reduce lactate levels by various mechanisms. It also reduces oxygen consumption in tissues and causes the use of glucose in the erobic pathway
	Linagliptin, sitagliptin	Since SARS-CoV-2 could use DPP4 receptor to invade cells, the inhibition of DPP4 could be useful in mild COVID-19 patients
Antimalarials	Artemisinin/artesunate	Anti-inflammatory activity, NF-κB-coronavirus effect and chloroquine-like endocytosis inhibition mechanism
	Atovaquone	It could inhibit SARS-CoV-2 through targeting of the viral RdRp or 3C-like protease
	Chloroquine, hydroxychloroquine	They showed interference with the glycosylation of ACE-2 receptors; they increase the pH of acidic cellular organelles, counteracting virus replication
	Mefloquine	It inhibited SARS-CoV-2 replication *in vitro* experimental models
Antitumorals	Plitidepsin	It could inhibit the multiplication and propagation of SARS-CoV-2
	Selinexor	It could inhibit the replication of SARS-CoV-2 and mediate anti-inflammatory and anti-viral effects
Antivirals	Atazanavir, danoprevir, darunavir	Potential SARS-CoV-2 protease inhibition
	Clevudine	It acts as a potent inhibitor of RdRp protein, preventing RNA replication
	Daclatasvir	It could target different proteins of the SARS-CoV-2 life cycle, affecting both viral RNA replication and virion assembly
	Emtricitabine	RNA synthesis nucleos(t)ide analogue inhibitors could have an effect against SARS-CoV-2 infection
	Favipiravir, galidesivir	They inhibit RdRp of RNA viruses, blocking SARS-CoV-2 replication
	Lopinavir/ritonavir	They could inhibit SARS-CoV-2 replication by blocking $3CL^{pro}$ and $PL2^{pro}$ proteases
	Nelfinavir	It may bind to the S trimer structure inhibiting the membrane fusion process
	Nitazoxanide	It exerts antiviral effects through the phosphorylation of protein kinase activated by double-stranded RNA, which leads to an increase in phosphorylated factor 2-alpha, an intracellular protein with antiviral effects
	Oseltamivir	It could inhibit virus replication and virion release
	Remdesivir	It could inhibit the RNA synthesis of SARS-CoV-2
	Ribavirin	It could inhibit SARS-CoV-2 replication
	Sofosbuvir	It is a chain terminator for SARS-CoV-2 RNA polymerase. In human brain organoids, it protected from SARS-CoV-2-induced cell death
	Tenofovir alafenamide	It could inhibit SARS-CoV-2 RdRp
	Umifenovir	It could block trimerization of the spike glycoprotein, essential for host cell adhesion
Immunosuppressants	Cyclosporine	It can block viral replication and thus transcription of pro-inflammatory cytokines
	Leflunomide	In *vitro* studies have shown antiviral effects of leflunomide against SARS-CoV-2
	Sirolimus	It could block viral protein expression and virion release
	Tacrolimus	It inhibited SARS-CoV-2 replication *in vitro*
Interferons	Alpha and beta interferons	Interferons exhibit both direct inhibitory effects on viral replication and supporting an immune response to clear virus infection
	Peginterferon lambda-1A	It inhibits viral replication without and does not trigger cytokine storm. It helps the body's natural immune system into action
Other	Amiodarone	It could reduce the internal acidity of endosomes and lysosomes affecting cell activities important for an efficient viral entry
	Bicalutamide, bromhexine, camostat mesilate, nafamostat	Inhibition of TMPRSS2, an enzyme facilitating SARS-CoV-2 cell penetration
	Chlorpromazine	It inhibits clatrine-mediate endocytosis by interacting with dynamin
	Estradiol patch	It could down-regulate ACE2 receptors in kidneys
	Famotidine	It could bind papain-like protease, responsible for initial processing of the SARS-CoV-2 polyprotein into active subunits
	Isotretinoin, retinoic acid	They can down-regulate ACE2 receptors; they are potential protease inhibitors; they could increase CD4 counts
	Ivermectin	It inhibits the replication of SARS-CoV-2 *in vitro*
	Niclosamide	It could block endocytosis of SARS-CoV-2 and prevent its autophagy by inhibition of S-Phase kinase associated protein 2
	Spironolactone	It could, theoretically, reduce ACE-2 expression on lung-cell surfaces
	Verapamil	It could interfere with coronavirus entry and amplification by blocking ion channels

Notes: Drugs involved in safety studies were not included; only drugs approved by regulatory agencies were included. Abbreviations: AAK1, Adaptor-associated protein kinase 1; ACE, Angiotensin Converting Enzyme; COVID-19, coronavirus disease 2019; DPP4, dipeptidyl-dipeptidase four; RdRp, RNA-dependent RNA polymerase; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; TMPRSS2, Transmembrane protease serine 2.

TABLE 2 | Overview of drugs proposed to potentially counteract the effects of SARS-CoV-2 infection and undergoing experimental studies at July 2, 2020—source: clinicaltrials.gov.

Pharmacological class	Drug	Proposed mechanism in SARS-CoV-2 infection
NSAIDs	Acetylsalicylic acid	It can inhibit virus replication and platelet aggregation, it has anti-inflammatory and could prevent lung injury
	Indomethacin	It could reduce symptoms in COVID-19 patients
	Naproxen	It could reduce symptoms in COVID-19 patients
Kinase inhibitors	Abivertinib, acalabrutinib, ibrutinib, ruxolitinib, anubrutinib	To counteract hyper-inflammatory symptoms caused by cytokine storm
	Pacritinib	It could prevent the development of an inflammatory response to the coronavirus infection and pulmonary failure
Anaesthetics	Isoflurane	In *vivo* studies volatile anaesthetics reduce the severity of ARDS compared to intravenous sedation
	Ketamine	It may be able to interrupt the inflammation that causes COVID-19 symptoms
	Sevoflurane	It has anti-inflammatory properties. In *vivo* studies volatile anaesthetics reduce the severity of ARDS
Antibacterials drugs	Azithromycin, clarithromycin	Macrolides can reduce the inflammatory process and modulate the immune system
Antidepressant drugs	Fluoxetine, fluvoxamine	To counteract hyper-inflammatory symptoms caused by cytokine storm
Anti-thrombotic drugs	Alteplase	Targeting the coagulation and fibrinolytic systems could limit ARDS progression and reduce ARDS-induced death
	Bemiparin, heparin, rivaroxaban, tinzaparin	Prevention of deep vein thrombosis and venous thromboembolism in COVID-19 patients
	Bivalirudin	Potential option to maintain systemic anticoagulation during extracorporeal membrane oxygenation
	Clopidogrel, prasugrel	They could prevent cardiovascular complications in COVID-19 patients
	Defibrotide	To treat endothelial inflammation in severe COVID-19 patients
	Dipyridamole	It could reduce D-dimers concentrations and increase lymphocyte and platelet recovery in the circulation
	Enoxaparin	It reduces D-dimers concentrations and prevents hemostasis abnormalities
	Pentoxifylline	It inhibits the synthesis of pro-inflammatory cytokines; it inhibits platelet aggregation and promotes the fibrinolytic activity
	Plasminogen activator	Targeting the coagulation and fibrinolytic systems could limit ARDS progression and reduce ARDS-induced death
Oncologic drugs	Duvelisib	PI3K inhibition with duvelisib could potentially quell aberrant hyper-activation of the innate immune system, preferentially polarize macrophages, reduce pulmonary inflammation, and limit viral persistence
	Bevacizumab	Suppression of pulmonary edema in COVID-19 patients with ARDS
	Etoposide	To counteract hyper-inflammatory symptoms caused by cytokine storm
	Melphalan	Ultra-low doses of melphalan have local and systemic anti-inflammatory effects and decrease the activation of lymphocytes
	Nivolumab	Nivolumab-induced immunity normalization could stimulate anti-viral response and prevent ARDS development
	Tetrandrine	It could inhibit pulmonary fibrosis
	Thalidomide	It could reduce the persistent cough and reduce the lung damage by blocking the inflammatory response
	Thymalfasin	Administered to individuals with end-stage renal disease, it could reduce the rate and severity of SARS-CoV-2 infection
Antivirals	Isoprinosine	It stimulates a non-specific immune response that is independent of the specific viral antigen
	Maraviroc	It may reverse lymphoid depletion and alter cell trafficking of inflammatory cells, both increasing viral control capacity and dampening damage to lung tissue, respectively
Glucocorticoids	Budesonide, ciclesonide, dexamethasone, hydrocortisone, methylprednisolone, prednisone	To reduce systemic inflammatory response to SARS-CoV-2 infection
Immunosuppressants	Fingolimod	It may confine the over-exuberant inflammatory response and slow down the progress of lung injury
	Levamisole	It can increase lymphocytes and empower the immunity of the body. It can bind to the SARS-CoV-2 protease and can decrease the levels of TNF-α and IL-6
	Methotrexate	It may reduce the over-exuberant inflammatory response and slow down the progress of lung injury
	Olokizumab	To counteract hyper-inflammatory symptoms caused by cytokine storm
	Ozanimod	Its immune-modulating activity could mitigate the morbidity and mortality of COVID-19
Interleukin inhibitors	Anakinra, apilimod, canakinumab, clazakizumab, sarilumab, siltuximab, tocilizumab	To counteract hyper-inflammatory symptoms caused by cytokine storm
Opioids	Naltrexone	It can reduce production of multiple cytokines, inhibit cellular proliferation of T- and B- cells and block Toll-like receptor 4
	Tramadol	It has anti-inflammatory effect decreasing plasma level of TNF-α, which may result in a subsequent increase in T cell numbers

(Continued on following page)

TABLE 2 | (*Continued*) Overview of drugs proposed to potentially counteract the effects of SARS-CoV-2 infection and undergoing experimental studies at July 2, 2020—source: clinicaltrials.gov.

Pharmacological class	Drug	Proposed mechanism in SARS-CoV-2 infection
Other	Almitrine	To treat COVID-19-induced hypoxic acute respiratory failure
	Aviptadil	It is a vasoactive intestinal polypeptide (VIP) analogue. In the lug, VIP prevents the activation of caspases, inhibits IL-6 and TNF-α production and protects against HCl-induced pulmonary edema
	Atorvastatin	Inhibition of virus proliferation; it reduced lung virus titers and reduced TNF-α, IL-6 in supernatants of infected cells
	Botulinum neurotoxin	It could attenuate chronic cough, dyspnoea, pneumonia, acute respiratory failure, abnormal circulation, cardiac defects and various neurological deficits
	Cholecalciferol	Its immunomodulatory effects could prevent the occurrence of respiratory derangement and other adverse clinical events
	Colchicine	It reduces cytokine levels as well as the activation of macrophages, neutrophils and the inflammasome
	Conestat alfa	It may dampen uncontrolled complement activation and collateral lung damage and reduce capillary leakage and subsequent pulmonary edema by direct inhibition of kinin-kallikrein system
	Crizanlizumab	It can decrease inflammation by binding to P-selectin, blocking leukocyte and platelet adherence to the vessel wall
	Deferoxamine	Since severe cases of COVID-19 pneumonia have similar clinical presentations of iron overload, it seems that deferoxamine could be a supportive therapy for resolving the complications of COVID-19 pneumonia
	Dornase alfa	It could break down the DNA backbone of neutrophil extracellular traps in the COVID-19 lung which will promote the degradation of pro-inflammatory extracellular histones and prevent the amplification of the inflammatory response and the resultant lung dam
	Eculizumab	It might work as an emergency therapy for the treatment of patients with severe pneumonia or ARDS associated with COVID-19 infection
	Emapalumab	To counteract hyper-inflammatory symptoms caused by cytokine storm
	Human immunoglobulin	It provides passive immune protection against a broad range of pathogens
	Ibudilast	To decrease COVID-19-related acute respiratory distress syndrome
	Iloprost	It may improve inflammation and oxygenation in suspected or confirmed COVID-19 patients with respiratory failure
	Infliximab	To counteract hyper-inflammatory symptoms caused by cytokine storm
	Lanadelumab	Blocking the bradykinin 2 receptor and inhibiting plasma kallikrein activity might have an ameliorating effect on early disease caused by SARS-CoV-2 and might prevent acute respiratory distress syndrome
	Leronlimab	To counteract hyper-inflammatory symptoms caused by cytokine storm
	Lucinactant	It may be able to benefit patients with acute respiratory distress syndrome, improving oxygenation and lung compliance
	Montelukast	It can inhibit the signaling of NF-κB, such as interleukin-6,8,10, TNF-α, MCP-1, and other pro-inflammatory mediators
	N-acetylcysteine	It can reduce the formation of pro-inflammatory cytokines and also has vasodilator properties by increasing cyclic GMP levels and by contributing to the regeneration of endothelial-derived relaxing factor
	Nintedanib	To treat pulmonary fibrosis in patients with moderate to severe COVID -19
	Pamrevlumab	It could reduce edema and block fibrotic degeneration of lung tissue in patients with bilateral COVID-19 pneumonia
	Pirfenidone	It could inhibit apoptosis, down-regulate ACE receptors expression, decrease inflammation, ameliorate oxidative stress and protect pneumocytes and other cells from SARS-CoV-2 invasion and cytokine storm
	Poractant alfa	It could improve oxygenation and survival in COVID-19 patients with acute distress respiratory syndrome
	Prazosin	It prevents cytokine storm and markedly increased survival following inflammatory stimuli in preclinical models
	Progesterone	It could reduce the immunity response
	Pyridostigmine	Acetylcholine-esterase inhibitors may act as immunomodulators during viral infections, potentially reducing the inflammatory cascade observed in critically ill COVID-19 patients
	Ravulizumab	It could counteract hyper-inflammatory symptoms caused by cytokine storm
	Sargramostim	It may confer benefit to patients with ARDS due to COVID-19 exposure, who are at significant risk of mortality
	Sildenafil citrate	It inhibits inducible nitric oxide synthase, an enzyme activating a cascade of inflammatory processes
	Tranexamic acid	It reduces the elevated levels of plasmin/plasminogen
	Ulinastatin	It could counteract hyper-inflammatory symptoms caused by cytokine storm
	Zilucoplan	It can inhibit acute lung injury post COVID-19 and can promote lung repair mechanisms

Notes: Drugs involved in safety studies were not included; only drugs approved by regulatory agencies were included. Abbreviations: ACE, Angiotensin Converting Enzyme; ARDS, Acute respiratory distress syndrome; COVID-19, coronavirus disease 2019; IL, Interleukin; MCP-1, Monocyte chemoattractant protein-1; PI3K, phosphoinositide 3-kinase; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; TNF-α, Tumor necrosis factor-alfa.

Studies aiming to evaluate drug safety were excluded, as were all studies concerning vaccines. Studies were included irrespectively of whether they were planned, ongoing or completed, and also irrespectively of whether the drugs were intended to prevent or treat COVID-19. For each drug being considered as a treatment in a clinical trial, the mechanism of action was identified through a literature search.

Overall, drugs currently being tested for repositioning in COVID-19 can be distinguished as 1) drugs potentially able to inhibit one or more steps of the coronavirus lifecycle (**Table 1**) and 2) drugs potentially able to counteract the effects of SARS-CoV-2 infection, such as the amplified immune response and the massive cytokine release, which both lead to severe complications such as coagulopathy and acute respiratory distress syndrome (ARDS) (**Table 2**). To date, only remdesivir has been approved to treat COVID-19.

Drugs Inhibiting One or More Steps of SARS-CoV-2 Lifecycle

Virus Attachment and Entry

The first targetable step of SARS-CoV-2 life cycle is the entry of the virus in the host cells. The virus can enter the cells via endocytosis or via plasma membrane fusion through the interaction between the Spike (S) protein of the virus and angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) at target cell (Fehr and Perlman, 2015). Specifically, it has been demonstrated that SARS-CoV-2 binds to ACE2 receptors for entry and uses TMPRSS2 for S protein priming, which is essential for the binding to its receptor (Hoffmann et al., 2020). A number of molecules hypothesized to down-regulate ACE2 receptors such as estradiol, spironolactone, isotretinoin and retinoic acid, as well as the TMPRSS2 inhibitors bicalutamide, camostat mesilate and nafamostat were therefore proposed as potential treatments for COVID-19 patients. Furthermore, much attention is being paid to two antiviral drugs potentially inhibiting the binding of SARS-CoV-2 to host cells: umifenovir, which could block the trimerization of the S protein, and nelfinavir, which may bind to the S trimer structure inhibiting the membrane fusion process. It has also been hypothesized that human dipeptidyl peptidase 4 (DPP4) could be a functional receptor for the S protein of SARS-CoV-2, which in turn led to the hypothesis that DPP4 inhibitors may play a role in preventing and reducing the risk and progression of COVID-19 (Iacobellis, 2020). As stated above, SARS-CoV-2 can also enter the cells via endocytosis, a transport mechanism by which the virus is enveloped by the cell membrane and enters the cell within a vesicle. Drugs potentially inhibiting the endocytosis, e.g., the antimalarials chloroquine, hydroxychloroquine, amodiaquine, artemisinin and artesunate baricitinib, chlorpromazine, niclosamide, imatinib and amiodarone and the antimalarials have been therefore proposed to inhibit the entry of SARS-CoV-2 in the host cells. The main proposed mechanisms for the inhibition of endocytosis are: 1) the inhibition of endocytic proteins (e.g., clathrins, adaptor-associated protein kinase 1 and dynamin); 2) the accumulation in acid vesicles and, therefore, the inhibition of viral entry when the endocytosis is pH dependent.

Viral Replication

The second step in SARS-CoV-2 lifecycle is the replication of viral RNA from an RNA template, catalyzed by the RNA-dependent RNA polymerase (RdRp) (Fehr and Perlman, 2015). Drugs able to inhibit this enzyme, such as the antiviral drugs favipiravir, galidesivir, tenofovir, sofosbuvir and clevudine, and antivirals inhibiting the replication of RNA, e.g., remdesivir, emtricitabine have been proposed as candidates for repurposing in COVID-19. Interferons (alfa interferon, beta interferon and peginterferon lambda) are also currently being evaluated as viral replication inhibitors and promoters of immune response to clear virus infection. Viral RNA replication is followed by RNA translation and proteolytic processing of viral proteins. Protease inhibitors currently used to treat human immunodeficiency virus and acquired immune deficiency syndrome (HIV/AIDS) such as atazanavir, danoprevir, darunavir, lopinavir and ritonavir and the immunosuppressant levamisole have been therefore proposed as potential treatments to inhibit SARS-CoV-2 replication. Tetracycline derivatives such as doxycycline have also been proposed as candidates for repurposing in COVID-19, due to their potential to chelate metalloproteinases used by coronavirus for cell fusion and virus replication.

Virion Assembly and Release

Once the viral structural proteins are synthetized and processed, they are assembled to form new virus particles called virions. They are subsequently transported to the cell surface in vesicles and then released by exocytosis (Fehr and Perlman, 2015). Thus, antiviral drugs acting on this step of viral replication such as oseltamivir and daclatasvir have been proposed for repurposing for COVID-19 treatment, along with other drugs like the immunosuppressant sirolimus.

Drugs Potentially Counteracting the Effects of SARS-CoV-2 Infection

As stated above, SARS-CoV-2 infection can be associated with amplified immune and inflammatory response leading to an uncontrolled cytokine release, known as cytokine storm, especially in its severe form. The cytokine storm is in turn associated with complications like ARDS, macrophage activation syndrome (MAS), lymphopenia and coagulopathy, representing one of the most studied targets to find an effective treatment for COVID-19 patients (Crisafulli et al., 2020). This is the reason why a number of anti-inflammatory and immunomodulatory drugs like non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, kinase inhibitors and interleukin antagonists are being evaluated to be repositioned in COVID-19. Specifically, these drugs could reduce systemic inflammatory symptoms and counteract cytokine storm effects. Dexamethasone in particular has been evaluated for its ability to counteract the effects of SARS-CoV-2 infection and was the subject of several trials. Indeed, a meta-analysis of seven trials found that dexamethasone was associated with a lower risk of mortality at 28 days (odds ratio = 0.64 [95% confidence interval: 0.50–0.82]) in critically ill COVID-19

patients, compared to placebo or standard of care (Sterne et al., 2020). Anti-inflammatory and immunomodulatory effects have been postulated also for macrolide antibiotics such as azithromycin and clarithromycin (Sultana et al., 2020a).

To treat respiratory complications due to SARS-CoV-2 infection such ARDS, several drugs approved for the treatment of idiopathic pulmonary fibrosis such as nintedanib, pirfenidone and pamrevlumab have been proposed. Other drugs currently being evaluated as candidates for repositioning in COVID-19-induced ARDS are bevacizumab, aviptadil, eculizumab and conestat alfa, due to their potential to reduce pulmonary edema. Coagulopathy is another of the main complications of COVID-19-triggered cytokine storm, suggesting the potential role of anti-thrombotic agents to prevent venous thromboembolism. Coagulation is activated by the inflammatory response through several pro-coagulant pathways (Connors and Levy, 2020) and it presents with a considerable increase of D-dimer levels that could be ascribable to the attempt of the fibrinolytic system to remove fibrin and necrotic tissue from the lung parenchyma (Medcalf et al., 2020). Indeed, it has also been suggested that fibrinolytic therapy may be an effective pharmacological strategy to treat acute lung injury in COVID-19 patients (Liu et al., 2018). Based on these considerations, tissue-plasminogen activator and alteplase are currently being investigated in experimental studies for repurposing in COVID-19 patients. Finally, it is interesting to note that some approved drugs with mechanism of actions that are not immediately associated with COVID-19 have also been proposed as candidates for repositioning. Examples include general anaesthetics ketamine, sevoflurane and isoflurane, which are hypothesized to reduce systemic inflammation and ARDS severity. Further examples include the antidepressants fluoxetine and fluvoxamine, which are thought to be potentially able to counteract hyper-inflammatory symptoms caused by cytokine storm. Another drug proposed as a potential therapy for COVID-19, although not yet evaluated in a clinical trial, is the mood stabilizer lithium, whose antiviral activity was demonstrated at preclinical level, but it was not confirmed in clinical settings (Murru et al., 2020; Rajkumar, 2020).

SOURCES OF EVIDENCE FOR THE BENEFIT-RISK EVALUATION OF DRUGS FOR REPURPOSING IN COVID-19

Pre-Clinical Studies

Pre-clinical studies are those studies which are conducted *in vitro* (in a pathogen, animal or human cells) or *in vivo* through animal models prior to the initiation of clinical studies in patients, whether healthy or sick. They include studies which are conducted in silico (Elmezayen et al., 2020), *in vitro*, such as receptor-binding assays to identify drug ligands and studies using cell lines or tissues excised from animals or humans representative of a specific disease, to evaluate how these biological milieus of varying complexity respond to a drug. Pre-clinical studies also include those studies which are conducted *in vivo*, where animals are exposed to a particular drug. The animals treated with a drug are then tested for various biological and/or behavioral parameters and may be sacrificed for histological examinations. Such animals may be small models, such as mice or ferrets, or larger models, such as non-human primates. A compendium of existing animal models used for COVID-19 research is available in the public domain (National Center for Advancing Translational Sciences, 2020). Taken together, pre-clinical studies can be useful in drug repurposing in the phase of hypothesis generation. Through these studies, the pharmacological mechanism of a drug in a novel context, in this case in SARS-CoV-2 infection can be better understood. This is important to attribute a degree of biological plausibility to the hypothesized effect of a drug in a new indication, as indeed, all indications of repurposed drugs are *de facto* new.

Ideally, pre-clinical studies in a repurposing context should specifically concern the effects of a drug in the disease for which repurposing is being attempted. For example, pre-clinical studies to support the repurposing of an anti-viral drug for SARS-CoV-2 should be conducted using microbiological assays, or cell lines and animal models which have been infected with the agent of interest, i.e., SARS-CoV-2. However, this presupposes an accurate knowledge of the disease pathology and symptoms, which may be problematic in a novel disease such as COVID-19. If the biological and clinical aspects of the disease which are critical determinants of clinical outcomes are not known, they cannot be measured and identified as therapeutic targets, even in a pre-clinical setting. Direct pre-clinical evidence of the infectious agent of interest may also be limited during a pandemic for several reasons. First, the virus has to be isolated, described and cultured before it can be sent to laboratories, unlike other microbial agents which are already known and available *in vitro*. Secondly, while *in vivo* experiments are higher up the evidence hierarchy than *in vitro* experiments because they represent a complex living organism rather than isolated cells of tissues, the pathology of SARS-CoV-2 may be different in animals as compared to humans. As a result, the safety and efficacy of drugs *in vivo* may not be generalizable to humans. Some animal models may need to be specifically developed to address a particular hypothesis and may not be available on large scale. One example is the transgenic mouse model which produces the human ACE2 protein, needed to conduct pre-clinical studies concerning renin-angiotensin-aldosterone system (RAAS) inhibitors, such as angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) (Callaway, 2020). Once developed, the effect of the virus in the mouse model needs to be described in detail for it to be accepted as a valid model of the disease (Bao et al., 2020; Jiang et al., 2020). Only then can pharmacological testing be considered potentially informative. In the absence of *in vitro* and *in vivo* models of SARS-CoV-2, models of other related infective agents can be and have been considered but their relevance to the disease of interest may be ambiguous. An example is how to support the biological plausibility of azithromycin, an antibiotic, being clinically useful in COVID-19, caused by a virus, is based on its ability to modify the reaction of the immune system and to reduce inflammation

(Kanoh and Rubin, 2010; Parnham et al., 2014; Cramer et al., 2017) in a pre-clinical setting. This information was not derived directly from SARS-CoV-2 infections. The effectiveness of azithromycin in a clinical setting has not been proved to date (Sultana et al., 2020a).

Pre-clinical studies can be useful because they can provide results in a relatively short time compared to clinical studies concerning whether a particular pharmacological approach is likely to be worth pursuing. However, the information they can provide on drug safety and efficacy is very limited because pre-clinical studies necessarily provide an incomplete picture of disease pathology as well as potentially generating contrasting findings. The real value of pre-clinical evidence must be proven in a clinical setting. ACEIs and ARBs are a good example of how pharmacological evidence derived from a pre-clinical setting is not always directly translatable to the clinical setting. Pre-clinical evidence on these drugs was not consistent to begin with, with some research suggesting that based on their pharmacological interaction with the virus, that they will worsen prognosis after SARS-CoV-2 infection and/or increase the risk of infection (Fang et al., 2020; Gurwitz, 2020; Zheng et al., 2020), and other research findings concluding that they are likely to be protective (Gurwitz, 2020). Ultimately, in several observational studies of infected patients it was seen that ACEIs and ARBs are likely to be neither (Li et al., 2020a; de Abajo et al., 2020; Feng et al., 2020; Fosbøl et al., 2020; Gao et al., 2020; Mancia et al., 2020; Mehta et al., 2020; Meng et al., 2020; Reynolds et al., 2020; Selçuk et al., 2020; Yang et al., 2020; Zhang et al., 2020). Another example concerns hydroxychloroquine, which was found to inhibit SARS-CoV-2 *in vitro* (Liu et al., 2020) but not found yet to be effective in clinical practice (Geleris et al., 2020), either as treatment or as post-exposure prophylaxis (Boulware et al., 2020). Another important role of pre-clinical studies is to evaluate the pharmacokinetic profile of a potential drug candidate, in order to ensure that the effective doses in animal models can be translated safely to humans.

Clinical Trials

Clinical trials, ranging from single-arm open-label trials to randomised controlled trials (RCTs) provide information on drug tolerability and, most importantly, efficacy. RCTs are considered the gold standard of evidence generation on drug efficacy because randomization of treatment randomly distributes potential confounders among treated and untreated patients. The masking of participants, investigators, and/or outcomes assessor to treatment assignment further increases the reliability of results by preventing bias. The clinical information that trials can provide concerning potentially repurposable drugs for SARS-CoV-2 varies widely and can refer to very specific indications such as post-exposure prophylaxis (NCT04321174 - lopinavir/ritonavir RCT), treatment of mild COVID-19 (NCT04342663 - fluvoxamine RCT) and treatment of hospitalized patients with COVID-19 (NCT04280705 - remdesivir RCT) as well as critically ill hospitalized patients specifically (NCT04244591 - methylprednisolone RCT). This has implications for the generalizability of study results and the application of findings in clinical practice. To address this issue, both the FDA and the World Health Organization (WHO) have published guidance on the recommended standards in drug development and clinical research in COVID-19 (Food and Drug Administration, 2020b; Marshall et al., 2020b).

Although serendipitous drug rediscovery is possible, it is perhaps more likely that drugs with a consolidated pharmacological target in the disease of interest have a higher chance of successfully getting through trials, not least because this may impact the choice of appropriate outcomes. An example of a drug which is not supported by a strong pharmacological rationale is fluvoxamine, currently being tested for the treatment of non-hospitalized persons with COVID-19 (NCT04342663 - fluvoxamine RCT). Another example is estradiol, where evidence is still emerging (Li et al., 2020b), currently being tested among persons with suspected or confirmed COVID-19 to evaluate whether it reduces the severity of symptoms (NCT04359329 - estradiol patch RCT). These examples highlight the complementarity of pre-clinical and clinical studies in drug repurposing as in drug discovery, because pre-clinical evidence can suggest the biological plausibility of a drug being effective while the clinical evidence is needed to confirm that a drug is effective and safe.

Conducting methodologically sound trials during a pandemic can be challenging, for scientific, logistical and ethical reasons (Angus, 2020) and rapid dissemination of poor quality studies can have serious consequences (Kim et al., 2020). Some issues highlight the need to interpret the available clinical trials with great care. Not all trials are created equal and there is an accepted hierarchy of quality. Open-label trials, such as an ongoing trial for ruxolitinib in combination with simvastatin (NCT04348695 - ruxolitinib in combination with simvastatin RCT) in such a highly charged situation as a pandemic may lead to bias, as compared to double-blind trials (Wang et al., 2020). Similarly, clinical studies lacking randomization (NCT04304313 - sildenafil non-randomized trial) are considered much less reliable than studies randomizing treatment (NCT04350593 - dapagliflozin RCT).

Patient recruitment can be more problematic when organizing a trial during a pandemic than it would otherwise be. The size of a study population can limit the scope of causal inference if a trial is underpowered. There are currently ongoing trials to repurpose drugs which aim to recruit as little as 10 patients (NCT04304313 - sildenafil non-randomized trial). Restrictive inclusion and exclusion criteria may not only lead to an under-powered study but also to a patient population that is not representative of the real-world setting. For example, in a randomized double-blind trial of fluvoxamine in COVID-19, patients with severe underlying respiratory disease were excluded, as were patients with dementia, as these could not provide informed consent (NCT04342663 - fluvoxamine RCT). A broader population would only be included in phase III trials. While such exclusion criteria are necessary for the purpose of causal inference and to respect patients' right to self-determination, lack of this data in phase III confirmatory trials may limit the generalizability of study findings by excluding patients who are at highest risk of contracting COVID-19

and/or developing severe symptoms as well as of developing adverse drug effects. Even patient follow-up can be problematic during a pandemic. Some researchers have resolved this issue by conducting trials remotely, for example completely avoiding face-to-face encounters, with study material, including the study drug, being delivered to patients' homes (NCT04342663 - fluvoxamine RCT). This could be a problem for taking into account patient compliance and is likely to be more viable for cases of COVID-19 which are treated at home, however use of digital communications such as text messaging, teleconference programs and couriers has largely overcome this. While ideally a clinical trial should be conducted according to a strict protocol, there are ethical implications in continuing to treat patients with a comparator drug if the main study drug appears to be effective. Indeed, some trials explicitly state that should interim results suggest a study drug is effective, it will be considered the new control (NCT04280705 - remdesivir RCT). Small controlled trials are considered to be less useful when evaluating drug safety but this is potentially less of a problem with drugs which have already been approved, because their safety profile is likely to have already been well-described. Indeed, this is the main advantage of repurposing drugs as opposed to *de novo* drug discovery. However, because of their limitations clinical trials must be complemented by observational studies conducted in a real-world setting.

A variant of clinical trials worth mentioning, a hybrid between trial design and observational study design, is the adaptive trial. In adaptive trials, a review and adapt approach is used while the trial is being conducted, as opposed to the linear approach used in classical trials, where the trial occurs in three distinct phases, i.e., trial design, implementation and analyses (Pallmann et al., 2018). As a result, certain changes can be made to trial design and analytic plans based on preliminary data. Such a study design can be very valuable in the pandemic setting because new findings are constantly emerging and standards of care are rapidly changing. Some examples of adaptive trials being carried out to evaluate drug efficacy and safety for COVID-19 treatment include the REMAP-CAP (Angus et al., 2020), WHO SOLIDARITY trial (World Health Organization, 2020) and RECOVERY trials (Horby et al., 2020).

There is a notable interplay between regulatory and government bodies and researchers conducting clinical trials, as such entities have a role in regulating clinical trials. An example is the ACTIV/Operation Warp Speed groups in the United States that are coordinating pre-clinical studies and clinical evaluation of interventions to advance the most promising candidates given the limited resources available, including animals for pre-clinical evaluation and patient population for clinical evaluation.

Observational Studies

While clinical studies are useful to evaluate drug efficacy, they may not be able to provide evidence rapidly and on a large scale, as they are contingent on the speed of prospective data collection and analysis. During a pandemic, time is of essence and public health authorities cannot wait for weeks until trial results get published. Observational studies have an important role to play in this regard because they rely either on data which has already been collected or on data which is collected quickly in a prospective manner using previously established data systems and can therefore be conducted rapidly. They should be conducted alongside prospective controlled clinical trials which establish pharmacological basis of evidence. The main role of observational studies in evidence generation concerns drugs which are used off-label for COVID-19. For example, a retrospective observational study was carried out in a hospital in Lombardy, Italy, to evaluate the effectiveness of anakinra in improving clinical outcomes among patients infected with COVID-19, finding that this IL-1 antagonist was effective (Cavalli et al., 2020). Similarly, an observational study was conducted in hospitals of the Bologna and Emilia-Romagna areas in Italy, to assess the effectiveness of tocilizumab in improving clinical outcomes among patients with COVID-19, also finding that this drug is potentially effective (Campochiaro et al., 2020). It was also observational studies which were able to rapidly shed light on lack of effectiveness of RAAS inhibitors in improving clinical outcomes in COVID-19 (Li et al., 2020a; de Abajo et al., 2020; Feng et al., 2020; Fosbøl et al., 2020; Gao et al., 2020; Mancia et al., 2020; Mehta et al., 2020; Meng et al., 2020; Reynolds et al., 2020; Selçuk et al., 2020; Yang et al., 2020; Zhang et al., 2020). Another example yet is an observational study which showed that drugs used to treat prostate cancer, androgen-deprivation therapies, may have a protective effect among persons with COVID-19 (Montopoli et al., 2020). Observational studies have other advantages in addition to the speed of data collection and large sample sizes. The populations identified in these studies are a reflection of real-world patients, including those patients who are likely to be excluded by trials—children, the very old, persons with serious underlying respiratory diseases and persons with a large number of concomitant medications (Sultana et al., 2013).

To date, there are 50 studies concerning COVID-19 recorded in the EU post-authorization study (PAS) register, the official European repository of PAS. The PASs for a repurposed product in a pandemic depend on the potential and identified risks, as well as the important missing information identified during pre-marketing. While the gaps in the safety and effectiveness information at the time of licensing are greater for a repurposed product that has been developed expeditiously, the principles are similar to conventional products. Research questions need to be formulated based on the public health needs and the available pre-marketing data. The next task will be selecting the most appropriate study approach (primary or secondary data collection), study setting or data resource and study design (cohort, case-control, nested case-control, cross sectional or in some cases simple randomised clinical trial). Open extensions of post-marketing clinical trials are an important method which will be used frequently. Situations where the incidence and prevalence of cases is low present a challenge; a solution is to conduct the studies in countries with higher incidence of the infection or extending the study population by conducting multi-country studies. The sample size of studies spanning several countries is typically larger than those restricted to a single country, meaning that the

target recruitment is generally achieved more quickly and results are available faster. Multinational studies do not only report the effect of the drug but also reflect the nature and delivery of the healthcare system in the different countries. This in turn has an impact on the conduct of the studies, as certain types of health information may not be available in the same way in all study countries. This includes information such as exposure, outcomes, confounders and previous medical history. However, this is not an insurmountable issue because one of the key points of studies conducted in a network of countries is that the master study protocol can be modified to take account of different data sources. Depending on the heterogeneity or similarity of the information available, results can be meta-analyzed or pooled with data from different sources with the same objectives. Multi-database studies are becoming increasingly important as their potential in reaching large sample sizes over a short observation period is recognized (Gini et al., 2020). Indeed, it is internationally acknowledged that having a stable multi-database network to rapidly provide almost real-time healthcare information to regulatory and public health agencies is very important (Sultana et al., 2020b). This potential is highlighted in a public health crisis such as the COVID-19 pandemic.

Of course, observational studies also have their limitations. Since they are often conducted using sources of secondary data, i.e., data not intended primarily for research purposes, the quality of the data must be thoroughly checked as it can contain errors in data entry, such as incomplete data, Anatomic Therapeutic Chemical (ATC) classification codes at a lower level than the fifth level, missing data, such as sex, or clearly implausible data, such as date of birth set in the future. Furthermore, diagnosis data in secondary data sources may be of limited reliability and there may be underestimation of certain diseases based on the context in which coding is assigned. For example, acute conditions are likely to be better captured in secondary data sources such as hospital claims while chronic conditions are likely to be better captured in primary care electronic records (Trifirò et al., 2019). In the case of COVID-19 specifically, observational studies may be limited by the extent to which they can reliably identify patients who have a microbiologically ascertained diagnosis of COVID-19. Finally, unlike classical clinical trials, observational studies cannot ascertain that a prescribed or dispensed drug was truly administered. This is assumed to be the case, but in the context of a pandemic, it is possible that patients may not actually go to fill their prescriptions.

CONCLUSION

Several drugs are currently being studied as potential repurposing candidates in clinical trials, but to date only remdesivir has been approved for the treatment of COVID-19. Although drug repurposing has the potential to decrease the time usually required for a drug to reach the market, it is a process that is still associated with many challenges, whether from a regulatory or a scientific perspective. Close collaboration between various stakeholders is needed to leverage and critically evaluate existing evidence and strategically plan the generation of new pre-clinical, clinical and observational evidence to investigate the efficacy/effectiveness and safety of drug for potential repurposing. One of the main objectives of such a collaboration should be to avoid duplication of studies and plan studies in such a way that the outcomes evaluated can be compared. Pre-clinical, clinical and observational research all generate complementary information which is necessary in building the case for drug repurposing.

AUTHOR CONTRIBUTIONS

GT conceived the study. JS, SC, FG, EL, and SS wrote the first draft of the paper.

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REVIEW
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Repurposing Sigma-1 Receptor Ligands for COVID-19 Therapy?

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Outbreaks of emerging infections, such as COVID-19 pandemic especially, confront health professionals with the unique challenge of treating patients. With no time to discover new drugs, repurposing of approved drugs or in clinical development is likely the only solution. Replication of coronaviruses (CoVs) occurs in a modified membranous compartment derived from the endoplasmic reticulum (ER), causes host cell ER stress and activates pathways to facilitate adaptation of the host cell machinery to viral needs. Accordingly, modulation of ER remodeling and ER stress response might be pivotal in elucidating CoV-host interactions and provide a rationale for new therapeutic, host-based antiviral approaches. The sigma-1 receptor (Sig-1R) is a ligand-operated, ER membrane-bound chaperone that acts as an upstream modulator of ER stress and thus a candidate host protein for host-based repurposing approaches to treat COVID-19 patients. Sig-1R ligands are frequently identified in in vitro drug repurposing screens aiming to identify antiviral compounds against CoVs, including severe acute respiratory syndrome CoV-2 (SARS-CoV-2). Sig-1R regulates key mechanisms of the adaptive host cell stress response and takes part in early steps of viral replication. It is enriched in lipid rafts and detergent-resistant ER membranes, where it colocalizes with viral replicase proteins. Indeed, the non-structural SARS-CoV-2 protein Nsp6 interacts with Sig-1R. The activity of Sig-1R ligands against COVID-19 remains to be specifically assessed in clinical trials. This review provides a rationale for targeting Sig-1R as a host-based drug repurposing approach to treat COVID-19 patients. Evidence gained using Sig-1R ligands in unbiased *in vitro* antiviral drug screens and the potential mechanisms underlying the modulatory effect of Sig-1R on the host cell response are discussed. Targeting Sig-1R is not expected to reduce dramatically established viral replication, but it might interfere with early steps of virus-induced host cell reprogramming, aid to slow down the course of infection, prevent the aggravation of the disease and/or allow a time window to mature a protective immune response. Sig-1R-based medicines could provide benefit not only as early intervention, preventive but also as adjuvant therapy.

Keywords: COVID-19, SARS-CoV-2, anti-viral, repurposed drugs, drug repurposing, sigma-1 receptor, ER stress, viral replication

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INTRODUCTION

The newly emerged 2019 novel coronavirus (CoV), named as severe acute respiratory syndrome CoV-2 (SARS-CoV-2), has been associated with high infection rates and has spread rapidly to become a pandemic (COVID-19 pandemic) since its identification in patients with severe pneumonia in Wuhan, China. Unfortunately, no vaccine has yet been approved to treat human CoVs and the discovery and development of new drugs will require years. Accordingly, repurposing of approved drugs or drugs in clinical development has emerged as a feasible approach to reduce the time compared to *de novo* drug discovery and ultimately to provide a faster treatment option for COVID-19 patients.

CoV replication is structurally and functionally associated with the endoplasmic reticulum (ER) (Sola et al., 2015), and CoV infection is well known to activate pathways to facilitate adaptation of ER stress to viral needs. These embrace hijacking the host cell ER stress responses to modulate protein translation, ER protein folding capacity, ER-associated degradation (ERAD), including autophagy, and apoptotic cell death (Fung and Liu, 2014; Fung et al., 2014a). Therefore, modulation of ER stress response might be pivotal in elucidating CoV-host interactions and might provide the rationale for new therapeutic approaches.

The sigma-1 receptor (Sig-1R) acts as an upstream modulator of ER stress. Sig-1R is a ligand-operated, membrane-bound chaperone that normally reside at the ER-mitochondrion contact called the (mitochondrion-associated ER membrane (MAM), where it regulates ER-mitochondrion signaling and ER-nucleus crosstalk (Hayashi, 2019). Mitochondrial function regulation by Sig-1R includes bioenergetics and free radical generation/oxidative stress. When cells undergo stress, Sig-1R translocates from the MAM to the ER reticular network and plasma membrane to regulate a variety of functional proteins. Via its molecular chaperone activity, the Sig-1R regulates protein folding/degradation, calcium (Ca^{2+}) homeostasis, ER stress responses, autophagy, and ultimately cell survival (Hayashi and Su, 2007; Su et al., 2010; Schrock et al., 2013; Vollrath et al., 2014; Hayashi, 2019; Delprat et al., 2020). Interestingly, its chaperone activity can be activated or inhibited by synthetic Sig-1R ligands in an agonist-antagonist manner.

As it regards to its potential antiviral activity, Sig-1R ligands are frequently identified in in vitro drug repurposing screens aiming to identify antiviral compounds against SARS-CoV-2 and other CoVs. Mechanistically, Sig-1R is involved in cellular stress pathways which are used by viruses to promote viral replication (Vasallo and Gastaminza, 2015). Accordingly, Sig-1R has been shown to colocalize with viral replicase proteins in membranous compartments (Friesland et al., 2013), and it has been recently reported that the non-structural (NS) SARS-CoV-2 protein Nsp6 directly interacts with Sig-1R (Gordon et al., 2020). Sig-1R is expressed at substantial density in rodent (Lever et al., 2015) and human (Stone et al., 2006) lungs.

Here pharmacological and genetic data supporting a role for Sig-1R in viral infection are collected and summarized, with a focus on CoV in general and SARS-CoV-2 in particular. Targeting Sig-1R is identified as a potential drug repurposing approach to treat COVID-19 patients that, unlike virus-targeted antiviral agents, addresses adaptive cellular mechanisms of host cells that are crucial for viral infection.

SIGMA-1 RECEPTOR LIGANDS EXERT ANTIVIRAL ACTIVITY

Pharmacology Findings Against Non-Coronaviruses

The first insight about a potential role for Sig-1R ligands as antivirals was probably published in 1984 (Nemerow and Cooper, 1984). In this study, several phenothiazines, including trifluoperazine, chlorpromazine, prochlorpromazine and promethazine as well haloperidol (non-phenothiazine but butyrophenone) were shown to inhibit infection of B lymphocytes by a human herpesvirus, Epstein-Barr virus (EBV). By this time sigma was just starting to be considered a separate binding site from phencyclidine and mu and delta opioid receptors to which (+)-[^{3}H]SKF10,047 binds (Su, 1982; Tam, 1983). Also by this time, different non-selective neuroleptics including haloperidol, trifluoperazine, chlorpromazine and promethazine were shown to bind this sigma site (Su, 1982; Tam and Cook, 1984) (**Table 1**), but this was twelve years before the Sig-1R was first cloned (Hanner et al., 1996). Accordingly, authors did not mention sigma mechanisms and attributed the antiviral efficacy of these drugs to effects on calmodulin-regulated cellular endocytic processes involved in early stages of EBV infection. These non-selective Sig-1R ligands were found later to exert antiviral activity against other viruses, including coronaviruses (**Table 1**).

Drugs binding Sig-1R showing antiviral activity were identified in three *in vitro* screening studies aiming to discover inhibitors targeting different steps of Hepatitis C virus (HCV) infection. In the first study, a set of 446 compounds from the National Institutes of Health Clinical Collection were assayed for their ability to inhibit HCV infection of human hepatocarcinoma Huh-7 cells *in vitro* (Gastaminza et al., 2010). Compounds were screened in a cell-based assay in an unbiased manner, independent on target specificity or mechanism of action. Among the 446 clinically approved small molecules assayed, 33 compounds displayed antiviral activity (>85% reduction in HCV infection of Huh-7 cells, as compared to the vehicle DMSO control) in the absence of cytotoxicity at low micromolar and submicromolar concentrations. Compounds targeted several aspects of HCV infection, including entry, replication, and assembly. Some of the active antiviral compounds were already known to have antiviral activity, but the ability of most of them to inhibit HCV infection was unexpected. Among the 33 active compounds, 19 compounds (cyproheptadine, toremifene, fluphenazine, trifluoperazine, CGS 12066B, prochlorperazine, doxepin, ketotifen, amiodarone, lofepramine, rimcazole, clobenpropit, salmeterol, azelastine, desloratadine, indatraline, haloperidol, benproperine and carvedilol) bind to Sig-1R with high to moderate affinity (**Table 1**). All of them are non-selective and bind primarily to molecular targets other than Sig-1R, but it is remarkable that near

TABLE 1 | Drug class.

Intended therapeutic effect	Compound	Sigma-1 receptor affinity		Antiviral activity	
		Ki or IC50[a] (nM)	References	Virus	References
Antiarrhythmic	Amiodarone	1.4–2.1	Moebius et al., 1997	EBOV	(Madrid et al., 2015)
		335[a]	Buschman, 2007/Internal data		(Gehring et al., 2014)
					(Salata et al., 2015)
					(Dyall et al., 2018)
				HCV	(Gastaminza et al., 2010)
					(Chockalingam et al., 2010)
					(Cheng et al., 2013)
				SARS-CoV	(Stadler et al., 2008)
				SARS-CoV-2	(Mirabelli et al., 2020)
Antidepressant Anxiolytic	Amitriptyline	287	Werling et al. 2007	FLUAV (H5N1)	(Huang et al., 2020)
		216[a]	Buschman, 2007/Internal data	HCV	(Chockalingam et al., 2010)
		300[a]	Weber et al., 1986		
Antimalarial	Amodiaquine	355[a]	Buschman, 2007/Internal data	EBOV	(Madrid et al., 2013)
				DENV	(Boonyasuppayakorn et al., 2014)
				MARV	(Madrid et al., 2013)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV	(Dyall et al., 2014)
				SARS-CoV-2	(Jeon et al., 2020; Weston et al., 2020)
Antihistaminic	Astemizole	43[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV	(Dyall et al., 2014)
Antiallergic	Azelastine	274[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)
Antitussive	Benproperine	19[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)
Antiparkinsonian Treatment dystonia and extrapyramidal side effects of antipsychotics	Benztropine	65[a]	Buschman, 2007/Internal data	EBOV	(Madrid et al., 2015)
					(Johansen et al., 2015)
				HCV	(Chockalingam et al., 2010)
					(Mingorance et al., 2014)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV SARS-CoV-2	(Dyall et al., 2014; Weston et al., 2020)
Antiarrhythmic and antianginal	Bepridil	365[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
Antihypertensive	Carvedilol	1570[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)
Serotonergic	CGS 12066B	1180[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)

(Continued on following page)

TABLE 1 | (*Continued*) Drug class.

Intended therapeutic effect	Compound	Sigma-1 receptor affinity		Antiviral activity	
		Ki or IC50[a] (nM)	References	Virus	References
Antimalarial	Chloroquine	108.6	PDSP Ki Database certified data	CCHFV	(Ferraris et al., 2015)
		2300[a]	Buschman, 2007/Internal data	CHIKV	(Bassetto et al., 2013)
					(Pohjala et al., 2011)
				DENV	(Farias et al., 2013)
				EBOV	(Madrid et al., 2013)
					(Madrid et al., 2015)
				FLUAV (H1N1 and H3N2)	(Ooi et al., 2006)
				FLUAV (H5N1)	(Yan et al., 2013)
				HCoV-229E	(de Wilde et al., 2014)
				HCoV-OC43	(Keyaerts et al., 2009)
				HIV-1	(Savarino et al., 2001)
				MARV	(Madrid et al., 2013)
				MERS-CoV	(de Wilde et al., 2014)
					(Dyall et al., 2014)
				SARS-CoV	(de Wilde et al., 2014)
					(Keyaerts et al., 2004)
					(Dyall et al., 2014)
				SARS-CoV-2	(Yao et al., 2020)
					(Jeon et al., 2020; Weston et al., 2020)
Antihistaminic Antiparkinsonian	Chlorphenoxamine	1760[a]	Buschman, 2007/Internal data	MERS-CoV	(Dyall et al., 2014)
				SARS-CoV	(Dyall et al., 2014)
Antipsychotic	Chlorpromazine	146	Tam and Cook, 1984	CCHFV	(Ferraris et al., 2015)
		200[a]	Lang et al., 1994	CHIKV	(Pohjala et al., 2011)
		1070[a]	Buschman, 2007/Internal data	EBV	(Nemerow and Cooper, 1984)
				FLUAV	(Nugent and Shanley, 1984)
				HCoV-229E	(de Wilde et al., 2014)
				HCV	(Mingorance et al., 2014)
				MERS-CoV	(Dyall et al., 2014)
					(de Wilde et al., 2014)
				SARS-CoVSARS-CoV-2	(Dyall et al., 2014)
					(Jeon et al., 2020; Weston et al., 2020)
Antihistaminic Antivertigo	Cinnarizine	22	Klein and Musacchio, 1989	HCV	(Chockalingam et al., 2010)
		119[a]	Buschman, 2007/Internal data		
Antihistaminic	Clemastine	67	Gregori-Puigjané et al., 2012	EBOV	(Johansen et al., 2015)
		505[a]	Buschman, 2007/Internal data	SARS-CoV-2	(Gordon et al., 2020)
Neuroprotectant	Clobenpropit	1080[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)
Estrogen receptor modulator	Clomiphene	4.7–12	Moebius et al., 1997	EBOV	(Madrid et al., 2015)
Ovulation stimulator		195[a]	Buschman, 2007/Internal data		(Johansen et al., 2013; Johansen et al., 2015)
				HCV	(Murakami et al., 2013)
					(Mingorance et al., 2014)
Antidepressant	Clomipramine	195[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
				HCV	(Mingorance et al., 2014)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV SARS-CoV-2	(Dyall et al., 2014; Weston et al., 2020)
Antihistaminic Antitussive	Cloperastine	20	Gregori-Puigjané et al., 2012	SARS-CoV-2	(Gordon et al., 2020)
		277[a]	Buschman, 2007/Internal data		

(Continued on following page)

TABLE 1 | (*Continued*) Drug class.

Intended therapeutic effect	Compound	Sigma-1 receptor affinity		Antiviral activity	
		Ki or IC50[a] (nM)	References	Virus	References
Antifungal	Cycloheximide	1030[a]	Buschman, 2007/Internal data	MERS-CoV	(Dyall et al., 2014
				SARS-CoV	(Dyall et al., 2014
Antiallergic Antihistaminic	Cyproheptadine	284[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)
		930	He et al., 2012		(Chockalingam et al., 2010)
Antidepressant	Desipramine	1190[a]	Buschman, 2007/Internal data	HCV	(Mingorance et al., 2014)
		1987	Narita et al., 1996		
Antiallergic	Desloratadine	1510[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)
Antidepressant	Doxepin	394[a]	Buschman, 2007/Internal data	CHIKV	(Pohjala et al., 2011)
				HCV	(Gastaminza et al., 2010)
Selective sigma-1 receptor antagonist	E-52862 (S1RA)	17	Romero et al., 2012	SARS-CoV-2	(Mirabelli et al., 2020)
Antimigraine	Flunarizine	28[a]	Buschman, 2007/Internal data	HCV	(Chockalingam et al., 2010)
Antidepressant	Fluoxetine	240	Narita et al., 1996	HCV	(Mingorance et al., 2014)
		949[a]	Buschman, 2007/Internal data	HCV	
Antipsychotic	Flupentixol	70[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
Antipsychotic	Fluphenazine	13	Tam and Cook, 1984	EBOV	(Johansen et al., 2015)
		62	Largent et al., 1984	HCV	(Gastaminza et al., 2010)
		109[a]	Buschman, 2007/Internal data	MERS-CoV	(Chockalingam et al., 2010)
					(Dyall et al., 2014)
				SARS-CoV SARS-CoV-2	(Dyall et al., 2014; Weston et al., 2020)
Antipsychotic	Fluspirilene	150	Schotte et al., 1996	MERS-CoV	(Dyall et al., 2014)
		380[a]	Contreras et al., 1990	SARS-CoV	(Dyall et al., 2014)
		563[a]	Buschman, 2007/Internal data	SARS-CoV-2	(Weston et al., 2020)
Antipsychotic	Haloperidol	0.2	Hanner et al., 1996	EBV	(Nemerow and Cooper, 1984)
		1.1	Schotte et al., 1996	HCV	(Gastaminza et al., 2010)
		1.1[a]	Lang et al., 1994	MERS-CoV	(Dyall et al., 2014)
		1.2	Akunne et al., 1997	SARS-CoV	(Dyall et al., 2014)
		2.4	Largent et al., 1984	SARS-CoV-2	(Gordon et al., 2020)
		3	Tam and Cook, 1984		
		6.5–7.3	Su, 1991		
		17[a]	Weber et al., 1986		
		73[a]	Buschman, 2007/Internal data		
Antimalarial	Hydroxychloroquine	2120[a]	Buschman, 2007/Internal data	DENV	(Wang et al., 2015)
				HIV-1	(Sperber et al., 1993)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV	
				SARS-CoV-2	(Gordon et al., 2020)
					(Yao et al., 2020)
					(Mirabelli et al., 2020; Weston et al., 2020)
Antiallergic	Hydroxyzine	342[a]	Buschman, 2007/Internal data	HCV	(Mingorance et al., 201)4
Antihistaminic		46	Klein and Musacchio, 1989		
Anxiolytic					
Neuroprotectant Anticonvulsant Antihypertensive	Ifenprodil	1.02	Gitto et al., 2014	FLUAV (H5N1)	(Zhang et al., 2019)
		2–2	Hanner et al., 1996	HCV	(Chockalingam et al., 2010)
		5.5[a]	Buschman, 2007/Internal data		
		28–34	Su, 1991		
Antidepressant	Imipramine	343	Narita et al., 1996	HCV	(Mingorance et al., 2014)
		529[a]	Buschman, 2007/Internal data		

(Continued on following page)

TABLE 1 | (*Continued*) Drug class.

Intended therapeutic effect	Compound	Sigma-1 receptor affinity		Antiviral activity	
		Ki or IC50[a] (nM)	References	Virus	References
Antiadictive	Indatraline	737[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)
Antihistaminic	Ketotifen	3800[a]	Buschman, 2007/Internal data	HCV	Gastaminza et al., 2010)
Antidepressant	Lofepramine	2520	PDSP Ki Database certified data	HCV	(Gastaminza et al., 2010)
		100% inh. at 10 μM	Buschman, 2007/Internal data		
Antimigraine	Lomerizine	37[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
Antidiarrheal	Loperamide	271	Buschman, 2007/Internal data	HCoV-229E	(de Wilde et al., 2014)
				MERS-CoV	
				SARS-CoV	
				SARS-CoV-2	(Jeon et al., 2020)
Antidepressant	Maprotiline	37[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
Antimalarial	Mefloquine	2560[a]	Buschman, 2007/Internal data	JCV	(Brickelmaier et al., 2009)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV	(Dyall et al., 2014)
				SARS-CoV-2	(Jeon et al., 2020; Weston et al., 2020)
Antihistaminic	Methdilazine	167[a]	Buschman, 2007/Internal data	CHIKV	(Pohjala et al., 2011)
Sigma ligand	PB28	0.38	Abate et al., 2011	SARS-CoV-2	(Gordon et al., 2020)
		10–13	Azzariti et al., 2006		
Sigma ligand	PD-144418	0.08	Akunne et al., 1997	SARS-CoV-2	(Gordon et al., 2020)
		0.46	Lever et al., 2014		
Antipsychotic Antiemetic Anxiolytic	Perphenazine	8	Tam and Cook, 1984	CHIKV	(Pohjala et al., 2011
		13	Largent et al., 1984	HCV	(Chockalingam et al., 2010)
		21[a]	Weber et al., 1986		(Mingorance et al., 2014)
		45–53	Su, 1991		
		104[a]	Buschman, 2007/Internal data		
Antipsychotic Treatment of Tourette syndrome and resistant tics	Pimozide	139	Tam and Cook, 1984	EBOV	(Johansen et al., 2015)
		508	Largent et al., 1984	HCV	(Chockalingam et al., 2010)
		337[a]	Buschman, 2007/Internal data		
Antipsychotic	Piperacetazine	823[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
Antipsychotic Antiemetic Anxiolytic	Prochlorperazine	232[a]	Buschman, 2007/Internal data	EBOV	(Madrid et al., 2015)
					(Johansen et al., 2015)
				HCV	(Gastaminza et al., 2010)
					(Chockalingam et al., 2010)
Endogenous steroid Menopausal hormone therapy	Progesterone	173–196	Su, 1991	SARS-CoV-2	(Gordon et al., 2020)
		1960[a]	Buschman, 2007/Internal data		
		260–338	Hanner et al., 1996		
Antiallergic Antihistaminic	Promethazine	857[a]	Buschman, 2007/Internal data	EBV	(Nemerow and Cooper, 1984)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV SARS-CoV-2	(Dyall et al., 2014; Weston et al., 2020)
Antidepressant	Protriptyline	307[a]	Buschman, 2007/Internal data	HCV	(Chockalingam et al., 2010)
Antimalarial	Quinacrine	953[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
Antiarrhythmic	Quinidine	570	Musacchio and Klein, 1988	HCV	(Chockalingam et al., 2010)
		5480[a]	Buschman, 2007/Internal data		
Serotonergic	Quipazine-6N	1250[a]	Buschman, 2007/Internal data	HCV	(Chockalingam et al., 2010)
Estrogen receptor modulator Treatment of osteoporosis in postmenopausal women	Raloxifene	38	Laggner et al., 2005	HCV	(Chockalingam et al., 2010)
		122[a]	Buschman, 2007/Internal data		(Mingorance et al., 2014)
					(Murakami et al., 2013)

(Continued on following page)

TABLE 1 | (*Continued*) Drug class.

Intended therapeutic effect	Compound	Sigma-1 receptor affinity		Antiviral activity	
		Ki or IC50[a] (nM)	References	Virus	References
Sigma ligand	Rimcazole	260[a]	Lang et al., 1994	HCV	(Gastaminza et al., 2010)
		500[a]	Ferris et al., 1986		
		820	Gilligan et al., 1994		
		908	Husbands et al., 1999		
Antitumoral (breast cancer)	Ritanserin	190[a]	Buschman, 2007/Internal data	HCV	(Chockalingam et al., 2010)
Antiasthmatic	Salmeterol	151[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)
Antidepressant Anxiolytic	Sertraline	57	Narita et al., 1996	EBOV	(Johansen et al., 2015)
		260[a]	Buschman, 2007/Internal data		
Sigma ligand	Siramesine	17[a]	Perregaard et al., 1995	SARS-CoV-2	(Gordon et al., 2020)
Estrogen receptor modulator	Tamoxifen	34–26	Moebius et al., 1997	HCV	(Mingorance et al., 2014)
Antitumoral (breast cancer)		367[a]	Buschman, 2007/Internal data	HSV-1	(Murakami et al., 2013)
					(Zheng et al., 2014)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV SARS-CoV-2	(Dyall et al., 2014; Weston et al., 2020)
Antifungal	Terconazole	159[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2015)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV SARS-CoV-2	(Dyall et al., 2014; Weston et al., 2020)
Antiemetic	Thiethylperazine	528[a]	Buschman, 2007/Internal data	CHIKV	(Pohjala et al., 2011)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV SARS-CoV-2	(Dyall et al., 2014; Weston et al., 2020)
Antipsychotic	Thioridazine	286[a]	Buschman, 2007/Internal data	CHIKV	(Pohjala et al., 2011)
				EBOV	(Johansen et al., 2015)
Antipsychotic	Thiothixene	353[a]	Buschman, 2007/Internal data	MERS-CoV	(Dyall et al., 2014)
				SARS-CoV	(Dyall et al., 2014)
Estrogen receptor modulator	Toremifene	220[a]	Buschman, 2007/Internal data	EBOV	(Johansen et al., 2013; Johansen et al., 2015)
Antitumoral (breast cancer)				HCV	(Gastaminza et al., 2010)
				MERS-CoV	(Dyall et al., 2014)
				SARS-CoV SARS-CoV-2	(Dyall et al., 2014; Weston et al., 2020)
Antipsychotic	Trifluoperazine	15–21	Hanner et al., 1996	EBV	(Nemerow and Cooper, 1984)
		54	Tam and Cook, 1984	HCV	(Gastaminza et al., 2010)
		125[a]	Buschman, 2007/Internal data		(Chockalingam et al., 2010)
		345[a]	Weber et al., 1986		
Antipsychotic Antiemetic	Triflupromazine	154	Tam and Cook, 1984	HCV	(Chockalingam et al., 2010)
		470[a]	Buschman, 2007/Internal data	MERS-CoV	(Dyall et al., 2014)
		605[a]	Weber et al., 1986	SARS-CoV	(Dyall et al., 2014)
Antihypertensive	Verapamil	258[a]	Buschman, 2007/Internal data	FLUAV	(Nugent and Shanley, 1984)
Antiarrhythmic				SARS-CoV-2	(Mirabelli et al., 2020)
Antipsychotic	(–)-Butaclamol	40	Tam and Cook, 1984	HCV	(Gastaminza et al., 2010)
		95.7	Akunne et al., 1997		
		157	Largent et al., 1984		
		183[a]	Weber et al., 1986		
Antipsychotic	(±)-Butaclamol	343[a]	Buschman, 2007/Internal data	HCV	(Gastaminza et al., 2010)

[a]*IC50. CHIKV, Chikungunya virus; HCV, Hepatitis C virus; FLUAV, Influenza A virus; H5N1, Avian influenza A H5N1 virus, other subtypes; CCHFV, Crimean-Congo hemorrhagic virus; HSV-1, Herpes simplex virus type 1; JCV, JC (John Cunningham) virus; DENV, Dengue virus; HCoV-229E, Human coronavirus strain 229E; MARV, Marburg hemorrhagic fever virus; MERS-CoV, East respiratory syndrome coronavirus; SARS-CoV, Human coronavirus strain OC43 (HCoV-OC43) Severe acute respiratory syndrome coronavirus; EBOV, Ebola virus; HIV-1, Human immunodeficiency virus type 1; SARS-CoV-2, Severe acute respiratory syndrome coronavirus type 2; EBV, Epstein-Barr virus.*

60% of active compounds inhibiting >85% HCV infection had known affinity for Sig-1R. Most of these compounds inhibited HCV entry and display selective anti-HCV activity relative to vesicular stomatitis virus (VSV)-pseudotypes. In another unbiased cell-based screening, a chemical library of 281 clinically approved drugs prescribed for non-HCV applications were assayed. Twelve compounds reduced HCV infection by more than one order of magnitude without significantly reducing cell biomass (Mingorance et al., 2014). Surprisingly, all of them (chlorpromazine, clomipramine, desipramine, perphenazine, imipramine, raloxifene, tamoxifen, clomiphene, hydroxyzine, benztropine and fluoxetine) bind to Sig-1R with significant affinity (**Table 1**). Hydroxyzine and benztropine were selected to define the step of the replication cycle they target. Both HCV inhibitors interfered with an early step of the infection, at a step downstream viral particle attachment and internalization but previous to the establishment of persistent RNA replication and infectious virus production. Together, results reinforced the notion that compounds inhibit an early step of HCV RNA replication. The involvement of Sig-1R was not discussed in these papers, but authors noted that affinity for this molecular target was shared by a significant number of active compounds, which evoked studies addressing specifically the role of Sig-1R in HCV infection (Friesland et al., 2013).

In the third of these screening studies, 1280 compounds, many in clinical trials or approved for therapeutic use, were assayed for their ability to alleviate the HCV-induced cytopathic effect on the engineered cell line n4mBid (Chockalingam et al., 2010). They found >200 hits able to increase n4mBid cell viability relative to untreated cells. Of the 55 leading hits, 47 compounds inhibited one or more aspects of the HCV life cycle (entry, replication or infectious virus assembly/release) by >40%. Interestingly, significant affinity for the Sig-1R has been reported for 19 of them: amiodarone, amitriptyline, benztropine, butaclamol, cinnarizine, cyproheptadine, flunarizine, fluphenazine, ifenprodil, prochlorperazine, perphenazine, pimozide, protriptyline, quinidine, quipazine-6N, raloxifene, ritanserin, trifluprom azine and trifluoperazine (**Table 1**). Interaction with Sig-1R has also been suggested for biperiden (Yoshida et al., 2000) and SKF-38393 has been described as allosteric modulator of the Sig-1R (Guo et al., 2013). That is, 21 out of 55 leads identified in the cell protection small-molecule screen against HCV were known sigma-1 binders. All of them are non-selective and typically known by their activity on molecular targets other than Sig-1R. Affinity data for Sig-1R are unknown or have not been reported for the rest of identified anti-HCV compounds and thus the possibility that Sig-1R-mediated mechanisms contribute to their effect cannot be ruled-out. Inhibition of entry and infectious virus production, assembly and release accounted for the protective effect of most known Sig-1R ligands, although some of them also inhibited HCV replication. The potential contribution of Sig-1R was not discussed.

Changes in cell death induced by avian influenza A (FLUAV) H5N1 virus in A549 lung epithelial cells were explored using RNA interference (RNAi) screening methods. These screens identified multiple genes for which knockdown altered cell viability and drugs targeting some of these genes were assayed for their potential antiviral activity. The neurological drug ifenprodil increased cell viability *in vitro* and markedly decreased leukocyte infiltration and lung injury, and improved survival of mice infected with H5N1 (Zhang et al., 2019), the most lethal influenza virus strain. The effect of ifenprodil was discussed in the context of its antagonism at the N-methyl-D-aspartate (NMDA) receptor as overstimulation of the NMDA receptor can trigger lung injury. In another study sharing authors with the previous one, genes and pathways differentially expressed in A549 cells upon FLUAV H5N1 virus infection were identified and some drugs were assayed as potential treatments (Huang et al., 2020). Amitriptyline increased viability of A549 cells infected with H5N1 for 48 h when assayed 1 h before infection or at 3 h after infection, and reduced the infiltrating cell count, decreased lung injury, improved lung edema and survival of H5N1 virus-infected mice. The involvement of Sig-1R in mediating the effects of these drugs on influenza A H5N1 virus infection was not discussed, although ifenprodil shows high affinity for Sig-1R (Hashimoto and London, 1995; Gitto et al., 2014). Amitriptyline, a non-selective antidepressant binding to multiple receptors and transporters, also binds to Sig-1R with moderate affinity (Werling et al., 2007). Note that ifenprodil and amitriptyline were previously shown to inhibit the HCV-induced cytopathic effect (Chockalingam et al., 2010).

Regarding filoviruses, a systematic *in vitro* screen of FDA-approved drugs was performed to identify compounds with antiviral activities against the Ebola virus (EBOV) (Madrid et al., 2015). Assays were conducted in the Vero cell line. Active compounds (>50% viral inhibition and <30% cellular toxicity) at a single concentration were tested in dose-response assays. On the basis of the approved human dosing, toxicity/tolerability and pharmacokinetic data, seven *in vitro* hits were selected and evaluated for their *in vivo* efficacy. Five of the seven (chloroquine, amiodarone, prochlorperazine, benztropine, and clomiphene) hit compounds show affinity for the Sig-1R (**Table 1**), although the contribution of Sig-1R-mediated mechanisms was not discussed in the paper. When administered *in vivo* in a mouse model, azithromycin (100 mg/kg, twice daily, i.p.), chloroquine (90 mg/kg, twice daily, i.p.), and amiodarone (60 mg/kg, twice daily, i.p.) increased survival of infected mice, but only chloroquine gave significant reproducible efficacy with this dosing regimen. Azithromycin and chloroquine were also tested in a guinea pig model of EBOV infection, but none of the tested doses increased survival. In a separate study, also testing FDA-approved drugs (~2600 drugs and molecular probes) in an *in vitro* infection assay using the type species Zaire EBOV, selective antiviral activity was found for 80 drugs spanning multiple mechanistic classes (Johansen et al., 2015). A set of 30 active compounds was prioritized. A good number of them (17 out of 30: astemizole, benztropine, bepridil, clemastine, clomiphene, clomipramine, flupentixol, fluphenazine, lomerizine, maprotiline, piperacetazine, prochlorperazine, quinacrine, sertraline, terconazole, thioridazine and toremifene) are known to display affinity for Sig-1R and most of them were indeed identified in previous studies with other viruses (**Table 1**). Interestingly, results in a murine EBOV infection model confirmed the

protective ability of several drugs, notably bepridil and sertraline, which both bind Sig-1R with remarkable affinity (**Table 1**). Viral entry assays indicated that most of these antiviral drugs block a late stage of viral entry.

Finally, inhibition of the cytopathic effect induced by Chikungunya virus and other alphaviruses (Semliki Forest virus and Sindbis virus) was found for chlorpromazine, doxepin, methdilazine, perphenazine, thiethylperazine, thioridazine and chloroquine (Pohjala et al., 2011), all of them non-selective Sig-1R ligands that also exhibit antiviral activity against other viruses (**Table 1**).

Pharmacology Findings Against Coronaviruses

SARS-CoV-2 (severe acute respiratory syndrome-related CoV type 2), the causative virus of COVID-19 pandemic, belongs to the broad family of positive-sense single-stranded RNA (+ssRNA) CoV. Other CoV also cause illnesses ranging from common cold to more severe diseases such as Middle East respiratory syndrome (MERS). It is the seventh known CoV to infect people, after 229E, NL63, OC43, HKU1, MERS-CoV, and the original SARS-CoV (Zhu et al., 2020). Phylogenetic analyses revealed conserved evolutionary relationship between SARS-CoV-2 and SARS-CoV (79.7% nucleotide sequence identity) (Zhou et al., 2020).

In this section, data supporting the involvement of Sig-1R and therapeutic potential of Sig-1R ligands against CoV infection is summarized.

Chloroquine and hydroxychloroquine bind to Sig-1R (**Table 1**). These antimalarial drugs have shown antiviral activity against different viruses (Sperber et al., 1993; Savarino et al., 2001; Madrid et al., 2013; Ferraris et al., 2015; Wang et al., 2015). There was also evidence supporting the efficacy of chloroquine and hydroxychloroquine against other members of the Coronaviridae family before COVID-19 pandemic. Chlroroquine was described to show antiviral activity against human CoV strain OC43 (HCoV-OC43) (Keyaerts et al., 2009). HCoV-OC43 together with HCoV-229E are responsible for 10 to 30% of all common colds, and infections occur mainly during the winter and early spring (Larson et al., 1980). Chloroquine inhibited HCoV-OC43 replication in HRT-18 cells and prevented HCoV-OC43-induced death in newborn mice when mothers were treated daily with chloroquine (15 mg/kg). On these bases, authors suggested that chloroquine might be considered as a future drug against HCoVs (Keyaerts et al., 2009). Indeed, chloroquine also inhibited the replication of SARS-CoV *in vitro* (Keyaerts et al., 2004) and a number of subsequent studies have confirmed its antiviral activity against SARS-CoV (de Wilde et al., 2014; Dyall et al., 2014) and recently against SARS-CoV-2 (Jeon et al., 2020; Yao et al., 2020).

Chloroquine and its hydroxy analog were by far the most popular drugs proposed initially for treatment and prophylaxis of COVID-19: 208 interventional clinical trials registered on the NIH site involve treatment with these drugs, alone or in combination (ClinicalTrials.gov query, 2020). *In vitro*, both drugs inhibit SARS-CoV-2 infection in Vero cells, but hydroxychloroquine (EC_{50} = 0.72 μM) is more potent than chloroquine (EC_{50} = 5.47 μM) (Yao et al., 2020). The benefits of this treatment have been investigated during the course of this pandemic, yet no scientific evidence supports the widespread use of these medications. In fact, results of the first clinical studies evaluating the effect of hydroxychloroquine do not support efficacy of this drug in COVID-19 patients (Geleris et al., 2020; Mitjà et al., 2020; Roustit et al., 2020). Yet, preliminary studies aroused considerable media interest, raising fears of massive and uncontrolled use of these drugs, inexpensively produced in several countries. On the other hand, serious adverse drug reactions have been reported in patients with COVID-19 receiving hydroxychloroquine. Side effects of both antimalarial drugs are well established, including serious retinopathies and cardiopathies associated with bioaccumulation of the drugs (Palmeira et al., 2020). Recently (June 15, 2020), FDA has revoked the emergency use authorization to use hydroxychloroquine and chloroquine to treat COVID-19 based on findings from a large, randomized clinical trial in hospitalized patients showing no benefit for decreasing the likelihood of death or speeding recovery (FDA communication, 2020). The mechanism of action of these aminoquinolines is thought to depend on their capacity to increase the endosomal pH to inhibit lysosomal enzymes. This prevents enveloped viruses from entering and releasing their genetic material into the host cells (Tripathy et al., 2020). Binding to a ganglioside-binding domain at the N-terminal domain of the SARS-CoV-2 S protein has also been suggested as a mechanism of chloroquine and hydroxychloroquine to inhibit attachment of the virus to lipid rafts and contact with the ACE-2 receptor for entry (Fantini et al., 2020). The only reference to Sig-1R comes from an unrelated study describing protection by chloroquine against glutamate-induced cell death through a Sig-1R-mediated mechanism (Hirata et al., 2011). The eventual contribution of Sig-1R to the antiviral effects of chloroquine and hydroxychloroquine is just starting to be recognized (Gordon et al., 2020; Mirabelli et al., 2020), but they are non-selective Sig-1R ligands and their affinities for this molecular target are suboptimal.

The antiarrhythmic amiodarone, a non-selective but high affinity sigma-1 ligand, was reported to inhibit the spreading *in vitro* of SARS-CoV in Vero cells (Stadler et al., 2008). Amiodarone reduced the virus titer in a concentration-dependent manner, at concentrations at which it has no effect on cell viability. Direct interaction with the SARS-CoV or impairment of virus entry did not account for its antiviral activity, but amiodarone interfered with the SARS-CoV life cycle after delivery of its genome in the cytosol. As a cationic amphiphilic drug, amiodarone (and its main metabolite MDEA) accumulates into late endosomes/lysosomes and reduces their lumenal acidity, precluding acidic cleavage of viral proteins and interfering with the endocytic pathway (Salata et al., 2015). However, amiodarone displayed antiviral activity even when SARS-CoV has delivered its genome into the cytoplasm, thus involving additional mechanisms at a

post-endosomal level (Stadler et al., 2008). The contribution (or not) of sigma-1-mediated mechanisms to the antiviral activity of amiodarone was not discussed in the publications. Amiodarone was also shown to inhibit the HCV-induced cytopathic effect on the engineered cell line n4mBid (Chockalingam et al., 2010), HCV entry and assembly steps in Huh-7.5.1 cells (Cheng et al., 2013), and EBOV cell entry in a variety of cultured cell lines (Gehring et al., 2014; Salata et al., 2015; Dyall et al., 2018). Despite promising *in vitro* results, amiodarone failed to protect guinea pigs from a lethal dose of EBOV (Dyall et al., 2018). In the clinical setting, in December 2014, approximately 80 patients in Ebola treatment units in Freetown, Sierra Leone, received amiodarone as a compassionate therapy at doses up to 30 mg/kg per day (ClinicalTrials.gov Identifier NCT02307591, 2014). A decrease in case fatality rate was reported when compared with local historical data. Unfortunately, the study was not a formal clinical trial, and the statistical significance of this result is not known (Turone, 2014; Gupta-Wright et al., 2015). Recently, the case of a patient affected by COVID-19-related respiratory failure who recovered after only supportive measures and off-label short therapy with amiodarone (starting on the second day from admission and lasting 5 days; administered on day 1 as a 15 mg/kg/24 h intravenous infusion, followed by oral administration of 400 mg twice daily) has been reported (Castaldo et al., 2020). Accordingly, amiodarone, widely prescribed to treat both ventricular and supraventricular arrhythmias, has been proposed as a possible therapy (alone or as part of a combination regimen) to prevent SARS-CoV-2 infection rather than to treat symptomatic or severe COVID-19 patients (Aimo et al., 2020; Sanchis-Gomar et al., 2020).

A set of 348 FDA-approved drugs was screened in cell cultures infected with MERS-CoV (de Wilde et al., 2014). Four compounds (chloroquine, chlorpromazine, loperamide, and lopinavir) inhibited MERS-CoV replication in the low-micromolar range (IC_{50s} 3 to 8 μM). These compounds also inhibited the replication of SARS-CoV and HCoV-229E. Interestingly, chloroquine but also chlorpromazine and loperamide bind to Sig-1R (**Table 1**). Time-of-addition experiments suggested that chloroquine, chlorpromazine and loperamide inhibit an early step in the replicative cycle whereas lopinavir inhibits a post-entry step. This finding is congruent with previous findings showing that Sig-1R regulates early stages of HCV RNA replication (Friesland et al., 2013).

In another study, a library of 290 compounds with FDA approval or in advanced clinical development was screened for antiviral activity against MERS-CoV and SARS-CoV (Dyall et al., 2014). Twenty seven compounds displayed *in vitro* activity against both MERS-CoV and SARS-CoV. Among the 27 active compounds, at least 19 bind with significant affinity to Sig-1R (chloroquine, hydroxychloroquine, mefloquine, amodiaquine, tamoxifen, toremifene, terconazole, cycloheximide, benztropine, fluspirilene, thiothixene, fluphenazine, promethazine, astemizole, chlorphenoxamine, chlorpromazine, thiethylperazine, triflupromazine and clomipramine) (**Table 1**), though their antiviral activity was not discussed to be related to Sig-1R. Recently, authors prioritized 20 drugs from this previous screening and found that 17 of the 20 tested drugs that inhibited SARS-CoV and MERS-CoV also inhibited the cytopathic effect of SARS-CoV-2 on Vero E6 cells, with similar IC_{50} values and at non-cytotoxic concentrations (Weston et al., 2020). All (amodiaquine, benztropine, chloroquine, chlorpromazine, clomipramine, fluphenazine, fluspirilene, hydroxychloroquine, mefloquine, promethazine, tamoxifen, terconazole, thiethylperazine and toremifene) but two are known to bind Sig-1R (**Table 1**). Two of them, chloroquine and chlorpromazine, were evaluated *in vivo* using a mouse-adapted SARS-CoV model. Drug treatments did not inhibit virus replication in lungs, but did protect mice from clinical disease (Weston et al., 2020). Note that repurposing not only of chloroquine but also of the antipsychotic chlorpromazine has been proposed to treat COVID-19 (Nobile et al., 2020; Plaze et al., 2020).

In a recent repositioning study, 48 FDA-approved drugs, including 35 drugs pre-selected by their activity against SARS-CoV snd 13 drugs recommended from infectious diseases specialists, were assayed for their antiviral activity against SARS-CoV-2 in Vero cells (Jeon et al., 2020). Infected cells were analyzed by immunofluorescence using an antibody against the viral N protein of SARS-CoV-2. Among the 48 drugs evaluated, 24 showed potential anti-SARS-CoV-2 activity, with IC_{50} values between 0.1 and 10 μM. Three of them, loperamide, mefloquine and amodiaquine, in addition to chloroquine, are known to bind Sig-1R (**Table 1**). All of them were previously shown to be effective against other CoV, including MERS-CoV and SARS-CoV (Dyall et al., 2014).

In a recent paper, targeting Sig-1R was highlighted based on findings of a SARS-CoV-2 protein interaction map and pharmacological data (Gordon et al., 2020). Screening a subset of drugs identified two sets of pharmacological agents effectively reducing SARS-CoV-2 infectivity in Vero-6 cells: inhibitors of mRNA translation and predicted regulators of the sigma-1 and sigma-2 receptors. Non-selective Sig-1R ligands including haloperidol, PB28, PD-144418 and hydroxychloroquine, and subsequently clemastine, cloperastine, progesterone and siramesine (**Table 1**) were found to exert antiviral effects. Hydroxychloroquine was among the less potent antiviral of the assayed Sig-1R ligands, which correlated with its lower affinity for this molecular target. Authors discussed the involvement of sigma receptors. They noted that these molecules are also active against other receptors, but the only shared among all of them are the sigma receptors. For instance, the antipsychotic haloperidol inhibits the dopamine D2 and histamine H1 receptors, while clemastine and cloperastine are themselves antihistamines, but all three molecules are Sig-1R ligands and exert antiviral activity. In contrast, the antipsychotic olanzapine, which also inhibits H1 and D2 receptors, has no significant Sig-1R activity and is not antiviral. Authors also noted that the widely used antitussive dextromethorphan exerted proviral activity and stated that its use should merit caution and further study in the context of COVID-19. Dextromethorphan but also carbetapentane, another commonly used antitussive (Brown et al., 2004), the narcotic analgesic pentazocine (particularly its active (+)-pentazocine enantiomer) (Tam and Cook, 1984) and some antidepressants

(Narita et al., 1996), among some other marketed compounds, are considered prototype Sig-1R agonists/positive modulators. Thus, should caution be extended to the use of other potential, although non-selective Sig-1R agonists? In this way, cocaine is a non-selective Sig-1R agonist and exposure to cocaine has been shown to enhance HIV infection by activating Sig-1R (Roth et al., 2005). Cocaine use/abuse could thus be a risk factor but, to my knowledge, the effect of cocaine on CoV infections has not been investigated.

Finally, in a recent publication, quantitative high-content morphological profiling coupled with an AI-based machine learning strategy was applied to identify efficacious single agents against SARS-CoV-2 (Mirabelli et al., 2020). This assay detected multiple antiviral mechanisms of action, including inhibition of viral entry, propagation, and modulation of host cellular responses. Viral growth kinetics were assayed at a multiplicity of infection of 0.2 in Huh-7 cells, with peak viral titers at 48 h post infection. From a library of 1,441 FDA-approved compounds and clinical candidates, 15 dose-responsive compounds with antiviral potency below 1 μM and devoid of cytotoxicity were identified. Three of them, amiodarone, verapamil and E-52862 (S1RA) were known to bind Sig-1R (**Table 1**). Interestingly, E-52862 (S1RA) is a selective Sig-1R antagonist (Romero et al., 2012). It exerted potent activity against SARS-CoV-2 in Huh-7 cells (IC_{50} = 222 nM) and iPSC-derived alveolar epithelial type 2 cells (iAEC2s) (IC_{50} = 1 μM), with limited cell toxicity (CC_{50} > 5000 nM). E-52862 (S1RA) depleted infected cells and induced cellular changes suggestive of a host-modulation mechanism, which led to suggest that the activity of S1RA is dependent on host cell mechanisms (presumably active in Huh-7 and iAEC2s cells but not in Vero-6 cells, which are highly permissive to viral growth) and, promisingly, that human cells may be more responsive to this compound. This (and differences in other experimental conditions) could explain way E-52862 was devoid of activity when assayed in the Vero E6 cell line (Gordon et al., 2020).

MECHANISM OF ACTION

Sigma-1 Receptor and Viral Entry

Inhibition of viral entry has been reported for non-selective sigma-1 ligands in a number of studies (Chockalingam et al., 2010; Gastaminza et al., 2010; Cheng et al., 2013; Johansen et al., 2015; Dyall et al., 2018), but not in others (Nemerow and Cooper, 1984; Mingorance et al., 2014; Stadler et al., 2008; de Wilde et al., 2014; Gordon et al., 2020). Thus, it is unclear whether prevention of viral particle attachment or internalization accounts for Sig-1R-mediated antiviral effect of such drugs.

Inhibition of HCV entry into Huh-7 human hepatoma cells by sigma-1 ligands was demonstrated in pharmacology studies (Gastaminza et al., 2010), but downregulation of Sig-1R in Huh-7 cells did not affect HCV entry (Friesland et al., 2013). This might suggest that the deficiency of the modulatory sigma-1 protein (as in the case of gene silencing approaches) does not mimic the pharmacological inhibitory effect on viral entry elicited by an antagonist acting at the Sig-1R. Accordingly, absence of the regulatory mechanism in Sig-1R deficient cells would not be equivalent to the inhibitory effect promoted by a Sig-1R ligand on the target protein with which Sig-1R is interacting. This is possible due to the chaperone nature of the Sig-1R, which exerts its action through physical protein–protein interactions (Su et al., 2010; Pabba, 2013).

Sig-1R normally resides at the ER, typically at the MAM, but when cells undergo stress (as expected following viral infection) the Sig-1R translocates to the peripheral ER network and plasma membrane to regulate a variety of cell surface proteins (Su et al., 2010), which might account for ligand-operated, Sig-1R-mediated modulation of virus attachment or entry (**Figure 1**). In this way, Sig-1R associates to heavy chain binding immunoglobulin protein (BiP, also known as glucose regulating protein 78, GRP78; or heat shock 70 kDa protein 5, HSPA5) in the ER (Hayashi and Su, 2007). BiP also translocates upon cell stress from the ER to the cell surface, exposes multiple domains on the cell surface and assumes new functions (Zhang et al., 2010), including virus recognition by its substrate-binding domain and facilitation of entry of several viruses, including CoV (Chu et al., 2018) (**Figure 1**). The capacity of BiP to facilitate surface attachment and viral entry likely depends on its binding to surface S (spike) viral proteins, as demonstrated for MERS-CoV and bat CoV-HKU9, and predicted for SARS-CoV-2 (Ibrahim et al., 2020). Sig-1R is engaged in protein trafficking from the ER to the plasma membrane, binds to BiP and, like BiP, it trasnlocates to the cell surface upon ER stress (Hayashi, 2019), but the involvement of Sig-1R in the export of BiP to the plasma membrane has not been investigated. Sig-1R antagonists inhibit Sig-1R-BiP dissociation at the ER (Hayashi and Su, 2007; Hayashi, 2019) and this might prevent BiP trafficking, surface expression and ultimately CoV attachment via BiP. Unlike Sig-1R, BiP is described as a non-membrane-bound ER lumenal chaperone. Thus, the interaction with Sig-1R could allow BiP stabilization/anchoring to the plasma membrane, although putative transmembrane domains have been identified allowing its potential, autonomous cell surface relocalization (Zhang et al., 2010). Yet, no direct interaction of Sig-1R with BiP has been specifically described at the plasma membrane. Similarly, no direct interaction with other host membrane proteins involved in viral attachment/entry (e.g., ACE2 or TMPRSS2) or with structural viral envelope proteins has been described substantiating Sig-1R-dependent modulation of viral entry. Alternatively, as discussed later, Sig-1R might regulate early stages of RNA replication and host cell response but not viral entry, whereas structural features shared by a number of sigma-1 ligands, independent on their binding to Sig-1R, might account for viral entry inhibition.

Sigma-1 Receptor Ligands as Lysosomotropic Agents?

The endocytic pathway (receptor-dependent endocytosis) is a basic mechanism for entry of CoV, including SARS-CoV, MERS-CoV and SARS-CoV-2, into host cells (Glebov, 2020; Yang and Shen, 2020). Binding of the spike (S) protein of SARS-CoV-2 to its receptor exposes its cleavage sites to cellular proteases,

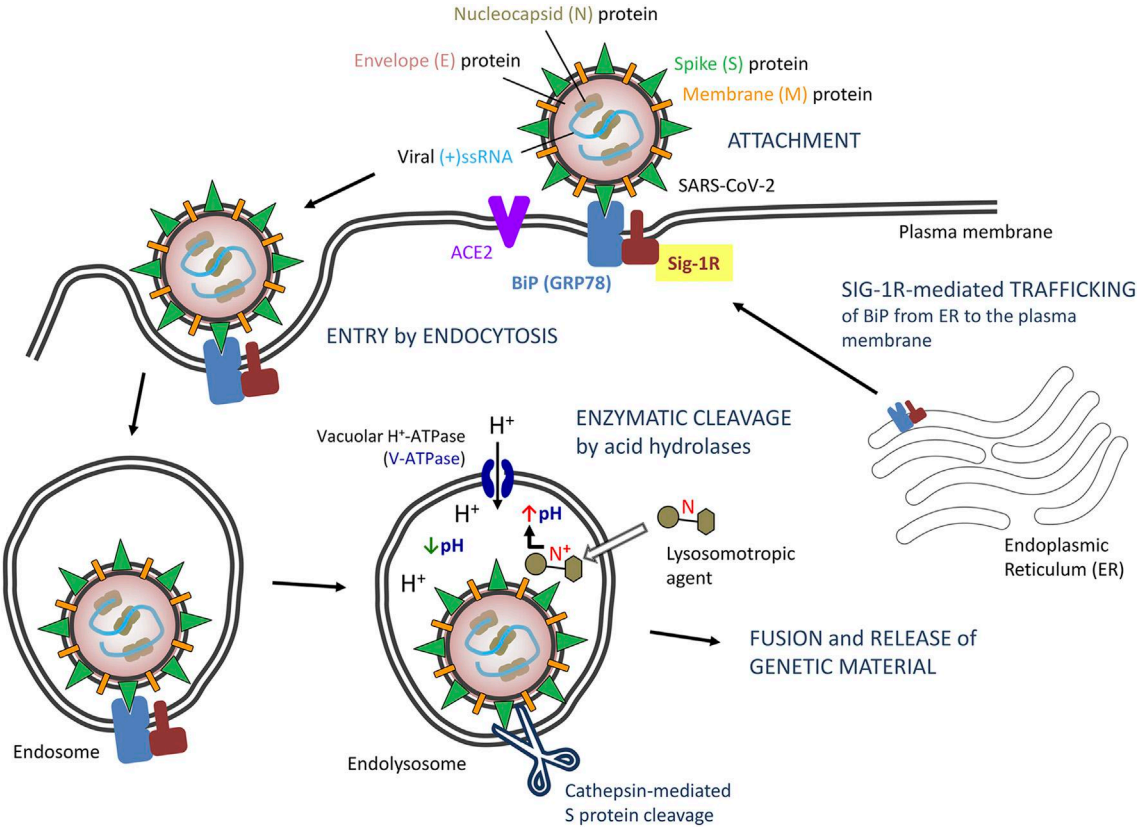

FIGURE 1 | Proposed model of severe acute respiratory syndrome CoV-2 (SARS-CoV-2) entry by endocytosis: Potential role of Sigma-1 receptors (Sig-1Rs) via BiP interaction. Abbreviations and bibliographic references for the role of Sig-1R are provided in the text.

including endosomal acid proteases involved in endocytic processing (Millet and Whittaker, 2015; Pillay, 2020). In particular, endosomal cathepsin-mediated S protein cleavage is considered a critical step for CoV entry and initiation of infection (Millet and Whittaker, 2015; Glebov, 2020; Wędrowska et al., 2020; Yang and Shen, 2020).

Endosomes and maturation of endosomes into a lysosome is featured by their acidic internal pH, which is required for acid proteases and critical for SARS-CoV-2 processing and internalization (Wędrowska et al., 2020). The plasma and lysosomal membranes are highly permeable to the unionized form of weak bases but are essentially impervious to the protonated form of the bases (Marceau et al., 2012; Homolak and Kodvanj, 2020). Accordingly, weak bases, unionized in the cytoplasm, can cross the lysosomal membrane and enter the lysosome. Once in the lysosome, they are rapidly protonated since the lysosomal pH is considerably lower than the cytosolic pH, and become trapped inside lysosomes. This results in intralysosomal accumulation (ion trapping) of the drug and increased lysosomal pH (i.e., neutralization of lysosomal pH) sufficient to block most lysosomal enzymatic activity. If the concentration of the protonated base inside the lysosome is high enough, water enter the lysosome osmotically and the lysosomes swell to form large vacuoles (i.e., lysosomal vacuolation), with the consequent loss of lysosomal function (Aki et al., 2012).

Some drugs recognized as antiviral agents are lipophilic amines/weak bases that accumulate and preclude acidification of lysosomes, thus inhibiting virus internalization and post-internalization trafficking to the site of replication (Sieczkarski and Whittaker, 2002; Kaufmann and Krise, 2007; Mercer et al., 2010). Such lysosomotropism is shared by some lipophilic amines and cationic amphiphilic drugs (Kaufmann and Krise, 2007), including chloroquine and other anti-malarial drugs (Homolak and Kodvanj, 2020). Indeed, lysosome targeting agents are considered a potential therapy for COVID-19 (Homolak and Kodvanj, 2020). However, the effectiveness of this mechanism of action to control viral infections is hampered by its low specificity, cell compensation mechanisms to lower/restore intralysosomal pH and egress entrapped amines from lysosomes (Goldman et al., 2009), and the need for high drug dosage to allow substantial drug accumulation and alkalinization inside lysosomes, which also raises safety concerns.

A variety of marketed drugs fit within general physicochemical properties of lysosomotropic agents. Essentially, drugs with a ClogP > 2 and pKa between 6.5 and 11 can accumulate into lysosomes (Nadanaciva et al., 2011), although other physicochemical features also affect lysosomotropism (Kaufmann and Krise, 2007). Pharmacophore models for sigma-1 ligands (both putative agonists and antagonists) specify a positive ionizable group (i.e., a basic nitrogen, usually

secondary or tertiary amine) flanked by hydrophobic regions (Pascual et al., 2019), which is coherent with potential lysosomotropism (**Figure 1**). This might have two implications. First, lysosomal sequestration might represent a barrier for a Sig-1R drug in reaching its intended target and would reduce its access to other cellular compartments (eg, ER, MAM or nuclear membranes) where Sig-1R is located for its antiviral effect to occur. Second, lysosomal trapping would result in unspecific, Sig-1R-independent defective acidification of lysosomes, and this off-target effect might be an added value for such drugs. It is clear that Sig-1R-mediated modulation of both viral replication and virus-induced ER-stress response might dependent on target-specific binding of ligands to Sig-1Rs in cellular compartments other than lysosomes, but it is presently unclear whether the pharmacophore-related, potential lysosomotropism of Sig-1R ligands actually hinders or contributes (and to what extent) to the activity reported for some Sig-1R ligands in antiviral drug screens.

Sigma-1 Receptor Regulates Early Steps of Viral RNA Replication

In this section, evidence gained through gene silencing approaches are discussed. The antiviral activity exerted by numerous sigma-1 ligands in drug repurposing *in vitro* screens was not invariably unnoticed. Following the trail of pharmacological findings described before (Gastaminza et al., 2010), the role played by Sig-1R in HCV infection was investigated. RNAi though lentivirus-delivered short hairpin RNA (shRNA) targeting Sig-1R mRNA was used to downregulate Sig-1R expression in Huh-7 human hepatoma cells (Friesland et al., 2013). Four different shRNAs caused Sig-1R protein silencing with different magnitudes as compared with control cells transduced with an irrelevant shRNA. Control and silenced cells were inoculated with infectious HCV virions and infection efficiency was monitored by measuring the production of intracellular and extracellular progeny infectious virus as well as intracellular HCV RNA. Downregulation of Sig-1R expression in Huh-7 cells caused a proportional decrease in susceptibility to HCV infection, as shown by reduced HCV RNA accumulation and intra- and extracellular infectivity in single-cycle infection experiments. That is, progeny virus production was proportional to cellular Sig-1R levels at 24 and 48 h postinfection. Experiments were also conducted to explore the underlying mechanisms and revealed that Sig-1R downregulation did not affect HCV entry and that its expression levels were not limiting for primary translation of viral RNA genome, persistent HCV RNA replication (steady-state HCV RNA replication) or particle assembly and secretion. However, sigma-1 expression was rate limiting for launching HCV RNA replication. The reduced accumulation of HCV RNA in Sig-1R-deficient cells in single-cycle infection experiments was due to a defect in the establishment of HCV RNA replication, downstream of primary translation. Accordingly, Sig-1R expression is rate limiting for RNA replication early after primary translation but it is dispensable once the viral replication machinery has been established and replication reaches steady-state levels, as observed in persistently infected cells. Another remarkable result in the study by Friesland et al. (2013) is that Sig-1R expression in Huh-7 cells was rate limiting for HCV infection but not for infection with negative-sense single-stranded RNA viruses such as influenza A virus (A/WSN/33) or VSV (Friesland et al., 2013). Accordingly, evidence on the role played in viral replication by host Sig-1R in cultured hepatoma cells (not the primary cell target of SARS-CoV-2) infected with HCV (a +ssRNA virus, but not SARS-CoV-2) in no case imply proven mechanistic correlates against SARS-CoV-2 on its natural target cells.

Overall, data from Sig-1R deficient cells indicate that Sig-1R is a host cellular factor recruited for HCV infection, downstream entry, delivery and primary translation of viral RNA genome that regulates early stages of HCV RNA replication (Friesland et al., 2013). This is consistent with pharmacology findings whereby Sig-1R ligands (unrecognized as active ligands at Sig-1R in most studies) where found to inhibit early steps of the replicative cycle, after viral particle attachment, internalization and delivery of its genome to the cytosol (Nemerow and Cooper, 1984; Mingorance et al., 2014; Stadler et al., 2008; de Wilde et al., 2014; Gordon et al., 2020).

Sigma-1 Receptor Colocalizes and Interacts with Non-Structural Proteins of the Viral Replicase/Transcriptase Complex

In this section, evidence gained from colocalization and interactome map studies are discussed. Sig-1R was found to colocalize with NS proteins of the HCV replication complex (Friesland et al., 2013). Cells were processed for double immunostaining with antibodies directed against components of the viral replicase (NS3, NS4B and NS5A) and against Sig-1Rs. In mock-infected Huh-7 cells, Sig-1R immunofluorescence revealed a predominant discrete cytoplasmic punctae localization that was juxtaposed to mitochondria as well as diffuse cytoplasmic pattern that colocalized with ER, the characteristic cellular distribution of Sig-1R in normal resting, unstressed cells (Su et al., 2010). During infection, the intracellular pattern of Sig-1R distribution changed: more that 70% of the infected cells displayed a diffuse perinuclear pattern 48 h postinfection. Interestingly, Sig-1R co-localized with viral NS3, NS4B and NS5A replicase components at perinuclear regions during early steps of viral infection. Later during infection (72 h), more than 60% of the infected cells displayed discrete cytoplasmic punctae that did not clearly colocalize with the bulk NS protein perinuclear signal, suggesting that a fraction of Sig-1R recovers the original pattern and that perinuclear colocalization of Sig-1R with viral replicase NS proteins observed at 48 h is transient. Overall, these results suggest that Sig-1R is recruited to perinuclear areas of the ER where NS proteins accumulate at early stages of viral infection to regulate the initiation of HCV RNA replication. Most Sig-1R and NS3 and NS5A were associated with detergent-resistant, cholesterol- and sphingolipid-rich intracellular membranes, further suggesting that Sig-1R and components of the HCV replicase target similar ER membrane environments, where Sig-1R likely exerts its proviral functions. Notably, such transient sigma-1

relocalization has been described during ER stress and proposed to contribute to the cellular response to stress (Hayashi and Su, 2007), suggesting that the virus takes advantage of host stress-related proteins to deploy a favorable cellular program. Cellular stress pathways induced by HCV infection to promote both viral replication and survival of the infected cell as well as the proviral role of Sig-1R in HCV infection have been reviewed (Vasallo and Gastaminza, 2015) and will not be reviewed further here.

Recently, a SARS-CoV-2 protein interaction map reveled a physical interaction with Sig-1R (Gordon et al., 2020). Authors cloned, tagged and expressed 26 of the 29 SARS-CoV-2 proteins and identified SARS-CoV-2-human protein-protein interactions using affinity-purification mass spectrometry. Approximately 40% of SARS-CoV-2 interacting proteins were associated with endomembrane compartments or vesicle trafficking pathways. In particular, the viral NS protein Nsp6 was specifically found to interact with Sig-1R. The SARS-CoV-2 genome encodes as many as 14 open reading frames (Orfs) (Masters, 2006; Chan et al., 2020; Gordon et al., 2020; Wu et al., 2020). The Orf1a/Orf1ab at the 5′ two-thirds of the genome encodes precursor polyproteins, which are auto-proteolytically processed into 16 NS proteins (Nsp1-16) that form the replicase/transcriptase complex. At the 3' end of the viral genome, as many as 13 additional Orfs are expressed from sub-genomic mRNAs encoding Spike (S), Envelope (E), Membrane (M) and Nucleocapsid (N) structural proteins and putative accessory proteins. The viral replication machinery is thought to localize in ER membranes thanks to Nsp3, Nsp4 and Nsp6. Nsp6 forms complexes with Nsp3 and Nsp4 to anchor the viral replicase/transcriptase complex to ER membranes (Oostra et al., 2008; Alsaadi and Jones, 2019). All three replicase proteins contain transmembrane-spanning sequences important for assembly of the viral replicase/transcriptase complex to the ER membrane (Oostra et al., 2008). Nsp6 was shown to contain seven hydrophobic domains but six transmembrane domains, with its amino and carboxy termini exposed in the cytoplasm, and a conserved hydrophobic domain in the C-terminal cytosolic tail (Oostra et al., 2008; Baliji et al., 2009). Two nsp6 products of approximately 23 and 25 kDa were identified by Western immunoblotting, although the reason for the existence of multiple forms of nsp6 is currently unknown (Baliji et al., 2009). In addition to its role in anchoring the replicase complex to ER membranes, Nsp6 has been found to induce double-membrane vesicles and autophagosome formation (Cottam et al., 2011).

Positive-strand RNA viruses, including HCV and SARS-CoV, sequester host cell ER membranes to assemble viral replication. A network of modified perinuclear rough ER that integrates convoluted membranes, interconnected double-membrane vesicles and vesicle packets has been described (Gosert et al., 2002; Knoops et al., 2008; Sola et al., 2015). The viral replicase subunits were most abundantly located in convoluted ER membranes, RNA replication (double-stranded RNA) localized in double-membrane vesicles, and vesicle packets appeared to result from the merge of double-membrane vesicles and develop into large cytoplasmic vacuoles containing (budding) virus particles. Ultimately, replication of the CoV genome requires continuous RNA synthesis (Sola et al., 2015) and the reticulovesicular network provides a structural and functional continuum that connects ER membrane structures involved in RNA synthesis to sites at which the assembly of new virions occurs (Knoops et al., 2008). According to previous studies, Sig-1R is required at early stages of replication but not for steady-state HCV RNA replication or infectious particle assembly and secretion (Friesland et al., 2013). Thus, internalization, delivery and primary translation of the viral RNA genome would precede the recruitment of Sig-1R, which complexes with newly synthesized viral replicase proteins at initial stages before the reticulovesicular network continuum has fully developed in persistent infections. Early and transient colocalization of Sig-1R with HCV replicase proteins (Friesland et al., 2013) and interaction of Sig-1R with Nsp6 SARS-CoV-2 replicase protein (Gordon et al., 2020) support this hypothesis. The functional purpose of this interaction is unknown. A prompt assumption is that Sig-1R might assist insertion of the viral replication machinery to ER (convoluted) membranes, as anchoring of the replicase/transcriptase complex to the ER membrane is the proposed role of its partner Nsp6. However, it might also allow proper folding or membrane orientation of nascent viral proteins to assist multiprotein assembly of the functional replicase/transcriptase complex, promote early ER remodeling and trafficking through the reticulovesicular network, and/or regulate ER-mitochondrion signaling and ER-nucleus crosstalk to couple host cell bioenergetics and biosynthetic machinery to early viral demands. All these functions are coherent with the role played by this resident ER chaperone/scaffolding and dynamic pluripotent modulator protein, involved in inter-organelle signaling, bioenergetics and cellular stress responses (Hayashi and Su, 2007; Su et al., 2010; Vollrath et al., 2014; Hayashi, 2019; Delprat et al., 2020).

A Role for Sigma-1 Receptor in Coronavirus-Induced Host Cellular Stress?

CoV infection of cultured cells causes ER stress and induces the unfolded protein response (UPR), the ER-specific stress response, and their downstream signals (Fung and Liu, 2014; Fung et al., 2014a). ER stress and UPR have been particularly involved in SARS-CoV-2 infection (Sureda et al., 2020) and combination therapies targeting COVID-19-mediated ER stress have been recently proposed (Banerjee et al., 2020). UPR aims to restore ER homeostasis and cell survival by global translation shutdown and increasing the ER folding capacity. The UPR signaling starts with the unfolded proteins activating three ER stress transducers: double-stranded RNA-activated protein kinase (PKR)-like ER protein kinase (PERK), activating transcriptional factor-6 (ATF6), or inositol-requiring enzyme (IRE1). Reversible dissociation from the ER lumenal chaperone BiP (also known as GRP78 or HSPA5) and interactions with other ER co-chaperones regulates the activation/deactivation dynamics of UPR transducers. BiP seems to be the direct ER stress sensor as it becomes activated by misfolded proteins. In unstressed cells, BiP binds to the ER lumenal domains of ER stress transducers and maintains them in an inactivated state (**Figure 2**). During ER

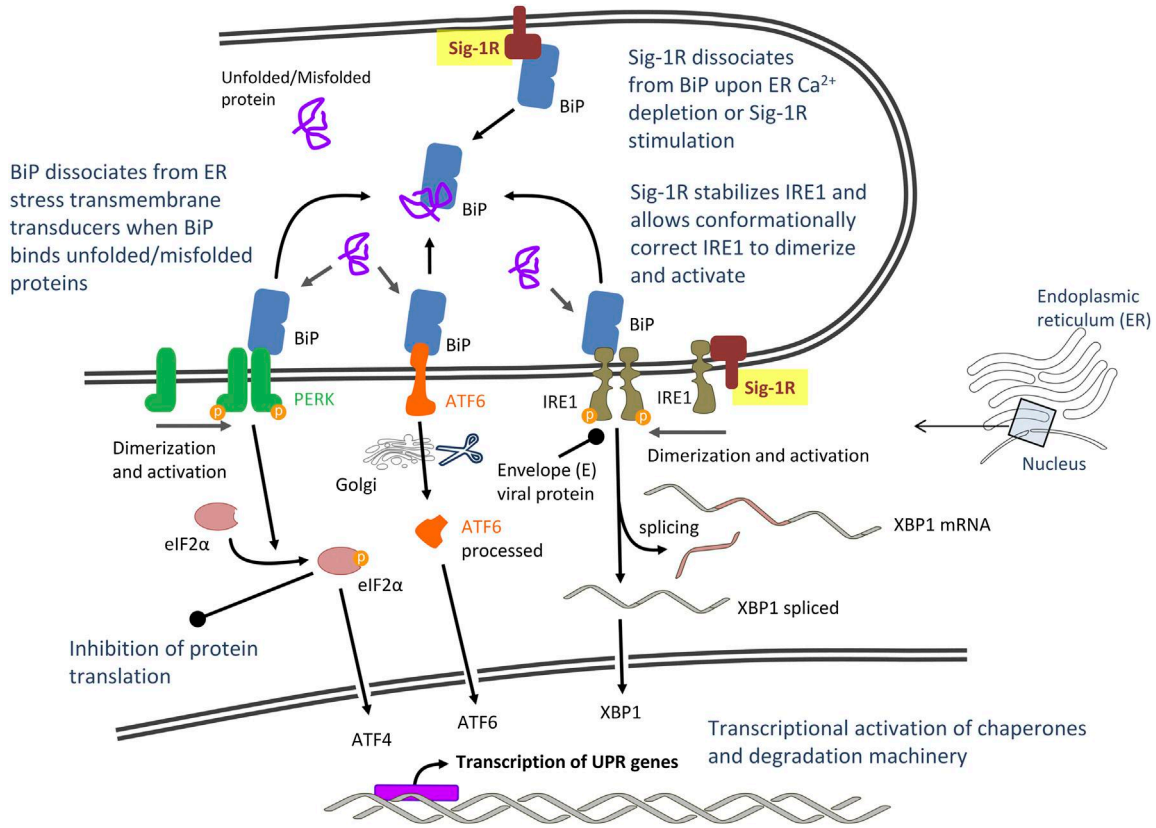

FIGURE 2 | Proposed model of severe acute respiratory syndrome CoV-2 (SARS-CoV-2)-mediated unfolded protein response (UPR) signaling: Potential role of Sigma-1 receptors (Sig-1Rs) via interaction with master UPR regulators. Abbreviations and bibliographic references for the role of Sig-1R are provided in the text.

stress, BiP preferentially binds to unfolded and misfolded proteins and dissociates from transmembrane transducers, facilitating their activation (Bertolotti et al., 2000; Kopp et al., 2019). Once activated, UPR transducers transmit the signal to the cytosol and the nucleus, and the cell responds by lowering the protein synthesis and increasing the ER folding capacity. The PERK/eIF2α (eukaryotic initiation factor 2α)/ATF4 pathway rapidly attenuates protein translation, whereas the ATF6 and the IRE1α/XBP1 (transcription factor X-box binding protein-1) cascades transcriptionally upregulate ER chaperone genes that promote proper folding (**Figure 2**). Accumulated unfolded proteins are either correctly refolded or unsuccessfully refolded and cleared via the ER associated degradation complex (ERAD) ubiquitin-proteasome pathway or via autophagy. However, under prolonged ER stress, UPR can also induce apoptotic cell death if homeostasis cannot be re-established and accumulation of misfolded protein becomes toxic. Apoptosis is triggered potentially via UPR-mediated and Ca^{2+}-mediated caspase activation pathways and recruitment of mitochondria (Kim et al., 2006; Fung and Liu, 2014; Karagöz et al., 2019). Indeed, Ca^{2+} homeostasis plays a major role in ER stress and UPR-mediated apoptosis induction. Depletion of ER Ca^{2+} stores has detrimental effects on ER-resident Ca^{2+}-dependent chaperones and protein folding, and undue Ca^{2+} transfer from ER to mitochondria at MAM (i.e., mitochondrial Ca^{2+} overload) leads to mitochondrial reactive oxygen species (ROS) production/oxidative stress and cytochrome C release (Carreras-Sureda et al., 2018). Finally, autophagy may also be activated under ER stress (ER stress-mediated autophagy) by pathways sharing common upstream signaling with UPR, including PERK, IRE1, ATF6 and Ca^{2+} (Song et al., 2017). Autophagy is characterized by the engulfment of cytoplasmic components in double-membrane-bound structures that are then delivered to lysosomes/vacuoles for degradation. Autophagosomes include worn-out proteins, protein aggregates and damaged organelles (Lee et al., 2015; Rashid et al., 2015; Song et al., 2017).

The burst of protein synthesis overloading the ER folding capacity, extensive rearrangement of the ER membrane during viral replication and viral proteins such as S (Siu et al., 2014) and 3a accessory (Minakshi et al., 2009) proteins of CoV cause ER stress, but viruses have evolved mechanisms to manage UPR signaling and create an environment favorable for its replication (Fung and Liu, 2014). Operative but hijacked UPR, with selective translational and transcriptional reprogramming but reduced susceptibility to cell death would contribute to host cell survival and sustain viral replication. Accordingly, CoV activate UPR transducers but induce minimal downstream induction of some UPR target genes. This favors a sustained shutdown of the synthesis of host cell proteins while the

translation of viral proteins escalates (Bechill et al., 2008). Also favoring viral infection, the envelope E protein of SARS-CoV has been shown to neutralize the IRE1α/XBP1 pathway of UPR and inhibit apoptosis (DeDiego et al., 2011). Note that apoptosis is a fatal fate for the infected cell, but it protects the host by limiting virus production and dissemination. However, not all the evidence has the same directionality and some findings support that ER stress, UPR and autophagy induction are innate responses in cell host's struggle with CoV. For instance, infection with the alphaCoV transmissible gastroenteritis virus (TGEV) activated all three UPR pathways (PERK, ATF6 and IRE1), but activation of the PERK/eIF2α axis inhibited TGEV replication through overall attenuation of protein translation (Xue et al., 2018). The PERK pathway was also activated in cells expressing the 3a accessory protein of SARS-CoV, a protein that is pro-apoptotic (Minakshi et al., 2009). Other studies point to a mix of positive and negative effects on viral replication. For instance, IRE1 RNase activity was reported to be unfavorable to viral replication whereas IRE1 kinase activity enhanced it (Su et al., 2017).

What about Sig-1R? ER stress/UPR induces Sig-1R expression through the PERK/eIF2α/ATF4 pathway (ATF4 binds to the 5' flanking region of Sig-1R gene to upregulate its transcription) (Mitsuda et al., 2011). In turn, Sig-1R upregulation, experimental overexpression or its ligand stimulation protects cells, which correlates with reduced ER stress and apoptosis in most studies (Mitsuda et al., 2011; Wang et al., 2012; Omi et al., 2014; Shimazawa et al., 2015; Cao et al., 2017; Ellis et al., 2017; Morihara et al., 2018; Zhai et al., 2019), but not all (Penas et al., 2011; Schrock et al., 2013; Alam et al., 2017). A biphasic role has also been described, with Sig-1R-mediated exacerbation followed by protection, concomitant with increased and reduced markers of ER stress and autophagy response, respectively (Yang et al., 2017). In paragraphs below, evidence supporting a role of Sig-1R in modulating several aspects of the ER stress response potentially relevant for CoV infection is reviewed and discussed.

Endomembrane Remodeling

ER remodeling is a key early element of ER stress response induced by CoVs. As discussed before, CoVs benefit from endomembrane compartments and induce the growth and remodeling of host cell ER membranes to form a reticulovesicular network (Knoops et al., 2008). Depletion of Sig-1R leads to abnormal ER morphology including loss of ER tethering and proliferation as well as mitochondrial abnormalities and mitophagy, suggesting a role of Sig-1R in maintaining structural and functional integrity of the ER and mitochondria (Vollrath et al., 2014). Thus, pharmacological blocking of Sig-1R might hinder ER remodeling and challenge mitochondrial energy supply, both required for viral replication. This is coherent with the finding that Sig-1R is required at early stages of HCV replication (Friesland et al., 2013), when ER remodeling and anchoring of the viral replicase complex occurs. Unfortunately, the role played by Sig-1R in architectonics of ER membranes during viral infection has not been investigated.

Calcium Homeostasis

Viruses have evolved mechanisms to disturb host cell Ca^{2+} homeostasis and increase intracellular Ca^{2+} as Ca^{2+} is essential for virus entry, replication, maturation and release (Olivier, 1996; Chen et al., 2019). Impeding virus-induced abnormal cytosolic Ca^{2+} increase by blocking Ca^{2+} release from the ER or Ca^{2+} entry through plasma membrane channels/pumps has emerged as a strategy to control viral infections (Chen et al., 2019). Accordingly, some Ca^{2+} channel blockers have been reported to improve mortality and decrease risk for intubation and mechanical ventilation in elderly patients hospitalized for COVID-19 (Solaimanzadeh, 2020). The Sig-1R regulates both Ca^{2+} entry at the plasma membrane level (via interaction with ligand- and voltage-gated Ca^{2+} channels) and Ca^{2+} mobilization from endoplasmic stores [via interaction with inositol-1,4,5 trisphosphate receptors, (IP_3Rs)] (Monnet, 2005). Under ER stress (Ca^{2+} depletion from ER stores), Sig-1R dissociates from BiP and chaperones IP_3R3, ensuring proper Ca^{2+} signaling from the ER into mitochondria (Hayashi and Su, 2001; Wu and Bowen, 2008) (Figure 3). Increased IP_3R3-mediated Ca^{2+} flow to mitochondria at MAM is fundamental for coupling cell physiology to energy demand, which is likely required for virus protein anabolism and RNA synthesis, but sustained/excessive Ca^{2+} influx into mitochondria results in excessive ROS, oxidative stress and apoptosis. Sig-1R agonists cause dissociation of Sig-1R from BiP, allow Sig-1R- IP_3R3 interaction and thus enhance IP_3R3-mediated Ca^{2+} flow to mitochondria whereas Sig-1R antagonists do not affect the Sig-1R-BiP association but inhibit the dissociation mediated by Sig-1R agonists. A Ying-Yang effect has been described for Sig-1R agonists, by increasing mitochondrial complex I activity and triggering moderate ROS increase in a Ca^{2+}-dependent manner as a physiological signal, but attenuating complex I and IV dysfunctions and promoting a marked anti-oxidant effect in pathological conditions (Goguadze et al., 2019). Treatment of mitochondrial membranes with the Sig-1R agonist (+)-pentazocine leads to phosphorylation of Bad and NADPH-dependent production of ROS through Rac1 signaling (Natsvlishvili et al., 2015). Immunoprecipitation techniques revealed that Sig-1R at MAM form complexes with Rac1, IP_3R and Bcl2, and Sig-1R agonists could induce mild oxidative stress through this IP_3R/Sig-1R/Bcl2/Rac1 multiprotein complex (Natsvlishvili et al., 2015). Altogether, both bioenergetic coupling and mitochondrial Ca^{2+} overflow-mediated apoptosis are dependent on Ca^{2+} signaling through IP_3R3 at MAM and are regulated by Sig-1R (Delprat et al., 2020). Ca^{2+} release from ER to cytosol via increased IP_3R1 activity also induces ER stress and Sig-1R binds to and regulates IP_3R1s as well (Kubickova et al., 2018) (**Figure 3**). Fine tune control (enough for enhanced energy supply but not too much to avoid host cell death) of these mechanisms might be essential for efficient viral infection, thus suggesting that pharmacological modulation of Sig-1R offers here a therapeutic opportunity to counteract the virus program (**Figure 3**).

Intreraction with Master Unfolded Protein Response Regulators

Sig-1R binds in a dynamic, reversible and Ca^{2+}-dependent manner to the ER lumenal chaperone and stress sensor BiP (Hayashi and Su,

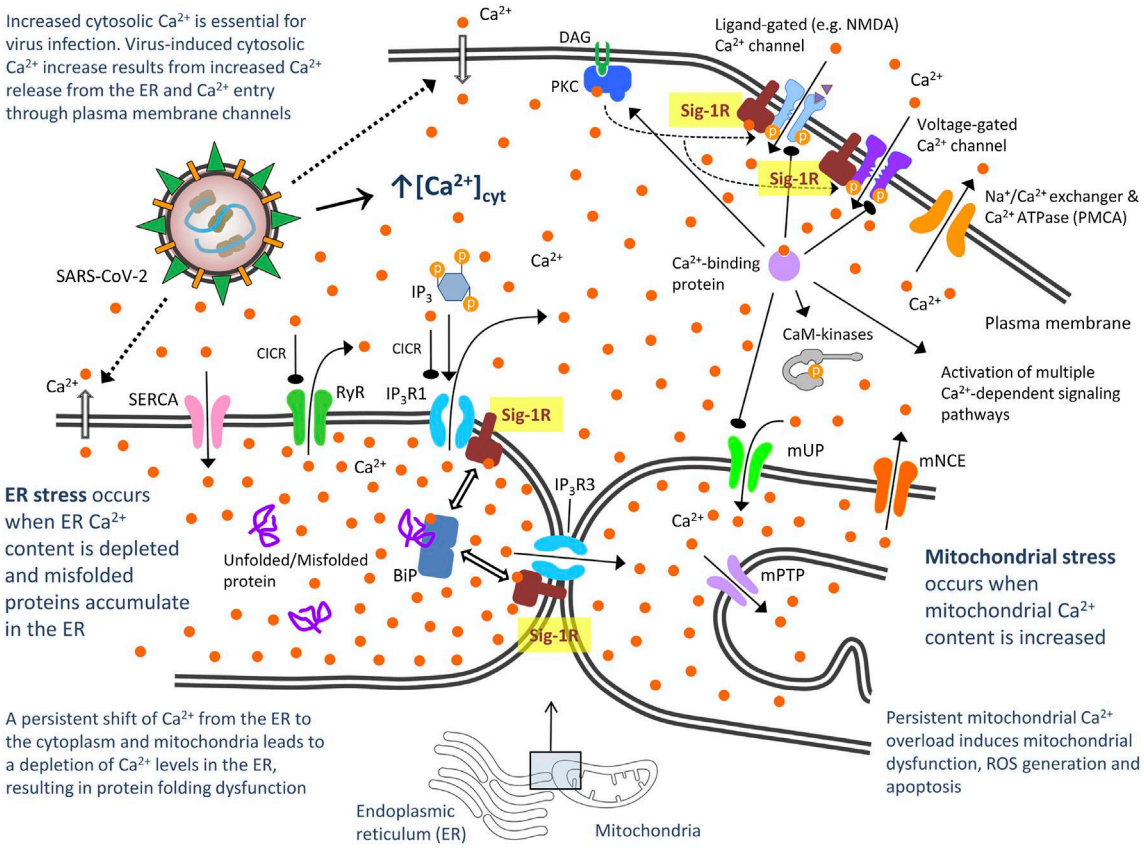

FIGURE 3 | Proposed model of severe acute respiratory syndrome CoV-2 (SARS-CoV-2)-mediated disturbance of host cell calcium (Ca^{2+}) homeostasis: Potential role of Sigma-1 receptors via interaction with Ca^{2+} channels in the plasma membrane and ER. CaM kinases, Ca^{2+}/calmodulin-dependent protein kinases; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; IP_3Rs, inositol 1,4,5-triphosphate receptor-gated channels; IP_3R1 mediates ca^{2+} release from ER to cytosol and IP_3R3 to mitochondria. They are activated not only by IP_3, but also by low Ca^{2+} concentrations through a process often referred to as Ca^{2+}-induced Ca^{2+} release (CICR). High cytosolic Ca^{2+} concentration can instead inhibit IP_3Rs; PKC, protein kinase C; mNCE, mitocondral Na^+/Ca^{2+} and $2H^+/Ca^{2+}$ exchangers. They slowly eject accumulated Ca^{2+} back into the cytosol; mPTP, mitochondrial permeability transition pore. Once intramitochondrial Ca^{2+} rises above a certain threshold, this voltage- and Ca^{2+}-dependent high-conductance channel in the inner membrane opens, activating cell death mechanisms; mUP, mitochondrial uniporter. It is gated by Ca^{2+} in a biphasic manner. Ca^{2+} uptake into mitochondria is facilitated by Ca^{2+}/calmodulin, but sustained cytosolic Ca^{2+} levels inactivate the uniporter, preventing further Ca^{2+} uptake; RyR, ryanodine receptor. It is a Ca^{2+}-gated Ca^{2+} channels (CICR); SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase. It restores ER with Ca^{2+}. Bibliographic references for the role of Sig-1R are provided in the text.

2007; Ortega-Roldan et al., 2013). BiP, also referred to as GRP78, is an important host factor for viral infection. A substantial amount of SARS-CoV S protein accumulates in the ER during infection and induces direct activation of BiP and UPR selective pathways (Chan et al., 2006). Targeting BiP has the potential to disrupt multiple stages of the viral life and it has recently proposed as a potential therapeutic approach for CoV infection (Ha et al., 2020). Sig-1R binds the nucleotide-binding domain of BiP though its bulky C-terminal lumenal domain (Ortega-Roldan et al., 2013). Dissociation of ER membrane-bound Sig-1R from lumenal BiP occurs upon Ca^{2+} depletion (indicative of ER stress) or pharmacological Sig-1R stimulation (**Figure 2**). BiP also binds to the ER lumenal domains of membrane-bound UPR transducers PERK and IRE1 and, when bounded, both BiP and UPR transducers remain in an inactive state. Recruitment of misfolded proteins to BiP substrate-binding domain during ER stress stimulates ATPase activity within its nucleotide-binding domain, enabling BiP to adopt an ADP-bound conformation that dissociates from PERK and IRE1 to allow their activation and initiation of UPR signaling cascades (Bertolotti et al., 2000; Kopp et al., 2019). The mechanistic details for Sig-1R modulation of UPR via interaction with BiP are unknown. Does Sig-1R-BiP dissociation act as a co-activator (together with misfolded proteins and ATP binding) to induce UPR? Does Sig-1R act as an allosteric inductor or compete with PERK and/or IRE1 for binding to the BiP nucleotide-binding domain? Despite these and other unanswered questions, evidence places Sig-1R as a sensor of ER stress (Ca^{2+} depletion) and upstream regulator of UPR. Does it support the antiviral effect of Sig-1R ligands in numerous cellular assays?

In addition to its interaction with its co-chaperone BiP, Sig-1R also chaperones the ER resident transmembrane protein IRE1 (Mori et al., 2013), one of the ER stress transducers, important for CoVs to adapt host cellular machinery to their demands and antagonize cell apoptosis (Fung et al., 2014b). Sig-1R stabilizes IRE1 when cells are under ER stress and such interaction allows

conformationally correct IRE1 to dimerize to the activated form (Mori et al., 2013) (**Figure 2**). IRE1 (alpha isoform, IRE1α) has RNase activity coupled to kinase activity. There are different models proposed for IRE1 activation and all of them involve dissociation from BiP, oligomerization and activation of its cytosolic kinase domain (Adams et al., 2019). This activation allows unconventional splicing of XBP1 mRNA and subsequent translation of an active transcription factor, XBP1s. XBP1s promotes expression of several targets including chaperones, foldases and components of the ERAD pathway in order to restore protein homeostasis (Smith et al., 2011). The envelope E protein of SARS-CoV has been shown to counteract the IRE1/XBP1 pathway of UPR (DeDiego et al., 2011), suggesting that inhibition of the IRE1/XBP1 pathway of UPR is important for CoV infection. Studies performed on the herpes simplex virus-1 replication showed an opposite action of IRE1 domains on viral replication, RNase activity being unfavorable to viral replication and kinase activity enhancing it (Su et al., 2017). IRE1 RNase activity activates the cellular protein degradation pathway (ERAD) that might lead to the degradation of viral proteins, which is unfavorable to viral replication (Su et al., 2017). Accordingly, in order to facilitate viral replication, IRE1 RNase activity was suppressed in infections by a variety of viruses, including CoV mouse hepatitis virus (MHV) (Bechill et al., 2008). The RNase activity of IRE1 may also target other genes via regulated IRE1-dependent decay (RIDD). RIDD is the mechanism by which IRE1 cleaves target transcript substrates that are degraded and contributes to the maintenance of ER homeostasis by diminishing ER protein load via mRNA degradation, but it has also been proposed to lead to cell death (Tam et al., 2014; Abdullah and Ravanan, 2018). Sig-1R associates to and restricts IRE1 endonuclease (RNAase) activity, needed for splicing the mRNA encoding XBP1 to produce active XBP1 protein in preclinical models of sepsis and inflammation (Rosen et al., 2019). Indeed, LPS-challenged Sig-1R knockout mice had increased hepatic XBP1 splicing when compared to WT mice. The mechanism by which the virus impairs IRE1 RNase activity is unknown, but pharmacological blocking of Sig-1R might promote IRE1 RNase activity and thus increase IRE1/XBP1-dependent degradation pathways. Is it contributing to the inhibitory effect of Sig-1R antagonist ligands on viral replication?

Autophagy

Finally, CoV infection (inclusive of SARS-CoV, MERS-CoV and the new SARS-CoV-2) has been demonstrated to induce autophagy (Maier and Britton, 2012; Yang and Shen, 2020). Interestingly, expression of viral Nsp6 from diverse CoVs induces autophagy (Cottam et al., 2011). Viral replication proteins from MHV and SARS CoVs have been shown to colocalize with autophagosome protein markers (Prentice et al., 2004a; Prentice et al., 2004b) and autophagy has been implicated in both the formation of double-membrane vesicles and replication of MHV (Prentice et al., 2004a). However, colocalization of autophagosome markers with specific replicase subunits of SARS-CoV was not observed in other study (Snijder et al., 2006) and a number of observations suggest that autophagy is not directly implicated in viral replication (Zhao et al., 2007). On the contrary, it was reported that MERS-CoV multiplication exerted an inhibitory effect on the autophagy process and that enhancement of autophagy reduced the replication of MERS-CoV (Gassen et al., 2019). Thus, it is controversial whether autophagy is used by viruses in their benefit or whether it actually represents a protective cellular response against CoV infections. Autophagosomes originate from the ER-mitochondria contact site (Hamasaki et al., 2013) and Sig-1R acts at this MAM intersection as an upstream modulator of autophagy (Schrock et al., 2013). Sig-1R agonists trigger autophagy after extended treatment, whereas Sig-1R antagonists and knockdown of Sig-1R suppresses autophagome formation (Schrock et al., 2013). Accordingly, loss-of-function mutations and Sig-1R deficiency are associated with defective autophagy, leading to accumulation of autophagic vacuoles. In contrast, re-expressing Sig-1R in the null background or its activation restores/induces autophagic activity (Vollrath et al., 2014; MacVicar et al., 2015; Christ et al., 2019; Yang et al., 2019; Christ et al., 2020). Sig-1R is not likely a core component of the general physiological autophagy machinery but it seems needed for cellular stress-induced autophagy (MacVicar et al., 2015). Despite the known interaction of transmembrane SARS-CoV-2 Nsp6 with host Sig-1R protein, the induction of autophagy by Nsp6 and the role played by Sig-1R in autophagy regulation, it is uncertain whether and how Sig-1R is implicated in autophagy induction secondary to CoV infection.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Hydroxychloroquine in COVID-19 Patients: Pros and Cons

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The pandemic of COVID-19, caused by SARS-CoV-2, has recently overwhelmed medical centers and paralyzed economies. The unparalleled public distress caused by this pandemic mandated an urgent quest for an effective approach to manage or treat this disease. Due to their well-established anti-infectious and anti-inflammatory properties, quinine derivatives have been sought as potential therapies for COVID-19. Indeed, these molecules were originally employed in the treatment and prophylaxis of malaria, and later in the management of various autoimmune rheumatic and dermatologic diseases. Initially, some promising results for the use of hydroxychloroquine (HCQ) in treating COVID-19 patients were reported by a few *in vitro* and *in vivo* studies. However, current evidence is not yet sufficiently solid to warrant its use as a therapy for this disease. Additionally, the therapeutic effects of HCQ are not without many side effects, which range from mild gastrointestinal effects to life-threatening cardiovascular and neurological effects. In this review, we explore the controversy associated with the repurposing of HCQ to manage or treat COVID-19, and we discuss the cellular and molecular mechanisms of action of HCQ.

Keywords: SARS-COV-2, COVID-19, hydroxychloroquine, chloroquine, drug discovery, drug repurposing

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INTRODUCTION

Coronaviruses (CoVs) belong to the Coronaviridae family, and usually cause mild acute respiratory illnesses or "common cold" (Su et al., 2016). In December 2019, pneumonia cases of unknown etiology were reported in China (Huang et al., 2020). Within a few weeks, the cause of these cases appeared to be a novel coronavirus (CoV). This novel virus shares around 96% with bat-CoV RaTG13 and around 80% sequence similarity with the SARS-CoV (Lu et al., 2020; Zhou et al., 2020b). Hence, it was given the name SARS-CoV-2, and the disease it causes was called coronavirus infectious disease 2019, or shortly COVID-19.

COVID-19 has imparted serious threat to the global economy and health system. At the time of writing this manuscript (August 11, 2020), over 20 million cases and more than 700,000 deaths related to COVID-19 infection were reported globally (WHO, 2020a). Research efforts linked the origin of SARS-CoV-2 to bat-to-human transmission through an unidentified intermediate host. Human-to-human transmission can then take place through respiratory droplets (Salata et al., 2019).

Abbreviations: HCQ, Hydroxychloroquine; LFTs, Liver function tests; AV block, Atrioventricular block.

SARS-CoV-2 infection may induce a wide spectrum of illnesses, with patient conditions range from being asymptomatic to severely ill. Indeed, various clinical symptoms with multi-organ involvement related to COVID-19 infection have been reported (Guan et al., 2020). These include respiratory, gastrointestinal, renal, neurologic and integumentary manifestations (Adhikari et al., 2020; Recalcati, 2020). Some of these are severe and life-threatening such as acute respiratory distress syndrome, acute kidney failure, stroke, arrythmias and heart failure (Adhikari et al., 2020; Bangalore et al., 2020; Cheng J. et al., 2020; Guan et al., 2020; Liu et al., 2020; Recalcati, 2020).

Treatment of COVID-19 infection is mainly symptomatic and is highly dependent on the severity of the disease. It includes hydration, pain control, fever treatment, oxygen supplementation, and invasive mechanical ventilation if needed (Cascella et al., 2020). As COVID-19 continues to be a source of global morbidity and mortality, urgent need of effective antiviral drug against COVID-19 appears. While numerous laboratories and clinical studies focused their efforts toward developing therapeutic and prophylactic interventions, repurposing an already known drug for use as an antiviral drug may be the fastest and least expensive. Indeed, recently, the anti-malarial agent hydroxychloroquine (HCQ) has gained attention as a potential drug that can be repositioned for the management of COVID-19. Below, we discuss the therapeutic value of this drug, along with its adverse effects.

PHARMACOLOGY OF CHLOROQUINE AND HYDROXYCHLOROQUINE

Chloroquine (CQ) and HCQ are produced and administered orally in tablet form (Pastick et al., 2020). CQ tablet consists of 500 mg of CQ phosphate. HCQ tablet is composed of 200 mg of HCQ sulfate (Pastick et al., 2020). The required dosage varies according to the treated disease (Sanofi-Aventis, 2019). For malarial prophylaxis, a weekly dosage of 6.5 mg/kg is prescribed to adult and pediatric patients (Sanofi-Aventis, 2019). However, a single dose should not exceed 400 mg. Patients are instructed to take two doses before travel to endemic countries and to continue the same dose until 1 month after return. A higher dosage of 2000 mg is used to treat acute malaria. On the contrary, a daily dosage of 200–600 mg is used to treat rheumatoid arthritis and systemic lupus erythematous (SLE) (Sanofi-Aventis, 2019).

For the treatment of COVID-19, the used daily dosages of HCQ have ranged between 800 and 1,600 mg. However, in one study, they defined the effective and safe dose of HCQ based on data reported by *in vitro* studies and clinical trials (Garcia-Cremades et al., 2020). They examined the relationship between viral load reduction and the dosing of HCQ in treated COVID-19 patients. In this study, it was concluded that a daily dose of HCQ should not exceed 800 mg (Garcia-Cremades et al., 2020). Higher dosages may lead to quicker reduction in viral load and clinical improvement. However, they may induce undesired side effects such as QT interval prolongation. For the therapy duration, the above-mentioned regimen should be given over 7 days (Garcia-Cremades et al., 2020).

HCQ and CQ bioavailability is around 70–80% (Furst, 1996). This makes their use in oral formulation appropriate for treating serious multi-organ diseases. Moreover, they are both recognized by their slowed clearance. CQ is cleared at a rate of 0.35–1 L/h/kg and HCQ is cleared at a rate of 96 ml/min (Ducharme and Farinotti, 1996; Furst, 1996). Their elimination half-lives are estimated at 40–50 days (Furst, 1996). CQ and HCQ are likely known to have large plasma volume of distributions of up to around 65,000 L and 44,257 L respectively (Browning, 2014). Given these pharmacokinetic properties of HCQ and CQ, the clinical course of patients treated with these medications might not be easily predicted particularly in patients with comorbid renal and liver diseases. In fact, these patients are prone to develop serious side effects owed to defective clearance and metabolism of CQ and HCQ. Similarly, CQ and HCQ may exert varying therapeutic effects in distinct patients depending on their renal and hepatic functions.

MECHANISM OF ACTION

Quinine along with its derivatives CQ and HCQ are weak bases that belong to the 4-aminoquinolines family (Savarino et al., 2003; Manohar et al., 2014). Both CQ and HCQ have common targets and similar mechanisms of action. Numerous mechanisms of action contribute to the role of these two drugs in a specific or a group of diseases (Yao et al., 2020). Below we discuss the mechanisms of action of CQ and HCQ which are classified into two groups based on their ultimate results: anti-inflammatory and anti-infectious.

Anti-infectious Activity of Chloroquine and Hydroxychloroquine

HCQ and CQ express anti-viral activity through interfering in various steps of the viral replication. One postulated mechanism is through impairing viral interaction with the target cell receptor by CQ thus hindering viral entry to the cell. This is accomplished by inhibiting the enzyme quinone oxidoreductase 2 (QR2) which is found in red blood cells (Kwiek et al., 2004). QR2 is vital for sialic acid biosynthesis which is a component of ligand recognition (Varki, 1997). Recent studies suggest that CQ and HCQ act by binding to both the sialic acids and the gangliosides, both of which are essential for SARS-CoV-2 entry to the host cell (Fantini et al., 2020). Besides, CQ alters viral and cellular protein glycosylation thus limiting viral-receptor interaction. This is thought to be the key mechanism by which CQ alters the interaction of SARS-CoV with the ACE2 receptor (Vincent et al., 2005). Furthermore, CQ interferes with the p38 mitogen-activated protein kinase (MAPK) pathway which is used by viruses for completion of viral replication cycle (Seitz et al., 2003; Wehbe et al., 2020).

Indeed, HCQ and CQ preferentially confine to acidic organelles (Manohar et al., 2014), and alkalinize the acidic vesicles needed for multiplication of some infectious agents. This effect was observed with multiple organisms including Tropheryma whipplei, *Coxiella* burnetii and others that need acidic environment for multiplication (Rolain et al., 2007). This increase in pH also impairs the function of several cellular enzymes affecting post-translational modification and limiting iron availability inside the cell (Manohar et al., 2014). Such a mechanism is used against retrovirus infection, where inhibition of post-translational glycosylation of the viral glycoprotein abrogates its interaction with the virus (Savarino et al., 2004). Correspondingly, proteolytic enzymes needed for viral protein processing are not activated in the presence of alkaline environment (Randolph et al., 1990). Similarly, by increasing lysosomal pH, CQ impairs endosome-dependent viral entry to the cell (Gay et al., 2012). This alkalinizing property was also found to constrain the uncoating process of some viral particles (Manohar et al., 2014). In addition, it appears that CQ boosts cytotoxic T lymphocyte response against viral infection through enhancing viral antigen presentation by dendritic cells (Accapezzato et al., 2005).

Anti-inflammatory Activity of Chloroquine and Hydroxychloroquine

The anti-inflammatory effects of CQ and HCQ are owed to their ability to modulate immune mechanisms. Indeed, CQ/HCQ elicit their effects by virtue of their ability to weaken the immune response. For instance, HCQ suppresses the release of several pro-inflammatory cytokines. Indeed, it abrogates the production of IL-6 in both monocytes and T-lymphocytes, and the production of IL-1 alpha in monocytes alone (Sperber et al., 1993). CQ also prevents the production of interleukin beta and Tumor Necrosis Factor-alpha (TNF-α) from macrophages (Jeong and Jue, 1997; Bondeson and Sundler, 1998). Interestingly, CQ represses TNF-α function by several mechanisms including decreased translation of its message (Bondeson and Sundler, 1998), post-translational change to soluble form (Jeong and Jue, 1997) or regulating the receptor expression (Jeong et al., 2002). CQ can also inhibit the activity of phospholipases A1 and A2 (Manku and Horrobin, 1976; Löffler et al., 1985). CQ negatively affects protein catabolism and antigen presentation while sparing phagocytic ability in macrophages (Ziegler and Unanue, 1982). HCQ also targets lymphocytes function by suppressing T-cell activation via inhibiting calcium signaling (Goldman et al., 2000). Besides, by virtue of their ability to abrogate toll-like receptor signaling, CQ and HCQ provide crucial immunosuppressive effect that is needed in the treatment of autoimmune diseases (Kyburz et al., 2006). To note, the immunomodulatory effects induced by CQ and HCQ are inferred from their therapeutic uses in rheumatic diseases such as rheumatoid arthritis and SLE.

The multiple cellular targets and effects of HCQ make it effective against many diseases. Despite some promising outcomes when used with COVID-19 patients, a clear mechanism of action in this particular disease has not yet been elucidated. However, based on the above-mentioned targets of HCQ in viral and autoimmune diseases, some potential cellular effects can be described (see **Figure 1**).

CLINICAL USES OF HYDROXYCHLOROQUINE

The medical use of quinine dates back to 1630 A.D. when the quinine powder, extracted from the tree of Cinchona was employed in the treatment of malaria (Schrezenmeier and Dörner, 2020). This was around 300 years before the medication and its derivatives, CQ and HCQ, were approved by the U.S. Food and Drug Administration (FDA), not only as treatment and prophylaxis for malaria, but also as treatment for rheumatic diseases (**Table 1**) (Schrezenmeier and Dörner, 2020). Currently, quinine derivatives are considered safe and well-tolerated medicines that are effective in treating a wide range of chronic autoimmune and rheumatic diseases such as anti-phospholipid syndrome, discoid or systemic lupus erythematous, Sjögren disease, juvenile idiopathic arthritis, psoriatic arthritis and rheumatoid arthritis, among others (Rynes, 1997; Lee et al., 2011; Al-Bari, 2015; Schrezenmeier and Dörner, 2020). CQ and HCQ are similarly effective in treating skin diseases such as dermatomyositis, cutaneous sarcoidosis, eosinophilic fasciitis, lichen planus and porphyria cutanea tarda (Rynes, 1997; Al-Bari, 2015). In the latter cases, they are used mainly when conventional therapies are contraindicated or ineffective (Rynes, 1997; Al-Bari, 2015).

A multitude of distinctive immunomodulatory and anti-inflammatory properties made HCQ a clinically attractive drug (Al-Bari, 2015; Schrezenmeier and Dörner, 2020). Nevertheless, additional distinguishing effects have also been reported in the literature. They include anti-thrombotic, anti-neoplastic, and anti-microbial effects (Al-Bari, 2015; Schrezenmeier and Dörner, 2020). In addition, the use of HCQ in systemic lupus erythematous and rheumatoid arthritis patients has been associated with diminished rates of cardiovascular morbidities and diabetes mellitus, shedding the light on added favorable properties that need to be further investigated (Al-Bari, 2015; Schrezenmeier and Dörner, 2020). Similarly, HCQ was associated with improved glycemic and lipid profiles in these patients, and thus in improved overall survival and life quality (Al-Bari, 2015; Schrezenmeier and Dörner, 2020).

Other uses for HCQ have also been reported. For instance, when co-administered with doxycycline, HCQ can be effective in treating Q fever endocarditis (Raoult et al., 1990; Raoult et al., 1999). This regimen results in quicker recovery rates and infrequent relapses when compared to the originally adopted regimen (Raoult et al., 1999). Similarly, HCQ appears to be adequate for the management of Whipple disease and *Tropheryma whipplei* endocarditis (Boulos et al., 2004; Fenollar et al., 2013; Lagier et al., 2014). Moreover, HCQ is effective against a multitude of other microbial agents such as giardia, Ebola virus, hepatitis C, HIV and chikungunya (Al-Bari, 2015).

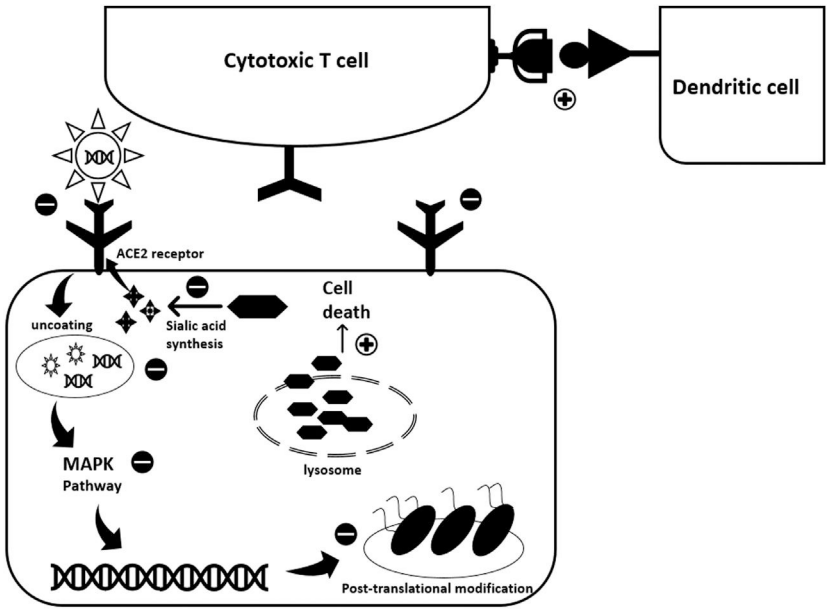

FIGURE 1 | Potential antiviral activity of Hydroxychloroquine against SARS-CoV-2. HCQ exhibits its antiviral activity through interfering with various steps of the viral replication cycle. While exact mode of action against COVID-19 is not totally revealed, experience with previous viral infections highlights possible scenarios. First, HCQ acts at the pre-entry level. It inhibits SARS-CoV-2 entry into the host cell through three different mechanisms: 1) It binds to the sialic acids and gangliosides, key components used by the spike protein for viral entry; 2) It binds to spike protein-ACE2 receptor complex; 3) It inhibits the activity of Quinone oxidoreductase 2 (QR2) which is essential for sialic acid biosynthesis. Sialic acid is important for ligand recognition. Second, once the virus enters the cell HCQ inhibits the pH-dependent uncoating process by alkalinizing the acidic endosomes. This constrains viral-endosome fusion and consequent viral DNA release into the cytoplasm. The pH-dependent viral entry to the host cell was encountered with previous coronaviruses. Besides, HCQ impedes viral replication by blocking the p38 MAPK cascade. Also, it acts at the viral protein level where it interfere with post-translational modifications such as protein glycosylation. This alters SARS-CoV-2 proteins, affecting viral ability to interact with future host cells. The effect of HCQ extends to induce infected cell death through increasing lysosomal membrane permeability and consequently allowing proteolytic enzymes leakage into the cytoplasm. Finally, HCQ promotes the immune response through enhancing antigen expression by the dendritic cells thus activating cytotoxic T-lymphocytes.

TABLE 1 | Key events portraying the well-approved clinical uses of hydroxychloroquine (HCQ) and chloroquine (CQ) along with the historical evolution of their utilization in the medical field (Schrezenmeier and Dörner, 2020).

Year	Event
1630	Quinine powder extracted from the tree of cinchona was used in the treatment of malaria
1934	Synthetic CQ was first produced
1949	The U.S Food and Drug Administration authorized the use of CQ in the management of rheumatic and skin diseases
1950	Synthetic HCQ was first produced
1955	The U.S Food and Drug Administration authorized the use of HCQ in the management of rheumatic and skin diseases

Hydroxychloroquine and COVID-19

Recently, the emergence of the COVID-19 pandemic prompted an increased quest for potential therapies that could prove effective in controlling or improving the outcomes of the disease. Owing to its anti-viral properties, especially ones which showed its efficacy in diminishing actions of SARS-CoV-1 (Keyaerts et al., 2004; Colson et al., 2020; Hashem et al., 2020), HCQ was thought of being repurposed for fighting SARS-CoV-2 and the consequent COVID-19.

Several *in vitro* studies were conducted to assess the anti-SARS-CoV-2 properties of CQ and HCQ (Hashem et al., 2020; Liu et al., 2020; Wang et al., 2020;Yao et al., 2020). Importantly, both drugs appear to significantly inhibit SARS-CoV-2 replication (Hashem et al., 2020; Liu et al., 2020; Wang et al., 2020;Yao et al., 2020). Additionally, combined HCQ and azithromycin treatment caused a synergistic anti-SARS-CoV-2 effect *in vitro* (Andreani et al., 2020). Here, we discuss the controversy associated with the use of HCQ in COVID-19 patients by exploring supportive and opposing evidence.

Pros

There are many advantages that make HCQ an attractive candidate. Not only it is safe, but it is also an effective medication with a broad spectrum of action covering various microbial and autoimmune diseases, likely by virtue of its ability to modulate the immune system (Wallace et al., 2012; Schrezenmeier and Dörner, 2020). Additionally, HCQ is a cheap medication with a good safety profile that has been garnered over hundreds of years of its use (Rynes, 1997; Savarino et al., 2003; Lee et al., 2011; Al-Bari, 2015; Schrezenmeier and Dörner, 2020; Zhou et al., 2020a). Importantly, it can be safely used in pregnant women as well (Costedoat-Chalumeau et al., 2003; Sperber et al., 2009).

The undesired side effects of this drug are also mild. They include gastrointestinal symptoms (nausea, vomiting and abdominal pain), along with cutaneous manifestations, and CNS symptoms (headache, dizziness, tinnitus and sleep disturbances) that are less frequently encountered (Rynes, 1997; Lee et al., 2011; Al-Bari, 2015; Littlejohn, 2020; Schrezenmeier and Dörner, 2020). Retinopathy is considered the most feared side effect of HCQ; yet, it is a rare manifestation that occurs mainly with prolonged use of high dosage therapy (Rynes, 1997; Wolfe and Marmor, 2010; Lee et al., 2011; Al-Bari, 2015; Littlejohn, 2020; Schrezenmeier and Dörner, 2020). Nevertheless, long-term monitoring and surveillance, and tight dosage regulation are associated with a reduced incidence of HCQ-induced retinopathy (Abdulaziz et al., 2018; Jorge et al., 2018). Other rare side effects, discussed in the following section, are encountered primarily in the presence of comorbid cardiovascular, renal and liver diseases (Gevers et al., 2020). Furthermore, HCQ is found to be less toxic and better tolerated than CQ (Rynes, 1997; Avina-Zubieta et al., 1998; Al-Bari, 2015; Liu et al., 2020; Schrezenmeier and Dörner, 2020; Zhou et al., 2020a).

Owed to its accessibility, effectiveness, and tolerability, HCQ has gained increased attention. It has been heavily examined in numerous studies as a potential treatment for the emerging pandemic of COVID-19. In this context, several studies, performed in different parts of the world, have discussed the benefits induced by the addition of HCQ to the conservative symptomatic therapies such as fluids, antipyretics and oxygen therapy (**Table 2**) (Gao et al., 2020; Gautret et al., 2020a; Gautret et al., 2020b; Million et al., 2020; Chen Z. et al., 2020).

Prior to the marked global propagation of the disease, trials using this drug has already started in China (Gao et al., 2020). In this context, it was reported that HCQ is more effective than conventional symptomatic treatment as per data derived from more than 100 patients (Gao et al., 2020). Indeed, treated patients had reduced disease severity, improved radiological findings, quicker virus clearance, and earlier recovery (Gao et al., 2020). Nonetheless, this study has several limitations. First, it is a non-randomized observational study with a limited number of participants. Second, the age and the pre-COVID-19 clinical status of the treated patients are not explicitly stated.

Similarly, *Zhaowei et al* assessed the efficacy of HCQ in a cohort of 62 SARS-CoV-2 positive patients (Chen Z. et al., 2020). Time to clinical recovery (TTCR), body temperature recovery time, and cough remission time were significantly reduced in the HCQ-treated group when compared to the control group (Chen Z. et al., 2020). Additionally, faster resolution of pneumonia was reported in 80.6% of the HCQ-treated patients vs. 54.8% of the control patients (Chen Z. et al., 2020). In this study, the diagnosis of COVID-19 was confirmed through several parameters including clinical, laboratory, physical and radiological findings. This minimizes the risk of missing a COVID-19 case and also the risk of misdiagnosing patients with COVID-19-like symptoms. The study is a randomized controlled clinical trial. However, the study is not blinded and is limited by the small number of participants. Similarly, patients with serious and critical COVID-19 were excluded as well as those with severe pre-existing medical conditions including arrhythmia, severe liver and renal diseases, and retinal diseases. This makes the selected cohort less susceptible to HCQ associated side effects.

A French trial included a total of 36 patients, 20 of whom received HCQ and 16 received control therapy (Gautret et al., 2020a). Azithromycin was added to the treatment of six HCQ-treated patients in order to avoid superimposed bacterial infection. These patients received daily echocardiographic monitoring (Gautret et al., 2020a). In this trial, HCQ was found to be superior to supportive therapy. After 7 days of treatment, the viral load was reduced in 70% of the HCQ-treated patients (Gautret et al., 2020a). Similarly, addition of azithromycin to HCQ resulted in quicker viral clearance when compared to HCQ alone. In fact, a synergistic reduction in the viral load was induced by this combination (Gautret et al., 2020a). In a second non-comparative study, *Gautret et al* revisited the benefit of HCQ incorporation in the management of COVID-19 patients, in a cohort of 80 patients (Gautret et al., 2020b). 81.3% of the patients had mild disease with favorable prognosis (Gautret et al., 2020b). 5% were asymptomatic and around 15% had moderate to severe disease requiring oxygen therapy. Three patients were admitted to the intensive care unit (Gautret et al., 2020b). A combination of azithromycin (500 mg on day 1, followed a course of 4 days of 250 mg daily) and HCQ (600 mg daily over 10 days) was given to all patients (Gautret et al., 2020b). After at least 3 days of treatment, 78 patients had improved clinical outcomes, early recovery, and reduced viral load. However, one patient died, and one remained in the intensive care unit despite treatment (Gautret et al., 2020b). The first study conducted by *Gautret el al* is a single-center non-randomized clinical trial limited by the lack of randomization and blinding and the minimal number of enrolled participants. It is likely limited by the lack of adequate follow up. Additionally, patients with retinopathy, QT prolongation and G6PD deficiency, who are prone to develop HCQ life-threatening side effects, were excluded. This reduces the incidence of serious side effects among the treated patients. Similarly, the second study is a non-randomized non-comparative observational study that is likely limited by the small number of participants and the imposed exclusion criteria. In fact, the evidence derived from these studies is not considered of high-quality owed to the limited sample size and the enhanced risk of selection bias.

Other trials examined the effectiveness of the same combination in a cohort of 1,061 confirmed inpatients (Million et al., 2020). In this study, the effect of this combination on mortality, recovery and viral shedding was determined. Findings showed that the virus cleared in 91.7% of the patients after less than 10 days of treatment. 4.4% of the patients, with a higher original viral load required a longer period of 10 days to clear the virus (Million et al., 2020). Unfavorable clinical outcome was noted in 46 patients (4.3%). Similarly, eight patients, accounting for 0.75%, died due to respiratory failure. Cardiac toxicity was not reported in any of the patients (Million et al., 2020). This study demonstrated that HCQ and azithromycin can be safely used in patients with early disease, particularly in the absence of associated complications. They accelerate recovery and improve overall clinical outcomes

TABLE 2 | Clinical trials supporting HCQ use. The following databases were searched: Cochrane, embase, Medline, New England Journal of Medicine and PubMed. A total of five trials supported the use of HCQ in patients with COVID-19. All of them except one were observational cohort studies.

Evidence supporting the use of HCQ

Study	Study type	Country	Population size	Results	Ref
Efficacy of hydroxychloroquine in patients with COVID-19: Results of a randomized clinical trial	Single-center RCT	China	62	A quicker recovery was noted in the HCQ-treated group compared to the control group. Studied outcomes included: • Time to clinical recovery • clinical progression • radiological progression	(Chen Z. et al., 2020)
Hydroxychloroquine and azithromycin as a treatment of COVID-19: Results of an open-label non-randomized clinical trial	Single-center, single-arm, non-randomized clinical trial	France	36	A quicker reduction in viral load was noted in the HCQ-treated group. A synergistic effect was prompted by the addition of azithromycin to the HCQ regimen. Studied outcomes included: • Virologic clearance achieved after 6 days of treatment • time to negative conversion • clinical progression • experienced side effects	(Gautret et al., 2020a)
Clinical and microbiological effect of a combination of hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a 6-day follow up: A pilot observational study	Single-center, uncontrolled, non-comparative observational study	France	80	Quicker reduction in viral load, shorter hospital stay, and improved outcomes were noted among the patients after an average of 5 days of treatment. Studied outcomes included: • Virologic clearance achieved up till day 12 of treatment • clinical outcomes • length of hospital stay	(Gautret et al., 2020b)
Early treatment of COVID-19 patients with hydroxychloroquine and azithromycin: A retrospective analysis of 1,061 cases in marseille, France	Single-center non-comparative, retrospective study	France	1,061	The use of HCQ combined with azithromycin was linked to improved mortality, clinical outcomes, and virologic clearance. Studied outcomes included: • Mortality clinical worsening (need for intensive care, and prolonged hospital stay) • virologic clearance	(Million et al., 2020)
Low dose of hydroxychloroquine reduces fatality of critically ill patients with COVID-19	Single-center comparative, retrospective study	China	550	The use of HCQ in critically ill COVID-19 patients was associated with improved survival, and reduced mortality rate. Studied outcomes included: • Length of hospital stay • mortality rate	(Yu, 2020)

(Million et al., 2020). Unlike the previously mentioned studies, this study has a larger cohort of participants. Plasma levels of medications were monitored adequately in most patients and the diagnosis of COVID-19 was based on sufficient clinical and laboratory evidence. However, just like all retrospective studies, the study is subjected to the inherent limitations of retrospective studies denoted by the lack of control and randomization and the biased selection of the participants. Additionally, patients susceptible to HCQ toxicities were likely excluded from the studied cohort.

The above-mentioned trials have triggered a call for further investigations. In fact, out of 688 and 2122 COVID-19 related ongoing trials registered in the Chinese clinical trial registry and the U.S. National Library of Medicine respectively, 11 and 218 trials aim to examine the effectiveness of HCQ in COVID-19 patients (Chinese Clinical Trial Registry, 2020; U.S. National Library of Medicine, 2020a; U.S. National Library of Medicine, 2020b; U.S. National Library of Medicine, 2020c; U.S. National Library of Medicine, 2020d). Ultimately, it is hoped that these trials will provide a clearer understanding of the therapeutic role of HCQ in curing SARS-CoV-2 infection, and also in averting its propagation.

Cons

Hydroxychloroquine and Viral Clearance in COVID Patients

Although HCQ is relatively safe to use in treating malaria and autoimmune diseases, COVID-19 patients may be more susceptible to its adverse reactions, in part because of the compromised function of vital organs secondary to SARS-CoV-2 infection (Gevers et al., 2020). Since HCQ is cleared by the kidney and the liver, severely ill patients, particularly ones with impaired renal or hepatic functions, are at increased risk of experiencing serious adverse reactions. Besides, drug-drug interactions are major causes of some of HCQ's adverse events (Gevers et al., 2020).

HCQ has an estimated half-life of around 2 months, and is inadequately distributed in adipose tissues. Thus, monitoring for side effects, over a long period of time, is highly advised particularly in the presence of severe comorbid conditions (Gevers et al., 2020). Furthermore, HCQ's adverse effects may mask or interfere with symptoms of specific illnesses such as COVID-19. This is especially important when evaluating their cardiovascular, neuropsychiatric and gastrointestinal side effects (Gevers et al., 2020).

Following the decision of The Health Ministry of France of permitting the use of HCQ to treat COVID-19, a prospective study assessed the outcomes of 11 patients that were hospitalized at Saint-Louis Hospital (Molina et al., 2020). Those patients received 600 mg/d of HCQ in combination with azithromycin for 10 days (500 mg day 1 and 250 mg days 2–5) (Molina et al., 2020). Among them, eight had underlying comorbid diseases (Molina et al., 2020). As the treatment started, 10 patients were febrile and on oxygen therapy. After 5 days of treatment, one patient passed away and two were moved to the intensive care unit. Furthermore, in one patient, the treatment was terminated after 4 days due to QT prolongation (Molina et al., 2020). After five to 6 days of treatment initiation, the nasopharyngeal swabs of eight patients were still positive for SARS-CoV-2 (Molina et al., 2020). However, these results contradicted the optimistic outcomes provided by an earlier study where 70% of patients treated with HCQ had negative PCR testing by day 6, compared to only 12.5% of patients in the control group (Gautret et al., 2020a). Congruently, no significant difference in the rate of viral clearance, hospital stay, radiologic findings or temperature regulation, between control and HCQ treated groups was also reported (Chen J. et al., 2020). Nevertheless, these two studies conducted by *Molina el al* and *Chen et al* are both limited by the small number of enrolled patients. The first study is likely subjected to several limitations owed to the lack of control and randomization and the inherent errors associated with observational studies.

The RECOVERY trial is a large multi-center randomized controlled trial that compares several treatments to standard management in patients with COVID-19 (Horby et al., 2020). Preliminary results depicting the difference between HCQ and standard care have shown no improvement in the clinical outcomes of the HCQ-treated group (Horby et al., 2020). HCQ was not linked with improved mortality in the treated group. Yet, it imposed an increase in the duration of hospitalization and an enhanced risk of deterioration and progression to assisted respiration (Horby et al., 2020). The SOLIDARITY trial is another multi-national multi-center randomized controlled clinical trial issued by WHO (WHO, 2020b). It compares several proposed anti-COVID-19 therapies to usual care. Owed to absence of or minimal benefit induced by HCQ, the committee has decided to discontinue the use of HCQ in this trial (WHO, 2020b). Furthermore, the ORCHID Study, a third multi-center placebo-controlled randomized clinical trial comparing HCQ to standard therapy, was also terminated by the National Institutes of Health due to the lack of benefit produced by HCQ (NIH, 2020).

Moreover, a recently published multicenter randomized clinical trial performed in Brazil has compared the efficacy of standard care alone to each of standard care plus HCQ and standard care plus combined HCQ and azithromycin in hospitalized patients with mild to moderate disease (Cavalcanti et al., 2020). 667 patients were randomly assigned to one of the three groups. HCQ was given at a dosage of 800 mg/day divided into two doses for 7 days. Azithromycin was given at a dosage of 500 mg once daily for 7 days. No significant difference in clinical status was noted among the three treated groups at 15 days of treatment initiation (Cavalcanti et al., 2020). Additionally, more side effects were encountered by the HCQ and the HCQ plus azithromycin treated groups as compared to the control group. Prolonged QT interval and hepatic injury were among the witnessed side effects in this study (Cavalcanti et al., 2020). This study was subjected to multiple limitations. First, the number of assessed outcomes was limited. Hence, the role of each of HCQ and azithromycin in treating COVID-19 cannot be objectively assessed and based on this study since unstudied benefits induced by HCQ and azithromycin may be easily missed. Second, no blinding was applied in this study. Finally, adherence to treatment regimen cannot be asserted owed to the

increased demand for and the lack of these medications in some of the enrolled hospitals. This may result in biased and inconsistent outcomes.

Besides, in a recently published study, SARS-CoV-2 infection was found to be resistant to CQ in lung cells positive for TMPRSS2, a cellular protease that facilitates the invasion of the cells by SARS-CoV-2 (Markus Hoffmann, 2020). The potential inhibitory effect induced by the expression of TMPRSS2 was not seen in non-pulmonary cell lines (Markus Hoffmann, 2020). This means that CQ, and likely HCQ, may not be effective in clearing SARS-CoV-2 infection in pulmonary tissues, and that initial *in-vitro* results supporting the use of HCQ in COVID-19 patients might have stemmed from experiments performed on non-pulmonary tissues (Markus Hoffmann, 2020). Congruently, use of HCQ for clearing SARS-CoV2 infection was not supported by preclinical evidence despite the different models employed such as mice or hamsters, or even *in vitro* studies (Funnell et al., 2020). This endorses the hypothesis suggested by Hoffman. In short, HCQ seems to be not suitable for treating human or human-like pulmonary tissues infected with SARS-CoV2 as concluded from these *in vitro* and *in vivo* studies.

As such, despite the positive outcome reported by some studies, whether HCQ is effective or not remains controversial. Furthermore, no solid evidence has validated the potent antiviral activity or clinical benefit of the combination of HCQ and azithromycin in curing hospitalized COVID-19 patients with moderate to severe disease. **Table 3** highlights the unfavorable results reported by some of the completed clinical trials.

Serious Side Effects of Hydroxychloroquine

In addition to its above-mentioned well-tolerated gastrointestinal and cutaneous adverse reactions, HCQ may lead to serious cardiotoxic, metabolic and neuropsychiatric manifestations, as depicted in **Figure 2**. Indeed, HCQ may lead to a multitude of cardiac events denoted by bundle branch block, complete AV block, QRS and QT prolongation, Torsades de pointes, and ventricular tachyarrhythmia (Jordan et al., 1999; Marquardt and Albertson, 2001; Chen et al., 2006; Kruisselbrink and Zaki Ahmed, 2010; Gevers et al., 2020). Similarly, patients on HCQ may develop cardiotoxicity secondary to HCQ-induced hypokalemia (Jordan et al., 1999; Marquardt and Albertson, 2001; Chen et al., 2006; Kruisselbrink and Zaki Ahmed, 2010). These patients may become prone to serious life-threatening hypotensive episodes especially in the setting of prolonged intake and overdose (Jordan et al., 1999; Marquardt and Albertson, 2001; Chen et al., 2006; Kruisselbrink and Zaki Ahmed, 2010).

Long-term use of HCQ has been associated, in rare cases, with advanced cardiomyopathy, as well as subsequent cardiovascular compromise and heart failure (Costedoat-Chalumeau et al., 2007; Hartmann et al., 2011; Muthukrishnan et al., 2011; Joyce et al., 2013; Zhao et al., 2018). Notably, HCQ cardiotoxicity is primarily encountered in patients with preexisting liver or kidney diseases, as well as in those taking medications that may affect HCQ metabolism or potentiate its side effects (Chen et al., 2006; Gevers et al., 2020). For instance, the risk of QT prolongation is greater when HCQ is added to other QT-prolonging drugs such as macrolides (Gevers et al., 2020). Besides, pediatric patients are also susceptible to HCQ's pro-arrhythmic effects, even if only small doses are used (Erickson et al., 2020). Hence, precise dosing and careful monitoring are both required to avoid fatal cardiotoxicities of HCQ.

In a recent study, male gender, older age and concurrent intake of NSAIDs were identified as potential risk factors for HCQ cardiotoxicity (Cohen et al., 2020). In addition, it appears that CQ confers a higher risk of cardiotoxicity as compared to HCQ (Cohen et al., 2020). Contextually, HCQ cardiotoxicity becomes of utmost relevance in COVID-19 patients. Indeed, two separate studies show that cardiac involvement is a predictor of mortality in COVID-19 patients (Guo et al., 2020; Shi et al., 2020). This explains the higher incidence of complications and adverse events in the group of HCQ-treated COVID-19 patients, as evidenced by an open label randomized clinical trial of 150 Chinese patients (Tang et al., 2020).

Hypoglycemia is yet another serious side effect exerted, albeit rarely, by HCQ (Cansu and Korkmaz, 2008; Sheikhbahaie et al., 2016; Richard De-Heer, 2018). Indeed, evidence stemming from both *in vitro* and clinical studies underscores the role of HCQ in reducing blood glucose levels. This is primarily achieved through the potentiation of the hypoglycemic effects of insulin (Cynober et al., 1987). It appears that HCQ increases plasma levels of insulin via the downregulation of its intracellular breakdown as well as the enhancement of intracellular accumulation (Cynober et al., 1987). Additionally, HCQ elicits this hypoglycemic effect by reducing the rate of glucose receptor recycling, and also by promoting insulin-dependent cellular uptake of glucose (Cynober et al., 1987).

Other undesired outcomes such as psychiatric and neuromuscular adverse effects have also been associated with HCQ use, particularly with prolonged use of increased doses. Some of these adverse effects include anxiety, agitation, depression and personality changes (Manzo et al., 2017; Gevers et al., 2020). Moreover, confusion, headache, neuropathy, seizure, visual disturbances and weakness represent reversible CNS manifestations of high dose-use of HCQ (Estes et al., 1987; Stein et al., 2000; Kwon et al., 2010; Vinciguerra et al., 2015; Gevers et al., 2020).

Table 4 depicts side effects experienced by COVID-19 patients as well as the incidence of these adverse effects among the treated patients. Overall, the incidence of side effects has ranged between 0.06% and 33.67%. This depends largely on the administered dose of HCQ and the co-existence of cardiac, hepatic and renal diseases that might potentiate the toxicity of HCQ. Most side effects were mild cutaneous, gastrointestinal and neurologic. Nonetheless, serious life-threatening side effects such as torsades de points and QT interval prolongation were likely encountered by these patients.

Hydroxychloroquine as Pre- and Post-exposure Prophylaxis for COVID-19

To date, no medication has been approved for pre- and post-exposure prevention of COVID-19. Adequate quarantine and

TABLE 3 | Clinical trials against HCQ use. The following databases were searched: Cochrane, Embase, Medline, New England Journal of Medicine and PubMed. A total of four trials (two randomized controlled trials (RCT) and two cohort studies) showed no significant improvement in clinical outcome and mortality when comparing the HCQ-treated group to the control group.

Evidence against the use of HCQ

Study	Study type	Country	Population size	Results	Ref
A pilot study of hydroxychloroquine in treatment of patients with common coronavirus disease-19 (COVID-19)	Single-center RCT	China	30	No significant prognostic difference was noted between the HCQ-treated group and the control group. Comparable adverse events were experienced by each group. Studied outcomes included: • Time to negative conversion • time for body temperature normalization • radiological progression • adverse events experienced by both groups	(Chen J. et al., 2020)
Hydroxychloroquine in patients with mainly mild to moderate coronavirus disease 2019: Open label, randomised controlled trial	Multi-center RCT	China	150	No significant prognostic difference was noted between the HCQ-treated group and the control group. More adverse events were observed in the treated group. Studied outcomes included: • Time to negative conversion • adverse events experienced by both groups	(Tang et al., 2020)
Association of treatment with hydroxychloroquine or azithromycin with in-hospital mortality in patients with COVID-19 in New York state	Multi-center retrospective cohort study	United States, New York	1,438	There was no significant improvement in mortality rate in hospitalized COVID-19 patients treated with HCQ, azithromycin or HCQ + azithromycin. Studied outcomes included: • In-hospital mortality • incidence of fatal cardiac events	(Rosenberg et al., 2020)
Observational study of hydroxychloroquine in hospitalized patients with Covid-19	Single-center, observational study	United States, New York	1,446	The use of HCQ had no significant effect on clinical outcomes and in-hospital mortality. Studied outcomes included: • Clinical worsening (denoted by the need for intubation) • death	(Geleris et al., 2020)

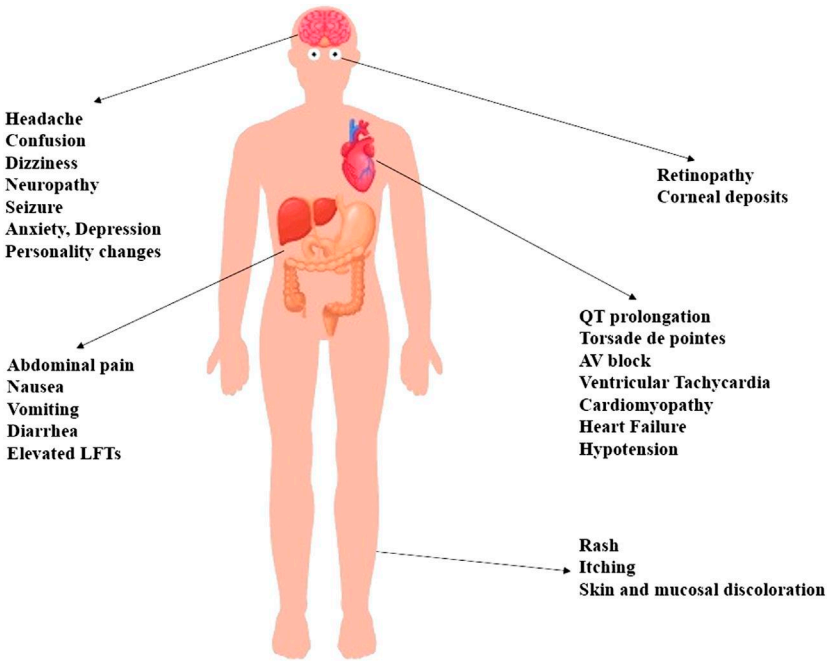

FIGURE 2 | A multitude of side effects may be elicited by the use of HCQ. Most of the experienced side effects are mild and well-tolerated ones that include cutaneous, gastrointestinal and neurological symptoms denoted by itching, skin rashes, abdominal pain, nausea, vomiting, diarrhea, headache, and dizziness. Life-threatening cardiovascular side effects are particularly encountered in critically ill patients with comorbid hepatic or renal diseases. Similarly, the toxicity of HCQ can be potentiated by the coadministration of drugs that may affect the metabolism of HCQ, or enhance its pro-arrhythmic effects. Retinopathy constitutes another feared side effect associated with chronic use of high-dose HCQ. Furthermore, patients may experience serious gastrointestinal events such as liver injury, and neuropsychiatric events such as confusion, seizure, mood changes and psychosis.

TABLE 4 | Side effects experienced by COVID-19 patients and their incidence among the treated patients.

Study	Side effects	Incidence of side effects	Ref
Efficacy of hydroxychloroquine in patients with COVID-19: Results of a randomized clinical trial	Skin rash (1) headache (1)	6.5% (2/31)	(Chen Z. et al., 2020)
Clinical and microbiological effect of a combination of hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a 6-day follow up: A pilot observational study	Blurry vision (1) nausea/Vomiting (2) diarrhea (4)	8.75% (7/80)	(Gautret et al., 2020b)
Early treatment of COVID-19 patients with hydroxychloroquine and azithromycin: A retrospective analysis of 1,061 cases in marseille, France	Abdominal pain (3), diarrhea (12), nausea (1), vomiting (1), headache (3), insomnia (2), blurry vision (2), skin rash (2)	2.35% (25/1,061)	(Million et al., 2020)
No evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection	QT interval prolongation (1)	9.1% (1/11)	(Molina et al., 2020)
A pilot study of hydroxychloroquine in treatment of patients with moderate COVID-19	Diarrhea (4) elevated liver enzymes (4)	26.67% (4/15)	(Chen J. et al., 2020)
Effect of hydroxychloroquine in hospitalized patients with COVID-19: Preliminary results from a multi-centre, randomized, controlled trial	Torsades de pointes (1)	0.06% (1/1,561)	(Horby et al., 2020)
Hydroxychloroquine with or without azithromycin in mild-to-moderate Covid-19	QT interval prolongation (13), arrhythmia (3), bradycardia (1), supraventricular tachycardia (2), pneumothorax (1), nausea (9), anemia (14), bloodstream infection (1), elevated liver enzymes (17), itching (1), hypoglycemia (1), elevated bilirubin level (5), thrombocytopenia (14), leukopenia (3), lymphopenia (17)	33.67% (67/199)	(Cavalcanti et al., 2020)

TABLE 5 | | Ongoing clinical trials. Owed to the scarcity of reliable evidence, hundreds of clinical trials were initiated in many parts of the world. Here, we searched the databases of Cochrane, embase, Medline, New England Journal of Medicine and PubMed along with the clinical trial registry (ClinicalTrial.gov), and we selected randomly some ongoing trials.

Ongoing clinical trials

Study	Study type	Country	Population size	Results	Ref
Azithromycin added to hydroxychloroquine for patients admitted to intensive care due to coronavirus disease 2019 (COVID-19)-protocol of randomised controlled trial AZIQUINE-ICU	Multi-center, double-blind, RCT	Czech republic	Not yet indicated	Outcomes to be studied: • Clinical worsening • mortality	(Duska et al., 2020)
Treatment with hydroxychloroquine vs hydroxychloroquine + nitazoxanide in COVID-19 patients with risk factors for poor prognosis: A structured summary of a study protocol for a randomised controlled trial	Single-center, single-blind, RCT	Mexico	86	Outcomes to be studied: • Need for mechanical ventilation • death	(Calderon et al., 2020)
Test and treat COVID 65 plus - hydroxychloroquine vs. placebo in early ambulatory diagnosis and treatment of older patients with COVID19: A structured summary of a study protocol for a randomised controlled trial	Multi-center, double-blind, RCT	Germany	300–400	Outcomes to be studied: • Need for hospitalization • death	(Gopel et al., 2020)
The COVIRL-001 trial: A multicentre, prospective, randomised trial comparing standard of care (SOC) alone, SOC plus hydroxychloroquine monotherapy or SOC plus a combination of hydroxychloroquine and azithromycin in the treatment of non- critical, SARS-cov-2 PCR-positive population not requiring immediate resuscitation or ventilation but who have evidence of clinical decline: A structured summary of a study protocol for a randomised controlled trial	RCT	Ireland	351	Outcomes to be studied: • Time to progression to intubation or non-invasive ventilation • need for high-dose corticosteroids • death	(Feeney et al., 2020)
Use of hydroxychloroquine in patients with COVID-19: A randomized controlled clinical trial	RCT	Saudi Arabia	200	Outcomes to be studied: • Time to viral clearance • mortality	(U.S. National Library of Medicine, 2020b)
Single-center, phase II, randomized double-blind, placebo-controlled study of hydroxychloroquine compared to placebo as treatment for severe acute respiratory syndrome coronavirus 2 (SARS-cov-2) infection	Single-center, double-blind, RCT	United States, New York	120	Outcomes to be studied: • Clinical improvement • need for mechanical ventilation	(U.S. National Library of Medicine, 2020d)
Hydroxychloroquine in SARS-cov-2 (COVID-19) pneumonia trial	Single-center RCT	United States, Washington	120	Outcomes to be studied: • Change from baseline oxygenation • length of ICU/hospital stay • need for oxygen therapy/mechanical ventilation • mortality • incidence of fatal cardiac events	(U.S. National Library of Medicine, 2020c)

(Continued on following page)

TABLE 5 | (*Continued*) | Ongoing clinical trials. Owed to the scarcity of reliable evidence, hundreds of clinical trials were initiated in many parts of the world. Here, we searched the databases of Cochrane, embase, Medline, New England Journal of Medicine and PubMed along with the clinical trial registry (ClinicalTrial.gov), and we selected randomly some ongoing trials.

Ongoing clinical trials					
Study	**Study type**	**Country**	**Population size**	**Results**	**Ref**
Norwegian coronavirus disease 2019 (NO COVID-19) pragmatic open label study to assess early use of hydroxychloroquine sulfate in moderately severe hospitalised patients with coronavirus disease 2019: A structured summary of a study protocol for a randomised controlled trial	Single-center double-blind, RCT	Norway	202	Outcomes to be studied: • Rate of viral load reduction • need for intensive care • length of hospital stay • mortality • clinical status reached after 14 days of study initiation • laboratory profile	(Lyngbakken et al., 2020)

monitoring of clinical symptoms remain the mainstay of post-exposure prophylaxis. Similarly, appropriate practicing of social distancing and proper utilization of personal protection equipment, including face masks and googles, continue to be the core means of COVID-19 pre-exposure prevention. Nonetheless, the role of HCQ in preventing COVID-19 pre- and post-exposure has been tackled in various clinical studies. *Boulware et al* performed a double-blinded randomized clinical trial involving 821 American and Canadian individuals who were exposed to a confirmed case of COVID-19 at home or occupation (Boulware et al., 2020). The participants were divided into two groups based on the degree of personal protection at the time of exposure: 1) group of high-risk exposure and 2) group of moderate risk exposure. Exposed Individuals with and without a face mask were considered at moderate- and high-risk respectively (Boulware et al., 2020). Furthermore, participants were given randomly placebo or HCQ. A cumulative HCQ dose of 3,800 mg, divided over five days, was provided to the treated group. After 14 days of follow up, there was no significant difference in the number of newly diagnosed COVID-19 cases among the placebo and the treated groups. Additionally, treated patients were subjected to more side effects with most side effects being self-limited gastrointestinal and neurologic effects (Boulware et al., 2020).

Interestingly, this trial had several limitations including inadequate confirmation of exposure, and inappropriate diagnosis of COVID-19 based on clinical symptoms in the absence of molecular confirmation. Indeed, participants who developed clinical symptoms similar to those of COVID-19 were considered SARS-CoV2 positive. Their infection was not proven positive through SARS-CoV2 polymerase Chain Reaction (PCR) testing. Furthermore, the median age of the enrolled participants was 40 years and most were aged between 33 and 50 years. This means that most of the enrolled participants were healthy young individuals. As a result, the prophylactic effect of HCQ can be better assessed through larger randomized clinical trials that involve older patients with pre-existing comorbid conditions.

The pre-exposure prophylactic effect of HCQ has been likely investigated in a multitude of complete and ongoing studies. In one double-blinded randomized clinical trial, the efficacy of HCQ in preventing COVID-19 was examined among 1,483 American and Canadian healthcare workers who are significantly exposed to COVID-19 patients in high-risk areas such as emergency departments, COVID-19 units and intensive care units (Rajasingham et al., 2020). The participants were assigned to three groups: 1) HCQ group 1, provided with a dose of 400 mg once weekly for 12 weeks, 2) HCQ group 2, provided with a dose of 800 mg twice weekly for 12 weeks, and 3) placebo group (Rajasingham et al., 2020). After 12 weeks of follow up, no significant difference in the incidence of COVID-19 was detected among the three groups (Rajasingham et al., 2020). However, just like the previous study, this study was limited by the lack of adequate PCR testing, and also by the inherent error associated with the use of PCR in confirming COVID-19. Similarly, the diagnosis of COVID-19 in many

participants was made based on clinical judgment and was not confirmed through laboratory testing.

CONCLUSION

Evidence on the effectiveness and safety of HCQ in treating COVID-19 infection is still controversial. Most of the available studies are non-randomized with preliminary results. We argue that multi-center placebo-controlled randomized clinical trials are urgently needed to assess the efficacy, safety as well as determining the best dosing regimen of HCQ. It is also essential to assess longer-term effects, and thus a thorough examination of upcoming results reported by high-quality ongoing trials is much needed (see **Table 5**).

AUTHOR CONTRIBUTIONS

MA, FB and AE developed the idea and the review framework. NY, RZ, SA wrote the first draft of the article. AE did the final editing. All authors contributed to corrections and adjustment of subsequent iterations of the article. All authors approve and agree with the content.

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Rational Drug Repurposing: Focus on Lysosomotropism, Targets in Disease Process, Drug Profile, and Pulmonary Tissue Accumulation in SARS-CoV-2 Infection/COVID-19

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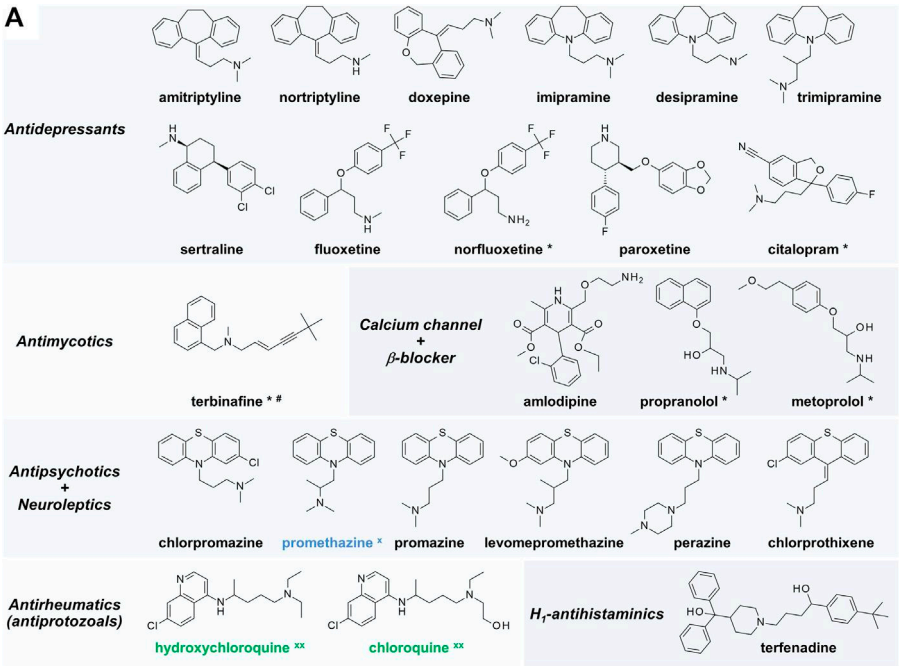
INTRODUCTION

The pandemic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified as the disease-causing pathogen of Coronavirus disease 2019 (COVID-19). (Pre) clinical research to identify rapidly available small molecules for the treatment of SARS-CoV-2 infections/COVID-19 has focused to date on the approved lysosomotropic antimalarials chloroquine and hydroxychloroquine, the investigational remdesivir (GS-5734, compassionate use), and the anti-inflammatory corticosteroid dexamethasone (COVID-19 Treatment Guidelines Panel, 2020). Lopinavir/ritonavir and other HIV protease inhibitors, however, were discontinued as treatment options in COVID-19 demonstrating no clinical benefit in clinical trials.

Despite encouraging results in treating hospitalized patients with COVID-19 requiring supplemental oxygen, mechanical ventilation, or extracorporeal membrane oxygenation (ECMO) with remdesivir and dexamethasone, there is still a lack of active compounds exhibiting pan-coronavirus antiviral activity, tackling or preventing host cell infection, forming syncytia, endotheliitis, or the cytokine release syndrome (CRS)/cytokine storm syndrome in COVID-19. Target-oriented and in particular site of action-oriented drug repurposing of small molecules has the potential to close the gap in prophylaxis and treatment of mild and moderate COVID-19 and to reduce mortality in severe cases.

OXIDATIVE STRESS, APOPTOSIS, MULTINUCLEATE SYNCYTIA, HOST CELL ENTRY, AND CYTOKINE STORM SYNDROME DEFINE DRUG REPURPOSING TARGETS

Oxidative stress (e.g., enhanced ROS levels) has been demonstrated in animal models of SARS (Delgado-Roche and Mesta, 2020) and serves as a possible explanation why SARS-CoV-2 patients with Glucose-6-phosphate dehydrogenase (G6PD) deficiency develop intravascular hemolysis and methemoglobinemia (Palmer et al., 2020). Both, chloroquine and hydroxychloroquine, are supposed to



B

	Cellular Target	Cellular Effect	SARS-CoV-(2)/COVID-19
1	(Unspecific) inhibition of **cathepsin L** and other lysosomal enzymes	Increase of (endo)lysosomal pH	Inhibition of viral fusion with host cells and viral replication Inhibition of terminal glycosylation of ACE2
2	**Gene expression** of inflammation-relevant genes	Gene expression of **viral** inflammation-relevant genes	Prevention of cytokine release syndrome (CRS)/cytokine storm ° Reduction of endothelial stress response in systemic inflammation °
		Gene expression of **bacterial** inflammation-relevant genes	Prevention of TLR4 related cytokine outburst that results in an acute pulmonary inflammation °°
3	Inhibition of stress related **C_{16}-/(C_{18}-)ceramide synthesis** Inhibition of apoptosis	Inhibition of substrate supply of reverse activity of lysosomal aCERase (C_{16}-/C_{18}-ceramide synthesis)	Blocking of synthesis of proapoptotic C_{16}-ceramide in lysosomes Blocking of synthesis of sycytia forming C_{18}-ceramide in lysosomes Blocking of endothelial apoptosis and inflammatory cell death

FIGURE 1 | (A) Variety of approved lysosomotropic compounds for various indications (Kornhuber et al., 2008; Blaess et al., 2018). Achievement of the desired lysosomotropic effect depends on the active compound, the dosage, and accumulation in lysosomes. Unless indicated, maximum daily doses are split into three applications. *Lysosomotropism very likely, but not yet confirmed, lysosomal drug concentration (effect) within the therapeutic margin expected; dosage: #single dose per day; x*in vitro* anti-SARS-CoV tested, xx*in vitro* anti-SARS-CoV and anti-SARS-CoV-2 tested (Vincent et al., 2005; Kornhuber et al., 2008; Dyall et al., 2014; Zhou et al., 2016; Blaess et al., 2018; Liu et al., 2020; Weston et al., 2020). **(B)** Cellular targets, cellular effects, and effects related effects of lysosomotropic active compounds in SARS-CoV-2 infection/COVID-19 (Vincent et al., 2005; Masters, 2006; Mingo et al., 2015; Zhou et al., 2016; Blaess et al., 2018; Varga et al., 2020; Zhou et al., 2020). Lysosomotropic compounds target in mammalian cells three major targets related to SARS-CoV-2 infection/COVID-19: cathepsin L (1), gene expression of inflammation-relevant genes (2), C_{16}-ceramide and C_{18}-ceramide synthesis, and apoptosis of host cells (3). Addressing targets 1–3 results in various disease process interfering effects supposed to improve SARS-CoV-2 infection/COVID-19 outcome; (°) in viral infection and bacterial superinfection, (°°) only in bacterial superinfection.

trigger severe drug-induced hemolytic anemia in G6PD-deficient COVID-19 patients (Beauverd et al., 2020; Kuipers et al., 2020).

Severe COVID-19 is associated with an atypical diffuse alveolar damage, ending in the acute respiratory distress syndrome (ARDS) (Huang et al., 2020), most likely accompanied by occurrence of syncytia as a result of a direct infection of cells by an infected neighboring cell without releasing a complete virus (Ou et al., 2020).

Ceramides, in particular C_{18}-ceramide, are present in (sepsis-induced) cardiac dysfunction (Chung et al., 2017), and are effective in triggering exocytosis in rat PC12 cells (Tang et al., 2007); further they may contribute to SARS-CoV-2-related

cell–cell fusion by exocytosis of viral S protein fractions and development of multinucleate syncytia.

Non-structural protein nsp2 of SARS-CoV-2 was associated with host cell cell cycle progression, and apoptosis in host cells, suggesting an impact on disrupting the host cell environment (Yoshimoto, 2020) and apoptosis of endothelial cells (Varga et al., 2020).

According to current knowledge, cleavage-mediated fusion of viral S protein with host cells can occur either immediately at the cell surface by TMPRSS2 or within the lysosome catalyzed by lysosomal cathepsin L (Belouzard et al., 2012). The lysosomal cathepsin L induced fusion of SARS particles bound to ACE2 with host cells (Millet and Whittaker, 2015) is sensitive to lysosomal pH. Hence both, TMPRSS2 and cathepsin L, display promising targets of prophylaxis and treatment of SARS-CoV-2 infection/COVID-19.

In severe COVID-19, SARS-CoV-2 is likely to cause both, pulmonary and systemic inflammation, thus leading to multi-organ dysfunction in high risk populations. Significantly higher concentrations of IL-8, TNFα, and IL-6 in deceased patients (Chen et al., 2020) are suggesting a rapid and severe deterioration during SARS-CoV-2 infection associated with CRS/cytokine storm syndrome (Mehta et al., 2020).

LYSOSOMOTROPIC (ACTIVE) COMPOUNDS ARE VALUABLE DRUG CANDIDATES

Lysosomotropism is a biological characteristic of small molecules and always present in addition to intrinsic pharmacological effects. Various well-known approved drugs such as amitriptyline, chlorpromazine, sertraline, and imipramine share lysosomotropic characteristics (**Figure 1A**) (Kornhuber et al., 2008; Blaess et al., 2018). Regardless of their pharmacological effects, they are accumulating in lysosomes raising the lysosomal pH from 4.5–5 to 6–6.5, beyond the optimum of most of the lysosomal enzymes, including cathepsin L. Since no effects of lysosomotropic aminoglycoside antibiotics on free cathepsin L (Zhou et al., 2016) or other lysosomotropic drugs on lysosomal enzymes such as acid sphingomyelinase exist (Blaess et al., 2018), a selective inhibition is unlikely.

Lysosomotropic compounds are not limited to mediate inactivation of cathepsin L **Figure 1B**. Moreover, lysosomotropic compounds are assumed to suppress the CRS/cytokine storm syndrome and to attenuate the transition from mild to severe SARS-CoV-2 infection/COVID-19 (Zhou et al., 2020). Data of the lysosomotropic model compound NB 06 in LPS-induced inflammation in monocytic cells (Blaess et al., 2018) supports the hypothesis. NB 06 affects gene expression of the prominent inflammatory messengers IL1B, IL23A, CCL4, CCL20, and IL6; likewise, it has beneficial effects in (systemic) infections involving bacterial endotoxins by targeting the TLR4 receptor pathway in sepsis. Similarly, desipramine reduces endothelial stress response in systemic inflammation (Chung et al., 2017).

Apoptosis of (infected) mammalian cells is characterized by an increase in C_{16}-ceramide (Thomas et al., 1999) and can be blocked via lysosomotropic compounds such as NB 06, chlorpromazine, and imipramine (Blaess et al., 2018). Furthermore, C_{18}-ceramide triggered exocytosis and forming of syncytia is blocked by chlorpromazine as well (Garner et al., 2010).

SUITABLE DRUG PROFILES AND ROUTES OF ADMINISTRATION

According to current knowledge, in therapy inhibition of lysosomal pH dependent processes (e.g., cathepsin L dependent viral entry into host cells) can be obtained only through off-label use of lysosomotropic drugs. Systemic application in lysosomotropic drug concentrations and obtaining an efficacious blood level is sometimes accompanied by severe adverse effects and/or (in this case) undesirable (intrinsic) pharmacological effects. Chloroquine was among the first lysosomotropic active compounds exerting antiviral effects on SARS-CoV-2 (Liu et al., 2020) and during SARS-CoV pre- and post-infection conditions (Vincent et al., 2005). Owing to an unfavorable drug profile (G6PD patients, insufficient lysosomotropism, elimination half-life of 45 ± 15 days), a recommendation against (hydroxy)chloroquine, but not against lysosomotropic active compounds in principle was issued (COVID-19 Treatment Guidelines Panel, 2020).

Chlorpromazine displayed anti-SARS-CoV-2 effects *in vitro* (Weston et al., 2020) and protective effects on COVID-19 in patients in a psychiatry hospital (NCT04366739). Consequently, chlorpromazine is rated as a promising candidate in COVID-19/CRS treatment. In case of treatment of people without mental illness, however, a premature termination of treatment due to severe side effects by systemic application of chlorpromazine is extremely likely. This raises the question of how to handle this issue to provide well tolerated lysosomotropic drugs in SARS-CoV-2 infection/COVID-19.

PERSONALIZED BENCH TO BEDSIDE TREATMENT CONCEPT

Numerous available approved drugs with lysosomotropic characteristics permit tailor-made therapy. The individual pre-existing conditions are a criterion for the selection and combination of lysosomotropic drugs. For choosing suitable lysosomotropic drugs some issues have to be considered:

Tolerable Intrinsic Pharmacology and Drug Profile

Various lysosmotropic drugs in **Figure 1A** demonstrated anti-SARS-CoV(-2) efficacy (Dyall et al., 2014; Zhou et al., 2016; Liu et al., 2020; Weston et al., 2020), offer a more favorable drug profile than the initially investigated chloroquine and hydroxychloroquine.

Accumulation In Lysosomes of Pulmonary Tissue

Imipramine and chlorpromazine are accumulating in isolated perfused lung tissue and imipramine in alveolar macrophages (Wilson et al., 1982; Macintyre and Cutler, 1988) suggesting that lysosomotropic drug concentrations in pulmonary alveoli and protective effects on SARS-CoV-2 infection of particular drugs are likely. Of the lysosomotropic *in vitro* anti-SARS-CoV-2 antibiotics teicoplanin, oritavancin, dalbavancin, and telavancin (Zhou et al., 2016), solely teicoplanin and telavancin are in accumulating pulmonary tissue and are expected to be a treatment option.

Additional Therapeutic Benefits In Sars-Cov-2 Infection/Covid-19

Beside lysosomotropism certain intrinsic pharmacological effects are advantageously in SARS-CoV-2 infection/COVID-19. The incidence of CRS/cytokine storm syndrome associated with secondary gram-positive bacterial infections is likely to be minimized by using the pulmonary tissue accumulating antibacterials teicoplanin and telavancin or the antifungal itraconazole in systemic mycoses in appropriate systemic drug levels.

Choosing A Suitable Route of Administration

Systemic application of chlorpromazine (NCT04366739) and fluoxetine (NCT04377308) as lysosomotropic drugs may provoke severe and unfavorable adverse effects in mental healthy patients. Since the respiratory tract is both, the gateway for SARS-CoV-2 infection/COVID-19 and an internal surface, the expedient is a local application in the airways and/or the respiratory tract. Local application of small molecules is possible, preferably as inhalant or via nebulizers to avoid (undesirable) systemic effects. The majority of lysosomotropic drugs should be suitable for inhalation.

Combination With Antivirals and Tmprss2 Inhibitors

COVID-19 originates from a SARS-CoV-2 infection that could not be tackled successfully by the immune system. The antiviral remdesivir proved to be effective in infection prophylaxis (phase 0) (de Wit et al., 2020) and viral (SARS-CoV-2) infection (phase 1) within a limited period (5–6 days), shortly after the symptoms emerge and viral shedding occurs (Mitjà and Clotet, 2020). In severe COVID-19 neither a lower mortality nor a faster clearance of viruses was observed (Wang et al., 2020). As soon as the infection initiates a CRS/cytokine storm, it is likely that the transition toward COVID-19 (phase 2), a disseminated intravascular coagulation/thrombotic microangiopathy, or a bacterial secondary infection occurs. An effective multi-drug therapy, focusing on the progression of COVID-19 and emerging severe complications, can be implemented by lysosomotropic drugs, TMPRSS2 inhibitors and antivirals.

NAFAMOSTAT: AN *IN VIVO* TMPRSS2 INHIBITOR?

Nafamostat is an approved protease inhibitor that inhibits TMPRSS2 (*in vitro*) (Hoffmann et al., 2020), prevents (sepsis-related) disseminated intravascular coagulation, and thrombotic microangiopathy (Okajima et al., 1995; Levi and Thachil, 2020), appears to be useful in SARS-CoV-2 infection and prophylaxis, and for patients subjected to extracorporeal circulation such as ECMO (Han et al., 2011). It is doubtful, however, whether the pulmonary concentration in therapeutically dosage (Ono Pharmaceuticals, 2020) is sufficient to generate a TMPRSS2 inhibition *in vivo* as demonstrated *in vitro* due to poor accumulation in pulmonary tissue (Midgley et al., 1994).

SINGLE OR MULTI TARGET APPROACH: LYSOSOMOTROPIC DRUGS VS. ANTIBODIES

Various clinical trials are currently under way using immunomodulatory IL-1 and IL-6 inhibitors or anti-IL-6R antibodies (anakinra, tocilizumab, siltuximab, and sarilumab) in patients with COVID-19 (COVID-19 Treatment Guidelines Panel, 2020); limited data, however, is yet available. In a retrospective study using tocilizumab and hydroxychloroquine, both demonstrated a limited benefit in survival (Ip et al., 2020). Tocilizumab shortens mechanical ventilation and hospital stay in severe COVID-19 (Eimer et al., 2020), while tocilizumab is often accompanied by bacterial pneumonia 2 days after application (23%) (Pettit et al., 2020).

To improve outcome, antibody cocktails consisting of anti-IL-6, IL-1 receptor blocker, IL-1 type 1 receptor, and TNF-α are suggested (Harrison, 2020), irrespective of the risk of serious adverse effects (e.g., bacterial pneumonia) due to more pronounced interference with the immune defense. Such cocktails are intended to tackle the release of pro-inflammatory cytokines IL-1β and IL-6 mediating lung and tissue inflammation, fever, and fibrosis, as they are supposed to be responsible for the emergence of COVID-19.

Although lysosomotropic drugs likewise interfere with the immune defense, such adverse effects are not reported. In contrast to antibodies, however, only the resynthesis of IL-6 and thus the available amount is reduced, but not completely obstructed, still allowing a moderate immune response. Multitargeting on core processes of the viral infection addressing the formation of multinucleate syncytia and alteration of tissue structure, ceramide metabolism, and the release of virions could be a key advantage of lysosomotropic drugs compared to current strategies.

FUTURE DIRECTIONS

Daunting results of (hydroxy)chloroquine in clinical trials are closely related to their drug profile and minor lysosomotropism, but not to the mode of action (lysosomotropism) in general. Observations in patients treated with chlorpromazine and the extensive accumulation of imipramine in alveolar macrophages and of both, imipramine and chlorpromazine in isolated perfused lung tissue supports the benefits of lysosomotropic drugs that are accumulating in pulmonary tissue in SARS-CoV-2 infection/COVID-19.

Promising candidates among lysosomotropic drugs in fact require more than adequate lysosomotropism; accumulation in pulmonary tissue is a prerequisite as well. It is, however, likely irrelevant whether the drug or its metabolite(s) is accumulating given the broad structural requirements for this activity. Since a large number of compounds has not yet been evaluated for lysosomotropism, many compounds beside those listed in **Figure 1A** are expected to meet the requirements described here and may (partially) be responsible for background immunity to SARS-CoV infection.

AUTHOR CONTRIBUTIONS

MB conceived the work. MB, LK, OS, SR, RC, and H-PD wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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OPINION
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Dexamethasone Treatment for Covid-19, a Curious Precedent Highlighting a Regulatory Gap

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INTRODUCTION

The centralized marketing authorization issued by the European Medicines Agency (EMA) valid in all European Union (EU) Member States has been introduced with the Council Regulation (EEC) No. 2309/93 (Council Regulation, 1993), and improved and amended by the Regulation (EC) No. 726/2004 (Regulation EC, 2004), which introduced the obligation of the procedure for some products, including treatment for viral diseases. Therefore, all the drugs and vaccines developed for Covid-19 must be approved according to this approach to be marketed throughout the EU.

Moreover, in order to deal with the emergency and expedite drug and vaccine development for Covid-19, EMA implemented several rapid procedures in addition to those already provided with the standard "accelerated assessment" (**Supplementary Material A1**).

Conversely, EMA has no power in pricing and reimbursement decisions, which remain the responsibility of the national competent authorities, due to the heterogeneity of the specific national health system (NHS) organization and financial resources.

According to the Article three of the Regulation (EC) No. 726/2004, application for each marketing authorization shall be submitted by the company to the Agency which will issue an opinion through the Committee for Medicinal Products for Human Use (CHMP). This Committee is solely responsible for releasing the opinions (publicly accessible) on all matters about medicinal products for human use.

Moreover, the Article five of the Regulation provides for the possibility that the Executive Director of the European Medicines Agency (EMA) or the European Commission (EC) or a Member State can request the CHMP to formulate an opinion on issues of particular relevance concerning medicinal products for human use.

Supplementary Material A2 shows EMA opinions issued till October 2020 according to Article five of Regulation 726/2004. Almost 60% of the queries concerned safety issues, in general or in special populations (e.g., elderly, pediatric patients, pregnant women).

More than half of the assessments were required by the Executive Director of the EMA or by EC and about 40% by national authorities.

On July 2020, CHMP started to review the data concerning the use of dexamethasone in patients with Covid-19 (EMA, 2020a) at the request of the EMA Executive Director following a discussion with European experts belonging to the Covid-19 EMA pandemic task force (COVID-ETF).

In the light of the results of the review, the Committee concluded that dexamethasone can be considered a treatment option for patients who require oxygen therapy (including supplemental oxygen and mechanical ventilation; EMA, 2020b).

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Evidence Supporting Dexamethasone Use in Covid-19 and European Positive Opinion

The available data assessed were those of the RECOVERY (Randomised Evaluation of COVid-19 thERapY) study arm, which provided for the use of dexamethasone as add-on therapy to the standard of care of hospitalized patients with Covid-19.

The RECOVERY study (University of Oxford, 2020) is a randomised, controlled, open-label, multicenter (involving 176 National Health Service organizations in the United Kingdom), adaptive trial designed to assess the effects of potential treatments in adults patients hospitalized with Covid-19, receiving invasive or non-invasive ventilation, and those receiving or not oxygen. The study is supported by the National Institute for Health Research-Clinical Research Network (NIHR-CRN), which funds high-quality health and care research in England.

According to the adaptive design, an independent Committee was responsible for the assessment of the interim trial results, which would be made available to the public in case of strong evidence on mortality. Moreover, in this case or if other candidate therapeutics with supporting evidence should be evaluated, the trial arms would have been amended accordingly.

One of the first version of the protocol provided the following arms (RECOVERY, 2020a):

- no additional treatment
- lopinavir-ritonavir
- interferon β
- low-dose corticosteroids
- hydroxychloroquine.

In a subsequent version the interferon β arm has been deleted and replaced by azithromycin one (RECOVERY, 2020b). In addition, the new protocol allowed a second randomization (no additional treatment vs. tocilizumab) for patients with evidence of hyper-inflammatory state (RECOVERY, 2020c).

Then the trial design was further modified, and eligible patients were allocated simultaneously to no additional treatment vs. convalescent plasma vs. synthetic neutralizing antibodies (RECOVERY, 2020d).

Finally on June 2020, the interim analysis showed important (and opposite) results which led to the withdrawal of three arms (RECOVERY, 2020e):

- Dexamethasone arm due to the demonstration of death reduction by up to one third in hospitalized patients with severe respiratory complications of Covid-19.
- Lopinavir-ritonavir and hydroxychloroquine due to the lack of clinical benefit (RECOVERY Collaborative Group, 2020).

The trial continues randomization to groups receiving azithromycin, tocilizumab, or convalescent plasma.

Overall 6,425 patients (89% with a laboratory-confirmed SARS-CoV-2 infection) were enrolled and randomized to receive either dexamethasone (2,104 patients) or usual care alone (4,321 patients) (RECOVERY Collaborative Group, 2020).

Among randomized patients, 60% required oxygen therapy, 16% invasive mechanical ventilation or extracorporeal membrane oxygenation, and 24% neither.

The primary endpoint was the mortality at 28 days was significantly lower in the dexamethasone group (22.9% death) than in the comparator one (25.7%; rate ratio, 0.83; 95% confidence interval [CI], 0.75 to 0.93; $P < 0.001$). In particular, the difference between groups was clear for patients receiving invasive mechanical ventilation (29.3 vs. 41.4%; rate ratio, 0.64; 95% CI, 0.51–0.81) and in those receiving supplementary oxygen without invasive mechanical ventilation (23.3 vs. 26.2%; rate ratio, 0.82; 95% CI, 0.72–0.94).

On the contrary, no reduction in the risk of death was obtained with the administration of dexamethasone in patients who were not receiving any respiratory support (17.8 vs. 14.0%; rate ratio, 1.19; 95% CI, 0.91–1.55). Moreover, the duration of hospitalization in the dexamethasone group was shorter than those in the usual care group especially among patients mechanically ventilated at randomization (rate ratio 1.48; 95% CI 1.16, 1.90), or receiving oxygen (rate ratio, 1.15; 95% CI 1.06–1.24), with no benefit in patients not receiving oxygen (rate ratio, 0.96; 95% CI 0.85–1.08).

These results are supported by additional published data, including a meta-analysis conducted by the World Health Organization (WHO), reporting data from seven clinical studies about the use of corticosteroids for the treatment of patients with Covid-19 (WHO Rapid Evidence Appraisal for Covid-19 Therapies (REACT) Working Group, 2020). The analysis included a total of 1703 patients randomized to receive systemic corticosteroids (dexamethasone, hydrocortisone, or methylprednisolone; n = 678) or usual care or placebo (1,025 patients). The primary endpoint was all-cause mortality at 28 days after randomization.

The study results show a reduced risk of death at 28 days among patients randomized to corticosteroids compared with standard of care or placebo [summary OR, 0.66 (95% CI, 0.53–0.82); $P < 0.001$ based on a fixed-effect meta-analysis].

Therefore, an inexpensive and commonly used steroid is the first drug showing to prevent deaths from Covid-19 (Ledford 2020).

Based on the data described above, EMA endorsed the use of dexamethasone (oral or injectable) in adults and adolescents (from 12 years of age and weighing at least 40 kg) who require supplemental oxygen therapy, at the recommended dose of 6 mg once a day for up to 10 days (EMA, 2020c).

From European Approval to Patients' Access

The procedure under the Article five of the Regulation has allowed to recommend an extension of the use of a product already on the market. This is the first time that a new indication is approved through this procedure. Previously the review of the risk-benefit profile has led to the recommendation of use and dosage in special populations, eg for antiviral and anti-tubercular drugs (EMEA, 2009; EMA, 2012).

However, this recommendation does not translate into an automatic update of the Summary of Product Characteristics

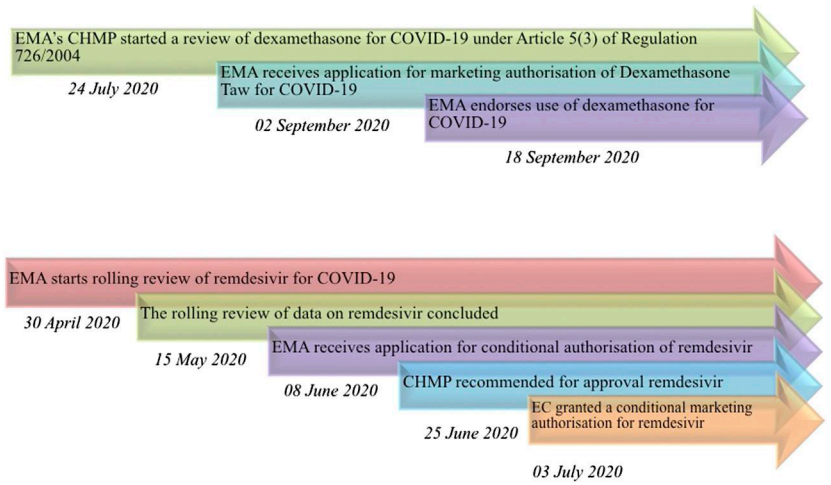

FIGURE 1 | Timeline of drugs' assessment performed by EMA for Covid-19. **(A)** desamethasone; **(B)** remdesivir. *EMA: European Medicines Agency; CHMP: Committee for Medicinal Products for Human Use; EC: European Commission*.

(SmPC), and the marketing authorization holders can request to add the new therapeutic use to their product's license by submitting an application to national regulatory authorities or EMA.

To date EMA received an application from Taw Pharma for the authorization of an injectable dexamethasone for treating hospitalized patients with Covid-19 (EMA, 2020d). The application will be evaluated by the CHMP according to an accelerated assessment. This will enable to issue an opinion within the shortest possible time.

The procedure allowed to deliver the opinion in less than two months (**Figure 1A**). On the other hand, the rapid assessment of the only drug approved for Covid-19, remdesivir, starting with the emergency procedure of the rolling review, has been completed in almost the same timeframe (**Figure 1B**). In this case, after the issue of conditional approval, EMA also implemented the *Emergency Support Instrument* (ESI) and subsequently a joint procurement contract in order to guarantee access to the drug throughout Europe (EC, 2020a; EC 2020b).

After CHMP opinion issued about dexamethasone on September 18th' 2020, the European national authorities have not implemented yet any procedure to guarantee access to the drug. In particular, waiting for the conclusive EMA approval following the companies' application for the final authorization of the new indication, some Member States have the possibility to recognize the nation-wide off-label use according to specific laws. For example, in Italy it is possible to include the off-label use in Covid-19 patients into the List of drugs reimbursed according to Law 648/96, whereas France may start a Temporary recommendation for use (RTU) program. Currently, no such action has been taken.

On October 6th the Italian Medicines Agency (AIFA) published a document with information useful to guide the prescription of corticosteroid in patients with Covid-19 (AIFA, 2020). However, the use has not been officially approved by the same Agency, so the treatment still falls within the scope of the off-label legislation, the so-called "*Di Bella Law*" (Gozzo et al., 2020; Di Bella, 2010), which allows the physicians to perform off-label prescriptions *in individual and exceptional cases*, unless the local Health Director of the hospital formally authorize case by case the use. Anyway, the NHS does not cover the cost of the treatment. The only case in which an off-label use can be reimbursed in Italy is about drugs included in specific lists under the Law 648/1996 (Law 648, 1996). In this context, during the emergency AIFA provisionally endorsed the use and reimbursement of some drugs, such as hydroxychloroquine/cloroquine, lopinavir/ritonavir, and darunavir/cobicistat, despite the non-applicability of the Law 648/1996, subsequently revoked due to the lack of data supporting a favorable risk-benefit profile (Gozzo et al. 2020).

Similarly, two decrees have been published by the French Ministry of Solidarity and Health, governing the prescription, dispensing and administration of hydroxychloroquine for patients with Covid-19:

The Decree n. 2020–314 of March 25, 2020 and n. 2020–337 of March 26, 2020 authorized the prescription, dispensation and administration of hydroxychloroquine and the combination of lopinavir/ritonavir "*under the responsibility of a doctor to patients affected by Covid-19, in the health establishments which take charge of them", "in particular, for patients with oxygen-demanding pneumonia or organ failure*" (Ministère Des Solidarités Et De La Santé, 2020a; Ministère Des Solidarités Et De La Santé, 2020b).

In this case, these drugs were supplied and paid by health institutions.

Finally, even the French government revoked the decrees that allowed to prescribe hydroxychloroquine, due to the lack of proof of benefit and the health risks (Ministère Des Solidarités Et De La Santé, 2020c).

Given the positive and well-established findings that the drug is currently the only one preventing the mortality of patients,

actions to ensure uniform and controlled access to corticosteroids for Covid-19 should be put in place as soon as possible.

The need to fill this regulatory gap is even stronger in light of the recently published interim results of the Solidarity Trial (Dyer, 2020; WHO, 2020; WHO Solidarity trial consortium, 2020).

The study supported by the World Health Organization is one of the largest international randomized trials for Covid-19 treatments, enrolling almost 12,000 patients in over 30 countries and evaluating the effect of drugs on important outcomes such as mortality, need for assisted ventilation and duration of hospitalization.

The preliminary results show little or no effect on these hard endpoints for the four treatments evaluated (remdesivir, hydroxychloroquine, lopinavir/ritonavir and interferon).

These findings confirm that till now only corticosteroids have proven effective in severe and critical Covid-19 patients. It is noteworthy to emphasize that these data come from well-designed clinical trials that it was possible to rapidly start and efficiently conduct despite the emergency status, giving the first specific and evidence-based (although adjustable following future studies) guidance to clinicians on how to manage patients with Covid-19.

CONCLUSION

A lot of molecules have been tested in Covid-19 patients, but few positive results have been obtained.

Regulatory authorities react to the emergency adopting a number of measures in order to accelerate drug development and assessment process of available results.

The European procedure regulated by the Article five of the Regulation (EC) No 726/2004 allows to start independently from company interest the assessment of drugs potentially useful for unmet need, such as Covid-19.

However, currently this advantage in terms of time and resource seems to be lost due to the lack of an automatic transferability for prescription in clinical practice, in particular in this emergency situation.

It is dramatically important to rapidly overcome this regulatory gap to made widely available dexamethasone and corticosteroid in general, the only therapeutic option which demonstrated clinical relevant results in Covid-19 so far.

AUTHOR CONTRIBUTIONS

LG wrote the first draft of the manuscript. FD checked and revised the draft manuscript. All authors contributed read, revised, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.621934/full#supplementary-material

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Rationale for COVID-19 Treatment by Nebulized Interferon-β-1b–Literature Review and Personal Preliminary Experience

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The inflammatory response to COVID-19 is specifically associated with an impaired type I interferon (IFN) response and complete blockade of IFN-β secretion. Clinically, nebulization of IFN-α-2b has been historically used in China to treat viral pneumonia associated with SARS-CoV. Very recent data show that the use of inhaled type I IFN is associated with decreased mortality in Chinese COVID-19 patients. However, IFN nebulization is currently not standard in Europe and the United States. Therefore, our group has set up a project aimed to evaluate the possibility to nebulize IFN-β-1b (a drug currently used in Europe to treat multiple sclerosis via subcutaneous injections) and to assess the safety of this new mode of administration in SARS-CoV-2 infected patients. We present here literature data that allowed us to build our hypothesis and to develop collaboration between clinical pharmacists, intensivists and nebulization engineers in order to gain first pre-clinical and clinical experience of IFN-β-1b nebulization. After validation of the nebulization method and verification of droplet size compatible with nebulization, the method has been applied to four intensive care patients treated at our university hospital, for whom none of the COVID-19 therapies initially used in France led to significant clinical improvement. All patients exhibited negative viral carriage and experienced clinical improvement 7–16 days after having initiated nebulized IFN-β-1b inhalation therapy. No side effects were observed. All patients were alive within a 90-days follow-up. Although it is not possible to draw firm conclusions on treatment efficacy based on this case report, our study shows that pulmonary IFN-β-1b administration is feasible, with a good safety profile. This procedure, which presents the advantage of directly targeting the lungs and reducing the risks of systemic side effects, may represent a promising therapeutic strategy for the care of patients with severe COVID-19. However, our preliminary observation requires confirmation by randomized controlled trials.

Keywords: COVID-19, SARS-CoV-2, nebulization, inflammation, interferon-β-1b

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INTRODUCTION

The emergence of SARS-CoV-2 and the resulting COVID-19 pandemic has caused the most serious health crisis of this century. SARS-CoV-2 is responsible for respiratory disease which frequently leads to severe forms of pneumonia and acute respiratory distress syndromes (ARDS) (Zhu et al., 2020). In addition, many other vital organs may be damaged as well. Overall case-fatality is estimated to be around 1%, but can reach 25–60% in patients requiring intensive care (Armstrong et al., 2020; Quah et al., 2020; Wu and McGoogan, 2020). The mortality risk depends on many different factors, including advanced age, male gender and presence of comorbidities such as chronic obstructive pulmonary disease (COPD), obesity, diabetes, cardiovascular disease, kidney disease and cancer (Docherty et al., 2020; Parohan et al., 2020), and last but not least type of patient management (Gupta et al., 2020).

The pathological process can be explained by a two-phase course of SARS-CoV-2 infection. The first phase corresponds to viral proliferation, associated with contamination of the bronchial tree by an interaction between the spike proteins of the virus and ACE2 receptors (Hoffmann et al., 2020). Beyond the viral infection per se, accumulating evidence suggests that a subgroup of patients with severe COVID-19 develop a severe inflammatory syndrome characterized by a dramatic rise of circulating pro-inflammatory cytokines IFN-γ, TNF-α, IL-6, IL-1 (Yang A.-P. et al., 2020; Yang L. et al., 2020) that attack cells in the pulmonary alveoli (Bradley et al., 2020; Carsana et al., 2020). The inflammatory reaction associated with SARS-CoV-2 infection contributes to the severity of the disease, and is associated with thrombotic and respiratory manifestations that require, in the most severely affected patients, intensive oxygen therapy, or even intubation leading to prolonged stays in intensive care units (Yuki et al., 2020). The viral invasion and inflammation may also be responsible for a multisystem disease, including cardiac, renal and neurological damage (Zhang B. et al., 2020; Chen T. et al., 2020; Helms et al., 2020).

To date, the management of patients showing the most severe forms of the disease remains essentially symptomatic and mainly consists in supply of oxygen (Phua et al., 2020), preventive anticoagulation adapted to disease severity (Atallah et al., 2020), and the use of low-dose corticosteroid therapy (RECOVERY Collaborative Group et al., 2020a). Despite intensive research and debate about the most efficient therapeutic approach leading to decreased viral shedding and improved patient outcomes, no antiviral treatment has as yet been convincingly shown to reduce COVID-19 mortality, be it hydroxychloroquine (hCQ) (RECOVERY Collaborative Group et al., 2020b; Fiolet et al., 2020), remdesivir (Beigel et al., 2020) or lopinavir/ritonavir (Cao et al., 2020; RECOVERY Collaborative Group, 2020; WHO, 2020).

In this context, our group highlighted early on promising signals from China as regards the potential therapeutic interest of pulmonary type I interferon (IFN) administration (Mary et al., 2020). Type I IFNs are cytokines which represent a major innate anti-viral defense. Type I IFN includes IFN-α, mainly produced by macrophages, and IFN-β produced by bronchial epithelial cells in response to viral infection. They display the ability to bind the surface of infected and neighboring cells and promote the induction of more than 1,000 different IFN-inducible genes (ISGs) that ultimately prevent virus protein trafficking, virus RNA synthesis or virion assembly and release (Hesse et al., 2009; Rauch et al., 2013; Schreiber and Piehler, 2015) (**Figure 1A**).

Although the use of IFN-α-2b nebulization is widespread in China for the control of respiratory viral diseases (Mary et al., 2020) currently such nebulization is not standard in Europe and United States. Moreover, IFN-α-2b is no longer marketed in many European countries since the end of 2019. Therefore, our group set up a project aimed to evaluate the possibility of administering nebulized IFN-β-1b by inhalation and to assess the safety of this new route of administration in SARS-CoV-2 infected patients. Note that IFN-β-1b is currently used in Europe to treat multiple sclerosis via sub-cutaneous injection. The present manuscript reports how a collaborative effort involving a clinical pharmacy department and an intensive care unit enabled the treatment of four patients suffering from severe COVID-19 with inhaled IFN-β-1b, as well as investigations carried out in collaboration with an industry company to better characterize this off-label route of administration.

The first section of the manuscript is devoted to literature reports that drove us to develop the hypothesis that type I IFN inhalation (and in particular INF-β) may reduce the duration of viral carriage and improve major symptoms in COVID-19 patients. The second part of the manuscript presents *in vitro* data on the feasibility of IFN-β-1b nebulization. In this section, we discuss the choice of the nebulization solvent and excipients, and present data on the stability of the product once reconstituted. We then report the aerodynamic size of particles obtained when the solution of IFN-β-1b was nebulized in a cascade impactor mimicking lung nebulization. The last part of the manuscript focuses on the clinical course of four intensive care patients, who received inhalation of IFN-β-1b as developed by our group, combined or not with lopinavir/ritonavir. This treatment was given on a compassionate basis after failure of the initial COVID-19 therapy (including antibiotic treatment with cefotaxime/azithromycin either alone or combined with hCQ). The final discussion focuses on the achievement of therapeutic targets with the inhalation system, as well as future prospects to better determine the potential therapeutic role of inhaled IFN in the treatment of COVID-19.

TYPE I INTERFERON RESPONSE AND COVID-19: A REVIEW OF THE LITERATURE

COVID-19 Patients Show a Delayed Type I Interferon Response

Since our first report (Mary et al., 2020) a variety of data have been published, presenting additional arguments supporting the usefulness of the type I IFN pathway in treating COVID-19 patients. In particular, an impaired type I IFNs response in lung

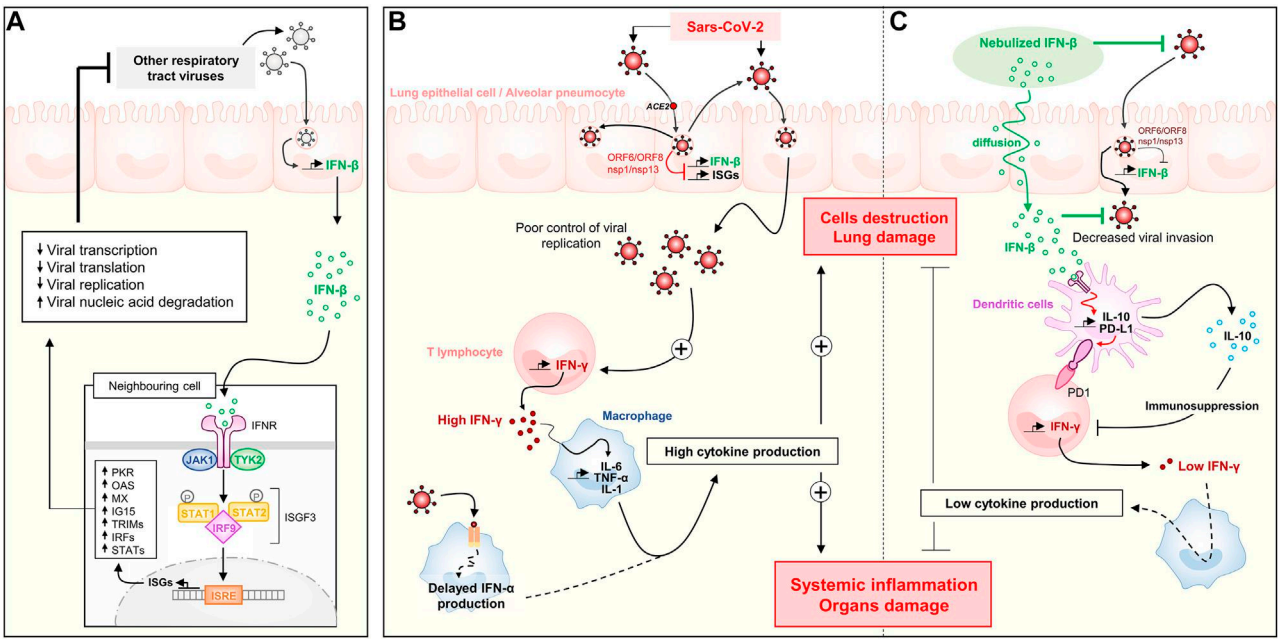


FIGURE 1 | Type I interferon response and the reduction of viral shedding: the impact of SARS-CoV-2 and key role of inhaled IFN-β. **(A)** Usual response to a respiratory virus such as influenza. **(B)** Immune response described in the severe COVID-19, with impaired response to type I IFN and excessive pro-inflammatory cytokine production. **(C)** Hypothesis on the therapeutic value of IFN-β nebulization on viral control and inflammation of COVID-19. IFN, Interferon; IL, Interleukine; TNF, Tumor Necrosis Factor; ISG, Interferon-stimulated genes; ISRE, Interferon-stimulated response element.

tissue has been identified in COVID-19 and analyzed in detail (Park and Iwasaki, 2020).

In fact, Blanco-Melo et al. have shown that SARS-CoV-2 induced only a very weak type I and III IFN response, juxtaposed to a dramatic inflammation characterized by excessive serum IL-6 and TNF-α levels (Blanco-Melo et al., 2020) (**Figure 1B**). Such a weak type I IFN response has also been reported by others (Trouillet-Assant et al., 2020a; Hadjadj et al., 2020). It is associated both with longer carriage time and severity of inflammation. This suggests compensation for type I IFN deficit by other immunological routes probably responsible for the frequent observation of a cytokine storm. It should be noted that in these three studies, serum concentrations of IFN-β were undetectable in COVID-19 patients (Trouillet-Assant et al., 2020a; Blanco-Melo et al., 2020; Hadjadj et al., 2020), suggesting absent IFN secretion by the pulmonary epithelia.

This has been confirmed in Calu3 cells, primary human airway epithelial cells (pHAE), alveolar epithelial type 2 cells (AT2s), A549 lung alveolar cells and in a reconstituted human bronchial epithelium model (MucilAir™ model), where SARS-CoV-2 infection failed to induce an appropriate type I and III IFN response (Blanco-Melo et al., 2020; Huang et al., 2020; Robinot et al., 2020; Shuai et al., 2020; Vanderheiden et al., 2020). According to recent reports, COVID-19 also causes an impaired type I IFN response in the periphery. Indeed, Arunachalam et al. showed reduced production of IFN-α in response to TLR stimulation in the plasmacytoid dendritic cells of infected individuals compared with those of healthy controls (Arunachalam et al., 2020), which suggests that plasmacytoid dendritic cells, the primary producers of type I IFNs, are impaired in COVID-19 infection.

SARS-CoV-2 has developed several mechanisms to hijack the innate immune system via both its structural and non-structural proteins. Among them, viral ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, nsp1, nsp3, nsp5, nsp6, nsp12, nsp13, nsp14, nsp15, nucleocapsid (N) and membrane (M) proteins were reported to be potential inhibitors of type I interferon production and/or type I IFN signaling pathway (Jiang H.-W. et al., 2020; Konno et al., 2020; Lei et al., 2020, 2; Li et al., 2020; Mu et al., 2020; Wu et al., 2020; Xia et al., 2020; Yuen et al., 2020). Viral ORF6 showed the most potent inhibition on IFN-β promoter and also inhibits the interferon-stimulated response element after Sendai virus infection (Lei et al., 2020, 2; Li et al., 2020; Xia et al., 2020; Yuen et al., 2020). Recently, Thoms et al. demonstrated the role of SARS-CoV-2 nsp1 protein in evading the immune system, including its ability to totally abrogate the ribosomal translation induced by IFN-β promoters (Thoms et al., 2020). The impaired IFN-β response and the specific type of inflammation induced by Sars-CoV-2 should be analyzed taking into account the capacity of IFN-β to exert immunosuppressive actions, as observed in multiple sclerosis (Reder and Feng, 2014). Specifically, type I IFNs are able to suppress inflammatory cytokine release, by decreasing IFN-γ production and by promoting IL-10 production (McNab et al., 2014). The hypothesis of compensating the IFN-β deficiency of pulmonary origin by excessive lymphocyte IFN-γ production could help to solve the apparent antinomy with the inflammatory transcriptomic profile of monocytes and lymphocytes (with a

marked TNF-α and IL-1β signature and an unconstant ISG signature) (Gardinassi et al., 2020; Lee et al., 2020; Maucourant et al., 2020; Wen et al., 2020; Wilk et al., 2020).

The dramatic pulmonary SARS-CoV-2 invasion observed in deceased COVID-19 patients (Bradley et al., 2020; Hanley et al., 2020), reinforces the hypothesis that the disease severity is associated with an immune system failure to clear the virus. In line with this hypothesis it has been shown that the response to type I IFNs is impaired in subgroups of patients at high risk of mortality from COVID-19. This is the case of elderly people (Abb et al., 1984), especially men with an "X chromosome monosomy (XCM)" linked to Y chromosome loss (Perez-Jurado et al., 2020). Interestingly Zhao et al. observed an impressive association between administration of inhaled IFN-α-2b and survival in people over 65 years of age (0.29 [95% CI 0.17–0.51], $p < 0.001$), suggesting that the elderly might represent a subgroup of patient particularly responsive to the treatment (Mei et al., 2020). Similarly, obese patients at risk for severe COVID-19 are known to have delayed responses to type I IFN (Teran-Cabanillas et al., 2013). In contrast, the mild forms of COVID-19 seen in children are likely to be related to their ability to produce type I IFN more efficiently than elderly persons (Trouillet-Assant et al., 2020b). In accordance with these data, type I IFN signaling pathway has been shown to be significantly up-regulated in lungs of juvenile macaques infected with SARS-CoV2, as compared to old infected macaques (Rosa et al., 2020). In SARS-CoV-infected aged macaques, the severity of the disease is associated with an increase in the expression of genes associated with inflammation and reduced expression of IFN-β as compared to young adult animals, who display a mild form of the disease (Smits et al., 2010). Treatment of old macaques with type I IFN reduced pro-inflammatory gene expression and disease severity.

On the other hand, genetic mutations may explain specific susceptibility to COVID-19 dependent on IFN pathways. This is true for mutations in genes encoding interferon-induced transmembrane proteins type 3 (IFITM3) (Zhang Y. et al., 2020), i.e., vesicular proteins promoting viral trafficking to lysosomes (Spence et al., 2019). It has also been suggested that the black African population is more sensitive to SARS-CoV-2 due to a polymorphism in the gene encoding IFIH1 (InterFeron-Induced helicase 1), a host protein that senses the presence of viral RNA and subsequently promotes IFN production (Maiti, 2020). Interestingly, van der Made et al. found a unique loss-of-function variants in X-chromosomal TLR7 (with an impaired transcriptional host type I IFN response downstream) responsible of severe COVID-19 in four young men (van der Made et al., 2020). More recently, Zhang et al. identified inborn errors of TLR3-and IRF7-dependent type I IFN immunity in 3.9% patients with life-threatening COVID-19 pneumonia (Zhang Q. et al., 2020). *In vitro*, human fibroblasts with mutations affecting this pathway are vulnerable to SARS-CoV-2, suggesting that inborn errors of TLR3-and IRF7-dependent type I immunity can underlie critical COVID-19. Other genetic studies are underway to assess potential associations between patient genotypes and COVID-19 severity. They could determine the place of others polymorphisms of IFN pathways in the severity of COVID-19 (COVID-19 Host Genetics Initiative, 2020).

Beyond genetic susceptibility, a study recently reported in *Science* showed that 13.7% of patients with life-threatening COVID-19 pneumonia have autoantibodies against IFN-ω and/or IFN-α able to neutralize the ability of the corresponding type I IFNs to block SARS-CoV-2 infection (Bastard et al., 2020, 19). Interestingly, only 1.9% of these critical patients had IFN-β autoantibodies of which only 10% displayed neutralizing properties *in vitro*. Collectively, these data suggest that an early treatment with IFN-α is unlikely to be beneficial in this patient group. On the opposite, treatment with IFN-β may have beneficial effects, since autoantibodies against IFN-β appear to be rare in patients with autoantibodies directed against type I IFNs.

Collectively, the available experimental and clinical data make us hypothesize that COVID-19 severity is at least partially caused by an insufficient innate IFN-β response of the pulmonary epithelium. This defect is subsequently overcompensated by an excessive leukocyte response promoting (among others) the secretion of type II IFN at the origin of the described interferonopathy of COVID-19 (**Figure 1B**). IFN-β in particular could thus represent a solid etiological therapeutic path, as also proposed by the Belardelli team (Aricò et al., 2020) (**Figure 1C**). We therefore focused on the therapeutic approach of treating COVID-19 by IFN, in particular with the β isoform, and the way to optimize a clinical answer.

In Vitro Efficacy of Type I Interferon in a SARS-CoV-2 Infected Cellular Model

Recent *in vitro* studies have confirmed the potential inhibitory effect of type I Interferon on SARS-CoV-2. In Vero cells, the virus is more sensitive to pretreatment with IFN-β than with IFN-α, with a particularly low EC50 at 0.76 IU/ml (Mantlo et al., 2020). Pretreatment of a reconstituted human bronchial epithelium (MucilAir™ model) with IFN-β is also efficient against Sars-CoV2 in decreasing viral RNA levels by two logs (Robinot et al., 2020). In a curative model, the inhibitory potency of IFN-β decreases over time, from 0.4 to 4.9 IU/ml after 48 h to an EC50 of 3.5–6.0 IU/ml at 96 h (Clementi et al., 2020). At 96 h, the maximum effect (variation of 10 CT) is only obtained for high concentrations, greater than or equal to 50 IU/ml (Clementi et al., 2020). Ruxolitinib, an inhibitor of IFN-triggered janus kinase 2 (JAK)/signal transducer and activator of transcription (STAT) signaling, enhances SARS-CoV-2 proliferation in IFN competent Calu three cells (Felgenhauer et al., 2020). In a comparative study, IFN-β-1b showed a most potent anti SARS-CoV-2 effect than IFN-β-1a in VeroE6 cells, characterized by higher affinity (EC50 = 31.2 IU/ml vs. 70.8 IU/ml, respectively) and a higher selectivity index (selectivity index: 1,602.2 vs. 706.2, respectively) (Yuan S. et al., 2020). In the more physiological primary human airway epithelial (pHAE) model, pretreatment or post-treatment with IFN-β at 100 IU/ml reduced viral load by more than 90% (Vanderheiden et al., 2020).

Impact of Type I Interferon Subcutaneous Injections on COVID-19 Patients

Based on these observations, a therapeutic approach with IFN was selected in the Solidarity and Discovery clinical trials, using subcutaneous IFN-β-1a administration every 3 days. To date, an IFN treatment arm remains in Solidarity, together with remdesivir. In Hong Kong, the team of Hung et al. conducted an open label, randomized phase 2a trial in 127 patients with mild to moderate forms of COVID-19 using the triple combination of subcutaneous IFN-β-1a, lopinavir/ritonavir and ribavirin. They found a significant reduction in viral carriage with a median from 12 to 7 days, complete symptom remission with a median from 8 to 4 days, as well as a reduction in the length of hospital stay (Hung et al., 2020). In Tehran, Davoudi et al. evaluated in 81 severe patients the impact of subcutaneous injection of IFN-β-1a (three times weekly for two consecutive weeks) to the treatment composed of hCQ + lopinavir/ritonavir or atazanavir/ritonavir. They observed a significant decrease in mortality at 28 days (19% vs. 43.6% $p = 0.015$), and an increase in discharge rate at 14 days (66.7% vs. 43.6%) (OR = 2.5; 95% CI: 1.05–6.37) (Davoudi-Monfared et al., 2020). More recently, the same group evaluated in 66 adults with severe COVID-19 the impact of subcutaneous injection of IFN-β-1b (every other day for two consecutive weeks) compared to the national protocol medications (lopinavir/ritonavir or atazanavir/ritonavir plus hydroxychloroquine for 7–10 days) (Rahmani et al., 2020). They observed that the time to clinical improvement in the IFN group was significantly shorter than in the control group ([9 (6–10) vs. 11 (9–15) days respectively, $p = 0.002$, HR = 2.30; 95% CI: 1.33–3.39]) and that the percentage of discharged patients at 14 days was significantly higher in the IFN group than in the control group (78.79% vs. 54.55%, respectively; OR = 3.09; 95% CI: 1.05–9.11, $p = 0.03$). ICU admission rate in the IFN group was significantly lower than the control group (42.4% vs. 66.7%, $p = 0.04$). In this trial, the treatment with IFN-β-1b did not impact all-cause 28-days mortality (6.06% in the IFN group vs. 18.18% in the control group, $p = 0.12$). In these trials, the efficacy of IFN was more marked when administered early during the disease course. In addition, investigators from Cuba also presented (pre-published) encouraging data on the use of IFN-α-2b by subcutaneous route in a prospective observational study, but this study contained several confounders which were not corrected by multivariable analysis (Pereda et al., 2020).

Toward the Use of Type I Interferon Via Inhalation

Subcutaneous injection of IFN-β is suitable for obtaining prolonged immunomodulatory effects, but allows achievement of a maximum concentration of at best 5 IU/ml, with a half-life of about 5 h (Munafo et al., 1998), (Salmon et al., 1996). Note that upon intravenous injection of IFN-α, the bioavailability reached at the pulmonary level is only around 50% (Greig et al., 1988). *In vitro* data suggest that maximum concentrations of 2.5–5 IU/ml do not appear to be sufficient to achieve an optimal antiviral effect. This limit was also highlighted by Jalkanen et al., who suggested to use the parenteral route in order to achieve more efficacious concentrations (Jalkanen et al., 2020). Higher, more specifically pulmonary concentrations could be obtained with inhaled IFN, but available pharmaceutical forms and pharmacokinetic data on this method of administration remain limited in the Western world (Aricò et al., 2020).

Impact of Type I Interferon Nebulization on COVID-19 Patients: Experience From China

Pulmonary administration of type I IFN may have the advantage of significantly reducing systemic adverse effects, while increasing its concentration in the infected epithelium (Mary et al., 2020). The Chinese Center for disease Control and Prevention (China CDC) early proposed the use of IFN-α-2b to treat COVID-19, as it has been historically used in China to treat viral pneumonia associated with SARS-Cov and middle east respiratory syndrome coronavirus (MERS). The first published data in favor of the use of inhaled IFN-α-2b date from February-March 2020 (Mary et al., 2020). First Liu et al. suggested that combination therapy involving IFN-α-2b inhalation combined with lopinavir/ritonavir and low-dose corticosteroids contributed to the observed 0% mortality in their COVID-19 patients, but without reporting any numerical data (Liu et al., 2020). More recently, the retrospective cohort from Chongqing public Health Medical Center presented the same 0% mortality in 217 patients (including 34 with severe forms), of which 99.5% were treated with therapy including IFN-α-2b (Ouyang et al., 2020). In the retrospective cohort study from Tongji University, there was a significant association between IFN-α-2b use and patient survival (OR2.32 IC95% [1,36; 3,97]) (Chen T. et al., 2020). Subsequently, reports have reinforced the potential of nebulized IFN to reduce mortality and viral carriage. Thus in Wuxi, China zero mortality was also observed in 55 COVID-19 patients, including 8 with a severe form of the disease, using a strategy including IFN-α-2b nebulization (Jiang X. et al., 2020), while in Wuhan, China a retrospective study performed in four different hospitals (including Tongji) found an independent association between IFN-α-2b inhalation and survival (3.45 [95% CI 1.96–0.51 5.88], $p < 0.001$) (Mei et al., 2020). In Anhui, China the early use of IFN-α-2b was independently associated with a reduction of viral carriage duration of SARS-CoV-2 (HR 1.649; 95% CI, 1.162–2.339) (Zuo et al., 2020). In Wuhan, China Zhou et al. showed in collaboration with E. Fish (Canada) that IFN-α-2b inhalation therapy combined with arbidol was associated with a significantly shorter duration of viral shedding compared to patients receiving arbidol alone (21 vs. 28 days; $p = 0.002$). Inhaled IFN-α-2b with or without arbidol also decreased serum IL-6 and CRP concentrations (Zhou et al., 2020). However, the initial characteristics of the patients (age, comorbidity, days from symptom onset to treatment) differed among the groups making any firm conclusion difficult (Zhou et al., 2020).

In other studies using inhaled IFN-α-2b, relatively short shedding times have been reported such as respectively in Shenzhen, China for moderate forms of COVID-19 (percentage of SARS-CoV-2 negative shift: 66% on day 6 and 95% on day 15) (Yuan J. et al., 2020) and in Shanghai, China for

mild forms (90% on day 7) (Chen J. et al., 2020). Of note, the addition of other therapeutic agents to IFN-α-2b nebulization did not result in significant changes in viral carriage time, be it hCQ (Chen J. et al., 2020), ribavirin (Yuan J. et al., 2020) or arbidol (Xu et al., 2020). Finally, in a prospective trial carried out in Hubei, China the prophylactic administration of IFN by the nasal route combined with barrier measures was reported to lead to a zero incidence of COVID-19 among caregivers (Meng et al., 2020). The interest of IFN was reinforced by a study carried out in Changsha, China where the administration by inhalation of Novaferon (an non-natural IFN, modified to gain affinity) in an open randomized trial conducted in 89 hospitalized COVID-19 patients led to a faster reduction of viral carriage than lopinavir alone (with a median viral carriage time from nine down to 6 days), and no transition to a severe disease form in the Novaferon group, as compared to 14% in the lopinavir group alone (Zheng et al., 2020). More recently, Fu et al. reported that aerosol inhalation of type I IFN-κ plus TFF2 (a healing and anti-inflammatory polypeptide) in combination with standard care is safe and superior to standard care alone in shortening the time up to viral RNA negative conversion in patients with moderate COVID-19 (Fu et al., 2020).

Collectively, the encouraging results obtained with IFN-α-2b in COVID-19 patients in China, together with the complete absence of IFN-β release, the greater sensitivity of SARS-CoV-2 to IFN-β *in vitro* and the fact that auto-antibodies against IFN-β appear to be rare in severe COVID-19 patients gave a reason for hope regarding superior clinical efficacy of inhaled IFN-β.

Current Data on the Safety of Inhaled Type I Interferon

Early studies on type I IFN nebulization showed that it was necessary to exceed 18 MIU (Million International Unit) of IFN-α-2b and 100 MIU of IFN-β to reach detectable concentrations of these compounds in blood (Halme et al., 1994). Similar data were obtained in non-human primates who exhibited acceptable local tolerance after direct instillation of 12 MIU of IFN-β-1a into the lungs and developed only mild subchronic alveolitis (Martin et al., 2002). This relatively good tolerance in preliminary studies was encouraging.

The only complete, currently available Treatment Emergent Adverse Events (TEAE) study with IFN-β inhalation comes from Synairgen, a drug discovery and development company founded by the University of Southampton, United Kingdom who developed SNG001, an aerosolizable form of IFN-β-1a. SNG001 has been used in phase 1 and 2 clinical trials with the aim to reduce rhinovirus-related symptoms in asthma patients. The drug allowed limited use of corticosteroids and presented few side effects (limited to 6.9% heart palpitations) (Djukanović et al., 2014). Moreover, no fever was observed although it was found in almost half of the patients when the product was administered subcutaneously (Limmroth et al., 2011). Psychiatric side effects were limited to rare sleep disturbances, and there was no significant hematological or hepatic toxicity, consistent with poor systemic passage of IFN aerosols (Djukanović et al., 2014). In view of this favorable risk/benefit balance, we decided to develop a nebulization protocol for IFN-β-1b administration. We present here the methods of IFN-β-1b administration, and the results obtained in four patients after administration on a compassionate basis.

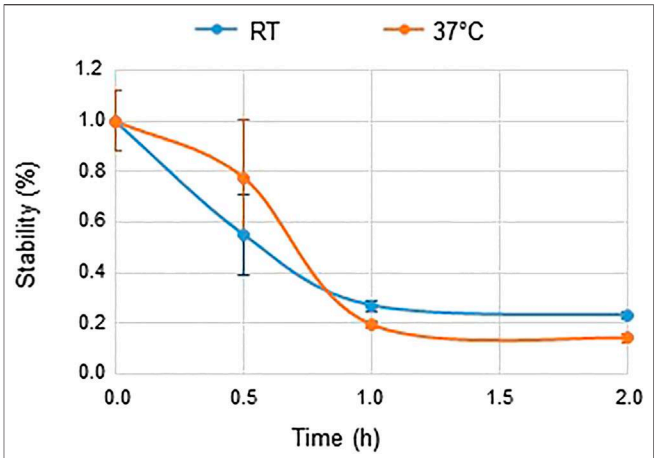


FIGURE 2 | Stability of IFN-β-1b. Results represent the mean ± SD of three experiments performed in duplicate. Two way ANCOVA. Time effect, $p < 0.0001$. Temperature effect, $p = 0.82$. RT: Room Temperature 20°C.

CHARACTERISTICS OF THE SOLUTION DEVISED TO NEBULIZE INTERFERON-β-1B

Choice of Solvents and Excipients

Many excipients contained in commercially available IFN formulations are at high risk of irritation. Well-known examples are acetic acid (Amdur, 1961) and benzyl alcohol (Wibbertmann et al., 2000). On the European market, only two type I IFNs have excipients which are fully compatible with the inhalation route: Betaferon® (marketed by Bayer Healthcare) and its biosimilar Extavia® (marketed by Novartis) which are both IFN-β-1b agents. These formulations are free from irritant compounds and contain albumin as well as mannitol, both of which are excipients recommended for dry inhalation (Bosquillon et al., 2001; Chew and Chan, 2002). Experiments presented in this work were undertaken with Extavia®. Extavia® is sold with a vial of 1.2 ml of solvent containing 0.54% NaCl that is necessary to reconstitute the product. Considering China CDC's recommendation to use 2 ml of water for injection (WFI), and the aqueous formulation of SNG001, we considered WFI to be suitable as a solvent, without organoleptic properties, for IFN-β-1b.

In Vitro Assessment of Stability

The stability of IFN-β-1b was tested at 30 min, 1 h and 2 h after reconstitution in 2 ml of WFI. Experiments were performed either at room temperature (RT, 20°C) or at 37°C. The concentration of IFN-β-1b was determined by immunoassay (R&D systems: DY008) according to the manufacturer's recommendations. The absorbance was read by spectrophotometry at 450 nm. When reconstituted at RT, a rapid decrease in IFN-β-1b mass was observed in the solution

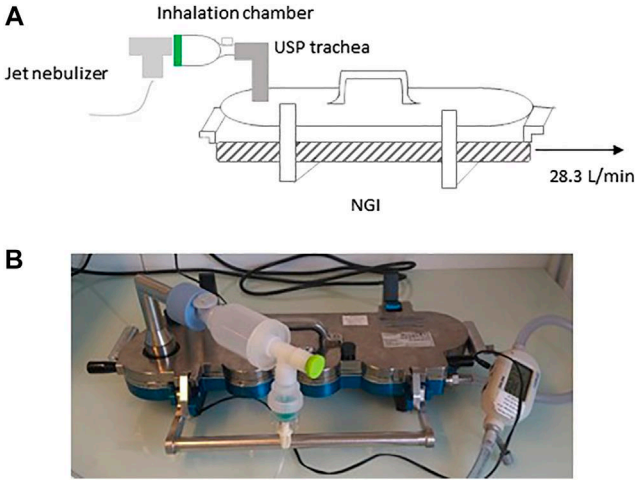


FIGURE 3 | Schematic representation **(A)** and picture **(B)** of the NGI cascade impactor used to determine particle size of IFN-β-1b. NGI, Next Generation Impactor.

(**Figure 2**). Specifically, 45% of the product was lost 30 min after reconstitution at RT. Stabilization was observed 1 h after reconstitution with only 25% of IFN-β-1b remaining in the solution. Same data were obtained when the product was reconstituted at 37°C (**Figure 2**).

Nebulization of Interferon-β-1b in a Cascade Impactor: Studies of Particles Size, Content and Distribution

To determine whether the size of IFN-β-1b-containing aerodynamic particles is compatible with deposition in the bronchial tree, the solution of IFN-β-1b reconstituted in 2 ml WFI was nebulized in a new generation cascade impactor (NGI, Copley Scientific). Particle size, content and distribution were then studied. To do so, the impactor was connected to the USP (United States Pharmacopeia) trachea and the HCP5 pump (Copley Scientific) at a constant flow rate of 28.3 L/min according to USP <1602>. Leakage was checked using a 4,043 flowmeter (Copley Scientific) before each series of measurements. The measured flow rate did not exceed a 5% error from the desired flow rate. A 10-min nebulization was performed with jet nebulizer MICROMIST® (Hudson RCI, ref. 41,745). A schematic representation of the assembly is depicted in **Figure 3**. Inside each impactor stage, trachea, and nebulizer chamber, the amount of IFN-β-1b (deposition or residual quantity) was determined by immunoassay (R&D systems: DY008) according to the manufacturer's recommendations. The absorbance was read by spectrophotometry at 450 nm.

The characteristics of the particles obtained by jet nebulizer are shown in **Figure 4**. Despite an initial quantity of 300 μg, only 114.25 ± 21.45 μg (mean ± SD) of IFN-β-1b was detected in the entire set-up after nebulization, consistent with the previously observed stability reached at 30 min. Although at the end of the 10 min nebulization no residual volume was visible in the nebulization chamber, there were still 57.66 ± 23.12 μg (mean ± SD) of IFN-β-1b remaining in the chamber, suggesting IFN-β-1b adhesion to chamber surfaces.

Inside the impactor we measured 41.63 ± 2.00 μg of IFN-β-1b, with a majority of fine particles ≤5 μm (91.38 ± 2.26%), and a mean particle size of 1.24 μm. Particle sizes between 4 and 0.54 μm (compatible with alveolar and bronchial deposition) accounted for the majority of the aerosolized IFN-β-1b, i.e., 32 μg (**Figure 4**). The jet nebulizer also allowed some deposition in the upper respiratory tract, of around 2.5 μg. In summary, our IFN nebulization technique allowed a topical delivery directly into the lungs of at least 10% of the total administered dose. In parallel to these *in vitro* studies, we have been able to treat four patients suffering from severe COVID-19 with IFN-β-1b inhalation, thereby providing an initial clinical feedback (see below).

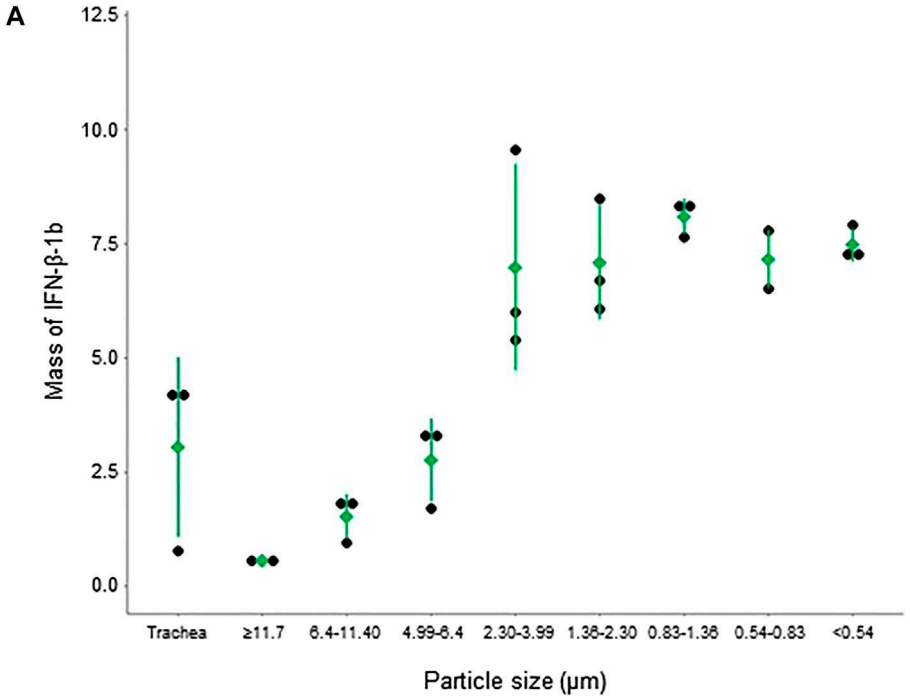
CASES-REPORT: COMPASSIONATE APPLICATION OF INTERFERON-β-1B INHALATION TO FOUR INTENSIVE CARE PATIENTS WITH SEVERE COVID-19

Given the therapeutic rationale for IFN-β-1b aerosol, and the first results in favor of an acceptable particle size for local deposition in the lungs, an intervention consisting in the inhalation of IFN-β-1b for the treatment of four patients with a severe form of COVID-19 was suggested by the clinical pharmacist to the intensive care unit team. Consent of the patients or their support person was obtained before proceeding to this off-label therapy and informed written consent of the patients was obtained to collect retrospectively individual data to follow CARE guidelines. **Table 1** presents the demographic and clinical characteristics of the four patients on critical care admission.

The medical history of patients 1 and 2 and patients 3 and 4 from time of hospital admission to time of hospital discharge is shown in **Figures 5**, **6**, respectively. Before treating the patients with inhaled IFN-β-1b, particle size was checked *in situ*, directly in patient's room, using an inverted microscope (Leica DMIL). Observed particles size was between 1.5 and 4.5 μm (**Supplementary Figure S1**).

Demographic and Clinical Characteristics of Patients on Critical Care Admission

The four patients (three men and one woman), aged 56–66 years, were admitted to the intensive care unit between april 4 and april 12, 2020. The main reason for admission was COVID-19 pneumopathy for patients 2 and 3 (**Table 1**). All of them presented fever >38°C at admission (**Table 1**). Patient one was admitted for decompensation of an atrioventricular block, which required emergency implantation of a pacemaker. Patient four was admitted with Grade E acute pancreatitis of biliary origin with criteria of a Systemic Inflammatory Response Syndrome, and the notion of a 48-h exposure to a room neighbor who proved to be SARS-CoV-2 positive. No patient presented chronic kidney disease or hypertension, and none reported smoking. The diagnosis of COVID-19 was made rapidly after ICU



B

Mass of IFN-β-1B in the vial (µg)	300
Mass in the impactor (µg)	41.63 ± 2.00
Mass in the nebulizer (µg)	57.66 ± 23.12
Total mass (µg)	114.25 ± 21.45
Dose of IFN-β-1b (µg) contained in fine particles ≤ 5µm	38.25 ± 3.74
Dose of IFN-β-1b (µg) contained in fine particles ≤ 3µm	33.87 ± 3.37
Fraction of fine particles ≤ 5µm (%)	91.38 ± 2.26
Fraction of fine particles ≤ 3 µm (%)	81.25 ± 2.25
Median aerodynamic diameter (MAD) (µm)	1.224 ± 0.030
Total duration of the measure (min)	25 ± 5

FIGURE 4 | Distribution of IFN-β-1b in fine particles. **(A)** Mass of IFN-β-1b in particles of different aerodynamic sizes. Means ± standard deviation are shown in green. Each black dot represents one experiment (n = 3). **(B)** Mass of IFN-β-1b in fine particles and in residue. Data are expressed as mean ± standard deviation (n = 3). Total duration of the measurements: 25 ± 5 min.

admission, except for patient two whose diagnosis was made prior to transfer (**Figures 5**, **6**). The diagnosis was confirmed by both RT-PCR on nasopharyngeal swab and chest CT. The RT-PCR method was performed based on recommendations by the National Reference Center for Respiratory Viruses, Institut Pasteur, Paris, using nCoV_IP2 and CoV_IP4 sequences (Institut Pasteur, 2020). The CT scans showed characteristic images of SARS-CoV-2 infection including bilateral frosted glass opacities of the lung parenchyma for all and bilateral pleural effusion for patient 4. On entry into critical care, all patients also had serum CRP levels >100 mg/L, and abnormal white blood cell counts with lymphocytopenia.

Initial Strategy of Care

The decision to treat these patients with IFN-β-1b was taken because they did not meet the inclusion criteria of other ongoing clinical trials such as Discovery (cardiac contraindication, or RT-PCR > 72 h). The initial strategy of care for these patients respected the recommendations made during this period in France. For severe forms of COVID-19, the HCSP (Haut Conseil de Santé Publique) suggested considering (case by case and according to a collegiate body's opinion) either the use of lopinavir/ritonavir or hCQ with close cardiological monitoring (HCSP, 2020). Only patient 2 started treatment targeting Sars-CoV-2 before she went into intensive care. At the

TABLE 1 | Demographic and clinical characteristics of patients on critical care admission.

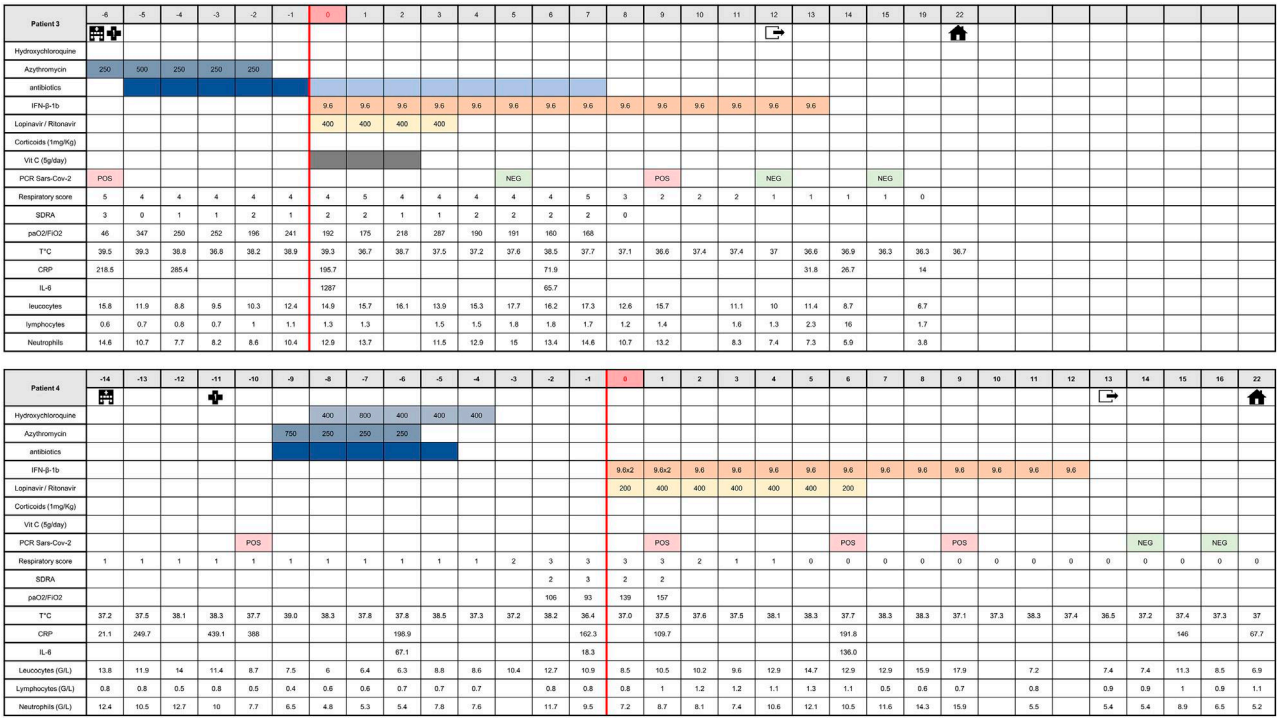
	Patient 1	Patient 2	Patient 3	Patient 4
Age (years)	59	56	66	56
Sex	Male	Female	Male	Male
Body mass index	42.9	39.3	27.3	25.3
Medical history	T2DM (NID) dyslipidemia	Hypothyroidism dyslipidemia	Total thyroidectomy T2DM (NID) schizophrenia	Benign prostate hyperplasia
Symptoms on admission	Atrioventricular block fever	ARDS fever	ARDS collapsus oligoanuria tachycardia fever	Abdominal pain acute peritonitis fever

T2DM, type 2 diabetes mellitus; NID, non-insulin dependent; ARDS, acute respiratory distress syndrome.

FIGURE 5 | Medical history of patients one and two from admission time to hospital discharge. This table shows clinical course of major symptoms and signs, blood biochemistry and viral shedding on initial therapy (azithromycin and/or hydroxychloroquine) as well as changes of these parameters upon start of IFN-β-1b therapy until hospital discharge. Respiratory scores: 0, No oxygen; 1, oxygen through nasal cannula or mask; 2, mask with high O2 concentration; 3, optiflow and/or NIV (Non-Invasive Ventilation); 4, mechanical ventilation with PEP ≤ 6 cm H2O or FiO2 ≤ 0.6; and 5, mechanical ventilation with PEP > 6 cm H2O and FiO2 > 0.6. *p*, prone therapy (18 h). ARDS scores: 0, no ARDS; 1, light ARDS; 2, moderate ARDS; 3, severe ARDS (corresponding to Berlin ARDS definition). CRP, C-reactive protein; ARDS, acute respiratory distress syndrom; T°C, temperature; G, giga.

time of COVID-19 diagnosis, lopinavir/ritonavir could not be prescribed to these patients because the product was out of stock. Instead all patients received antibiotic coverage with cefotaxime and azithromycin for severe community-acquired pneumonia. This treatment was combined with hCQ for patients 2 and 4, but not for patients 1 and 3 for whom the use of hCQ was contraindicated. Oxygen therapy was regularly adapted to the need of each patient, combined with 18 h prone therapy for patients 1 and 2 (**Figures 5, 6**).

Despite this management, the respiratory status of all patients deteriorated, with the appearance of severe ARDS with paO2/FiO2 <100 mmHg according to Berlin criteria. Patient 4 received high-flow oxygen therapy (Optiflow™) > 50 ml/min, considered as an application of PEEP >5 mmHg (Chertoff, 2017).



Patient 3	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	19	22
Hydroxychloroquine																								
Azythromycin	250	500	250	250	250																			
antibiotics																								
IFN-β-1b							9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6				
Lopinavir / Ritonavir							400	400	400	400														
Corticoids (1mg/Kg)																								
Vit C (5g/day)																								
PCR Sars-Cov-2	POS											NEG				POS			NEG			NEG		
Respiratory score	5	4	4	4	4	4	4	5	4	4	4	4	4	5	3	2	2	2	1	1	1	1	0	
SDRA	3	0	1	1	2	1	2	2	1	1	2	2	2	2	0									
paO2/FiO2	46	347	250	252	196	241	192	175	218	287	190	191	160	168										
T°C	39.5	39.3	38.8	36.8	38.2	38.9	39.3	36.7	38.7	37.5	37.2	37.6	38.5	37.7	37.1	36.6	37.4	37.4	37	36.6	36.9	36.3	36.3	36.7
CRP	218.5		285.4				195.7						71.9							31.8	26.7		14	
IL-6							1287						65.7											
leucocytes	15.8	11.9	8.8	9.5	10.3	12.4	14.9	15.7	16.1	13.9	15.3	17.7	16.2	17.3	12.6	15.7		11.1	10	11.4	8.7		6.7	
lymphocytes	0.6	0.7	0.8	0.7	1	1.1	1.3	1.3		1.5	1.5	1.8	1.8	1.7	1.2	1.4		1.6	1.3	2.3	16		1.7	
Neutrophils	14.6	10.7	7.7	8.2	8.6	10.4	12.9	13.7		11.5	12.9	15	13.4	14.6	10.7	13.2		8.3	7.4	7.3	5.9		3.8	

Patient 4	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	22
Hydroxychloroquine							400	800	400	400	400																					
Azythromycin						750	250	250	250																							
antibiotics																																
IFN-β-1b															9.6x2	9.6x2	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6	9.6					
Lopinavir / Ritonavir															200	400	400	400	400	400	200											
Corticoids (1mg/Kg)																																
Vit C (5g/day)																																
PCR Sars-Cov-2					POS											POS					POS			POS					NEG		NEG	
Respiratory score	1	1	1	1	1	1	1	1	1	1	1	2	3	3	3	3	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
SDRA													2	3	2	2																
paO2/FiO2													106	93	139	157																
T°C	37.2	37.5	38.1	38.3	37.7	39.0	38.3	37.8	37.8	38.5	37.3	37.2	38.2	36.4	37.0	37.5	37.6	37.5	38.1	38.3	37.7	38.3	38.3	37.1	37.3	38.3	37.4	36.5	37.2	37.4	37.3	37
CRP	21.1	249.7		439.1	388				198.9					162.3		109.7					191.8											67.7
IL-6									67.1					18.3							136.0											
Leucocytes (G/L)	13.8	11.9	14	11.4	8.7	7.5	6	6.4	6.3	8.8	8.6	10.4	12.7	10.9	8.5	10.5	10.2	9.6	12.9	14.7	12.9	12.9	15.9	17.9		7.2		7.4	7.4	11.3	8.5	6.9
Lymphocytes (G/L)	0.8	0.8	0.5	0.8	0.5	0.4	0.6	0.6	0.7	0.7	0.7		0.8	0.8	0.8	1	1.2	1.2	1.1	1.3	1.1	0.5	0.6	0.7		0.8		0.9	0.9	1	0.9	1.1
Neutrophils (G/L)	12.4	10.5	12.7	10	7.7	6.5	4.8	5.3	5.4	7.8	7.6		11.7	9.5	7.2	8.7	8.1	7.4	10.6	12.1	10.5	11.6	14.3	15.9		5.5		5.4	5.4	8.9	6.5	5.2

FIGURE 6 | Medical history of patients three and four from admission time to hospital discharge. This table shows clinical course of major symptoms and signs, blood biochemistry and viral shedding on initial therapy (azithromycin and/or hydroxychloroquine) as well as changes of these parameters upon start of IFN-β-1b therapy until hospital discharge. Respiratory scores: 0, No oxygen; 1, oxygen through nasal cannula or mask; 2, mask with high O2 concentration; 3, optiflow and/or NIV (Non-Invasive Ventilation); 4, mechanical ventilation with PEP ≤ 6 cm H2O or FiO2 ≤ 0.6; and 5, mechanical ventilation with PEP > 6 cm H2O and FiO2 > 0.6. *p*, prone therapy (18 h). ARDS scores: 0, no ARDS; 1, light ARDS; 2, moderate ARDS; 3, severe ARDS (corresponding to Berlin ARDS definition). CRP, C-reactive protein; ARDS, acute respiratory distress syndrom; T°C, temperature; G, giga.

Due to marked respiratory status deterioration of these four patients, the medical team proposed to start a treatment with inhaled IFN-β-1b.

Inhalation of Interferon-β-1b and Associated Therapies

IFN-β-1b (reconstituted with 2 ml WFI) was administered with MICROMIST® jet nebulizer at a dosage of 9.6 MIU (entire vial containing 8 MIU with 20% overage to facilitate reconstitution) per day, with a loading dose at 9.6 MIU bid for 48 h given to patient 4. Concomitantly, lopinavir/ritanovir (with dosage adjustment to serum concentrations) was administered to patients 2, 3 and 4 (**Figures 5, 6**). The main data in favor of the recommendation to use lopinavir/ritonavir in hospitalized patients came from a report by Cao et al. (Cao et al., 2020). In front of the general deterioration of the patients' health, and given the availability of lopinavir/ritonavir at the time when patients 2, 3, and 4 entered the compassionate protocol, the team decided to follow the Shenzen recommendations to associate corticosteroids (for patients with a high inflammatory status), lopinavir/ritonavir and inhalation of IFN in order to improve the chance of survival (Liu et al., 2020). Patient 1 did not receive lopinavir/ritonavir because the product remained out of stock when he entered the ICU one week before patients 2, 3, and 4. Vitamin C (5 g/day for 3 days) as anti-oxidant therapy was given to patients 1, 2, and 3. Methylprednisolone (1 mg/kg) as anti-inflammatory therapy was administered to patients 1 and 2 (**Figures 5, 6**). All patients also received anticoagulation therapy by sodic heparin (curative dose) during resuscitation. Patient 1 subsequently received argatroban due to heparin-induced thrombocytopenia. IFN-β-1b aerosol treatment was administered for 10–14 days.

Impact of Interferon-β-1b Inhalation on Major Clinical Parameters, Blood Biochemistry and Viral Shedding

The comparison of clinical and biochemical parameters between start of IFN-β-1b treatment and after 15 days of management is presented in **Table 2**. All patients showed an improvement in respiratory status, with only one patient still meeting mild ARDS criteria. Two patients were extubated, and in particular patient 4, after his loading doses, showed a resumption of respiratory secretions which allowed mobilization by physiotherapy, followed by early respiratory weaning, when the patient's intubation was about to be performed. Such rapid resumption of pulmonary mucus secretion following inhalation of IFN-β-1b was also observed in patient 1. It could not be evaluated in the two other patients who had a bacterial infection with purulent secretions.

TABLE 2 | Impact of IFN-β-1b inhalation on major symptoms, duration of viral shedding and outcomes in four patients hospitalized with COVID-19. If values were not available at d15 and d-1, the closest values available before treatment and toward the end of treatment.

IFN-β-1b therapy	Patient 1		Patient 2		Patient 3		Patient 4	
	d-1	d15	d-1	d15	d-1	d15	d-1	d15
Respiratory score	5	4	5	4	4	1	3	0
ARDS	2	1	2	0	1	0	3	0
T°C	39.2	38.3	37.6	37.2	38.9	36.3	36.4	37.4 (d12)
CRP	440.6	145 (d10)	91.5 (d-4)	24.5 (d8)	285.4 (d-4)	26.7 (d14)	162.3	146
IL-6	204	43 (d9)	166.5 (d-5)	26.8 (d5)	1,287 (d0)	65.7 (d7)	18.3	136.0 (d6)
Leukocyte count (G/L)	10.7	17.8	10.9	14.7	12.4	8.7 (d14)	10.9	11.3
Lymphocyte count (G/L)	0.9	1.4	1.7	3.1	1.1	1.6 (d14)	0.8	1
Neutrophill count (G/L)	9.4	15.5	8.6	10.3	10.4	5.9 (d14)	9.5	8.9
First negative PCR	Day 15		Day 6		Day 12		Day 14	
Discharge from resuscitation	Day 34		Day 21		Day 12		Day 13	
Discharge from home	Day 58		Day 30		Day 22		Day 22	

For all patients, the respiratory score improvement was concomitant with normalization of body temperature (**Table 2**). The treatment with inhaled IFN was accompanied by a decrease in serum CRP in all patients. Serum IL-6 also decreased except for patient 4, and was fluctuating in patient 1, according to the use of continuous venous dialysis. At the same time, the white blood cell count normalized, with an increase in lymphocyte count except for patient 4 (**Table 2**). Patients received regular nasopharyngeal swabs, and negative portage was found in all after two successive negative PCRs, after 6–15 days of management.

In terms of other hospitalization complications, patient 1 had acute kidney failure requiring continuous veno-venous dialysis, and developed heparin-induced thrombocytopenia. Two patients had secondary infections requiring antibiotic therapy on a probabilistic or documented basis (*Citrobacter Koseri* pneumonia and methicillin resistant *Staphycoccus epidermidis* bacteremia in patient 1, and ventilation-acquired *Serratia marcescens* pneumonia in patient 3). Patient 2 had his broad-spectrum antibiotic coverage discontinued upon return of negative bacteriology results.

None of the patients deteriorated further, and after 13–34 days they were all discharged from intensive care. Oxygen withdrawal was achieved during hospitalization in three among the four patients. At 3 months' follow-up, all patients were alive.

DISCUSSION

We were able to treat with inhaled IFN-β-1b, on a compassionate basis, four patients with severe COVID-19 admitted in the intensive care unit. Neither antibiotic nor hCQ administration had provided a satisfactory therapeutic response in these patients with severe co-morbidities. They showed an improvement in clinical and biochemical parameters within an acceptable time frame, in the absence of treatment-related adverse events. All of them were discharged from hospital and subsequently resumed normal life after a period of rehabilitation. Based on these observations, it seems reasonable to assume that inhaled IFN-β has exerted an antiviral action by restoring deficient pulmonary innate immunity defenses. This hypothesis is strongly supported by very recent immunological data supporting a role of insufficient response of type I IFNs (in particular with absence of IFN-β secretion) in the severity of COVID-19 (Arunachalam et al., 2020; Blanco-Melo et al., 2020; Hadjadj et al., 2020). The rationale of this treatment is further supported by the efficacy of IFN against SARS-CoV-2, both *in vitro* and *in vivo,* based on evaluation in retrospective studies and three randomized trials (Davoudi-Monfared et al., 2020; Hung et al., 2020; Rahmani et al., 2020).

Although the use of the pulmonary route represents an asset in terms of therapeutic precision, lack of knowledge of IFN nebulization remains a major limitation (Aricò et al., 2020). In order to apply this therapeutic modality to COVID-19, we explored the possibility of nebulizing IFN-β-1b.

Our approach is currently unique. In China, several randomized clinical trials have been built around different forms of IFN-α-2b (Chen J. et al., 2020; Jiang X. et al., 2020; Liu et al., 2020; Mei et al., 2020; Zhou et al., 2020; Zuo et al., 2020). This approach is currently explored by Fish et al. in Canada with the development of AP003 (BetterLifePharma Inc) (Choudhury, 2020). The more direct approach of pulmonary IFN-β nebulization is presently explored by Wilkinson et al. in collaboration with Synairgen, in a clinical trial with SNG001, a nebulizable form of IFN-β-1a (NCT04385095). However, these approaches are moving toward patent-protected products, with limited supply. A main advantage of IFN-β-1b is its immediate supply as a biosimilar, with a formulation suitable for nebulization without the need of major modifications. IFN-β-1b differs from IFN-β-1a in the way it is produced. On the one hand, IFN-β-1b is synthesized by bacteria without glycosylation and with modifications of 2 amino-acids to maintain stability. IFN-β-1a, on the other hand, is produced by mammalian cells and has a structure identical to natural IFN-β. Their difference results in a decrease of pharmacodynamic activity of about 25% for IFN-β-1b, as compared to IFN-β-1a. The decreased activity is compensated by a higher dosage of IFN-β-1b per vial (Stürzebecher et al., 1999).

Using a cascade impactor, we found that our process allowed nebulization of approximately 1 MIU (32 μg) of IFN-β-1b along

the bronchial tree. The distribution of particle sizes evenly distributed between 0.54 and 5.0 μm makes it possible to cover both alveoli and bronchioles (Cheng, 2014). The volume of pulmonary surfactant being limited from 1 to 5 ml, the administration resulted in a local epithelial concentration ranging from 10^6 IU/ml to 2.10^5 IU/ml. This amount allowed the achievement of a local concentration higher than that for which IFN has been shown to be most effective against SARS-CoV-2 (>50 IU/ml) (Clementi et al., 2020) for at least 12 local half-life. These concentrations are well above the 2.5 IU/ml cmax achievable by the subcutaneous (SC) pathway (Greig et al., 1988; Salmon et al., 1996; Munafo et al., 1998). The SC route of administration was chosen in the Discovery and Solidarity trials before knowing the EC50, and the ideal targets for SARS-CoV-2. The SC route does not appear to be optimal because it exposes to more adverse effects (Djukanović et al., 2014), and does not achieve full virucidal concentrations observed in a curative model (Clementi et al., 2020). The SC route also exposes to an increased risk of neutralizing autoantibodies against IFN-β, which could compromise the efficacy of IFN-β-based therapeutics as previously described in patients with multiple sclerosis (Sominanda et al., 2010). The pulmonary route may help to overcome this issue. The action of the aerosol is mainly localized at the level of the epithelium, with uncertainties regarding the amount that diffuses into lung parenchyma. Nevertheless, in the context of SARS-CoV-2, this local effect is particularly adapted to the marked tropism of the virus for epithelial cells, compared to other respiratory viruses (Hui et al., 2020). The deposited amount of IFN-β-1b (although representing only 10% of the initial content) therefore appears to be sufficient for a topical pulmonary effect. This is consistent with the rapid pharmacodynamic effect observed in two of the four patients, with the resumption of bronchial mucus secretion.

Galenic optimization of dosage and composition would be welcome to use lower amounts of IFN-β-1b (produced by genetic engineering). For example with IFN-γ, the choice of nebulizer and the use of feedback systems can heavily influence the amount deposited in the lung, between 7 and 65% (Condos et al., 2004; Diaz et al., 2012; Sweeney et al., 2019). Beyond the choice of the nebulizer, we are also faced with the problem of stability of the reconstituted product. By analogy, IFN-α in solution is known to aggregate and adhere to surfaces, limiting monomeric forms to 25% after 25 min (Ip et al., 1995). Ionic strength did not influence this aggregation, (Ip et al., 1995). The aggregation constitutes a pre-existing constraint for IFN-β-1b, with an aggregation rate of about 15% upon reconstitution (Barnard et al., 2013). Galenic optimization for protein nebulization is complex, and could be achieved by different excipients such as PEG 8000, n-Dodecyl-β-D-maltoside, l-arginine and trehalose (Mahjoubi et al., 2015; Sécher et al., 2019). The use of pegylated IFNs could also be considered to both increase stability and extend action at the local level (Mcleod et al., 2015).

The issue of galenic optimization is closely linked to the optimization of administered doses to keep antiviral lung IFN concentration over time. In order to be more effective at treatment start, we propose inhalation bid for the first 48 h. The patient who received such high dosing was the one who had the best clinical outcome. In hepatitis C, the anti-viral action of IFN-β has been shown to be dose-dependent and more pronounced over 48 h, which reinforces the concept of the need for more aggressive dosing at treatment initiation (Hosseini-Moghaddam et al., 2009). However, pharmacokinetic studies are required to evaluate local concentrations, as well as the elimination time of the product to better determine the optimal dosing regimen.

Our four patients had all moderate to severe ARDS at the time of treatment initiation and improved respiratory function upon inhalation of IFN-β-1b. At 3 months of follow-up they were all alive. The probability of such a good outcome was estimated at 2.5–32%, considering an intensive care mortality of 25–60%. One should however also take into account the overall optimization of patient management, in particular anticoagulation (Atallah et al., 2020), frequent use of glucorticoids (RECOVERY Collaborative Group et al., 2020a) and vitamin C (Carr, 2020; Colunga Biancatelli et al., 2020), in these highly inflamed individuals, as well as an organization allowing prone therapy (Coppo et al., 2020). Based on recently published results from the Recovery study, it is unlikely that lopinavir/ritonavir contributed to patient improvement (RECOVERY Collaborative Group, 2020). However, the existence of a synergistic effect between IFN-β-1b and loponavir/ritonavir cannot be completely ruled out. In the same manner, given the recent data showing that the use of hCQ and/or azithromycin does not improve clinical outcomes in hospitalized patients (RECOVERY Collaborative Group et al., 2020b; Fiolet et al., 2020; Furtado et al., 2020), it is unlikely that the initial therapy given to these patients contributed to the observed clinical improvement. Although it is not possible to draw a firm conclusion on treatment efficacy based on our preliminary observations the safety and tolerance of IFN-β-1b inhalation are reassuring. Particular attention should be paid to the risk of secondary bacterial infections, especially in case of prolonged treatments (Boxx and Cheng, 2016). In addition, IFN therapy may have diminished efficacy or even become deleterious if administered too late against SARS-CoV-2 (Wang et al., 2020). Monitoring of inflammation and viral elimination therefore appears to be important parameters for individual adaptation of treatment.

In order to know the risk-benefit ratio of IFN-β-1b nebulization in the treatment of COVID-19 it is now important to evaluate this ratio in randomized controlled trials. Since IFN-β can act in both the viral and the inflammatory phases of COVID-19 further studies should evaluate the potential of inhaled IFN-β in all phases of the disease. The effect of SNG001 (inhaled IFN-β-1a) is currently evaluated in patients with no need of resuscitation. Our protocol was submitted to and approved by the regulatory authorities in France. It is registered under No. NCT04469491. We plan to include more patients with moderate and severe COVID-19 who are hospitalized and need oxygen therapy, whether in intensive care units or not.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical approval was not provided for this study on human participants because the results present medical record data, retrieved retrospectively. To follow CARE guidelines, Patients gave their written and informed consent to the use of their medical data to investigate the hypothesis described in the article. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM, LH, and MB developed the initial hypotheses and wrote the manuscript. AM, PYM, LB, and HD validated the proposed therapeutic approach and critically reviewed the manuscript. AM and AC were in charge of clinical data collection and microscopically checked particle size *in situ*. TP and ME performed *in vitro* experiments on the feasibility of IFN-β-1b nebulization. AM and LH designed the figures and tables.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.592543/full#supplementary-material

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A Retrospective Study on the Use of Chinese Patent Medicine in 24 Medical Institutions for COVID-19 in China

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Objective: This research aims to analyze the application regularity of Chinese patent medicine during the COVID-19 epidemic by collecting the names of the top three Chinese patent medicines used by 24 hospitals in 14 provinces of China in four time periods (January 20–22, February 16–18, March 01–03, April 01–03, 2020), and explore its contribution to combating the disease.

Methods: 1) We built a database of the top three Chinese patent medicines used by 24 hospitals. 2) The frequency and efficacy distribution of Chinese patent medicine were analyzed with risk areas, regions, and hospitals of different properties as three factors. 3) Finally, we analyzed the differences in the use of heat-clearing and non-heat-clearing medicines among the three factors (χ^2 test) and the correlation between the Chinese patent medicine and COVID-19 epidemic (correlation analysis) with SPSS 23.0 statistical software.

Results: 1) The heat-clearing medicine was the main use category nationwide during January 20–22, 2020. Meanwhile, there was a significant difference in the utilization rate of heat-clearing and non-heat-clearing medicine in different risk areas ($p < 0.01$). 2) The variety of Chinese patent medicine was increased nationwide during February 16–18, 2020, mainly including tonics, blood-activating and resolving-stasis, and heat-clearing medicines. Meanwhile, there was a significant difference in the utilization rate of heat-

clearing and non-heat-clearing medicine in the southern and northern regions ($p < 0.05$). 3) Tonics, and blood-activating and resolving-stasis medicines became the primary use categories nationwide during March 01–03, 2020. 4) The tonics class, and blood-activating and resolving-stasis medicine were still the primary categories nationwide during April 01–03, 2020. Meanwhile, there was a significant difference in the utilization rate of heat-clearing and non-heat-clearing medicine in different risk areas ($p < 0.01$).

Conclusion: Chinese patent medicine has a certain degree of participation in fighting against the COVID-19. The efficacy distribution is related to the risk area, region, and hospital of different properties, among which the risk area is the main influencing factor. It is hoped that future research can further collect the application amount of Chinese patent medicine used in hospitals all over the country, so as to perfectly reflect the relationship between Chinese patent medicine and the epidemic situation.

Keywords: Chinese patent medicine, COVID-19, application regularity, correlative factor, retrospective analysis

INTRODUCTION

COVID-19 is a contagious respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was named by the coronavirus study group of the International Committee on Taxonomy of Viruses on February 11, 2020 (Sun et al., 2020). Currently, this epidemic disease has spread all around the world. The number of cumulative confirmed cases and existing confirmed cases in the countries except for China still show a continuous growth trend (World Health Organization, 2020), so the global pandemic remains severe.

From the Western Han Dynasty to the late Qing Dynasty, there were at least 321 large plagues in Chinese history. Therefore, Chinese history also contains a history of traditional Chinese medicine (TCM) against plagues. Faced with the SARS in 2003, China set up two independent "TCM Zone", which achieved favorable results with the combination of Chinese and Western treatment. In the face of the COVID-19 pandemic, there is still a lack of effective drugs in the world. COVID-19 meeting of the Central Committee of China's Leading Group request: strengthen the integration of TCM and western medicine, promote the whole process of the deep intervention of TCM diagnosis and treatment, and extend the effective TCM prescription and Chinese patent medicine (Wang et al., 2020).

There are four main aspects of TCM's participation in the fight against COVID-19 (Zhang, 2020). First, providing TCM decoction to four quarantined groups of people, such as suspected and confirmed cases. Second, establishing Fangcang hospital, where nearly 10,000 patients almost entirely use TCM, and the coverage rate reached 95%. Third, for severe and critical patients, TCM also played an auxiliary role in improving oxygenation level and suppressing inflammatory factor storms. Finally, promoting recovery and reducing sequelae. TCM can remove residual evil, support vital qi, promote the absorption of pulmonary inflammation, and improve immune function. The proportion of TCM participating in the treatment of Hubei related hospitals was more than 2/3. The clinical practice data showed that the treatment of COVID-19 with integrated TCM and western medicine is effective (Yuan et al., 2020). TCM has shown remarkable effects in relieving fever symptoms, controlling disease progression, preventing disease transmissibility, reducing hormone dosage, decreasing complications, and preventing drug resistance (Chen et al., 2020).

The most obvious changes in the "sixth Trial Version of the Guidelines for the Diagnosis and Treatment of COVID-19" and later versions issued by the National Health Commission of China are the increased proportion of TCM therapeutic regimen, and the recommendation of Chinese patent medicine in different courses of COVID-19, especially the usage of TCM injections used for severe and critical patients. Twelve Chinese patent medicines are recommended for use in different stages of COVID-19 in the "seventh Trial Version of the Guidelines for the Diagnosis and Treatment of COVID-19" (National Health Commission National Administration of Traditional Chinese Medicine, 2020). Some studies have shown that Chinese patent medicines can significantly reduce the clinical manifestations of COVID-19 and play their pharmacological role in various mechanisms (Wang et al., 2020b; Zhang et al., 2020b).

This research aims to investigate the use of Chinese patent medicines used by 24 third-grade class-A hospitals in 14 provinces or cities of China during the epidemic from January to April, and analyze the usage characteristics, so as to have an in-depth understanding of the Chinese patent medicines' participation and the related factors affecting its usage during the COVID-19 epidemic.

MATERIALS AND METHODS

Data Sources

Data of the name of Chinese patent medicine ranked top three used in 24 third-grade class-A hospitals in four time periods were collected. The four periods are January 20–22, February 16–18, March 01–03, April 01–03, 2020. The 24 hospitals are distributed in 14 provinces or cities of China (Beijing, Tianjin, Jilin Province, Shanxi Province, Shaanxi Province, Gansu Province, Xinjiang Province, Hubei Province, Zhejiang Province, Guangzhou Province, Anhui Province, Shanghai Province, Jiangxi Province, Sichuan Province). The above four time points are

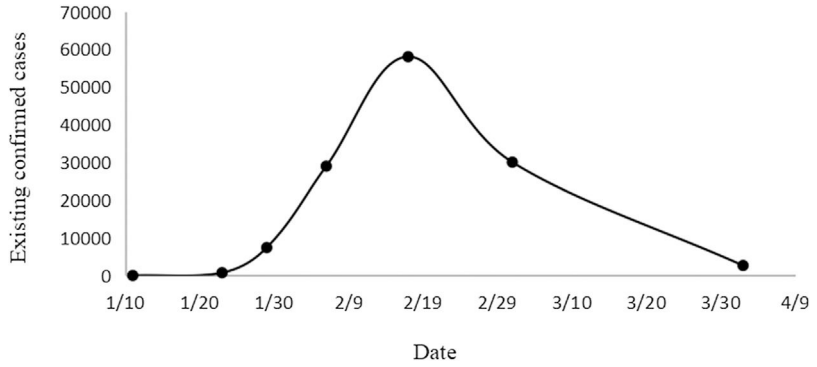

FIGURE 1 | Trends in the number of confirmed cases over time.

distributed in the initial stage, the highest peak, the fastest decline stage, and the end-stage, respectively, of the curve of the existing confirmed cases of the COVID-19 in China, aiming to fully reflect the drug use in all stages (**Figure 1**).

Statistical Analysis

Data were analyzed with SPSS 23.0 statistical software. Chi-square (χ^2) test was conducted when analyzing the difference of three factors in the frequency of heat-clearing and non-heat-clearing medicine. Regression analysis was taken when exploring the correlation between three factors and the epidemic situation. To avoid the influence of the uneven data on the analysis results, the frequency is weighted according to the proportion of the hospital with different properties when analyzing the hospital factor. Values of $p < 0.05$ and $p < 0.01$ were considered statistically significant differences and extremely significant differences separately.

Exclusion Criteria

(1) Specialist medicines;
(2) The medicines with an obscure name.

Medicines with the same ingredient but different dosage forms are considered to be the same type.

Classification Criteria of Three Factors

Risk area classification criteria: Risk regions were divided into high-risk areas (cumulative confirmed cases >500) and low-risk areas (cumulative confirmed cases <500). According to the distribution of COVID-19 up to April 14, 2020, China was divided into six levels according to the accumulated confirmed cases (as shown in **Figure 2**). We select the median 500 as the boundary of the high and low-risk areas based on the severity of the epidemic at that time.

Region classification criteria: According to the south or north of the Qinling Mountain-Huaihe River Line, the areas within the statistical scope are divided into the southern region and the northern region. Qinling Mountain-Huaihe River line is currently recognized as China's north-south geographical boundary. There were many differences on both sides of this line in the natural conditions, agricultural production, geographical features, and people's living customs (Sheng, 2008).

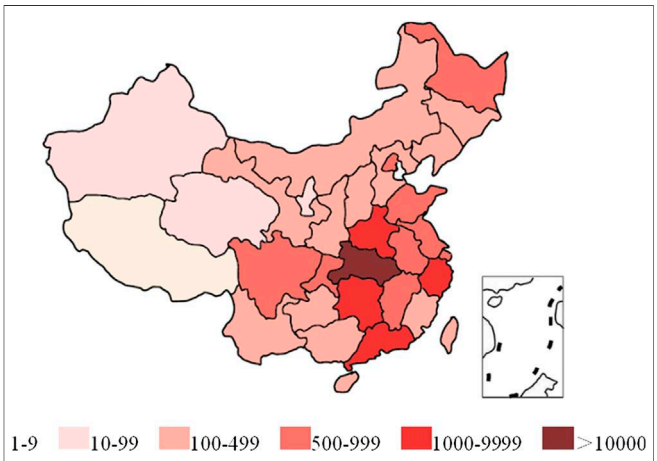

FIGURE 2 | Six levels of confirmed cases in China.

Hospital classification criteria: According to the official website of the hospitals and the management system of the National Administration of TCM, 24 hospitals are divided into traditional Chinese medical hospitals (TCM hospitals) and Western medical hospitals (note: integrative medicine hospitals are included in TCM hospitals for their same TCM treatment department setting).

Related Concepts

(1) TCM includes Chinese medicinal decoction pieces/TCM decoction and Chinese patent medicine.
(2) Chinese patent medicine is a kind of TCM product which is processed into a certain dosage form according to the prescription and preparation technology under the guidance of TCM theory and in order to prevent and treat diseases.
(3) Heat-clearing medicine: The properties of heat-clearing medicine is cold, whcih can clear the body's internal heat, including heat-clearing and detoxifying medicines, heat-

clearing and fire-purging medicines, heat-clearing and damp-drying medicines, heat-clearing and blood-cooling medicines, et al.

Non-heat clearing medicine: The main function of non-heat clearing medicine is not to clear away heat, in this article refers to the tonifying deficiency medicines or activating blood and resolving stasis medicines.

RESULTS

Data Statistics for January 20–22, 2020

Nationwide Data Statistics

The data of 24 hospitals nationwide were summarized and the name of Chinese patent medicines with a frequency of more than one was obtained. Among them, the top three Chinese medicines were Lianhua Qingwen granule (capsule), Lanqin oral liquid, and Jinhua Qinggan granule. It was shown that the medicine for clearing heat and removing toxicity is the main use category in the whole country from January 20 to January 22 (specific data is shown in **Supplementary Table S1**).

Data Statistics of Different Risk Areas

The usage frequency of Chinese patent medicine in high-risk and low-risk areas was counted (specific data is shown in **Supplementary Tables S2, S3**), respectively, and the distribution regularities of efficacy were analyzed (**Tables 1**, **2**). By comparing the high-risk and low-risk areas, it can be seen that the high-risk areas are concentrated in heat-clearing medicines with a frequency as high as 86%, while the heat-clearing drugs accounting for only 37%. The analysis results showed that the usage frequency of heat-clearing medicines and non-heat-clearing medicines was significantly different between high-risk and low-risk areas ($p < 0.01$, **Table 7**). Therefore, the higher the risk level of the epidemic situation, the stronger the pertinence of drug types to the disease.

Data Statistics of Different Regions

The usage frequency of Chinese patent medicine in southern and northern regions was counted (specific data is shown in **Supplementary Tables S4, S5**), respectively, and the distribution regularities of efficacy were analyzed (**Table 3**, **4**). By comparing the northern region and southern region, it can be seen that the efficacy of Chinese patent medicines used in the southern region is relatively concentrated, with heat-clearing drugs (70%) as the main type, while the efficacy of Chinese patent medicines used in the northern region is relatively dispersed, with heat-clearing drugs (59%) as the first one, followed by the medicine for activating blood and resolving stasis and the medicine for tonifying deficiency. The analysis results showed that there was no significant difference in the frequency of heat-clearing drugs and non-heat-clearing drugs between southern and northern regions ($p > 0.05$, **Table 7**).

The reason for the low utilization rate of heat-clearing medicines in northern regions may be related to the epidemic situation. The COVID-19 epidemic in southern China is extensive, so the used medicines focus on the prevention and treatment of pneumonia, while the used medicines in northern China focus on body regulation. Besides, considering that there is

TABLE 1 | Effect distribution of Chinese patent medicines in high-risk areas.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule (capsule) Lanqin oral liquid Jinhua Qinggan granule Huachansu capsule Antivirus oral liquid Banlangen granule Qingfei pill Feilike mixture Qingqiao Kangdu granule Jingyin mixture Chonglian oral liquid Compound shuanghua tablet Pudilan antiphlogistic oral liquid Huangkui capsule Compound Daqing granule Shufeng Jiedu capsule Xuanfei Zhisou mixture Compound Xianzhuli liquid Shiwei Longdanhua granule Maxing Huatan mixture	CLLLLLlearing heat	20	87%
Bailing capsule (tablet) Yupingfeng granule Shengxuebao mixture	Tonifying deficiency	3	13%

TABLE 2 | Effect distribution of Chinese patent medicines in low-risk areas.

Effect	Frequency	Percentage (%)
Clearing heat	7	37
Tonifying deficiency	5	26
Activating blood and resolving stasis	7	37

TABLE 3 | Effect distribution of Chinese patent medicines in southern regions.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule (capsule) Lanqin oral liquid Huachansucapsule Shufeng Jiedu capsule Antivirus oral liquid Pudilan antiphlogistic oral liquid Feilike mixture Maxing Huatan mixture Qingqiao Kangdu granule Jingyin mixture Chonglian oral liquid Xuanfei Zhisou mixture Compound Daqing granule	Clearing heat	13	70%
Bailingcapsule (tablet) Shengxuebao mixture Yupingfeng granule Huaier granule Naoxintong capsule	Tonifying deficiency	5	30%

TABLE 4 | Effect distribution of Chinese patent medicines in northern regions.

Name	Effect	Frequency	Percentage
Jinhua Qinggan granule	Clearing heat	17	59%
Lianhua Qingwen granule			
Lanqin oral liquid			
Antivirus oral liquid			
Banlangen granule			
Compound Xianzhuli liquid			
Shiwei Longdanhua granule			
Zukamu granule			
Qingfei pill			
Compound shuanghua tablet			
Honghua Qinggan thirteen pill			
Suhuang Zhike capsule			
Huangkui capsule			
Qingfei Huatan mixture			
Relinqing granule			
Modified Shuanghuanglian oral liquid			
Huachansu tablet			
Bailing capsule	Tonifying deficiency	6	21%
Shensong Yangxin capsule			
Jinshuibao pill			
Peiyuan Tongnao capsule			
Zhizhu Kuanzhong capsule			
Rougan Hepi pill			
Yinxing Mihuan oral liquid	Activating blood and resolving stasis	6	20%
Xuefu Zhuyu capsule			
Naoxintong capsule			
Compound Danshen dropping pill			
Guanxin Danshen dropping pill			
Danqi soft capsule			

no significant difference in the northern and southern regions, so it is considered that the main factor affecting the drug use in January is epidemic risk grade without obvious direct correlation with geographical location.

Data Statistics of Hospitals of Different Properties

The usage frequency of Chinese patent medicine in TCM and western medical hospitals was counted (specific data is shown in **Supplementary Tables S6, S7**), respectively, and the distribution regularities of efficacy were analyzed (**Table 5**, **6**). The comparison between TCM hospital and Western medical hospital shows that TCM hospitals mainly use heat-clearing medicines (60%), supplemented with Chinese patent medicines with different treatment principles such as tonifying deficiency, activating blood and resolving stasis. The purpose of treatment in western medical hospitals are relatively clear, and the frequency of using heat-clearing medicines is up to 78%. The analysis results showed that there was no significant difference between heat-clearing medicines and non-heat-clearing medicines between TCM hospitals and western medical hospitals ($p > 0.05$, **Table 7**).

The reasons for the differences in drug use may be related to the characteristics of different medical systems. TCM takes syndrome differentiation for treatment and body regulation as its primary treatment principles, so there were various kinds of medicines used. Western medical hospital emphasizes the symptomatic treatment, so the efficacy distribution of medicines was relatively narrow.

Data Statistics for February 16–18, 2020

National Data Statistics

The data of 24 hospitals nationwide were summarized and the name of Chinese patent medicines with a frequency of more than one was obtained. Among them, the top three Chinese medicines are the Bailing capsule (tablet), compound Danshen dropping pill, and Lianhua Qingwen capsule (granule). From February 16 to 18, it was shown that the types of Chinese patent medicine had increased nationwide, such as tonifying deficiency, activating blood and resolving stasis, and heat-clearing medicines. Meanwhile, the utilization rate of heat-clearing drugs was lower than that in January (specific data is shown in **Supplementary Table S8**).

Data Statistics of Different Risk Areas

The usage frequency of Chinese patent medicine in high-risk and low-risk areas was counted (specific data is shown in **Supplementary Tables S9, S10**), respectively, and the distribution regularities of efficacy were analyzed (**Table 8**, **9**). Comparing the high-risk and low-risk areas, the heat-clearing medicines in the high-risk areas still accounted for a large proportion (36%), while the low-risk areas were dominated by medicine for activating blood and resolving stasis (47%), with a significantly low utilization rate of heat-clearing medicines (18%). The analysis results showed that there was no significant difference between heat-clearing drugs and non-heat-clearing drugs in different risk areas ($p > 0.05$, **Table 14**).

TABLE 5 | Effect distribution of Chinese patent medicines in traditional Chinese medicine hospitals.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule (capsule) Jinhua Qinggan granule Shufeng Jiedu capsule Lanqin oral liquid Antivirus oral liquid Banlangen granule Compound Daqing granule Jingyin mixture Qingqiao Kangdu granule Pudilan antipyrotic oral liquid Chonglian oral liquid Huachansu capsule Qingfei pill Zukamu granule Huangkui capsule Qingfei Huatan mixture Maxing Huatan mixture Relinqing granule Honghua Qinggan thirteen pill Modified Shuanghuanglian oral liquid Xuanfei Zhisou mixture Suhuang Zhike capsule	Clearing heat	22	60%
Bailingcapsule (tablet) Yupingfeng granule Shensong Yangxin capsule Shengxuebao mixture Jinshuibao pill Rougan Hepi pill Zhizhu Kuanzhong capsule	Tonifying deficiency	7	20%
Naoxintong capsule Xuefu Zhuyu capsule Compound Danshen dropping pill Guanxin Danshen dropping pill Danqi soft capsule Yinxing Mihuan oral liquid	Activating blood and resolving stasis	6	20%

Data Statistics of Different Regions

The usage frequency of Chinese patent medicine in the southern and northern regions was counted (specific data is shown in **Supplementary Tables S11, S12**), respectively, and the distribution regularities of efficacy were analyzed (**Table 10**, **11**). Comparing the northern and southern region, the heat-clearing medicines were still dominant (41%) in the southern region. In northern regions, the medicine for activating blood and resolving stasis is the main type, and heat-clearing medicine accounts for the lowest proportion (17%). The analysis results showed that there were significant differences between heat-clearing medicines and non-heat-clearing medicines in different regions ($p < 0.05$, **Table 14**).

Analyzing the reasons for the significant difference of drug use in February between the northern and southern regions, it was concluded that, the characteristics of drug use in different regions are revealed with the development and research of the COVID-19. Therefore, the region became the main influence factor of drug use in February.

TABLE 6 | Effect distribution of Chinese patent medicines in western medical hospitals.

Name	Effect	Frequency	Percentage
Jinhua Qinggan granule Lianhua Qingwen granule Compound Xianzhuli liquid Compound shuanghua tablet Huachansu capsule Shiwei Longdanhua granule Feilike mixture	Clearing heat	7	78%
Huaier granule Peiyuan Tongnao capsule	Tonifying deficiency	2	22%

Data Statistics of Hospitals of Different Properties

The usage frequency of Chinese patent medicine in TCM and western medical hospitals was counted (specific data is shown in **Supplementary Tables S13, S14**), respectively, and the distribution regularities of efficacy were analyzed (**Table 12**, **13**). The comparison between TCM hospitals and western medical hospitals showed that the use of Chinese patent medicine in February in two kinds of hospitals was similar. Although both of them take medicine for activating blood and removing stasis as the primary use category, heat-clearing

TABLE 7 | Analysis of medication difference of three factors in January.

	Heat-clearing medicine (frequency)	Non-heat-clearing medicine (frequency)	*p*-value
High-risk area	20	4	0.002[a]
Low-risk area	7	12	
Sourthern region	13	5	0.345
Northern region	17	12	
Traditional Chinese medicine hospital	7	4	0.642
Western medical hospital	7	2	

[a]p < *0.01, High-risk area vs. Low-risk area.*

TABLE 8 | Effect distribution of Chinese patent medicines in high-risk areas.

Name	Effect	Frequency	Percentage
Lianhua Qingwengranule (capsule)	Clearing heat	15	36%
Xuebijing injection			
Xiyanping injection			
Jinhua Qinggangranule			
Lanqin oral liquid			
Chonglian oral liquid			
Jingyin mixture			
Banlangen granule			
Huangkui capsule			
Compound Daqing granule			
Maxing Huatan mixture			
Feilike mixture			
Compound Huangqi jiedu mixture			
Honghua Qinggan pill			
Babaodan capsule			
Compound Danshen dropping pill	Activating blood and resolving stasis	17	40%
Naoxintong capsule			
Linaoxin tablet			
Tiandan Tongluo capsule			
Yindan Xinnaotong soft capsule			
Tongluo Yiqi pill			
Naoshuantong capsule			
Xiaoshuan changyong capsule			
Qili Qiangxin capsule			
Xiaoshuan Tongluo capsule			
Xueshuan Xinmaining tablet			
Sanqi Shutong capsule			
Xiongdan capsule			
Ginkgo drop pill			
Xueshuantong granule			
Compound Xueshuantong capsule			
Shexiang Baoxin pill			
Bailingcapsule	Tonifying deficiency	10	24%
Jinshuibao pill			
Xinyuan capsule			
Yupingfeng granule			
Peiyuan Tongnao capsule			
Shenyan Kangfu tablet			
Zhenyuan capsule			
Kangfuxin liquid			
Yixinshu capsule			
Shenqi Gankang capsule			

medicines still account for a large proportion. Analysis results showed that there was no significant difference between heat-clearing medicines and non-heat-clearing medicines in different hospitals ($p > 0.05$, **Table 14**).

Data Statistics for March 01–03, 2020

National Data Statistics

The data of 24 hospitals nationwide were summarized and the name of Chinese patent medicines with a frequency of more

TABLE 9 | Effect distribution of Chinese patent medicines in low-risk areas.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule	Clearing heat	3	18%
Qingfei Huatan mixture			
Huangkui capsule			
Bailingcapsule	Tonifying deficiency	6	35%
Shensong Yangxin			
Qishen Yiqi drop pill			
Wenxin granule			
Jinshuibao pill			
Shenkangfu capsule 2			
Naoxintong capsule	Activating blood and resolving stasis	8	47%
Compound Danshen dropping pill			
Ginkgo tablet			
Naoxintong capsule			
Tongxinluo capsule			
Xuefu Zhuyugranule			
Yuxuebi capsule			
Compound Xueshuantong capsule			

TABLE 10 | Effect distribution of Chinese patent medicines in southern regions.

Name	Effect	Frequency	Percentage
Lianhua Qingwengranule (capsule)	Clearing heat	12	41%
Xiyanping injection			
Xuebijing injection			
Lanqin oral liquid			
Chonglian oral liquid			
Jingyin mixture			
Compound Huangqi jiedu mixture			
Compound Daqing granule			
Maxing Huatan mixture			
Feilike mixture			
Honghua Qinggan pill			
Babaodan capsule			
Compound Danshen dropping pill	Activating blood and resolving stasis	10	34%
Naoxintong capsule			
Ginkgo drop pill			
Shexiang Baoxin pill			
Yindan Xinnaotong soft capsule			
Naoshuantong capsule			
Tiandan Tongluo capsule			
Tongluo Yiqi pill			
Xiaoshuan changyong capsule			
Xiongdan capsule			
Bailing capsule	Tonifying deficiency	7	24%
Jinshuibao pill			
Yixinshu capsule			
Yupingfeng granule			
Shenqi Duotang oral liquid			
Shenyan Kangfu tablet			
Shenqi Gankang capsule			

than one was obtained. Among them, the top four Chinese patent medicines are the Bailing capsule (tablet), compound Danshen dropping pill, Naoxintong capsule, Lianhua Qingwen granules (capsule). During March 01–03, medicines for tonifying deficiency, and activating blood and resolving stasis became the main categories nationwide (specific data is shown in **Supplementary Table S27**).

Data Statistics of Different Risk Areas

The usage frequency of Chinese patent medicine in high-risk and low-risk areas was counted (specific data is shown in **Supplementary Tables S16, S17**), respectively, and the distribution regularities of efficacy were analyzed (**Table 15**, **16**). Comparing with the high-risk area and low-risk area, the utilization rate of heat-clearing medicines (33%) in the high-risk

TABLE 11 | Effect distribution of Chinese patent medicines in northern regions.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule	Clearing heat	5	17%
Huangkui capsule			
Jinhua Qinggangranule			
Qingfei Huatan mixture			
Banlangen granule			
Naoxintong capsule	Activating blood and resolving stasis	14	48%
Compound Danshen dropping pill			
Ginkgo tablet			
Linaoxin tablet			
Compound Xueshuantong capsule			
Xuefu Zhuyugranule			
Xueshuantong granule			
Tongxinluo capsule			
Qili Qiangxin capsule			
Sanqi Shutong capsule			
Yuxuebi capsule			
Tiandan Tongluo capsule			
Xiaoshuan Tongluo capsule			
Xueshuan Xinmaining tablet			
Bailingcapsule	Tonifying deficiency	10	34%
Jinshuibao pill			
Xinyuan capsule			
Shensong Yangxin			
Kangfuxin liquid			
Zhenyuan capsule			
Wenxin granule			
Qishen Yiqi drop pill			
Shenkangfu 2 capsule			
Peiyuan Tongnao capsule			

areas was about twice as much as that in the low-risk area (12%). The results showed that there was no significant difference between the use frequency of heat-clearing medicines and non-heat-clearing medicines in the high-risk and low-risk areas ($p > 0.05$, **Table 21**).

Data Statistics of Different Regions

The usage frequency of Chinese patent medicine in the southern and northern regions was counted (specific data is shown in **Supplementary Tables S18, S19**), respectively, and the distribution regularities of efficacy were analyzed (**Table 17**, **18**). It can be seen that the proportion of heat-clearing medicines in southern regions is the highest (40%). In the northern regions, the heat-clearing medicines account for the smallest proportion (20%), while activating blood and removing stasis is the category with the highest utilization rate (47%). The analysis results showed that there was no significant difference in the frequency of using heat-clearing medicines and non-heat-clearing medicines between southern and northern regions ($p > 0.05$, **Table 21**).

Data Statistics of Hospitals of Different Properties

The usage frequency of Chinese patent medicine in TCM and western medical hospitals was counted (specific data is shown in of **Supplementary Tables S20, S21**), respectively, and the distribution regularities of efficacy were analyzed (**Table 19**, **20**). It can be seen that both TCM hospitals and Western medical hospitals tend to use medicines for activating blood and removing stasis, and tonifying deficiency. The results showed that there was no significant difference in the usage frequency of heat-clearing medicines and non-heat-clearing medicines between different hospitals ($p > 0.05$, **Table 21**).

Data Statistics for April 01–03, 2020

National Data Statistics

The data of 24 hospitals nationwide were summarized and the name of Chinese patent medicines with a frequency of more than one was obtained. Among them, the top four proprietary Chinese medicines are the Bailing capsule, compound Danshen dropping pill, Naoxintong capsule, Jinshuibao tablet, and Lianhua Qingwen granules (capsules) (specific data is shown in Table 40 **Supplementary Table S22**).

Data Statistics of Different Risk Areas

The usage frequency of Chinese patent medicine in high-risk and low-risk areas was counted (specific data is shown in **Supplementary Tables S23, S24**), respectively, and the distribution regularities of efficacy were analyzed (**Table 22**, **23**). The high-risk areas in April were still dominated by heat-clearing medicines. In the low-risk areas, the medicines for activating blood and removing stasis, and tonifying deficiency have occupied the majority of the commonly used medicines, while the utilization rate of heat-clearing medicines has decreased significantly, accounting for only 5%. The analysis results showed that the usage frequency of heat-clearing medicines and non-heat-clearing medicines was

TABLE 12 | Effect distribution of Chinese patent medicines in traditional Chinese medicine hospitals.

Name	Effect	Frequency	Percentage
Lianhua Qingwengranule (capsule)	Clearing heat	12	32%
Huangkui capsule			
Xiyanping injection			
Xuebijing injection			
Maxing Huatan mixture			
Jingyin mixture			
Chonglian oral liquid			
Qingfei Huatan mixture			
Compound Daqing granule			
Honghua Qinggan pill			
Compound Huangqi jiedu mixture			
Babaodan capsule			
Compound Danshen dropping pill	Activating blood and resolving stasis	15	41%
Naoxintong capsule			
Compound Xueshuantong capsule			
Ginkgo tablet			
Tongxinluo capsule			
Tongluo Yiqi pill			
Linaoxin tablet			
Tiandan Tongluo capsule			
Naoshuantong capsule			
Xueshuantong granule			
Yuxuebi capsule			
Xiongdan capsule			
Shexiang Baoxin pill			
Xuefu Zhuyu granule			
Qili Qiangxin capsule			
Bailingcapsule (tablet)	Tonifying deficiency	10	27%
Jinshuibao pill			
Shensong Yangxin			
Shenqi Duotang oral liquid			
Wenxin granule			
Yupingfeng granule			
Qishen Yiqi drop pill			
Peiyuan Tongnao capsule			
Shenyan Kangfu tablet			
Shenkangfu capsule 2			

TABLE 13 | Effect distribution of Chinese patent medicines in western medical hospitals.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule	Clearing heat	5	31%
Jinhua Qinggan granule			
Lanqin oral liquid			
Banlangen granule			
Feilike mixture			
Sanqi Shutong capsule	Activating blood and resolving stasis	6	38%
Tiandan Tongluo capsule			
Xiaoshuan Tongluo capsule			
Xueshuan Xinmaining tablet			
Linaoxin tablet			
Yindan Xinnaotong soft capsule			
Xinyuan capsule	Tonifying deficiency	5	31%
Jinshuibao pill			
Kangfuxin liquid			
Zhenyuan capsule			
Yixinshu capsule			

TABLE 14 | Analysis of medication difference of three factors in February.

	Heat-clearing medicine (frequency)	Non-heat-clearing medicine (frequency)	*p*-value
High-risk area	15	27	0.172
Low-risk area	3	14	
Sourthern region	12	17	0.043[a]
Northern region	5	24	
Traditional Chinese medicine hospital	4	8	0.907
Western medical hospital	5	11	

[a]p < *0.05, Southern region vs. Northern region.*

TABLE 15 | Effect distribution of Chinese patent medicines in high-risk areas.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule (capsule)	Clearing heat	14	33%
Xiyanping injection			
Xuebijing injection			
Jinhua Qinggan granule			
Lanqin oral liquid			
Huangkui capsule			
Compound Huangqi jiedu mixture			
Compound Daqing granule			
Antivirus oral liquid			
Huachansucapsule			
Maxing Huatan mixture			
Honghua Qinggan pill			
Feilike mixture			
Zhenbao pill			
Bailing capsule (tablet)	Tonifying deficiency	15	34%
Jinshuibao pill			
Shensong Yangxin			
Shengxuebao mixture			
Shenqi Duotang oral liquid			
Dengzhan Shengmaicapsule			
Xinyuan capsule			
Wenxin granule			
Linglingcapsule			
Shenqi Gankang capsule			
Yishen Huashigranule			
Congrong Yishen granule			
Compound congrong Yizhicapsule			
Shenyan Kangfu tablet			
Shenshuaining capsule			
Compound Danshen dropping pill	Activating blood and resolving stasis	14	33%
Naoxintong capsule			
Ginkgo tablet (drop pill)			
Xintong oral liquid			
Xuefu Zhuyu capsule			
Compound Xueshuantong capsule			
Xueshuantong granule			
Qili Qiangxin capsule			
Sanqi Shutong capsule			
Xueshuan Xinmaining tablet			
Xiaoshuan changyong capsule			
Linaoxin tablet			
Tiandan Tongluo capsule			
Xiongdan capsule			

TABLE 16 | Effect distribution of Chinese patent medicines in low-risk areas.

Name	Effect	Frequency	Percentage
Yifei Jiedu granule	Clearing heat	2	12%
Yichuanping capsule			
Compound Danshen dropping pill	Activating blood and resolving stasis	7	41%
Naoxintong capsule			
Yuxuebi capsule			
Danqicapsule			
Tongxinluo capsule			
Xuefu Zhuyu capsule			
Compound Xueshuantong capsule			
Bailing capsule	Tonifying deficiency	8	47%
Shensong Yangxin capsule			
Jinshuibao pill			
Shenkangfu 2 capsule			
Xianling Gubaocapsule			
Fufang Xuanju capsule			
Rougan Hepi pill			
Zhizhu Kuanzhong capsule			

TABLE 17 | Effect distribution of Chinese patent medicines in southern regions.

Name	Effect	Frequency	Percentage
Lianhua Qingwengranule (capsule)	Clearing heat	12	40%
Xuebijing injection			
Xiyanping injection			
Lanqin oral liquid			
Antivirus oral liquid			
Compound Huangqi jiedu mixture			
Compound Daqing granule			
Maxing Huatan mixture			
Huachansucapsule			
Honghua Qinggan pill			
Huangkui capsule			
Feilike mixture			
Bailingcapsule (tablet)	Tonifying deficiency	10	33%
Jinshuibao pill			
Shengxuebao mixture			
Dengzhan Shengmai capsule			
Lingling capsule			
Shenqi Duotang oral liquid			
Wenxin granule			
Congrong Yishen granule			
Yishen Huashigranule			
Shenqi Gankang capsule			
Naoxintong capsule	Activating blood and resolving stasis	8	27%
Compound Danshen dropping pill			
Ginkgo drop pill			
Xiaoshuan changyong capsule			
Tiandan Tongluo capsule			
Shenshuainingcapsule			
Shenyan Kangfu tablet			
Xiongdan capsule			

significantly different between high-risk and low-risk areas ($p < 0.01$, **Table 28**).

Statistics of Different Regions

The usage frequency of Chinese patent medicine in the southern and northern regions was counted (specific data is shown in **Supplementary Tables S25, S26**), respectively, and the distribution regularities of efficacy were analyzed (**Table 24**, **25**). It can be seen that Chinese patent medicines mainly used in southern China are still heat-clearing medicine, followed by the medicine for tonifying deficiency, and activating blood and removing stasis. In

TABLE 18 | Effect distribution of Chinese patent medicines in northern regions.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule	Clearing heat	6	20%
Jinhua Qinggan granule			
Yifei Jiedu granule			
Yichuanping capsule			
Huangkui capsule			
Zhenbao pill			
Compound Danshen dropping pill	Activating blood and resolving stasis	14	47%
Naoxintong capsule			
Compound Xueshuantong capsule			
Ginkgo tablet			
Danqi capsule			
Sanqi Shutong capsule			
Xueshuantong granule			
Tongxinluo capsule			
Xueshuan Xinmaining tablet			
Qili Qiangxin capsule			
Linaoxin tablet			
Yuxuebi capsule			
Xintong oral liquid			
Xuefu Zhuyu capsule			
Bailing capsule	Tonifying deficiency	10	33%
Shensong Yangxin			
Jinshuibao pill			
Xinyuan capsule			
Xianling Gubao			
Shenkangfu capsule2			
Fufang Xuanju capsule			
Compound congrong Yizhi capsule			
Zhizhu Kuanzhong capsule			
Rougan Hepi pill			

northern China, the main category is the medicine for activating blood and removing stasis, followed by heat-clearing medicine and medicine for tonifying deficiency. The analysis results showed that there was no significant difference in the frequency of heat-clearing medicines and non-heat-clearing medicines between the southern and northern regions ($p > 0.05$, **Table 28**).

Data Statistics of Hospitals of Different Properties

The usage frequency of Chinese patent medicine in TCM and western medical hospitals was counted (specific data is shown in **Supplementary Tables S27, S28**), respectively, and the distribution regularities of efficacy were analyzed (**Table 26**, **27**). It can be seen from the comparison between TCM hospital and western medical hospital that the main treatment direction of TCM hospital is to tonify deficiency and promote circulation and remove stasis, which is in line with the characteristics of TCM for the recovery period. Although the western hospital still takes clearing heat as the primary treatment direction, the utilization rate of the other two kinds of medicine has increased, which is in agreement with the different stages of the epidemic situation in general. The analysis results showed that there was no significant difference between heat-clearing and non-heat-clearing medicines between two different types of hospitals ($p > 0.05$, **Table 28**).

DISCUSSION

According to the data and analysis results of this study, it is considered that the analysis method (χ^2 test) matches the type of data and research purpose (significant difference), and more scientific and reasonable explanations can be obtained through the analysis results.

Because the TCMs for prevention and treatment of COVID-19 are mainly heat-clearing medicines, so the analysis focuses on the difference between the use of heat-clearing and non-heat-clearing medicines. Synthesizing the above statistical results, it turned out that in January, the utilization rate of heat-clearing and non-heat-clearing medicine in high and low-risk areas was significantly different ($p < 0.01$). In February, the north and south region were significantly different ($p < 0.05$), and in April, the high and low-risk area was significantly different ($p < 0.01$). According to the analysis, at the end of January, COVID-19 was just in the initial phase, and there was no effective prescription or decoction. Therefore, Chinese patent medicine became the main force to resist COVID-19 in high-risk areas. At the same time, the number of confirmed cases in low-risk areas has not yet risen to a severe level, so the Chinese patent medicines were not widely used. Consequently, different risk areas became the main factors affecting drug use in January. In mid-February, the number of confirmed cases nationwide peaked, and local treatment programmes began to be rolled out in each region, making it

TABLE 19 | Utilization rate of Chinese patent medicine in traditional Chinese medicine hospitals.

Name	Effect	Frequency	Percentage
Lianhua Qingwengranule (capsule)	Clearing heat	10	25%
Xiyanping injection			
Xuebijing injection			
Compound Daqing granule			
Compound Huangqi jiedu mixture			
Maxing Huatan mixture			
Yichuanping capsule			
Yifei Jiedu granule			
Honghua Qinggan pill			
Huachansu capsule			
Compound Danshen dropping pill	Activating blood and resolving stasis	14	35%
Naoxintong capsule			
Compound Xueshuantong capsule			
Ginkgo drop pill (tablet)			
Danqicapsule			
Xuefu Zhuyu capsule			
Yuxuebi capsule			
Tongxinluo capsule			
Xiaoshuan changyong capsule			
Sanqi Shutong capsule			
Tiandan Tongluo capsule			
Xueshuantong granule			
Qili Qiangxin capsule			
Xiongdan capsule			
Bailingcapsule (tablet)	Tonifying deficiency	16	40%
Jinshuibao pill			
Shensong Yangxin			
Shenqi Duotang oral liquid			
Lingling capsule			
Shengxuebao mixture			
Wenxin granule			
Shenqi Gankang capsule			
Zhizhu Kuanzhong capsule			
Rougan Hepi pill			
Shenshuaining capsule			
Shenyan Kangfu tablet			
Shenkangfu capsule 2			
Fufang Xuanju capsule			
Xianling Gubao			
Yishen Huashi granule			

TABLE 20 | Effect distribution of Chinese patent medicines in western medical hospitals.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule	Clearing heat	6	33%
Jinhua Qinggan granule			
Lanqin oral liquid			
Antivirus oral liquid			
Zhenbao pill			
Feilike mixture			
Compound Danshen dropping pill	Activating blood and resolving stasis	5	28%
Naoxintong capsule			
Linaoxin tablet			
Xueshuan Xinmaining tablet			
Xintong oral liquid			
Bailing capsule	Tonifying deficiency	7	39%
Dengzhan Shengmai capsule			
Jinshuibao pill			
Shensong Yangxin			
Congrong Yishen granule			
Compound congrong Yizhicapsule			
Xinyuan capsule			

TABLE 21 | Analysis of medication difference of three factors in March.

	Heat-clearing medicine (frequency)	Non-heat-clearing medicine (frequency)	*p*-value
High-risk area	14	29	0.101
Low-risk area	2	15	
Sourthern region	12	18	0.091
Northern region	6	24	
Traditional Chinese medicine hospital	3	10	0.585
Western medical hospital	6	12	

TABLE 22 | Effect distribution of Chinese patent medicines in high-risk areas.

Name	Effect	Frequency	Percentage
Bailing capsule (tablet)	Clearing heat	15	39%
Lianhua Qingwen granule (capsule)			
Jinhua Qinggan granule			
Lanqin oral liquid			
Huachansucapsule			
Huangkui capsule			
Chonglian oral liquid			
Compound Daqing granule			
Jingyin mixture			
Feilike mixture			
Niuhuang Qingxin pill			
Longqing tablet			
Zhenbao pill			
Honghua Qinggan thirteen pill			
Weimaining capsule			
Jinshuibao pill	Tonifying deficiency	11	28%
Shengxuebao mixture			
Shensong Yangxin			
Longlu capsule			
Mingmu Yanggan pill			
Jiuwei Zhenxin granule			
Lishukang capsule			
Qiwei Wenyang capsule			
Congrong Yishen granule			
Wenxin granule			
Shenyan Kangfu tablet			
Naoxintong capsule	Activating blood and resolving stasis	13	33%
Compound Danshen dropping pill			
Maizhiling tablet			
Tiandan Tongluo capsule			
Huoxue Tongmaicapsule			
Salvia miltiorrhiza polyphenolic acid for injection			
Compound Xueshuantong capsule			
Xueshuan Xinmaining tablet			
Yindan Xinnaotong soft capsule			
Xiaoshuan Tongluo capsule			
Yuxuebi capsule			
Xiaoshuan changyong capsule			
Honghua Xiaoyao tablet			

a major influence on drug use in February. In March, the epidemic situation was in a stage of significant decline, TCM has explored more mature and diversified treatment schemes, and the participation rate of Chinese patent medicines dropped. Hence, there was no significant difference among the three factors. In April, the epidemic situation was basically under control. At that time, the low-risk areas relaxed their vigilance, making the usage rate of heat-clearing medicine into the lowest point, so there was a significant difference with high-risk areas.

As shown in **Figure 3**, from January to April, the usage rate of heat-clearing medicines was the highest during January 20–22, when COVID-19 was just beginning to spread, while the

TABLE 23 | Effect distribution of Chinese patent medicines in low-risk areas.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule	Clearing heat	1	5%
Zhizhu Kuanzhong capsule	Tonifying deficiency	9	45%
Bailing capsule			
Shensong Yangxin			
Jinshuibao pill			
Fufang Xuanju capsule			
Gujin pill			
Kangfuxin liquid			
Xianling Gubao capsule			
Longlu pill			
Compound Danshen dropping pill	Activating blood and resolving stasis	10	50%
Naoxintong capsule			
Tongxinluo capsule			
Guanxin Danshen dropping pill			
Yinxing Mihuan oral liquid			
Xuefu Zhuyu capsule			
Yuxuebi capsule			
Danqi soft capsule			
Moxa stick			
Suxiao Jiuxin pill			

TABLE 24 | Effect distribution of Chinese patent medicines in southern regions.

Name	Effect	Frequency	Percentage
Lianhua Qingwen capsule	Clearing heat	10	38%
Lanqin oral liquid			
Compound Daqing granule			
Chonglian oral liquid			
Jingyin mixture			
Feilike mixture			
Huachansu capsule			
Huangkui capsule			
Longqing tablet			
Weimaining capsule			
Bailingcapsule (tablet)	Tonifying deficiency	9	35%
Jinshuibao pill			
Shenyan Kangfu tablet			
Longlu capsule			
Lishukang capsule			
Qiwei Wenyang capsule			
Congrong Yishen granule			
Shengxuebao mixture			
Wenxin granule			
Compound Danshen dropping pill	Activating blood and resolving stasis	7	27%
Naoxintong capsule			
Honghua Xiaoyao tablet			
Qufeng Zhitong pill			
Tiandan Tongluo capsule			
Xiaoshuan changyong capsule			
Yindan Xinnaotong soft capsule			

remarkably decreased usage rates are shown in other three time points. Moreover, the usage rate of heat-clearing medicines in different risk areas, regions, and hospitals of different properties also showed the above trend, but did not show a significant correlation with the COVID-19 epidemic ($p > 0.05$, **Table 29**). On account of the above results, the following considerations are made: 1) The Chinese patent medicines mainly participate in the early stage of the COVID-19, and the participation decreases in the outbreak stage, the decline stage, and the end-stage. 2) It is speculated that there were many blind purchases or use of Chinese patent medicines in the early stage, due to the public's lack of understanding of COVID-19,

TABLE 25 | Effect distribution of Chinese patent medicines in northern regions.

Name	Effect	Frequency	Percentage
Lianhua Qingwen capsule	Clearing heat	8	23%
Jinhua Qinggan granule			
Huangkui capsule			
Lanqin oral liquid			
Huachansu capsule			
Zhenbao pill			
Honghua Qinggan thirteen pill			
Niuhuang Qingxin pill			
Jinshuibao pill	Tonifying deficiency	11	31%
Bailing capsule			
Kangfuxin liquid			
Shensong Yangxin			
Xianling Gubao capsule			
Longlu pill			
Gujin pill			
Fufang Xuanju capsule			
Mingmu Yanggan pill			
Jiuwei Zhenxin granule			
Zhizhu Kuanzhong capsule			
Naoxintong capsule	Activating blood and resolving stasis	16	46%
Compound Danshen dropping pill			
Yuxuebi capsule			
Compound Xueshuantong capsule			
Salvia miltiorrhiza polyphenolic acid for injection			
Xuefu Zhuyu capsule			
Danqi soft capsule			
Xueshuan Xinmaining tablet			
Tiandan Tongluo capsule			
Xiaoshuan Tongluo capsule			
Huoxue Tongmaicapsule			
Tongxinluo capsule			
Suxiao Jiuxin pill			
Guanxin Danshen dropping pill			
Yinxing Mihuan oral liquid			
Moxa stick			

panic mentality, and the pharmacy's lax control over the use of heat-clearing medicines. 3) In this survey, the collected time points are limited with a short period, which cannot fully reflect the specific change rule of utilization rate over time. More evidence is needed to verify the above speculations further. 4) In correlation analysis, the sample size is too small to fully explain the relationship between the epidemic situation and three factors.

CONCLUSION

At present, studies on Chinese patent medicines for COVID-19 mostly focus on therapeutic regimens, clinical observation, and pharmacological studies. There is almost no analysis of the overall use of Chinese patent medicines during COVID-19. This study was conducted to investigate the use of Chinese patent medicines in 24 third-grade class-A hospitals in 14 provinces or cities of China during the epidemic of COVID-19. And we have found that Chinese patent medicines play a role in the fight against COVID-19 and heat-clearing medicines were the most used weapons. Moreover, the risk area is the main influencing factor for the use of Chinese patent medicines.

Heat-clearing medicine, especially with the antiviral effect, has a high utilization rate during January-April, so it is considered that Chinese patent medicine has a certain degree of participation in the fight against COVID-19. On the whole, previous studies paid more attention to the medication difference of three-concerned therapy of "individual concerned therapy, environment concerned therapy, climate concerned therapy," and this study confirmed that the use of Chinese patent medicine was different in the regions to some extent. Furthermore, two other factors were considered in the investigation, namely, hospitals of different properties and different risk regions. It was found that different risk regions were the main factor affecting the utilization rate of heat-clearing drugs.

On January 31, 2020, COVID-19 was listed as a public health emergency of international concern by the World Health Organization, which seriously endangered people's health and public safety, and became one of the major epidemics after SARS in 2003 (Zhong et al., 2003; Cucinotta and Vanelli, 2020). So far, there is still no effective antiviral treatment for COVID-19 (Shereen et al., 2020; Stawicki et al., 2020). Symptomatic support therapy and comprehensive interventions are mainly used in clinical practice (Wu et al., 2020b).

TABLE 26 | Effect distribution of Chinese patent medicines in traditional Chinese medicine hospitals.

Name	Effect	Frequency	Percentage
Lianhua Qingwen capsule (granule)	Clearing heat	9	24%
Chonglian oral liquid			
Jingyin mixture			
Huangkui capsule			
Compound Daqing granule			
Huachansu capsule			
Honghua Qinggan thirteen pill			
Longqing tablet			
Weimaining capsule			
Bailing capsule (tablet)	Tonifying deficiency	14	38%
Jinshuibao pill			
Shensong Yangxin			
Longlucapsule (pill)			
Zhizhu Kuanzhong capsule			
Shengxuebao mixture			
Wenxin granule			
Shenyan Kangfu tablet			
Xianling Gubao capsule			
Lishukang capsule			
Fufang Xuanju capsule			
Mingmu Yanggan pill			
Gujin pill			
Kangfuxin liquid			
Naoxintong capsule	Activating blood and resolving stasis	14	38%
Compound Danshen dropping pill			
Yuxuebi capsule			
Danqi soft capsule			
Honghua Xiaoyao tablet			
Salvia miltiorrhiza polyphenolic acid for injection			
Guanxin Danshen dropping pill			
Tongxinluo capsule			
Tiandan Tongluo capsule			
Xuefu Zhuyu capsule			
Compound Xueshuantong capsule			
Suxiao Jiuxin pill			
Yinxing Mihuan oral liquid			
Moxa stick			

TABLE 27 | Effect distribution of Chinese patent medicines in western medical hospitals.

Name	Effect	Frequency	Percentage
Lianhua Qingwen granule	Clearing heat	7	41%
Lanqin oral liquid			
Jinhua Qinggan granule			
Huachansu capsule			
Niuhuang Qingxin pill			
Zhenbao pill			
Feilike mixture			
Qiwei Wenyang capsule	Tonifying deficiency	4	24%
Congrong Yishen granule			
Kangfuxin liquid			
Jiuwei Zhenxin granule			
Compound Danshen dropping pill	Activating blood and resolving stasis	6	35%
Huoxue Tongmaicapsule			
Xueshuan Xinmaining tablet			
Xiaoshuan Tongluo capsule			
Tiandan Tongluo capsule			
Yindan Xinnaotong soft capsule			

TABLE 28 | Analysis of medication difference of three factors in April.

	Heat-clearing medicine (frequency)	Non-heat-clearing medicine (frequency)	*p*-value
High-risk area	15	24	0.006[a]
Low-risk area	1	19	
Sourthern region	10	16	0.186
Northern region	8	27	
Traditional Chinese medicine hospital	3	9	0.367
Western medical hospital	7	10	

[a]p < *0.01, High-risk area vs. Low-risk area.*

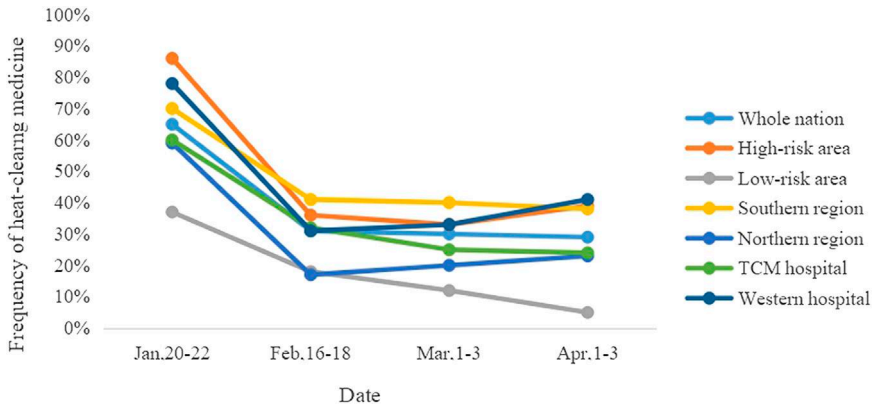

FIGURE 3 | Changes in the utilization rate of heat-clearing medicine.

TABLE 29 | Utilization rate of heat-clearing medicines of three factors.

Date	Existing confirmed cases (average)	Whole nation	High-risk area	Low-risk area	Southern region	Northern region	Traditional Chinese medicine hospital	Western medical hospital
20–22	434	65%	86%	37%	70%	59%	60%	78%
16–18	57,918	31%	36%	18%	41%	17%	32%	31%
1–3	30,030	30%	33%	12%	40%	20%	25%	33%
1–3	1717	29%	39%	5%	38%	23%	24%	41%

p > *0.05, Existing confirmed cases vs. three factors.*

Since the outbreak of COVID-19 (Wu et al., 2020b), TCM has been able to get involved in the whole process of treatment and achieved remarkable results (Li et al., 2020). TCM integrates its treatment principles (syndrome differentiation and three-concerned therapy of "individual concerned therapy, environment concerned therapy, climate concerned therapy") (Wu et al., 2020a) with the different stages of COVID-19. Clinical studies (Liu et al., 2020; Zhang et al., 2020a) showed that TCM could improve the symptoms, shorten the course of treatment, and prevent conversion to the severe state for ordinary patients. For severe and critical patients, TCM can reduce pulmonary exudation, control inflammatory overreaction, improve oxygenation level, stabilize blood oxygen saturation, and reduce the use of hormones and antibiotics to prevent the deterioration of the disease. For convalescent patients, the rehabilitation process can be promoted by TCM. Meanwhile, patients with COVID-19 also include the elderly, children, pregnant women, and those with basic diseases, whose medications have also been considered in clinical treatment. Besides, the syndrome characteristics of COVID-19 in different regions are "the same but different." Although the common characteristic is "wet," different regions have diverse pathogenesis due to the environmental aspect (Ma et al., 2020; Shi et al., 2020) and other factors. Therefore, multiple TCM medication plans have been introduced in different regions in China. Moreover, many studies (Bashir et al., 2020; Chen et al., 2020; Tosepu et al., 2020) have shown that climate change can affect the spread of the COVID-19 and the pathogenesis of the

human body, so the use of TCM should also take into account the influence of seasonal variations. Besides, some studies have suggested that the transmission of COVID-19 is related to air pollution and population density (Kadi and Khelfaoui, 2020; Martelletti and Martelletti, 2020). The cure rate and prognosis of COVID-19 are closely related to the underlying diseases such as cancer, hypertension, body mass index, and diabetes (Malik et al., 2020; Meng et al., 2020; Pugliese et al., 2020). These views have a high value of in-depth thinking and provide more direction for the research of drug use for COVID-19.

The TCMs involved in anti-epidemic include TCM decoction and Chinese patent medicine. It is well known that TCM decoction has played a great role in against the COVID-19 in China, while there are few reports and studies on Chinese patent medicine. The seventh Trial Version of the Guidelines for the Diagnosis and Treatment of COVID-19 by the National Health Commission of China recommends four oral Chinese patent medicines [Huoxiang Zhengqi capsule (pill, oral liquid, water), Jinhua Qinggan granule, Lianhua Qingwen capsule (granule), Shufeng Jiedu capsule (granule)] and eight TCM injections [(Xiyanping injection, Tanreqing injection, Xuebijing injection, Reduning injection, Xingnaojing injection, Shengmai injection, Shenfu injection, Shenmai injection)] respectively during the medical observation and clinical treatment period. According to some Chinese experts consensus and clinical experience, the intervention with Chinese patent medicine during medical observation can cut off the development of the disease in advance (Bao et al., 2020; Jin et al., 2020). Chinese patent medicine mainly plays two roles: on the one hand, it can provide symptomatic treatment; on the other hand, it can help strengthen immunity to resist the attack of the virus, so as to "prevent infection before illness" and "prevent transmission after illness" (Xiong et al., 2020). Clinical observation showed that (Duan et al., 2020; Yao et al., 2020), Jinhua Qinggan granules can significantly relieve the clinical symptoms of fever, cough, fatigue, and expectoration in mild COVID-19 patients. Lianhua Qingwen granules can significantly improve fever, cough, expectoration, and anhelation in COVID-19 patients, whose antifebrile time and the time of viral nucleic acid test turning negative were comparable to oseltamivir. Pharmacology experiments found that (Wang et al., 2020a) Chinese patent medicine showed a direct antiviral effect, could improve the inflammation caused by a virus infection, and have the function of the two-way adjusting the immune system. Furthermore, it can also impede or delay cytokine storm through the immunoregulation and anti-inflammatory action (Luo et al., 2020) and can suppress the occurrence or development of pulmonary fibrosis effectively. Huoxiang Zhengqi relieves symptoms through anti-inflammatory effects. Lianhua Qingwen defends the lung from COVID-19 by inhibiting pro-inflammatory cytokine production. Shufeng Jiedu plays roles in the COVID-19 through multiple targets and inflammatory signaling pathways. Xuebijing injection can reduce multiple organ damage by anti-inflammatory and improving immune function (Tong et al., 2020).

However, there are some limitations in this study. Firstly, the investigation only counted the names of the top three Chinese patent medicines used by hospitals, ignoring the specific application amount, which made it challenging to conduct more in-depth statistical analysis. Secondly, the distribution of the 24 hospitals investigated in this study is uneven across the country, which may affect the rigor of analysis results although the frequency was weighted in the analysis. Finally, the limited time points with a short time quantum cannot fully reflect the specific change rules of utilization rate over time, which need further in-depth discussion.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

YH, LL, XZ, XK, BZ, WY, YL, DD, MZ, YZ, LL, XW (16th author), JW, XL, HN, XW (20th author), HuW, FL, HoW, HuW, YL, LL, WZ, MY collected the hospital data. NZ drafted the manuscript. NS, SL, GL helped the data analysis. YW, HZ, YW revised the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.574562/full#supplementary-material.

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REVIEW
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Multidisciplinary Approach to the Diagnosis and In-Hospital Management of COVID-19 Infection: A Narrative Review

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Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2 or COVID-19 disease) was declared a pandemic on 11th March 2020 by the World Health Organization. This unprecedented circumstance has challenged hospitals' response capacity, requiring significant structural and organizational changes to cope with the surge in healthcare demand and to minimize in-hospital risk of transmission. As our knowledge advances, we now understand that COVID-19 is a multi-systemic disease rather than a mere respiratory tract infection, therefore requiring holistic care and expertise from various medical specialties. In fact, the clinical spectrum of presentation ranges from respiratory complaints to gastrointestinal, cardiac or neurological symptoms. In addition, COVID-19 pandemic has created a global burden of mental illness that affects the general population as well as healthcare practitioners. The aim of this manuscript is to provide a comprehensive and multidisciplinary insight into the complexity of this disease, reviewing current scientific evidence on COVID-19 management and treatment across several medical specialties involved in the in-hospital care of these patients.

Keywords: COVID-19, hospital care, multispecialist care, pandemic (COVID-19), hospital response capability

INTRODUCTION

Since COVID-19 has been declared a Public Health Emergency of International concern on the 30th January 2020, more than 39 millions of people worldwide have been infected (https://covid19.who.int/).

Hospitalized patients with COVID-19 require a multidisciplinary approach as the infection can lead to a plethora of clinical scenarios. Most commonly, patients are hospitalized for respiratory insufficiency, which requires oxygen administration delivered in several forms including mechanical ventilation in the most severe cases (Marini and Gattinoni, 2020). Indeed, a subgroup of infected individuals—i.e., approximately 5% of COVID-19 patients - rapidly progress to acute respiratory

distress syndrome (ARDS), often associated with multiple organ failure (MOF), sepsis and septic shock requiring admission to intensive care units (ICUs) (Marini and Gattinoni, 2020).

Central nervous system involvement in COVID-19 infection has been noticed since the early stages of the pandemic. In fact, symptoms such as anosmia or ageusia are relatively common among infected individuals (Guan et al., 2020). In addition, other neurological manifestations have been reported, such as meningoencephalitis and stroke, the latter being related to the prothrombotic state observed with this infection (Zhang et al., 2020a). Gastrointestinal symptoms (GIS), as nausea, vomiting, abdominal pain and diarrhea, may be an early manifestation of COVID-19 disease, and some studies suggest that the presence of GIS may indicate a higher probability of a severe course (Jin et al., 2020b; Guan et al., 2020).

Cardiovascular manifestations of COVID-19 such as myocarditis, arrhythmias, acute coronary syndromes and venous thromboembolism also dictate hospital admission and ad-hoc treatment (Clerkin et al., 2020). Eventually, a global burden of mental illness has been associated with the current pandemic. Indeed, these unprecedented circumstances have induced adverse psychological outcomes in the general population as well as among healthcare workers, which range from anxiety, depression or fear up to violence and suicidal ideation (Kisely et al., 2020; Rossi et al., 2020).

This paper reviews the available literature, guidelines and guidance models from multiple medical societies until 3rd October 2020. It aims to provide multidisciplinary guidance for hospital clinicians who are currently involved in the management of COVID-19 patients.

Management of COVID-19 Patients in the Emergency Department

Structural, Organizational and Logistical Response to the Pandemic

Emergency Departments (EDs) are playing on a global scale a pivotal role in providing an adequate response to healthcare demand during COVID-19 pandemic. Traditionally, EDs have to both guarantee emergency care to patients hospitalized via pre-hospital emergency services, and triage and treat a vast number of self-presenting patients on a 24/7 basis. In current pandemic times many EDs are experiencing an unprecedented surge of caseload, that can easily overwhelm ED's response capacity, already chronically affected by understaffing, overcrowding, limited resources and poor infrastructures. Moreover, ED's overcrowding during a pandemic can dramatically increase the risk of facilitating and catalyzing the spread of infection among patients and operators. Therefore, now more than ever efforts should be made at all levels to minimize inappropriate ED utilization (Karan, 2020) and to adequately triage patients suspected of SARS-CoV-2 identifying those requiring hospitalization.

The increased demand in health care and the need to guarantee workers and patients safety during epidemic has led to the need of structural, organizational and logistical changes of EDs. Resilience capacity and preparedness of EDs to adapt to the pandemic might be facilitated by the pre-existence of institutional mass casualties' incidents (MCI) protocols. However, several differences exist between MCI and a viral pandemic. Indeed, MCI typically have a finite number of cases concentrated in an initial peak followed by a progressive decline of visits and hospital admissions over time. In addition to that, managing patients during MCI usually does not require the rigid need of standard precautions as during COVID-19 pandemic. Nevertheless, when facing a viral outbreak, prediction models can help hospitals to adapt their response and the deployment of resources (Gagliano et al., 2020; Paganini et al., 2020).

Interestingly, during the ongoing pandemic similar solutions have been adopted by authors working in very different contexts across the globe. Tents or tent-like structures have been widely used in several countries. Typically positioned outside EDs, these structures offer a simple solution to expand ED's spaces and can serve as waiting areas as well as for the initial triage of patients (Chen et al., 2020a; Liang et al., 2020; Paganini et al., 2020).

In currently available literature, there is wide agreement on the necessity of creating two distinct and physically separated pathways for suspected COVID-19 patients (also defined as "dirty" or "red" or "infectious" pathway) from non-COVID-19 patients (often referred as "green" or "clean" pathway) (Chen et al., 2020a; Asperges et al., 2020; Gagliano et al., 2020; Liang et al., 2020; Paganini et al., 2020; Paglia et al., 2020). This can be achieved by readapting other hospital buildings in the hospital or alternatively by expanding ED's space into adjacent repurposed areas. Another possibility is represented by the creation of a filter zone inside ED's pre-existing spaces by using available construction plastic, similar to the barriers in the refrigeration compartment of stores that are suspended to guarantee a droplet barrier (Paganini et al., 2020).

Whatever areas are repurposed to serve as temporary COVID-19 EDs, it is essential to share decisions with all hospital stakeholders and particularly with hospital engineers: in fact, technical interventions may be needed to ensure availability of oxygen and compressed air, and correct functioning of vacuum and electrical circuits (Paganini et al., 2020). Each area of the ED, i.e., the red and green areas, should use dedicated equipment (e.g., ECG, ultrasound and x-ray machines etc.), to avoid risk of contact-related contagion. Moreover, using portable imaging devices will reduce the need to transfer patients through the hospital, thus helping to reduce droplet spreading (Asperges et al., 2020; Gagliano et al., 2020).

Eventually, during the course of the current COVID-19 epidemic some institutions have implemented strategies of technology-based clinical evaluation (Turer et al., 2020; Wittbold et al., 2020). The adoption of such methods of digital care delivery in Emergency departments is showing promising results, as they can help minimizing direct contact between operators and infectious patients, hence increasing operator's safety, at the same time also reducing the utilization of personal protective equipment.

Triage

One of the greatest difficulties encountered by ED's healthcare professionals during the current pandemic is certainly represented by triage. Indeed, COVID-19 patients can present with respiratory syndromes indistinguishable from other

common conditions. Moreover, respiratory symptoms may even be absent, with patients complaining only of fever and/or a number of other systemic symptoms (Lu et al., 2020a). The variegated clinical picture poses a challenge for early detection during triage at the emergency department (ED). Initially, the World Health Organization (WHO) triage recommendation focused on patients with pneumonia and a recent travel history to Wuhan, based on the knowledge of the outbreak at that time (Global Surveillance, 2020). These criteria were broadened from the 27th of February 2020, to include all patients with acute respiratory disease with no alternative etiology and a history of residence in any country reporting current outbreaks (Liang et al., 2020). Interestingly, a recent study from Singapore demonstrated that using broader ED's triage criteria as compared to official recommendations, can increase the sensitivity of detection of COVID-19 cases (Liang et al., 2020). Similarly, many healthcare trusts, hospitals and scientific medical societies worldwide have proposed a various number of different triage criteria (Paglia et al., 2020; RCEM Quality Policy, 2020). A higher sensitivity of screening of COVID-19 patient's at triage will automatically translate into increased inpatient resource utilization, in particular a higher requirement of hospital beds. However, such effort allows to guarantee adequate separation of patients into the most appropriate pathway and reduces the risk of nosocomial transmission of the virus (Liang et al., 2020). In any case, there will always be a risk of nosocomial transmission from asymptomatic COVID-19 patients admitted for other reasons. Hence, each patient accessing the hospital should be considered infectious until proven otherwise and should therefore be provided with a surgical mask (Paglia et al., 2020).

Laboratory Test

A baseline number of laboratory investigations, including full blood count, serum electrolytes, renal and hepatic function and coagulation study should be performed in all suspected COVID-19 cases (Paglia et al., 2020; RCEM Quality Policy, 2020). Patients usually show normal leukocytes count and lymphopenia even though leukopenia and leukocytosis have been reported (Rodriguez-Morales et al., 2020). C-reactive protein, hsTroponin, D-Dimer, serum ferritin and lactate dehydrogenase have shown to have prognostic value in initial studies (Tan et al., 2020; Zhou et al., 2020). Blood cultures and testing for atypical bacteria should also be considered (Turer et al., 2020; Rodriguez-Morales et al., 2020).

Procalcitonin is known to be a useful marker to guide initiation and duration of antibiotic treatment in respiratory infections, and preliminary experience in COVID-19 patients seems to confirm current knowledge (Zhou et al., 2020; Schuetz et al., 2017). In particular, dosage of procalcitonin can help in guiding antibiotic appropriateness and in reducing duration of treatments and antibiotic-related side effects (Schuetz et al., 2017). Pulse oximetry should be performed both at rest and after exercise (i.e., 6-min walking test), because a measurement of oxygen saturation at rest only may not detect an underlying respiratory insufficiency (Paglia et al., 2020; RCEM Quality Policy, 2020; FADOI, 2020). A more accurate and reliable assessment of the respiratory status and oxygen requirement can be easily obtained with an arterial blood gas analysis, which is recommended as a baseline test by several scientific societies and institutions (Paglia et al., 2020; RCEM Quality Policy, 2020; FADOI, 2020). Similarly, nasopharyngeal swabs should be routinely performed in all patients investigated for possible COVID-19 disease, as suggested by WHO guidelines (Paglia et al., 2020; RCEM Quality Policy, 2020; FADOI, 2020). SARS-CoV-2 can be detected 1–2 days before the onset of symptoms in upper respiratory tract samples and usually persist for 7–14 days, although cases of prolonged swab positivity have been reported (World Health Organization, 2020c).

Standard precautions assume that every person is potentially infected or colonized with a pathogen that could be transmitted in the healthcare setting.

The swab is also an effective tool for contact-tracing and it is useful to implement prevention and control measures.

In relation to each nation'|'s testing ability, European Center for disease Prevention and Control recommends nasopharyngeal swab in the following categories (presented in order of importance) (European Center for Disease Prevention and Control (ECDC), 2020a; European Center for Disease Prevention and Control (ECDC), 2020b; European Center for Disease Prevention and Control (ECDC), 2020c):

- Patients hospitalized with severe acute respiratory infection for a better clinical management and to provide the rapid patient isolation and implementation of individual protection measures.
- All cases of acute respiratory infection in hospitalized patients or long-term care facilities in order to draw up a prevention program for dedicated staff and for the early treatment of fragile patients.
- All patients admitted to sentinel hospitals with severe acute respiratory infection in order to assess virus circulation in the population.
- Elderly patients and patients with multiple comorbidities to prevent any worsening of the respiratory picture.

Imaging

Baseline chest radiographs have a sensitivity for the diagnosis of COVID-19 of 69% (Ambrose et al., 2019). As such, chest radiographs are of little diagnostic value in early stages and its routine use as a screening tool in the early course of the disease is not recommended, except in very exceptional resource-constrained environments (Salehi et al., 2019; Rubin et al., 2020). The main role of chest radiographs is played for assessing disease progression in hospitalized patients, bacterial superinfection, pneumothorax and pleural effusion (Salehi et al., 2019).

Chest CT has a very high sensitivity (97%) for the diagnosis of COVID-19 disease, but a low specificity (i.e., 25–56%), due to overlapping of imaging features of other viral or atypical pneumonia or with non-infectious diseases, such as vasculitis, dermatomyositis (Jin et al., 2020a; Caruso et al., 2020; Kooraki et al., 2020; Tao et al., 2020).

The main findings of COVID-19 patients on both chest radiographs and CT include bilateral pneumonia in the majority of hospitalized patients, with the most common

pattern being ground-glass opacities (GGO) with peripheral distribution and predominant involvement of the lower lung zones (Ambrose et al., 2019; Rodriguez-Morales et al., 2020). CT findings of COVID-19 pneumonia vary with time (Bernheim et al., 2020), from single or multiple focal GGO in the early stage, followed by multiple scattered patchy or agglomerated ground-glass opacities that may progress to multiple patchy consolidations (Jin et al., 2020a). In addition, CT angiography can play a role in identifying pulmonary embolism, whose occurrence seems to be higher in COVID-19 patients and it should be suspected especially with evidence of high D-dimer levels (Helms et al., 2020). However, it may be logistically difficult to follow up hospitalized patients with multiple CT scans.

Although there is limited experience at this time on lung ultrasound (LUS) in COVID-19 patients, abundant literature supports the utility of lung ultrasound for a variety of respiratory conditions, including ARDS (Chiumello et al., 2018; Mojoli et al., 2019). This imaging technique offers some advantages over CT: it can be used in the ED or in the prehospital setting for a rapid triage of suspected cases (rule-in/rule-out) and therefore aid decision making for "red" or "green" pathway; it can help to quantify the severity of the disease, thus allowing for prognostic stratification; it can be repeated on patients admitted to hospital to monitor the progression of the disease and efficacy of therapeutic measures (Soldati et al., 2020a); it can be used to diagnose or rule out pneumothorax at the bedside, which is a potential complication of non-invasive and invasive ventilation (Carron, 2020). The main LUS findings in COVID-19 patients are thickening and irregularities of the pleural line, B lines in a variety of patterns including (focal, multifocal, and confluent), consolidations and pleural effusions (the latter two mainly observed in case of superimposed bacterial pneumonia) (Peng et al., 2020).

Nevertheless, lung ultrasound should be performed by experienced physicians whose competencies have been objectively evaluated, and technique and reporting should be standardized as much as possible to facilitate reproducibility between physicians (Soldati et al., 2020b; Di Pietro et al., 2020).

All the above investigations, together with a thorough physical examination and history, will help the emergency physician to stratify the severity of COVID-19 patients and will aid decision making on admission and discharge. Based on current evidence and recommendations, patients should be discharged only when showing no signs of respiratory insufficiency and no requirement of oxygen, i.e., when normal arterial blood gas and saturation both at rest and after physical effort can be demonstrated. Beside the latter investigations, physicians should attentively observe and report the mechanics and work of breathing (Paglia et al., 2020; RCEM Quality Policy, 2020; FADOI, 2020).

Treatment and Palliative Care

Available treatments for the management of COVID-19 cases, including modalities of oxygen administration and ventilation as well as pharmacological interventions, will be discussed further below in this review.

In addition to therapeutic interventions, EDs and other wards involved in the care of COVID-19 cases should set up high-quality palliative care pathways to ensure adequate and compassionate end of life care. This should ideally be accomplished through a multidisciplinary cooperation involving experts from relevant specialties (Fausto et al., 2020; Hendin et al., 2020).

In-Hospital Infection Control

As a suspected or confirmed COVID-19 patient enters the hospital, prevention of infection spread must be assured. In this regard, the use of Personal Protective Equipment (PPE) is essential for healthcare personnel (HCP), together with general hygiene rules (such as emphasized hand hygiene) (Interim Infection Prevention, 2020).

Ideally, suspected cases should be isolated as soon as possible in separated and well-ventilated areas, preferably a private room with door closed and a private bathroom. Airborne Infection Isolation Rooms (AIIRs) should be used for aerosol generating procedures, however their availability is limited in many hospitals (Saravia et al., 2007). Other interventions, such as cancellation of elective surgical procedures and the implementation of telemedicine-based strategies can help to diminish the number of people accessing the hospital (https://www.cms.gov/files/document/cms-non-emergent-elective-medical-recommendations.pdf; https://www.ama-assn.org/system/files/2020-05/state-elective-procedure-chart.pdf).

A Multiorgan Disease: Pulmonary Involvement of COVID-19 Infection

COVID-19 is characterized in the majority of cases by a mild respiratory disease, while in approximately 15% of cases a severe pneumonia is observed. The latter can progress to bilateral multifocal pneumonia, leading in 5% of total cases to ARDS, sepsis and septic shock (Wu and McGoogan, 2020).

During the incubation and in non-severe stages, a specific adaptive immune response is activated to eliminate the virus, but the development of this response can be possible if the host is healthy and with an appropriate genetic background (e.g., HLA). Conversely, when immune response is impaired, the virus will propagate and massive destruction of the affected tissues will occur, especially in organs that have high ACE2 expression (Shi et al., 2020). The damaged cells induce innate inflammation in the lungs that is largely mediated by pro-inflammatory macrophages and granulocytes. Lung inflammation is the main cause of life-threatening respiratory disorders at a severe stage (Xu et al., 2020b).

As such, we can distinguish different stages of disease progression with different clinical syndromes:

Early infection phase the initial inflammatory response may cause in about 85% of cases mild illness with local or non-specific systemic symptoms such as fever (88–99% of cases), fatigue (38–70%), dry cough (59–68%), anorexia (40%), myalgias (15–35%), dyspnea (19–31%), sputum production (27–34%). Gastrointestinal symptoms (nausea, diarrhea), rhinorrhea, sore throat and pharyngalgia have also been reported (Lechien et al., 2020; Tinku, 2020). These patients usually show no hypoxia on blood gas analysis (BGA), present with a respiratory rate (RR) less than 22 breaths/minute (b/m) and a negative chest radiograph. Most of them do not progress beyond this phase and their

management should be assigned to general practitioners (GP) (Lechien et al., 2020; Lopes et al., 2020; Tinku, 2020).

Pulmonary phase SARS-CoV-2 shows on its surface a glycoprotein that binds angiotensin-converting enzyme 2 (ACE2), a receptor located on type 2 pneumocytes. Through this way the virus infiltrates the lung parenchyma and begins to proliferate (Li and Ma, 2020). Increased levels of ACE2 were found in SARS-CoV-2 infected cells, suggesting that ACE2 is also involved in post-infection regulation, including immune response, cytokine secretion, and viral genome replication (Li and Ma, 2020). About 15% of infected individuals develop a severe pneumonia with ARF requiring hospitalization and oxygen support. This group of patients need to be closely monitored as some of them may further exacerbate and develop a severe hyperinflammatory response (Tinku, 2020).

Hyperinflammatory phase in patients with severe clinical manifestation of COVID-19 a cytokine storm syndrome (CSS) may occur. The hallmark of CSS is an uncontrolled activation and amplification of the host immune system induced by SARS-Cov-2 infection, causing a systemic massive release of proinflammatory cytokines such as TNF-a, IL-1, IL-6 due to the lysis of cells (Heimfarth et al., 2020).

COVID-19 patients with severe symptoms exhibit an extreme decline in total $CD4^+$ and $CD8^+$ T cells in their circulation: IL-6 may induce apoptosis of T cells through the Fas/FaL pathway, while TNF-a and IFN-I may promote the attachment and retention of T cells in lymphoid organs (Fouladseresht et al., 2020).

CSS could also cause an increase in vascular permeability, resulting in severe damage of the alveolar cells and consequently development of acute respiratory failure (Leiva-Juárez et al., 2018; Zhang et al., 2020b).

Acute respiratory distress syndrome (ARDS) can be observed in these patients, which is characterized by several features, among which a P/F ratio <200 on BGA, increasing of RR above 30 b/m and bilateral opacities at imaging (Vernuccio et al., 2020). An in depth discussion of ARDS will be presented later in this review.

Bronchoscopic procedures SarS-CoV-2 can be detected on 93% of bronchoalveolar lavage samples, thus showing a high sensitivity (Wang et al., 2020b). However, its routinary use has been discouraged due to the high risk of contagion to healthcare professionals (Wang et al., 2020b). Nevertheless, bronchoscopy should be considered in specific circumstances such as massive hemoptysis, acute foreign body aspiration, severe central airway obstruction, neutropenic fever with infiltrates and no clinical diagnosis or improvement (Pritchett et al., 2020).

Oxygen and Ventilatory therapy According to the ITS- AIPO-SIC document (Harari et al., 2004) patients should be divided into four groups according to their respiratory status:

(1) green: SaO 2 > 94%, RR < 20 b/m: if no ARF on BGA, no oxygen needed;
(2) yellow: SaO2 <94%, RR > 20 b/m: oxygen supply (up to 10–15 L/min) improves saturation;
(3) orange: SaO2 <94%, RR > 20 b/m: poor response to oxygen 10–15 L/min and needing high flow nasal oxygen (HFNO), continuous positive airway pressure (CPAP), NIV with very high FiO2;
(4) red: SaO2<94%, RR > 20 b/m: no response to all previous treatments or presenting respiratory distress with PaO2/FiO2 <200 and needing endotracheal intubation (EI).

O2 saturation and RR should be re-evaluated no more than 2 h after therapy initiation and subsequently every 6 h (if target saturation and RR values are met and the patient remains stable) (Harari et al., 2004). High flow nasal cannula (HFNO) may be used as a bridge between oxygen and CPAP (continuous positive airway pressure) trial although this technique generates a relatively high amount of droplets (Harari et al., 2004). Ideally, CPAP should be delivered via a full-face non-vented mask, together with an expiratory viral filter and exhalation port; alternatively, an helmet can be used (as second choice) (Harari et al., 2004). Recommended values for positive end expiratory pressure (PEEP) are between 10 and 15 cmH2O (Harari et al., 2004; NHS Specialty Guides, 2020).

NIV should be used with a full-face non-vented mask and double circuit. Suggested initial settings are PS 8–10 cmH2 0 + 60–100% FiO2. NIV should be considered for hypercapnic respiratory failure or to prevent hypercapnia in COPD patients (NHS Specialty Guides, 2020). Ideally, this should be delivered with a full face non-vented mask and a double circuit, using values of pressure support between 8 and 10 cmH20 (Harari et al., 2004; NHS Specialty Guides, 2020).

In order to improve patient's comfort and compliance, administration of low doses of opioids can be considered. Humidification is generally discouraged, as it increases the quantity of droplet generation (NHS Specialty Guides, 2020).

Early intubation is mandatory if the patient does not respond adequately to CPAP or NIV (hypoxemia with P/F < 150–175 after 1 h of CPAP/NIV in absence of BGA improvement, RR > 30 b/m; SAPS score >34, intolerance to ventilation, clinical decline) (NHS Specialty Guides, 2020; Antonelli et al., 2001).

Preliminary experience with self-proning of awake non-intubated patients has shown promising results in terms of improving oxygenation levels, although these findings and the safety of the procedure need to be confirmed in further trials (Caputo et al., 2020).

Pharmacological Treatment

Numerous studies have been conducted to find potential curative agents against COVID-19 disease, and many trials are still ongoing. Researcher's attention has been mostly directed towards drugs with direct antiviral activity and to those with immune-modulating or immune-suppressive effects.

Among the antiviral agents, Remdesivir (200 mg loading dose on day 1, followed by 100 mg daily for up to nine additional days) has been demonstrated to be superior to placebo in shortening the time to recovery in adults hospitalized with COVID-19 and evidence of lower respiratory tract infection (Beigel et al., 2020).

A large United Kingdom multicentric study has investigated the role of dexamethasone in hospitalized COVID-19 patients. Investigators have demonstrated a reduction in the 28 days mortality in the intervention group that received 6 mg of

dexamethasone (oral or intravenous) for up to 10 days (Horby and Lim, 2020). Interestingly, a subgroup analysis showed that the effects are more pronounced in patients mechanically ventilated or with high oxygen requirements as compared to those with no oxygen requirement (Horby and Lim, 2020). These findings suggest that dexamethasone plays an important role in the modulation of the excessive immune response observed in some cases of COVID-19 (see above *Hyperinflammatory phase*).

The efficacy of several other drugs have been investigated, such as tocilizumab, azithromycin, hydroxychloroquine, however results have been inconclusive (Oldenburg and Doan, 2020; Sanders, 2020; Skipper et al., 2020).

Numerous randomized controlled trials are currently being conducted to assess the efficacy of convalescent plasma (Li et al., 2020a). Current evidence suggests the safety of this therapeutic strategy and has shown promising results, therefore its use has been approved by the FDA and in several european countries (Li et al., 2020b; Shen, 2020).

A Multiorgan Disease: Gastroenterologic Involvement in COVID-19

Gastrointestinal symptoms (GIS), as nausea, vomiting, abdominal pain and diarrhea, may be an early manifestation of SARS-CoV-2 infection (Wang et al., 2020a; Jin et al., 2020b; Huang et al., 2020). In fact, Huang et al. (2020) reported that GI involvement was present in 2–10% of patients with COVID-19. A systematic review evaluating GI involvement reported that the presence of GI symptoms had a great variability between 2 and 100%; in particular, according to a pooled analysis, 16.1% presented GIS, 8.3% diarrhea, 12% nausea-vomiting and 4% abdominal pain (Pamolona et al., 2020). Sometimes, GI symptoms may precede respiratory ones (Wang et al., 2020a). Some studies suggest that the presence of GIS may indicate a higher probability of a severe course (Jin et al., 2020b; Guan et al., 2020). A higher percentage of diarrhea was observed in patients with severe disease (5.8%) as compared to patients with a mild course of the disease (3.5%) (Guan et al., 2020). As for other organs, also in the GI system the ACE2 receptor plays a fundamental role. This protein, in fact, is expressed in gastric, intestinal and colonic cells, promoting virus infection (Wan et al., 2020a). Therefore, once the virus infects the human intestinal epithelia, it can potentially propagate via fecal-oral route (Wang et al., 2020a; Guan et al., 2020; Pamolona et al., 2020). Interesting, viral RNA is detected in the stool for a longer time than in the respiratory system (Pan et al., 2020; Wu et al., 2020b). Consequently, it has been suggested that its detection in fecal samples should be considered as one of the routine diagnostic tests to guide decision making on hospital discharge and the lifting of isolation measures (Pamolona et al., 2020).

A Multiorgan Disease: Cardiac and Cardiovascular Involvement of COVID-19 Infection

The myocardial tissue and the cardiovascular (CV) system can be affected by COVID-19 infection through a variety of mechanisms with an important role played by inflammatory cytokines (ESC Guidance, 2020). The main CV manifestations observed are myocarditis, cardiomyopathies, arrhythmias, acute coronary syndromes (STEMI and NSTEMI) and venous thromboembolism which can lead to acute heart failure with cardiogenic shock (Clerkin et al., 2020). Their occurrence is associated with an increased risk of inhospital mortality, so it is crucial to identify these patients as soon as possible. In COVID-19 infection, the severe hypoxia with subsequent increase of circulating catecholamines and the activation of T cells with an abnormal cytokines release (mainly IL-6 and IL-17) lead to oxidative stress and endothelial dysfunction with a consequent microangiopathy, vasospasm and myocardial ischaemia even in absence of coronary lesion. In addition, activation of the immune system leads to plaque instability in coronary arteries leading to coronary lesions, acute myocardial injury and arrhythmias as a consequence (Xu et al., 2020b; Madjid et al., 2020).

It has been reported a high prevalence of CV comorbidities (hypertension, atrial fibrillation (AF), DM, chronic heart failure (CHF) and kidney failure) in COVID patients. In a retrospective analysis carried out on 138 COVID-19 patients in Wuhan, one or more CV comorbidities were found in 50% of cases at least, rising 72% in severe cases (Zhou et al., 2020), with hypertension playing the main role (Wang et al., 2020a; Liu et al., 2020a; Zhou et al., 2020). This detail is important considering that ACE-2 receptor (located also in lungs, heart and vessels) is a part of the renin angiotensin system (RAS) and plays a main role in the development of COVID-19 CV involvement. SARS-CoV-2 infection appears to cause a loss of regulation of the RAS system, leading to upregulation of ACE-2 (Li, 2018; Walls et al., 2020; Zhou et al., 2020). This hypothesis might explain the datum of high prevalence of pre-existing hypertension in COVID-19 patients in ACEinhibitor (ACE-I) or angiotensin receptor blocker (ARBS) treatment, whose cardiac and vascular cells show a major expression of ACE-2 receptors compared to patients who do not assume these drugs (Walls et al., 2020; Zhou et al., 2020). In addition, ACE-2 up-regulation can also cause a direct myocardial injury secondary to an increased catecholamine level (Walls et al., 2020; Zhou et al., 2020).

As expected, common symptoms of CV involvement are represented by chest pain, breathlessness, tachycardia (ESC Guidance, 2020) and other varying signs and symptoms depending on the particular CV manifestation.

Myocardial injury might be due to myocarditis, characterized by infiltrates of interstitial mononuclear inflammatory cells (Xu et al., 2020b) and to a mismatch between oxygen supply and demand [type 2 classification according to the Fourth universal Definition (Thygesen et al., 2018)]. This second option may be secondary to the primary infection, hemodynamic and respiratory derangement.

A clinical and electrophysiological manifestation of myocarditis are arrhythmias, which have been reported in 16,7% of total patients and in 44% of ICU ones (Wang et al., 2020a). Sinus tachycardia is often linked to hypoxemia. The most common arrhythmia seems to be atrial fibrillation (new-onset or permanent with higher rate) which often appears in patients with

electrolyte disturbances, ischaemia or acute cor pulmonale (Huang et al., 2020). New onset atrial fibrillation has been associated with higher mortality (Walkey et al., 2014; Boriani et al., 2019). There have been recognized different causes for arrhythmias genesis: first, via cross-talk between immune cells and myocardial cells, resulting in fibrosis that creates slow conduction areas; second, via leukocytes interacting with conduction system cells; third, via antibodies and cytokines causing ionic channels dysregulation. Another CV manifestation is heart failure (HF) which holds the worst presentation and prognosis (Li et al., 2020c; Zhou et al., 2020). It can be due to different mechanisms, such as acute myocardial infarction, myocarditis, acute kidney damage, hypovolemia, dehydration with hypovolemia, Takotsubo cardiomyopathy and ARDS with hypoxemia (Guan et al., 2020). In rare cases, myocarditis may have a fulminant presentation (with cardiac symptoms, haemodynamic deterioration, arrhythmias, elevation of biomarkers and suggesting imaging) (Liu et al., 2020b). HF could evolve in cardiogenic shock.

Laboratory tests in patients at high risk of mortality show high levels of Troponin T, IL-6 (Zhou et al., 2020) and DDimer (Walkey et al., 2014). In addition, the dynamic variations of Troponin I and proBNP have to be considered to identify high risk patients (Liu et al., 2020a). Indeed, persistent elevation and dynamic changes of Troponin I is an independent risk factor of mortality especially in patients with previous cardiovascular diseases (Liu et al., 2020a). BNP and NT-proBNP are usually elevated in patients with severe respiratory distress but they may also express cardiac injury in COVID-19 patients (Christ-Crain et al., 2008). Finally, if D-Dimer level is >1,000 ng/dl it may indicate the presence of pulmonary embolism or disseminated intravascular coagulation in COVID-19 patients (Chen et al., 2020b).

In spite of what has been reported until now, multiple studies have found that the incidence of hospitalization for acute MI has decreased as much as 40–50% during the pandemic (De Filippo et al., 2020; Solomon et al., 2020).

This evenience could have two possible explanations: a patient avoidance of medical care secondary to the fear of being infected if hospitalized and redistribution of health care.

Treatment in patients with suspected SARS-CoV-2 infection and acute myocardial infarction, STEMI primary PCI might be postponed up to 60 min than the usual delay (120 min) in order to set all the protective measures; behind this delay, fibrinolysis should be considered. In acute myocardial infarction NSTEMI, cardiac CT should be considered for risk stratification in patients at intermediate and low risk. In patients with chronic coronary syndrome, aspirin should not be stopped because of its anti-inflammatory effect (Rauch, 2020). Statin therapy may be interrupted considering the elevated liver enzymes in some COVID-19 patients and the possible rhabdomyolysis occurring as an adverse event of statins (Xu et al., 2020a).

Hypertension treatment with ACEIs and ARBS is a subject of debate. On one side these drugs could increase the expression of ACE2 receptors, raising the risk of COVID-19 infections (Hamming et al., 2004; Chen et al., 2020f; Hoffmann et al., 2020); on the other side, studies on animal models have shown a protective role of ARBs for lungs affected by some viruses (Rodrigues Prestes et al., 2017). Therefore, right now, there is no evidence of benefit or harm by those drugs, consequently they should not be discontinued or contraindicated (Poissy et al., 2020).

The incidence of pulmonary embolism in COVID-9 patients is reported to be high (Danzi et al., 2020; Poissy et al., 2020) and all COVID-19 patients admitted in hospital should start anticoagulation at prophylactic dose. If clinical and radiological findings confirm pulmonary embolism an appropriate treatment should be started, represented by thrombolysis for patients in shock, anticoagulation with unfractionated heparin, LMWH for stable patients. Regarding NOACs, an interaction with COVID drugs (such as lopinavir/ritonavir via Cytochrome P450) has to be considered, causing an increased bleeding risk. Therefore, it is reasonable to consider to substitute NOAC with LMWH. Vitamin K antagonists should be discontinued and substituted with heparin and only considered in particular conditions such as mechanical valves implant (Guan Yap, 2003).

In COVID-19 patients with arrhythmias management and treatment are influenced by the clinical presentation and considering drug interactions. If allowed by haemodynamic conditions, antiarrhythmic drugs for AF and atrial flutter should be discontinued, due to interactions with azithromycin; therefore, minimal dosage of beta-blockers and calcium channel blockers should be preferred to gain rate control. Otherwise, in case of hemodynamic instability, electrical cardioversion does not seem effective in COVID-19 patients without treating underlying conditions (hypoxaemia, hypokalaemia, hypomagnesaemia, acidosis). When ventricular tachycardia, ventricular fibrillation, AF or atrial flutter occur in unstable patients' amiodarone can be considered the safest drug, due to his property to not cause QT dispersion. Sotalol and flecainide should not be administered.

QT prolongation, ventricular fibrillation, Torsades de Pointes (TdP) and sudden death are rarely due to a single administration of a drug (Chen et al., 2020e), and even when arrhythmias occur, they often disappear on their own. Hydroxychloroquine - which was mainly used in the early stages of the pandemics - causes significant QT prolongation in association with azithromycin, increasing the incidence of cardiac arrhythmias at 31% (Zhao et al., 2020a). In patients with ventricular tachycardia and QT prolongation electrical, cardioversion and lidocaine represent the treatment of choice especially in patients with antiviral therapy. Drugs inducing QT prolongation should be stopped in patients with QTc >500 ms (550 ms in presence of bundle branch blocks) or an increase >60 ms from the baseline ECG; negative chronotropic drugs (beta-blockers, digoxin, ivabradine and calcium channel blockers), inducing bradycardia, prolong QT interval and their interactions with antiviral drugs need to be monitored.

A Multiorgan Disease: Involvement of the Nervous System in COVID-19 Infection

Coronaviruses (CoVs) (including also SARS-CoV-2) may invade the central nervous system (CNS) causing neurological diseases.

Indeed, in order to gain cell entry, as it has already said, the virus binds to the ACE2 receptor which is also expressed in neurons, vascular endothelial and glial cells (Zhao et al., 2020a).

Two main routes through which the SARS-CoV-2 invades the nervous system have been proposed. Firstly, the dissemination of SARS-CoV-2 in the systemic circulation during an early or later phase can determine cerebral involvement (Baig et al., 2020). Secondly, increasing evidence shows that CoV may first invade peripheral nerve terminals and then gain access to the CNS via a synapse connected route (Li et al., 2012; Li et al., 2020d). Through the trans-synaptic transfer, CoV can access to the brainstem (including the nucleus of the solitary tract and the nucleus ambiguous, which have a fundamental role in control of heart and lung' function) and this can worsen the dysfunction of the respiratory system (Netland et al., 2008). Nevertheless, this hypothesis has been debated due to the fact that brain failure usually gives a pattern of respiratory failure different from that seen in patients with COVID-19 (Turtle, 2020).

Together with the acute pneumonia and severe respiratory distress symptoms, many patients with COVID-19 complain of neurological disturbances, ranging from headache, hyposmia, ageusia, muscle pain to conscious disturbance, skeletal muscle injury and seizures. Mao et al. (2020) reported that 36% of patients with a severe infection presented various neurologic manifestations involving CNS, PNS and skeletal muscles, mostly in old patients. Some of these neurologic symptoms might be foreseeable. Indeed, it is not uncommon that during an infective disease with high fever patients, especially the older, can manifest seizures. It has also been supposed that the severe hypoxia secondary to acute respiratory distress syndrome can enhance brain damage, being therefore the main reason for CNS involvement (Li et al., 2020d). As far as epilepsy is concerned, clinicians have to be careful in choosing the correct treatment in COVID-19 patients. In fact, they have to consider pharmacological interactions between antiepileptic drugs (AEDs) and COVID-19 drugs (Liverpool Drug Interaction Group, http://www.covid19-druginteractions.org/). The same interactions can underlie seizures occurrence in epileptic patients even if in appropriate treatment. For example, cases reported the association of seizures with chloroquine therapy in systemic lupus erythematosus patients (Krzeminski et al., 2018).

Another neurologic manifestation is delirium. Moreover, as far as COVID-19 is concerned, the use of total-body personal protective equipment by medical staff, artificial light, closed wards, isolation and the absence of relatives can exacerbate and early arise delirium symptoms. Because its presence is associated with a devastating impact in outcomes for critically ill patients it should be promptly recognised and treated, according to current guidelines (Burry et al., 2019). Medical treatment for delirium includes not only supportive medical care and non-pharmacological intervention (which, as said before, in the contest of COVID ward can be difficult), but also antipsychotic drugs (e.g., haloperidol, olanzapine and quetiapine), which need to be used with caution due to the QTc prolongation and their interaction with COVID-19 drugs. Adequate pain identification and management, both in ICU and non-ICU setting, is crucial in order to prevent this manifestation which itself is a robust prognostic indicator of worse survival immediately (Kotfis et al., 2020).

Alongside, cerebrovascular system is also involved, as reported from the description of strokes (both in the setting of critical illness and during hypotension), coagulopathy and antiphospholipid antibodies in patients with COVID-19 (Zhang et al., 2020a) and acute hemorrhagic necrotizing encephalopathy (Poyiadji et al., 2020).

Neurologist should also expect the occurrence of post infectious syndromes such as acute disseminated encephalomyelitis and Guillain-Barré syndrome; the latter has been described even if, actually, it is not known if it is a consequence or a coincidence of SARS-CoV-2 infection, because real-time polymerase-chain-reaction assay of the CSF was negative for SARS-CoV-2 (Toscano et al., 2020; Zhao et al., 2020b). An important fact is the time of onset, which is essential to distinguish acute polyneuropathy with COVID-19 from critical illness neuropathy and myopathy, which usually appear later in the course of intensive care unit recovery.

In addition (and differently from SARS infection), olfactory and taste disorders hold a special interest due to the fact that they have been complained of during the incubation period while sudden onset sensorineural hearing loss has been reported during the course of the Covid-19 (Koumpa et al., 2020; Guan et al., 2020). An Italian cross-sectional survey described that 20% of patients presented olfactory and taste disorders before the hospital admission and only 13% during the hospital stay; interestingly patients with these symptoms were younger than those without (Giacomelli et al., 2020). The exact pathogenesis of ageusia and anosmia is still unknown: it might be due to a direct damage inside the olfactory bulb from the coronavirus or it might express only the classical congestion which is seen also in other viral infections. Interestingly, COVID-19 patients do not report nasal obstruction, differently from flu.

Apart from the suspected neurotropism of SARS-CoV-2, neurologists are concerned about the impact that the infection can have in patients with chronic neurologic diseases (e.g., previous stroke or other neurodegenerative disorder) or in patients with diseases that need immune-modulatory drugs (for example multiple sclerosis, myasthenia gravis, and neuromyelitis optica). In the latter case, if taking off immune-modulatory drugs is not advisable due to the catastrophic complications that this can set off, a possible intervention is to reassess treatment, both in dosage and in frequency of infusion (e.g., natalizumab and fingolimod for multiple sclerosis) (Bomprezzi and Pawate, 2014; Ghezzi, 2019). Time-dependent treatment of acute patients (namely for ischemic stroke) should also be reorganized with the aim to appropriately deal with it and to not increase disability in human beings (Khosravani et al., 2020).

Finally, even if it is known that the most severe neurologic complication occurs later and in more severe patients, with the growing knowledge about SARS-CoV-2 infection, big data, strenuous surveillance and global cooperation in recognizing other acute or post-infectious conditions are needed in order to deal with this challenge in the possible best way.

A Multiorgan Disease: Psychiatric Implications of COVID-19 Pandemic

Maintaining a satisfactory mental health is a delicate balance that COVID-19 pandemic has undermined for the general population, health care workers, psychiatric patients and patients with COVID-19. During lockdown, the general population have experienced adverse psychological outcomes, such as anger, anxiety, boredom, confusion, fear, depression, emotional exhaustion, frustration, irritability, stress, avoidance behaviour and subthreshold symptoms of alcohol use disorder (Brooks et al., 2020; Pfefferbaum and North, 2020). Excessive concern for the pandemic with distressing somatic symptoms, detachment from others, post-traumatic stress disorder (PTSD), violence and suicidal ideation have also been described (Brooks et al., 2020; Pfefferbaum and North, 2020). Cross-sectional, self-report surveys from January to april 2020 found that these symptoms were clinically significant present in up to 36% of adults (Wang et al., 2020c). Among healthcare workers—who are at high risk of exposure—psychiatric problems, such as significant psychological stress and acute and/or PTSD were more common in workers exposed to the virus than in those who were not (Kisely et al., 2020). In particular, anxiety was present in 12–20%, depression in 15–25%, insomnia in 8% and traumatic distress in 35–49% (Rossi et al., 2020). Among patients with pre-existing psychiatric illness, infection with SARS-CoV-2 may exacerbate the pre-existing illness (Holmes et al., 2020). In addition to respiratory symptoms, COVID-19 patients may present neuropsychiatric syndrome in the acute phase of the illness, such as confusion and impaired consciousness, anxiety (35%) and depression (28%) (Rogers et al., 2020). The pathogenesis of psychiatric symptoms in previous healthy patients may include biologic and psychosocial factors. In fact, it is known that a combination of systemic infection, viral neurotropism and environmental stress facilitates induces development of psychiatric pathologies (Kisely et al., 2020). The "cytokine storm" secondary to viral infection, with high levels of circulating cytokine (IL-6, IL-1β, IL-2, TNF- α), is responsible of symptoms from apathy, motor inhibition to obsessive compulsive disorder, PTSD and schizophrenia (Steardo et al., 2020).

A Multiorgan Disease: Ocular Involvement of COVID-19 Infection

Ocular Findings and Early Diagnosis

According to recent reports, the only ocular clinical manifestation in patients with COVID-19 is acute viral conjunctivitis (Chen et al., 2020d; Wu et al., 2020a; Xia et al., 2020). SARS-CoV-2, as described by Wu et al. (2020a), can cause ocular involvement (32% of 38 COVID-19 patients) and sometimes it may represent the first symptom of COVID-19 disease. The acute nonspecific viral conjunctivitis is characterized by conjunctival hyperemia, chemosis, epiphora, foreign body sensation, tearing and secretions. Chen et al. (2020d) identified in a patient with COVID-19 the signs of the viral conjunctivitis through slit lamp examination: bilateral moderate conjunctival injection, watery discharge, inferior palpebral conjunctival follicles and tender palpable preauricular lymph nodes. Treatment is the same as common viral conjunctivitis. Ocular findings were found in patients with high levels of leukocytes, neutrophils, procalcitonin, CRP and lactate dehydrogenase suggesting a correlation between ocular involvement and a severe disease form (Wu et al., 2020a).

Moreover, in patients with positive nasopharyngeal swabs for SARS-CoV-2 conjunctival swab was performed resulting positive only in a small part of patients (5%) with conjunctivitis (Wu et al., 2020a). Additionally, Vinores et al. (2001) evaluated the tear and conjunctival secretions of COVID-19 patients with RT-PCR and only one swab on 30 tested positive for SARSCoV- 2.

Based on these results, SARS-CoV-2 can cause ocular complications and in some cases may represent the first symptom of disease, even if is not a common manifestation. Early screening of SARS-CoV-2 in patients with conjunctivitis by searching the virus in the tears and conjunctival secretions may be conceivable. However, since the viral RNA levels in conjunctival specimens are dramatically lower than those in respiratory samples (Chen et al., 2020d), the conjunctiva might not serve as an ideal site for early diagnostic tests of SARS-CoV-2 infection.

Regarding other ocular complications, since coagulation disorders are also common in patients with SARS-CoV-2 infection, recent studies have linked coronavirus infection with retinal disorders, such as microangiopathy (Invernizzi et al., 2020b), hemolytic uremic syndrome with retinal vessel occlusion (Greenwood, 2015) and impending central retinal vein occlusion (Invernizzi et al., 2020a). Marinho et al. (2020) reported an alteration of inner retinal layers, such as hyperreflective lesions, based on optical coherence tomography (OCT) scans. However some authors suggested a possible misinterpretation of these findings, which may represent an individual variability of normal retinal vessels (Vavvas et al., 2020). The "Screening the retina in patients with COVID-19" study (SERPICO-19) showed the presence of retinal findings in patients with COVID-19, including retinal haemorrhages (9.25%), cotton wools spots (7.4%), drusen (11.1%), dilated veins (27.7%) and tortuous vessels (12.9%) (Invernizzi et al., 2020b). However, concerns may be raised about the presence of bias in the sample enrolled, given the high prevalence of hypertension and diabetes in the cohort, which make these findings as possible incidental findings.

Further clinical studies are needed to evaluate the clinical spectrum of ocular diseases caused by SARS-CoV-2. Moreover, since ACE-2 is a cellular receptor for SARS-CoV-2 (Lu et al., 2020b) detected in the human retina (Wagner et al., 1996; Senanayake et al., 2007), a possible involvement of the internal ocular structures such as the retina cannot be excluded.

Transmission Through the Ocular Surface

The role of the eye in transmitting human SARS-CoV-2 is still under discussion.

Some authors have underlined that the transmission through the ocular surface should not be underestimated, since infectious droplets can easily contaminate the human conjunctival epithelium (Lu et al., 2020a). The detection of the SARS-CoV-2 in tears and conjunctival secretions confirms this hypothesis (Chen et al., 2020d; Wu et al., 2020a; Xia et al., 2020). However,

the low prevalence of SARS-CoV-2 in the ocular surface of patients with conjunctivitis and the absence in patients without ocular signs could mean that tears and conjunctival secretions of COVID-19 patients are not a common infectious route for SARS-CoV-2. Nevertheless, the risk of transmission could not be completely eliminated. As reported by Chen et al. (Chen et al., 2020d) the viral loads in conjunctival specimens of COVID-19 patients gradually decrease over time with less potential for transmissibility accompanied by improvement of the ocular symptoms. Therefore SARS-CoV-2 in conjunctival specimens may represent a source of spread, especially in the acute stage of ocular complications characterized by high viral load. Qing et al. (2020) stressed the role of lacrimal drainage as a route of SARS-CoV-2 transmission. Anatomically, the ocular surface and upper respiratory tract are connected by nasolacrimal duct. Therefore, it is possible that the virus reaches the tears through droplets, passing through the nasolacrimal ducts and then into the respiratory tract.

Precautionary Measures Needed for Physicians

Containing viral spread is the primary means by which we protect people from newly emerging infections (Sommer, 2020). Ophthalmologists are a high-risk category, not only because they have close contact with patients during the examination (conjunctival, tear secretions and aerosol secretions), but also because their daily outpatient clinic and emergency lists have a high patient volume (Lai et al., 2020a; Romano et al., 2020). In order to minimize transmission of COVID-19, some precautionary measures are mandatory for physicians when coming into contact with suspected or confirmed cases of COVID-19 (Lai et al., 2020a; Lai et al., 2020b; Li et al., 2020a; Mungmungpuntipantip and Wiwanitkit, 2020; Romano et al., 2020). These measures include:

- Protection of health workers with appropriate PPE: protective eyewear can prevent direct inoculation of respiratory droplets through the conjunctiva, and also indirect contamination of conjunctiva through inadvertent eye rubbing with a contaminated hand. During eye examination, a self-made transparent polycarbonate protector mounted to the slit lamp offers a physical barrier between the patient and physician (Wan et al., 2020b). Non-contact air-puff tonometry has been associated with a micro-aerosol formation (Wan et al., 2020b); therefore, other ways of intraocular pressure measurement, such as i-Care tonometry or Goldmann applanation tonometry should be used instead
- Appropriate environmental control: important to reduce the concentration of virus on contaminated surfaces. Considering that coronavirus can persist on inanimate surfaces up to 9 days (Kampf et al., 2020), it is crucial to perform an appropriate sanitation of the potentially contaminated environment. Equipment must be cleaned and disinfected after every clinic session.
- Reorganization of the workflow to minimize the risk of cross infections: non-urgent consultations and operations should be delayed. Urgent consultations (ocular trauma, acute glaucoma, retinal detachment, alkali chemical injury, etc.) should be attended with adequate PPE.

Management of COVID-19 Patients in the Intensive Care Unit

Hospitalization in ICU is required in about 5% of COVID-19 patients who can rapidly progress to ARDS, MOF, sepsis and septic shock. The primary reason for ICU admission is the patient's need for endotracheal intubation and mechanical ventilation (Grasselli et al., 2020).

COVID-19 patients mainly are affected by respiratory system failure whereas other organ functions are less involved. The most frequent clinical evolution during the hyperinflammatory phase is the development of ARDS. Nevertheless, not all the cases of severe ARF are considered as typical ARDS. For this reason, Marini et al., called ARDS COVID related as C-ARDS. There are differences between COVID-19-related ARDS and ARDS caused by other factors as defined by Berlin criteria, and, therefore, there are also differences in the treatment (Li and Ma, 2020).

ARDS can be classified on Berlin criteria in (The ARDS Definition Task Force, 2012):

- *Mild ARDS:* 200 mmHg < PaO2/FiO2 ≤300 mmHg (with PEEP or CPAP ≥5 cmH2O, orunventilated).
- *Moderate ARDS:* 100 mmHg < PaO2/FiO2 ≤200 mmHg (with PEEP ≥5 cmH2O, or unventilated).
- *Severe ARDS:* PaO2/FiO2 ≤100 mmHg (with PEEP ≥5 cmH2O, or unventilated). When PaO2 is not available, SpO2/FiO2 ≤315 suggests ARDS (including unventilated patients) (Circolare Ministeriale, 2020; World Health Organization, 2020a; European Center for Disease Prevention and Control, 2020; Ranieri, 2012; Arabi, 2020; World Health Organization, 2020b; Wax, 2020; BPC-PDTA, 2020).

Invasive Mechanical Ventilation

Patient selection for invasive mechanical ventilation (IVM) is clinically based on severe hypoxemia and dyspnea in patients previously treated by non invasive ventilation (NIV) or continuous positive airway pressure (CPAP) and, most of the time, the timing of IVM is very important.

Most of the patients in intensive care units shows the same clinical findings of acute respiratory distress syndrome but in some cases they do not have the same response to protective ventilation.

Mechanical ventilation in COVID-19 patients results in different respiratory patterns which can be challenging.

In april 2020, Marini and Gattinoni (2020) laid out a conceptual model to underline the role of a possible endothelial damage that disrupts pulmonary vasoregulation leading to a ventilation-perfusion mismatch and thrombogenesis. The endothelial damage could clinically translate into a particular pattern characterized by hypoxemia with normal pulmonary compliance, findings uncommon for ARDS patients (Gattinoni et al., 2020a).

This discrepancy between pulmonary compliance and hypoxemia may lead to different ventilation settings based on

the interactions between different factors: the phase of infection, the host response, and the time of NIV/CPAP.

The result of this interaction lead to a time-related disease spectrum within two primary "phenotypes" named (Gattinoni et al., 2020b): Type "L" patients, with Low elastance, Low ventilation to perfusion ratio, low lung weight, and low recruitability; IVM in this type of patients is aimed to minimize pulmonary stress, reduce hypoxemia and interrupt the vicious cycle that may lead to a ventilator-induced lung injury (VILI) (Marini and Gattinoni, 2020); Once intubated and sedated, these patients, present a good tolerance to Tidal Volume (TV) 7–8 ml/kg and they are low responsive to PEEP; a worsening of clinical symptoms and signs might be related with the negative intrathoracic pressure associated and the increased tidal volume in spontaneous breathing (Gattinoni et al., 2020a). Also, prone positioning should be used only as a rescue maneuver. Type "L" patients can evolve towards the phenotype "H". Type "H" patients are characterized by High elastance, High right-to-left shunt, High lung weight, and High recruitability. Type H patients should be treated as severe ARDS, including protective lung ventilation setting and higher PEEP, prone positioning and extracorporeal support (Brochard et al., 2017). The aim of mechanical ventilation in Type H patients is to minimize lung stress and ventilation-perfusion mismatch (Marini and Gattinoni, 2020).

Type L and Type H patients are best identified by CT scan and are affected by different pathophysiological mechanisms. Considering these assumptions, invasive ventilatory approach should be evaluated and above all, differentiated both in acute respiratory failure and in post acute phase. A clinical protocol should be applied in each COVID-19 center in order to differentiate patients that need invasive ventilation treatment and, above all, to choose which patients would benefit from invasive ventilation in relation to the stage of the disease and patient phenotype (Marini and Gattinoni, 2020).

Hemodynamic Support in Septic Shock

In septic COVID-19 critical patients, the illness is characterized by an organ dysfunction caused by a dysregulated response of the host to suspected or certain infection, with Sequential [Sepsisrelated] Organ Failure Assessment (SOFA) score of two points or more (Singer et al., 2016). The signs of organ dysfunction include altered mental status, difficult or rapid and superficial breathing, low oxygen saturation, oliguria, tachycardia, weak pulsations, cold extremities or hypotension, skin alterations, laboratory findings of coagulation alterations, thrombocytopenia, acidosis, elevated lactates or hyperbilirubinemia.

These COVID-19 critical patients may evolve to septic shock, defined as hypotension unresponsive to volume expansion, which requires vasopressors to maintain MAP ≥65 mmHg and serum lactate level ≥2 mmol.

The frequency of septic shock varies from 20 to 35% in ICU among patients affected by COVID-19 (Wang et al., 2020a; Yang et al., 2020). In some studies, the development of fulminant myocarditis has been possibly the dominant reason for 40% of ICU deaths (Ruan et al., 2020). Other studies also advise that risk factors to consider are older age comorbidities like diabetes and cardiovascular diseases including hypertension, lower lymphocyte count, higher D-dimer level, or possible cardiac injuries (Wang et al., 2020a; Yang et al., 2020).

The two mainstays of hemodynamic treatment have been increasing intravascular volume with fluids and by counteracting hypotension, as well as low cardiac output with vasoactive drugs with varying inotropic properties. The use of dynamic assessment should guide fluid therapy and it may reduce mortality, duration of mechanical ventilation and ICU length of stay (LOS). Within their respective limitations, the functional hemodynamic parameters which should be used to guide fluid therapy as part of goal directed therapy strategies are parameters such as stroke volume variation (SVV), pulse pressure variation (PPV). In contrast, assessing fluid responsiveness with passive leg raising manoeuvre, central venous pressure (CVP), and mean arterial pressure (MAP) may result in false-negative cases.

Moreover, early lactate clearance-directed therapy (even though a high lactate level does not always imply hypovolemia) may be linked to a reduction in mortality and LOS in ICU, when compared to the central venous oxygen saturation (ScVO2) guided therapy (Pan et al., 2019).

Fluid therapy used to correct circulatory failure is elementary and cheap. However, there are no indications that the fluids should be carefully prescribed in order to maximize their result or limit their side-effects. The use of dynamic assessment to guide fluid therapy has reduced both mortality and duration of mechanical ventilation (Bentzer et al., 2016; Bednarczyk et al., 2017). Although a review that compared restricted to liberal fluid volumes in the initial resuscitation of patients with sepsis has not found any statistically significant variation in mortality or serious adverse events (Meyhoff et al., 2020), we recommended an initial conservative approach to fluid resuscitation in COVID-19 patients with shock. There is no outcome that preferred the use of colloids when compared to the use of crystalloids in critically ill patients (Lewis et al., 2018). Knowing that some colloids are harmful, they are more expensive, and their availability can be limited. Therefore, we recommend the use of crystalloids for fluid resuscitation in COVID-19 patients with shock, using buffered/balanced crystalloids over the unbalanced ones, instead of choosing hydroxyethyl starches, gelatines, or dextrans. Also, the regular use of albumin for initial resuscitation is not linked to improved outcomes (Lewis et al., 2018).

The best first-line treatment on COVID-19 patients with shock is norepinephrine, alternatively, vasopressin or epinephrine should be considered (Gamper et al., 2016; Moller et al., 2016). Dopamine should be avoided, as it increases the arrhythmias risks. The targeted therapy based on the standard of care MAP targeted of 60–65 mmHg, titrating the vasoactive agents is recommended (Moller et al., 2018); moreover, it is also suggested to add a second-line agent (vasopressin) if the target is not achieved by norepinephrine itself (Honarmand et al., 2020). Furthermore, based on a physiological reason, the use of dobutamine in COVID-19

patients with shock and cardiac dysfunction, should be considered (Moller et al., 2018).

If available, Guideline recommends that all patients who require vasopressors have an arterial catheter placed as soon as practical.

For adults with COVID-19 and refractory shock, it is recommended the use of low-dose corticosteroid therapy ("shock-reversal") over no corticosteroid. A typical corticosteroid regimen in septic shock is intravenous hydrocortisone 200 mg per day administered either as an infusion or intermittent doses. The duration of hydrocortisone therapy is usually a clinical decision (https://www.covid19treatmentguidelines.nih.gov/critical-care/hemodynamics/; Rhodes et al., 2016; Bednarczyk et al., 2017).

Patient's Step-Down From the Intensive Care Unit

COVID-19 patients may be stepped-down from ICU to medical wards (or ad-hoc COVID-19 wards) when they show a non-critical condition and an improvement of clinical features and radiologic findings. The aims of the non-ICU department include the weaning from oxygen or from the use of CPAP/NIV or helmet CPAP, the prosecution of treatment of bacterial superinfection eventually contracted in ICU, the prevention of possible complication of Sars-CoV-2 infection, the follow-up of patients until hospital discharge. Whenever possible, patients should be discharged from hospital after recovery confirmed by the double consecutive negative swabs (Procedura Regionale Nuovo Coronavirus Sars, 2020).

Most patients admitted to ICU have a prolonged length of stay (on average 3 weeks), therefore requiring adequate rehabilitation once stepped-down to medical wards (Procedura Regionale Nuovo Coronavirus Sars, 2020). Despite the progressive clinical improvement of the respiratory disease, prolonged bed rest syndrome and invasive mechanical ventilation sequelae (such as iatrogenic post-intubation dysphagia, tracheostomy management) have been reported. Hence, it is advisable to promote a rehabilitation program into the non-ICU department (aerobic exercise, strength training for muscle weakness, bronchial clearance techniques in hyper-secretive patients) and to direct the frailest patients with severe sequelae to rehabilitation units (Brugliera et al., 2020).

Hospital Discharge

Different rules have been developed to decide whether or not patients should be discharged home after hospitalization. Generally, independently from the ICU or non-ICU stay, two main strategies are indicated (CDC Discontinuation, 2020):

Test-based strategy:

Resolution of fever without the use of fever-reducing medications and improvement in respiratory symptoms (e.g., cough, shortness of breath), and negative results of a COVID-19 molecular assay for detection of SARS-CoV-2 RNA from at least two consecutive nasopharyngeal swab specimens collected ≥24 h apart (total of two negative specimens).

Non-test-based strategy:

At least 3 days (72 h) have passed since recovery defined as resolution of fever without the use of fever-reducing medications and improvement in respiratory symptoms (e.g., cough, shortness of breath); and at least 7 days have passed since symptoms first appeared.

It is therefore specified, in accordance with what is outlined by the CDC, that meeting criteria for discontinuation of transmission-based precautions is not a prerequisite for discharge.

In Italy, a COVID-19 patient is considered cured after the resolution of symptoms and two negative tests for SARS-CoV-2 at 24-h intervals. In patients who clinically recover before 7 days after onset, an interval of 7 days between the first and the final test is recommended. For virus clearance it is defined as a negative viral RNA from body fluids of symptomatic and asymptomatic patients, accompanied by the appearance of specific IgG (Ministero della salute, 2020).

As a precautionary measure, in several countries patients are told to self-isolate once discharged from the hospital, even in case of swab negativity (Ministero della salute, 2020). Serological testing performed at time of discharge can provide important information on the immune response of infected individuals (Ministero della salute, 2020).

Nursing Role During COVID-19

In the setting of hospital care, all healthcare workers, including nurses, technicians, and drivers have played an important and variegated role during pandemic months. In regard to nurses, they helped doctors not only in treating COVID-19 patients, but also in supplying nosocomial infection prevention and surveillance (Chen et al., 2020c). Moreover, they provided health and screening education and support for the general population and high-risk categories (Chen et al., 2020c).

CONCLUSIONS

The COVID-19 pandemic has challenged healthcare systems on a global scale, requiring that hospitals make a significant effort to repurpose their services and healthcare delivery. As the pandemic has progressed, clinicians have developed a greater understanding of the multifaceted nature of COVID-19 disease, as well as its myriad presentations not limited to the respiratory tract. Given the complex nature of this new condition, assessment and treatment of hospitalized patients should involve the expertise of a range of specialties. Knowledge-sharing between specialists is undoubtedly required to determine the timing and setting in which proven treatments should be administered to manage patients suffering from COVID-19.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the review of literature and to the production of this manuscript.

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SYSTEMATIC REVIEW
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Repurposing Approved Drugs for Guiding COVID-19 Prophylaxis: A Systematic Review

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The SARS-CoV-2 outbreak originally appeared in China in December 2019 and became a global pandemic in March 2020. This infectious disease has directly affected public health and the world economy. Several palliative therapeutic treatments and prophylaxis strategies have been used to control the progress of this viral infection, including pre- (PrEP) and post-exposure prophylaxis. On the other hand, research groups around the world are still studying novel drug prophylaxis and treatment using repurposing approaches, as well as vaccination options, which are in different pre-clinical and clinical testing phases. This systematic review evaluated 1,228 articles from the PubMed and Scopus indexing databases, following the Kitchenham bibliographic searching protocol, with the aim to list drug candidates, potentially approved to be used as new options for SARS-CoV-2 prophylaxis clinical trials and medical protocols. In searching protocol, we used the following keywords: "Covid-19 or SARS-CoV-2" or "Coronavirus or 2019 nCoV," "prophylaxis," "prophylactic," "pre-exposure," "COVID-19 or SARS-CoV-2 Chemoprophylaxis," "repurposed," "strategies," "clinical," "trials," "anti-SARS-CoV-2," "anti-covid-19," "Antiviral," "Therapy prevention *in vitro*," in cells "and" human testing. After all protocol steps, we selected 60 articles that included: 15 studies with clinical data, 22 studies that used *in vitro* experiments, seven studies using animal models, and 18 studies performed with in silico experiments. Additionally, we included more 22 compounds between FDA approved drugs and drug-like like molecules, which were tested in large-scale screenings, as well as those repurposed approved drugs with new mechanism of actions. The drugs selected in this review can assist clinical studies and medical guidelines on the rational repurposing of known antiviral drugs for COVID-19 prophylaxis.

Keywords: SARS-CoV-2, prophylaxis, antiviral, drug repurposing, COVID-19

INTRODUCTION

The SARS-CoV-2 outbreak originally appeared in China in December 2019 and became a global pandemic in March 2020 (Xie et al., 2020). This infectious disease made a direct impact on global public health, and is still impairing the world economy (World Health Organization, 2020). In order to minimize and prevent the advance of COVID-19 and its effects, the world scientific community has been doing an unprecedented race in many research fields, resulting in many discoveries in viral biology, disease physiopathology, and new more effective and cost-beneficial therapeutic options to be used for the treatment of people affected by the new virus (Vellingiri et al., 2020).

Prophylactic drugs can be used both to block the pathogen's infectious cycle and/or to boost host immunity (Glushkov et al., 1999). There are two main categories of prophylaxis (I) pre-exposure prophylaxis (PrEP), which considers that treated individuals that had no contact with the pathogen (II) post-exposure prophylaxis (PEP), which includes individuals that may have been infected (e.g. contact with patients) but have not exhibited the disease symptoms (Zhang et al., 2020). These two models of prophylactic studies have been extensively used in endemic viral pathologies with high transmissivity, such as HIV (Krakower et al., 2015). Additionally, both methods exhibited success for other viral diseases with great global health impact (Mayer et al., 2015). PrEP and PEP have proven to be extremely effective strategies in viral transmission control for patients inside certain risk groups, such as those with comorbidities, and health professionals directly exposed to the risk of acquiring and transmitting Covid-19 (Rockstroh et al., 2020).

Prophylactic antiviral treatment is an important approach for rational drug administration since it can be used to block the disease evolution and to spread and reduce the risks of side and adverse effects, as well as toxicity in patients (Beigel et al., 2019; Cheng, 2019). Because of SARS-CoV-2 high degree of transmissivity, novel therapeutic ways that can reach the affected patients faster became necessary (Kang et al., 2020). Among many therapeutic strategies, drug repurposing has been reaching significant results against some pathogens (Cheng et al., 2016; Cheng, 2019). Drugs approved for human diseases can be repurposed for new targets in order to speed up the process of implementing these compounds in clinical protocols for the treatment and prophylaxis in the acute phase of viral diseases. Moreover, this approach is instrumental in preventing the viral transmission to healthy individuals (Zhou et al., 2020).

Currently, several preclinical studies, such as *in silico*, *in vitro* and *in vivo* trials have been guiding clinical decisions in choosing the best drug options for the treatment and prophylaxis against SARS-CoV-2 (Fragkou et al., 2020). Therefore, different drug classes with prophylactic properties have been repositioned in order to guarantee protection against viral transmission (Zhou et al., 2020). This could lead to an interesting strategy targeting COVID-19 since it can be used as an additional barrier to viral spreading, as well as preventing disease evolution, especially for patients inside the risk groups. In this review, we present a systematic analysis of the main antiviral drug agents for many diseases, which can be proposed as new prophylaxis in clinical trials against SARS-CoV-2 infection and other therapeutic interventions.

MATERIAL AND METHODS

This systematic review was conducted in five stages: planning, bibliographic search, initial selection, final selection, summary of data and results. All of these steps were performed based on the bibliographic search protocol model developed by Kitchenham (2004). We used two indexing databases for the bibliographic search: PubMed (www.ncbi.nlm.nih.gov) and Scopus (www.scopus.com), in order to retrieve papers related to the proposed theme of this review, and considered publications until June 30, 2020. On the PubMed database searching, we considered 38 strings with the terms: "Covid-19," "SARS-CoV-2," "Coronavirus," "2019 nCoV" and "Prophylaxis," combined with 38 drug names. The Scopus database search produced eight strings, constructed using the following words: prophylaxis, prophylactic, pre-exposure, COVID-19, SARS-CoV-2, Chemoprophylaxis, repurposed, strategies, clinical, trials, anti-SARS-CoV-2, anti-covid-19, antiviral, therapy prevention *in vitro*, in cells, and human testing. The detailed steps on sorting publications are described in the **Supplemental Material S1**.

The publications retrieved were imported to MEDLINE (PubMed) and BIBTEX (Scopus) formats, and submitted to the StArt (State of the Art through Systematic Review) program v. 3.3 Beta 03 (Fabbri et al., 2016), developed by the Federal University of São Carlos (UFSCar), and available for download on http://lapes.dc.ufscar.br/tools/start_tool. Furthermore, we excluded duplicated records, and then all the preselected publications were entered into an Excel spreadsheet model for the next steps.

The initial selection of publications was based on the inclusion and exclusion criteria described in the following protocol. Initially, all the review articles, case studies, clinical guidelines, research strategies, short communications and unfinished studies were excluded. In a second phase, we included only publications of *in vitro*, *in vivo* research, and randomized clinical studies on the use of drugs for pre- and post-exposure prophylaxis for the treatment of COVID-19. Additionally, until the end of the review process, we included novel pre-clinical and clincal SARS-CoV-2 drug repurposing studies as a way of complementing the discussion about drug prophylaxis against COVID-19.

The corresponding metadata of used drugs for each study, test phases (I, II, and III), number of patients, cell lineages used in *vitro* tests, as well as *in vivo* test details were extracted from each accepted publication, and this information is presented in the **Supplemental Material S2**, as well as all 1,288 reference articles used in this work are reported in the **Supplemental Material S3**.

Additional information about drug efficacy, half-life, toxicity, interactions, and side effects were obtained from the public domain database Drugbank (https://www.drugbank.ca/).

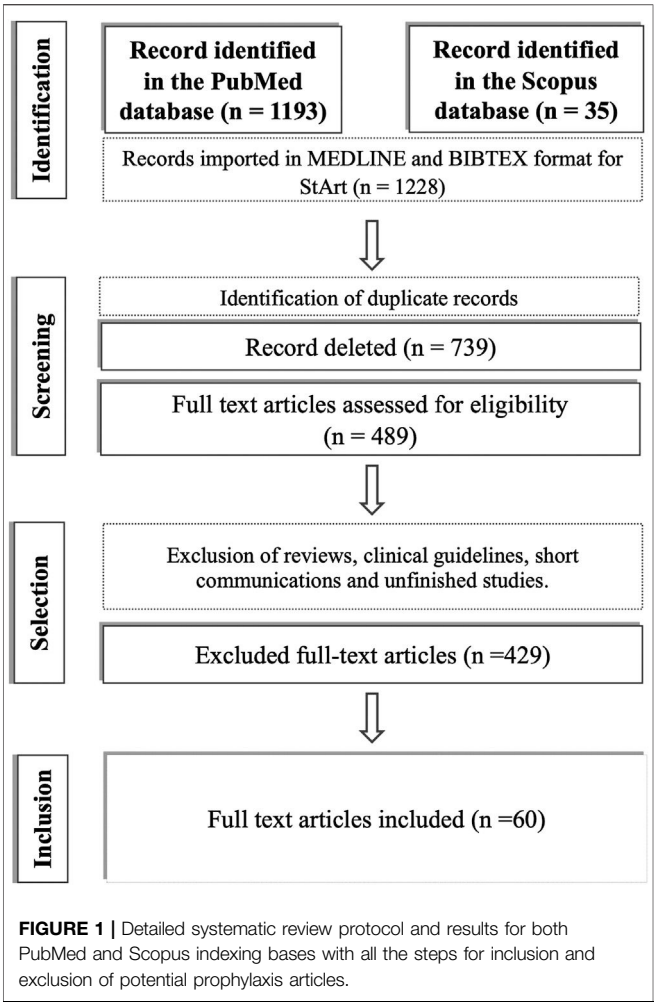

FIGURE 1 | Detailed systematic review protocol and results for both PubMed and Scopus indexing bases with all the steps for inclusion and exclusion of potential prophylaxis articles.

RESULTS

In this review, we reported a significant number of articles with human clinical studies, *in vivo* animal experiments, *in vitro* cell studies, and *in silico* approaches, mainly for drug repurposing strategies. Furthermore, we evaluated literature material of 1,228 article records. The screening, selection, and exclusion processes of all the publications are detailed in **Figure 1**.

After all the filtering steps included in our systematic protocol, we selected 60 articles to describe possible prophylaxis options for preclinical and clinical studies against SARS-CoV-2. The analysis of articles' contents indicated that: 15 studies were done with clinical data; 22 studies used *in vitro* approaches against pathogenic virus strains responsible for airway and pulmonary infections, such as influenza and SARS-CoV-2; seven studies used animal models; and 18 studies performed *in silico* experiments against viral targets. Furthermore, we included 37 complementary articles discussing 23 drug mechanism of action as additional prophylaxis options.

Usually, the studies did not discuss drug half-life, as well as other pharmacokinetic parameters, such as Cmax, Tmax, and renal clearance. All the drug doses reported were those used for daily treatment for parasitic and viral infections. On the other hand, by using the information from the *in vitro* tests, it is possible to predict a range of inhibitory concentrations, which could assist in extrapolating the concentration parameters in clinical studies with humans. Nonetheless, extrapolation of plasma dose and concentration should be assessed and monitored in blood plasma, as prophylactic studies have shown that some broad-spectrum antiviral drugs should be administered in concentrations greater than those provided for clinical protocols. Thus, it is possible to create more effective therapeutic responses against COVID-19; however, increasing doses and adjustment may induce potential adverse and side effects, as well as toxic and drug interactions, which has not been reported in most of the clinical studies considered in this review. All the reported drugs for the accepted papers are included in **Table 1**.

After completing the table, we generated a word map that reflects most important drugs and terms according to the frequency they appear in all the evaluated articles (**Figure 2**), as well as we showed a worldwide research distribution map (**Figure 3**) with all the antiviral drugs cited in the **Table 1**. Despite other terms, such as hydroxychloroquine and some immune, antibody, and anti-parasitic drugs also appeared in this word map, we only considered drugs with antiviral action. Furthermore, these other terms can be explored in other review and research studies.

DISCUSSION

In this section, we make a brief description of each selected drug from **Table 1**, and their mechanisms of action in order to support a possible repurposing of these approved drugs as new candidates on clinical trials studies as antiviral options against SARS-CoV-2, mainly for prophylaxis but not only restricted to it.

Amantadine is an M2 (Matrix protein 2) viral membrane protein inhibitor, necessary for the efficient release of the viral genome during virus entry (Jing et al., 2008). This drug has been used in the prophylactic or symptomatic treatment of influenza A but also acts as an antiparkinsonian (Lamb et al., 2005). The main hypothesis indicates that amantadine could interfere with the gene expression of endosomal cysteine protease (cathepsin L or B) in SARS-CoV-2 (Smieszek et al., 2020).

Amodiaquine is an aminoquinoline antimalarial drug, which has been used in other antiviral studies as a protease inhibitor, such as DENV2 and West Nile virus NS2B-NS3 protease using BHK-21 and Vero cells (Boonyasuppayakorn et al., 2014; D'alessandro et al., 2020), and Ebola virus (EBOV) by blocking viral replication in Huh seven and Vero E6 cells with IC50 = 2.8–3.2 μM and 9.5–11 μM, respectively. Additionally, amodiaquine present a synergic effect against viral replication of the SARS-CoV-2 in Vero E6 cells when it is combined with nelfinavir, and it presents higher synergic index when copared with other antimalarial drugs such as chloroquine, hydroxychloroquine, quinacrine and mefloquine (Ianevski et al., 2020). One theoretical pharmachophore modeling study published in Chemrxiv reinforces a possible action of this

TABLE 1 | Screened drugs with potential for prophylaxis studies, and their correspondent number of citations and mechanism of action.

	Drug	Citations	Mechanism of action
1	Aciclovir	World Health Organization (2020)	Nucleoside analog
2	Amantadine	World Health Organization (2020)	Interferes with transmembrane M2 protein
3	Amprenavir	World Health Organization (2020)	Protease inhibitor (HIV)
4	Baloxavir marboxil	Xie et al. (2020)	Endonuclease inhibitor—inhibits the initiation of mRNA synthesis
5	Darunavir	Zhang et al. (2020)	Second generation protease inhibitor
6	Entecavir	World Health Organization (2020)	Guanine analogue (HCV)
7	Faldaprevir	World Health Organization (2020)	HCV protease inhibitor
8	Favipariviir	Beigel et al. (2019)	Prodrug of a purine nucleotide, favipiravir ribofuranosyl-5′-triphosphate—RNA polymerase inhibitor
9	Galidesivir	Mayer et al. (2015)	Protease inhibitor—Adenine analog
10	GS-441524	Xie et al. (2020)	Adenisin nucleoside analog
11	Indinavir	World Health Organization (2020)	HIV protease specific inhibitor
12	Lopinavir	Beigel et al. (2019)	Aspartic acid protease (HIV) inhibitor
13	Nelfinavir	Vellingiri et al. (2020)	Protease inhibitor
14	Oseltamivir	Vellingiri et al. (2020)	Active neuraminidase inhibitor
15	Pleconaril	Xie et al. (2020)	Viral capsid inhibitor
16	Remdesivir	Cheng (2019)	Prodrug—active nucleoside analog C-adenosine triphosphate—(Ebola)
17	Ribavirin	Kang et al. (2020)	Nucleoside analogue (guanine)—inhibits viral RNA-dependent RNA polymerase
18	Sofosbuvir	Glushkov et al. (1999)	Nucleoside analog—hepatitis C virus NS5B polymerase inhibitor
19	Tenofovir	Glushkov et al. (1999)	Acyclic nucleoside analog adenosine monophosphate
20	Tipranavir	Xie et al. (2020)	HIV protease enzyme inhibitor
21	Umifenovir	Zhou et al. (2020)	Hemagglutinin inhibitor (influenza)
22	Zanamivir	World Health Organization (2020)	Neuraminidase inhibitor

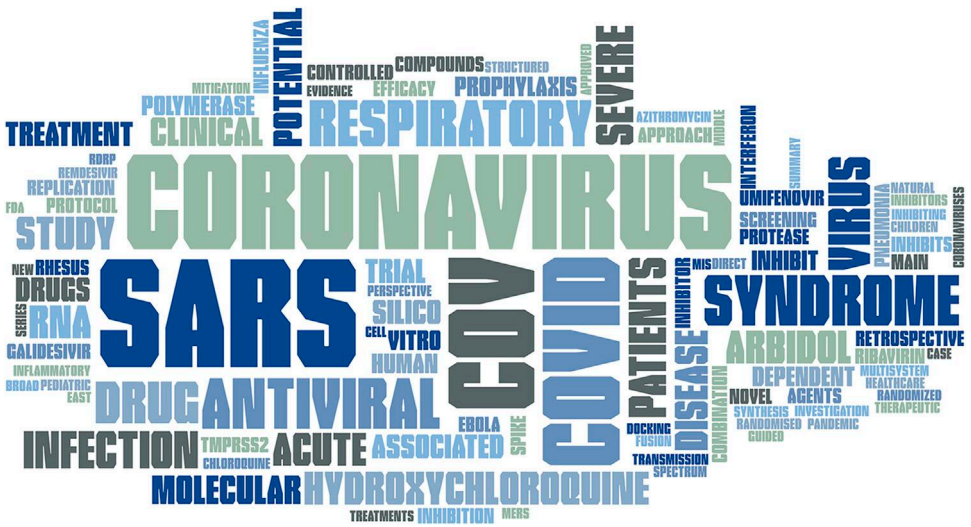

FIGURE 2 | Word map reflecting the most cited terms for all the evaluated articles used in the review processes.

aminoquinoline inhibitor against the SARS-CoV-2 virus, with a mechanism of blocking its main protease with a theoretical Ki of 0.073 μM(Acharya, 2020).

Amprenavir is a known inhibitor of the HIV protease enzyme, acting on the prevention of the gag-pol polyprotein cleavage and resulting in the formation of immature and non-infectious viral particles (Fung et al., 2000). Generally, HIV protease inhibitors are used in combination with at least two other anti-HIV drugs (Sadler et al., 2020). Recent *in vitro* studies demonstrated that amprenavir exhibited a considerable degree of inhibition against SARS-CoV-2 (Mugisha et al., 2020).

Apilimod is a known interleukin-13/23 production inhibitor by acting on the phosphatidylinositol-3-phosphate 5-kinase (PIKfyve) enzyme (Cai et al., 2013), as well is a safety drug for humans with a profile at doses of up to 125 mg twice daily and a peak serum concentration of 0.265 ± 0.183 μM (Huang et al., 2020). This drug was tested in *vitro* studies against EBOV and Marburg virus (MARV) using Vero E6, Huh seven cells and macrophages (hMDMs), and it was found that this inhibitor was capable of blocks both viral infection in all cell types (Nelson et al., 2017), as well as blocked EBOV particle entry (Nelson et al., 2017; White et al., 2018) and trafficking in cell cytoplasm (Nelson et al.,

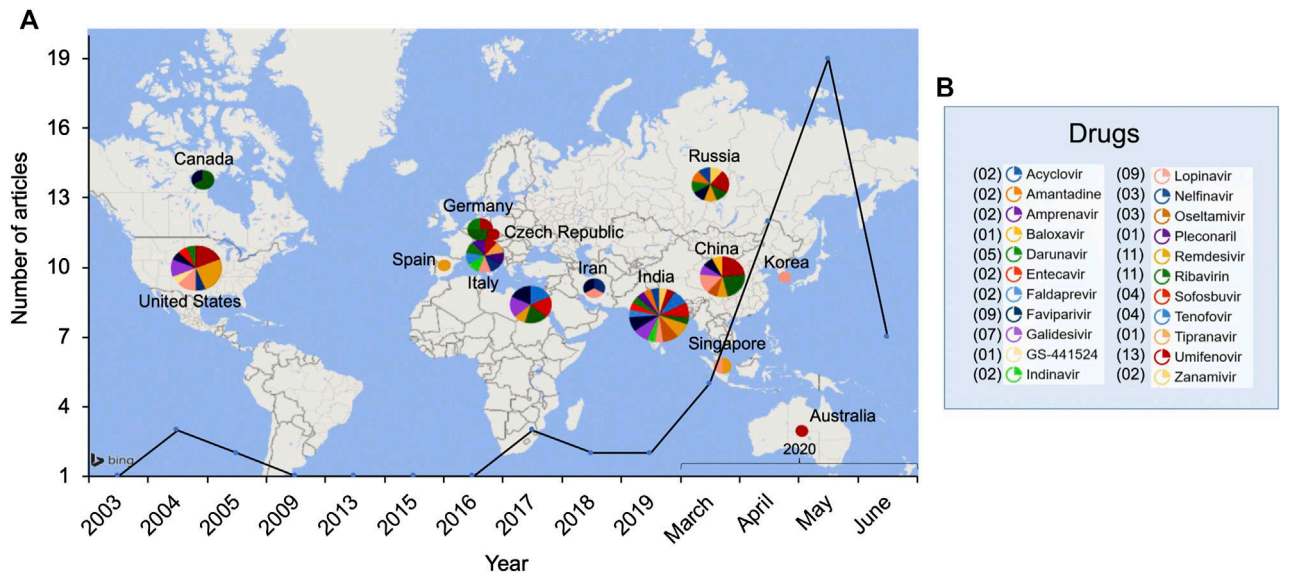

FIGURE 3 | Antiviral research distribution for the 22 selected drugs. **(A)** Most relevant countries for the selected drugs between 2003 and 2020. **(B)** Number of times cited per drug.

2017), with low IC_{50} values (Nelson et al., 2017; White et al., 2018). Additionally, apilimod has blocked Zaire ebolavirus (ZEBOV) and SARS-CoV-2 *in vitro* replication by acting on the PIKfyve kinase and reducing the intracellular trafficking of viral particles, as well as viral entry using Vero E6 cells with IC_{50} of 10 nM (Kang et al., 2020). Furthermore, a large-scale drug repurposing study apilimod was responsible for blocking viral replication in human pneumocyte-like cells derived from induced pluripotent stem cells with IC_{50} raging from 0.088 to 0.012 μM, as well as exhibited antiviral activity in a primary human lung explant model (Huang et al., 2020). Other study with Vero E6 cell viral replication monitored by QRT-PCR assays, indicated that apilimod potentially decreased the amount of SARS-CoV-2 RNA in cell culture supernatants, with an IC_{50} of 12 mM and low cell toxicity (Shema Mugisha et al., 2020).

Baloxavir marboxyl is an influenza A and B antiviral, which inhibits the cap-dependent endonuclease necessary for viral replication, and this is the first representative of influenza-type PB2 inhibitors. Baloxavir is under investigation in the clinical trial NCT04327791 since March 2020, and it has been used in a combined therapy with oseltamivir 1 with hospitalized patients with influenza infections (Reina and Reina, 2019).

Darunavir is an HIV protease inhibitor used against HIV infection, especially indicated to patients with a previous antiretroviral therapy history (Lu et al., 2020). *In silico* studies indicated that this drug could function as a protease inhibitor, as well as also interact with the 3C-like proteinase. Additionally, darunavir can also bind to the proteins of the SARS-CoV-2 replication complex (Beck et al., 2020). *In vitro* data for this drug showed potential for SARS-CoV-2 inhibition (Yamamoto et al., 2020). On the other hand, darunavir has not showed antiviral activity against SARS-CoV-2 at clinically relevant concentrations yet (De Meyer et al., 2020), but four other clinical trials are between phases 3 and 4 in order to evaluate the efficacy, safety, and pharmacokinetic characteristics of this drug in combination with other antiviral and anti-parasitic compounds against COVID-19 (Ciesek, 2020; Elmekaty, 2020; Lu, 2020; Gilead Sciences, 2020).

Entecavir is a guanine analogue that directly inhibits the replication process of hepatitis B virus (HBV) by blocking its reverse transcriptase mechanism (Huynh et al., 2020; Shah et al., 2020). This drug was considered a possible inhibitor of both RNA-dependent RNA polymerase and the main protease enzyme, from SARS-CoV and SARS-CoV-2 viruses (Shah et al., 2020).

Faldaprevir is a known Hepatitis C virus (HCV) NS3-4A protease inhibitor (HCV) (Kanda et al., 2015; Kanda et al., 2015). Its effectiveness was also confirmed when combined with other drugs such as pegylated interferon alfa-2a and ribavirin for chronic HCV infection treatment (Sulkowski et al., 2013). *In silico* experiments indicated a possible mechanism of action of faldaprevir for inhibiting the new coronavirus enzymes (Böcher et al., 2020; Shah et al., 2020), suggesting that this drug could be tested in the next preclinical and clinical trials.

Faviparivir is a pyrazine analogue that acts as a prodrug and inhibits the RNA-dependent RNA polymerase (RdRp) and, consequently, blocks viral transcription and replication (de Farias et al., 2017; Furuta et al., 2017). This drug was approved for therapeutic use in Japan in 2014 for influenza viruses. Nevertheless, because RdRp catalytic domain is conserved among several types of RNA viruses, its mechanism of action supports a wide spectrum of viral targets such those against Ebola and, more recently, SARS-CoV-2. (Furuta et al., 2017; Madelain et al., 2017; Raoul, 2020).

Galidesivir is an adenosine analog acting through the mechanism of inhibiting the viral RNA polymerase (Zhang et al., 2020). In this case, the nucleoside analog (NA) is incorporated into the viral RNA to exhibit its antiviral activity. Moreover, another mechanism of action is the recognition of this nucleotide analog as a substrate by the viral RNA polymerase, which blocks the RNA replication. This drug was initially used for treating HCV infections, but it has been reported as also efficient against Ebola, Zika and Yellow Fever viruses (De Clercq, 2019; Elfiky, 2020). Other NAs, such as remdesivir, favipiravir, and ribavirin were reported to have efficacy against SARS-CoV-2 by blocking RdRp activity (Zhang et al., 2020).

GC376 is a dipeptidyl bisulfite adduct salt that has been early used in cell-based inhibition assays against the picornavirus-like supercluster (picornaviruses, caliciviruses, and coronaviruses), with its mechanism of action on the inhibition of viral 3C-like proteases (Kim et al., 2012). This drug was used in a clinical study with a fatal coronavirus infection, caused by the feline infectious peritonitis virus (FIPV), and the authors have reported that GC376 significantly reduced viral load and symptoms, decreased the viral RNA levels in the macrophages from the cats that received the antiviral treatment, as well as returned all the individuals to their normal conditions by clinical observations and laboratory testing (Chang et al., 2016). Furthermore, it was reported that GC376 was capable to block the 3C-Like protease from the porcine epidemic diarrhea virus (PEDV) in cell-based assays using Vero cells, and they included a protein-inhibitor complex crystallization. In this case, the authors verified that in cell-based assays the IC_{50} was 1.11± 1.13 μM (Pedersen et al., 2020). Recently, this inhibitor has been tested against the SARS-CoV-2 in studies using fluorescence resonance energy transfer (FRET)-based screening assays targeting the Mpro enzyme, with molecular docking confirmation, as well as checking antiviral effects in infected Vero E6 cell cultures with an EC50 of 0.91± 0.03 μM (Hung et al., 2020). Other studies have reported GC376 as potent SARS-CoV-2 3C-like protease inhibitor in comparison to other drugs, such as Boceprevir, and describing their mechanism of interaction (Fu et al., 2020). In addition, other study compared the efficacy of the GC376 by generating its analogue the GC373 using FRET and cell-based studied, and including protein crystallization. The authors reported both molecules acted as potent inhibitors against SARS-CoV-2 Mpro, and presenting EC_{50} of 1.5 μM for GC373 and for 0.92 μM GC376 (Vuong et al., 2020).

GS-441524 is a remdesivir metabolite with activity against SARS-CoV-2 Mpro in molecular docking studies. In addition, it exhibited potent antiviral activity against several RNA viruses, including SARS infections (Cho et al., 2012). Besides, this drug could provide synergistic effects in combination with other RdRp antagonist drugs (Huynh et al., 2020).

Lopinavir is an antiretroviral protease inhibitor used in combination with other drugs for the HIV-7 treatment (Kim et al., 2020), such as ritonavir (Wada et al., 2020), which is a peptidomimetic inhibitor designed for inhibiting HIV-1 protease and is currently under investigation for the treatment of COVID-19 (De Clercq, 2009). Clinical studies indicated a potential benefit for patients infected with SARS-CoV-2 treated with lopinavir in the early stage of the disease (Li et al., 2020; Lim et al., 2020; Wu et al., 2020). Moreover, some authors suggested a protective effect of lopinavir on post-exposure prophylaxis for Middle East Respiratory Syndrome (MERS) (Li et al., 2020), as well as in combination with other antivirals for benefiting the treatment of SARS and MERS, including the reduction of acute respiratory distress syndrome (ARDS) incidences and mortality during early treatment (Chan et al., 2003).

Nelfinavir is an antiretroviral protease inhibitor with *in vitro* activity against the SARS-CoV 3CL protease, and *in silico*/*in vitro* activity against SARS-CoV replication (Yuen et al., 2005). *In silico* experiments indicated nelfinavir as a SARS-CoV-2 Mpro inhibitor, which could act in combination with cepharanthine on the control of disease progression and transmission risk (Chow et al., 2004; Yamamoto et al., 2004; Yuen et al., 2005). *In vitro* findings showed that cepharanthine reduced coronavirus cell entry (Yamamoto et al., 2019; Ohashi et al., 2020). Additionally, clinical data suggest that nelfinavir exhibits good pharmacokinetics characteristics in humans, and, thus, could be a potential drug candidate prophylaxis and treatment for COVID-19 patients (Bimonte et al., 2020; Yamamoto et al., 2020).

Oseltamivir is an influenza A and B approved drug with action inhibiting the viral neuraminidase, which decreases the release of viral particles from host cells and reduces viral spread in the respiratory tract (Rosa and Santos, 2020; Shah et al., 2020). This drug was used in the initial months of the COVID-19 outbreak, whether combined or not with antibiotics and corticosteroids, as well as with multiple combinations with chloroquine and favipiravir clinical trials. It is important to observe that clinical trials with oseltamivir at concentrations lower than 100 μM showed no apparent *in vitro* antiviral effect against the SARS-CoV-2 (Choy et al., 2020). In a clinical trial article, the authors indicated that this drug is not effective inhibiting SARS-CoV-2 even at its highest concentration; however, they do not show details of the trial, which is important to correctly determine the stage of infection at which the drug was administered and its effectiveness against COVID-19 (Rosa and Santos, 2020).

PF-00835231 is a ketone-based designed for inhibiting the SARS-CoV-1 virus (Yen et al., 2004). Recently, two studies revealed this drug as a potent inhibitor which were tested by FRET assay and for antiviral activity in Vero E6 cells with an IC50 0.00027 ± 0.0001 μM for 3C-like protease (Hoffman et al., 2020), as well as demonstrated to be statistically more potent than remdesivir in assays with infected SARS-CoV-2 $A549^{+ACE2}$ cells with an EC_{50} of 0.221 μM at 24 h, and 0.158 μM at 48 h without detectable cytotoxicity (de Vries et al., 2020).

Remdesivir is a nucleoside analog inhibitor of RNA polymerases with a large viral spectrum (Gordon et al., 2020), originally developed for the treatment against Ebola (Cheng et al., 2020), exhibiting antiviral effects against filoviruses, paramixoviruses, pneumoviruses, and coronaviruses (Wang et al., 2020). This drug has been tested both *in vitro* and *in vivo* experiments (mice and Rhesus monkeys) against SARS-CoV2 (Flanigan et al., 2020; Grein et al., 2020). Furthermore, clinical trials have been performed with SARS-CoV-2 infected adults and children in different dose ranges, and it has been demonstrating low toxicity (Hennon et al., 2020; Maharaj et al.,

2020). Additionally, double-blind, randomized, multicenter clinical studies observed a significant improvement in the reduction of viral load during the infection but without a considerable reduction in the mortality rate compared to patients who received placebo in the same period (Wang et al., 2020). Moreover, *in vitro* cell culture Vero-E6 tests showed antiviral activity of this drug in the post-entry stage of the cells, with an EC50 of 1.76 μM in EC50 (Wang et al., 2020).

Ribavirin is a guanosine analogue antiviral with activity against DNA and RNA viral polymerases that has been showing promising results against SARS-CoV-2 (Alhazzani et al., 2020; Khalili et al., 2020; Yousefi et al., 2020; Zhang et al., 2020). Although this compound is established among the first five antiviral drugs tested *in vitro* against SARS-CoV-2 due to its promising results against previous SARS and MERS infections (Morgenstern et al., 2005; Wohlford-Lenane et al., 2009; Khalili et al., 2020), some authors indicated a need of dose reduction on the new coronaviruses treatment (McCray et al., 2020). The prophylactic use of this drug includes the association with lopinavir and ritonavir, or interferon-α (INF-α), instead of monotherapy. Studies about ribavirin in the SARS-CoV-2 treatment still lack information about mechanisms of action, dose response, and different clinical aspects (Khalili et al., 2020; Zhang et al., 2020).

Tenofovir is a real nucleotide analog that has a phosphate group attached to a nitrogenous base (Härter et al., 2020). Once activated, tenofovir acts via different mechanisms, doing a potent reverse transcriptase inhibition and blocking the chain termination in the viral replication. This is an FDA approved drug for HIV and hepatitis C treatment, and it is currently used against the human herpes simplex virus, inhibiting the viral DNA polymerase (McConville et al., 2014). With the ongoing COVID-19 pandemic, this drug has been used in clinical studies with patient concomitant infected with HIV and SARS-CoV-2 (Härter et al., 2020).

Umifenovir (arbidol) is an indole-based derivative, which inhibits the influenza virus binding fusion proteins mechanisms (Hemagglutinin) (Blaising et al., 2014). It is a broad-spectrum oral antiviral used for the treatment and prophylaxis of influenza A and B and other respiratory infections (Blaising et al., 2014). As an oral antiviral, it has been used for the treatment and prophylaxis of influenza and other viral respiratory infections, licensed for use in Russia in 1993 and in China since 2006 (Liu et al., 2009; Mani Mishra et al., 2020). This drug has proven effectiveness *in vitro*, *in vivo*, and clinical studies for different viruses, including influenza A and B, Zika, as well as agents of acute respiratory tract infection: adenovirus, respiratory syncytial virus, coronavirus or SARS virus, rhinovirus, parainfluenza virus (Brooks et al., 2012; Tannock et al., 2016; Kadam & Wilson, 2017; Fink et al., 2018; Haviernik et al., 2018; Maleev et al., 2019). Some authors reported that this drug effectively inhibited SARS-CoV-2 when compared to other anti-influenza drugs of therapeutic use (Wang et al., 2020), and it was effective (EC50) in a range of inhibitory concentration against influenza and within a range of maximum plasma concentration estimated to be effective for SARS-CoV-2 (Sun et al., 2013). Currently, umifenovir is under clinical investigation as a potential agent for the treatment and prophylaxis of SARS-CoV-2 infections (Mani Mishra et al., 2020) and the early treatment with this drug can decrease the incidences of pneumonia in a high-risk hospitalized population (Beigel et al., 2019). In addition, it can be suggested that arbidol is associated with a decrease in infection among exposed individuals by COVID-19 (Zhang et al., 2020). *In silico* studies for this drug displayed activity with several SARS-CoV-2 targets, such as Mpro and Spike proteins (Vankadari, 2020; Zhao et al., 2020).

Several of the accepted papers in this review reported drugs with promising results, mainly with *in silico* and *in vitro* studies, which can be considered for further *in vivo* and clinical trials experiments. *Acyclovir* is a nucleoside analog that inhibits the action of viral DNA polymerase and DNA replication of different herpesviruses (O'Brien and Campoli-Richards, 1989); however, it did not show any effect against 2019-nCoV (Li et al., 2020). *Indinavir* is an antiretroviral protease inhibitor used against type 1 HIV infection. *In silico* studies reported that this drug could be used as a probable inhibitor of the SARS-CoV-2 Mpro, according to molecular docking experiments with the crystallized structures 5R7Z, 5R80, 5R81 and 5R82 (Shah et al., 2020). *Pleconaril* is a drug used for prevention of asthma, as well as common cold symptoms in asthmatic individuals exposed to respiratory infections. This drug acts against Picornaviridae viruses, and *in silico* molecular docking experiments indicated that pleconaril could be a SARS-CoV-2 spike protein blocker and may be selected for further preclinical and clinical experiments against this virus (Böcher et al., 2020). *Sofosbuvir* is a nucleoside analog used against HCV infections that acts inhibiting the viral RNA-dependent polymerase, and *in silico* studies demonstrated that this compound could complex with the SARS-CoV2 RNA polymerase (Elfiky, 2020), as well as other viral enzymes (Shah et al., 2020). *Tipranavir* is a non-peptide inhibitor of the HIV protease indicated for combined antiretroviral treatment. This drug has been repurposed in *silico* studies against 3CL SARS-CoV-2 proteases (Böcher et al., 2020). *Zanamivir* is a direct-acting antiviral drug that acts as a neuraminidase inhibitor against influenza A and B (Elfiky, 2020), and *in silico* studies demonstrated interaction with viral transcription proteases against SARS-CoV2 (Shah et al., 2020). Additional studies have been reporting the broad-spectrum anti-parasitic drug *ivermectin* as a SARS-CoV-2 replication inhibitor (Caly et al., 2020), since other studies have reported many antiviral actions against HIV (Wagstaff et al., 2012; Caly et al., 2020; Heidary and Gharebaghi, 2020), DENV (Xu et al., 2018; Caly et al., 2020; Heidary and Gharebaghi, 2020), ZIKA (Caly et al., 2020; Heidary and Gharebaghi, 2020), and Influenza A (Götz et al., 2016; Heidary and Gharebaghi, 2020). This drug is a macrocyclic lactone with its main antiviral mechanism of action in the nuclear transport role of the host importin α (IMPα) protein (Jans and Wagstaff, 2020; Schwemmle et al., 2020). Furthermore, one study has showed that this molecule presented antiviral effects against the SARS-CoV-2 in *vitro* assays with Vero/hSLAM cells with 5000-fold reduction in viral RNA after 48 h with IC50 varying from 2.2 to 2.5 μM (Caly et al., 2020; Sharun et al., 2020). *Teicoplanin* is a glycopeptide antibiotic with its main

activity to treat bacterial infection (Reynolds, 1989; Zhou et al., 2016). *In vitro* experiments demonstrated that this drug inhibited cell entry of the EBOV into primary human umbilical vein endothelial cells, A549 cells, HeLa cells, and THP-1 cells with an IC50 of 0.34 μM (Zhou et al., 2016), as well as its derivatives demonstrated antiviral activity against influenza strains, vaccinia, herpes, and human coronavirus (Bereczki et al., 2020). Recently, it was published that teicoplanin potently blocks the HIV-luc/2019-nCoV-S pseudoviruses entry into human A549 cells with a IC50 of 1.66 μM, suggesting this could be caused by the cathepsin L inhibition. Additionally, it was demonstrated that this drug repressed viral entrance into HEK293T and Huh7 cells (Zhang et al., 2020).

A recent study which performed a high-throughput screening using The ReFRAME library for drug repurposing against the SARS-CoV-2, investigated 11,987 FDA approved compounds in infected Vero E6 cell assays, and included a gene set enrichment analysis, which returned that they could be affecting viral replication and dynamics by acting in different targets, such as modulators of benzodiazepine receptors, aldose reductase, potassium channels, cholesterol homeostasis, serine proteases and retinoic acid receptor agonists. Furthermore, the authors realized an orthogonal validation of 300 active compounds with concentrations between 2.5 and 1 μM, and found 100 molecules that were capable to reduce viral replication, as well as several validated by the gene set enrichment analysis target classes. Compound efficacies were additionally checked with Huh-7 and HEK293T cells transduced with angiotensin-converting enzyme 2 (ACE2), and Thirteen compounds exhibited nanomolar EC_{50} values, including a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist DS-6930, the HIV-1 reverse transcriptase inhibitor R 82913, and the anti-mycobacterial clofazimine. Additionally, it was found that five of most potent inhibitors presented activity in the cell viral entry step but, on the other hand, it is suggestive that the protease inhibitors VBY-825, ONO 5334, Z LVG CHN2 and MDL 28170 are acting in host's proteases once they have not acted on both SARS-CoV-2 3C-like and papain-like proteases. The compound Z LVG CHN2 is probably acting as an endosomal protease inhibitor whereas ONO 5334 is a cathepsin K inhibitor, and VBY-825 a cathepsin protease inhibitor. Another assay with human induced pluripotent stem cell (iPSC)-derived pneumocyte-like cells indicated that the molecules ONO 5334, MDL 28170 and apilimod drastically reduced the number of infected cells. Furthermore, an *ex vivo* lung culture system assay showed apilimod as a potent antagonist to viral replication in comparison to the positive control remdesivir (Huang et al., 2020; Wec et al., 2020).

A second recent study with a large-scale molecule library, with approximately 1000 FDA approved drugs and 2,100 drug-like molecules with validated pharmacological activity purchased from Selleckchem, performed an antiviral screening using Lung epithelial Calu-3 cells, Vero E6 and Huh7.5 cells. Initially, the authors found that remdesivir and hydroxychloroquine were active in infected Vero E6, with IC_{50} of 0.46 and 1.32 μM, respectively, as well as other six drugs including the natural compound nanchangmycin, with IC_{50} of 0.01 μM. Additionally, Huh7.5 cell assays demonstrated that 33 drugs were active with IC50 below 0.5 μM, including remdesivir, hydroxychloroquine and nanchangmycin. On the other hand, since remdesivir was active in infected Calu-3 cells, hydroxychloroquine and its derivates presented none activity. This suggests that there is a different mechanism of endosomal acidification in this cell types which turns the mechanism of action of the hydroxychloroquine and its derivatives, as well as the drug Z-FA-FMK ineffective in these cells but active in Vero E6 and Huh7.5. Thus, the authors suggested the role of the he plasma membrane-associated serine protease (TMPRSS2), allowing the SARS-CoV-2 entry in Calu-3 cells, and proposed a specific inhibition action of the drug camostat with IC50 of 0.35 μM since it has not presented activity in Vero E6 and Huh7.5 cells (Walker et al., 2020). This mechanism was also demonstrated in another study using human bronchial epithelial cells (HBEC), primary type II alveolar epithelial cells (AECII), and Calu-3 cells for Influenza A and B Virus (Limburg et al., 2019). On the other hand, recent clinical studies with hydroxychloroquine indicated a lack of efficacy in acute infected hospitalized patients with COVID-19, but with small cardiac effects and cardiac deaths (Giovanna and Carlo, 2020; Pan et al., 2020), which could indicate that this drug have its main action as a prophylactic agent, as well as in non-severe cases. Additionally, similar results were achieved for remdesivir, lopinavir (Pan et al., 2020), as well as for the combination between lopinavir and ritonavir (Horby et al., 2020). Thus, this is suggestive that these drugs could be used as prophylactic mechanisms, as well as in the early stages of COVID-19 infection in non-hospitalized patients. Therefore, further prophylactic studies are required for hydroxychloroquine, remdesivir and lopinavir, before they can be largely used by the physicians. Other nine drug candidates presented selective antiviral index greater than 2 against SARS-CoV-2 Calu-3 cells: Salinomycin, Y-320, AZD8055, bemcentinib, dacomitinib, WYE-125132, ebastine, Dp44mT, and cyclosporine (Limburg et al., 2019).

The new coronavirus pandemic caused by the SARS-CoV-2 has provoked a global health and economic crisis in most countries, which has mobilized a great number of scientific research groups in many fields of study, especially with drug therapy and vaccination, in order to find prophylaxis and therapeutic alternatives against the COVID-19. Although many research results have revealed palliative drug treatment against this infection focused in human targets, many other researches have been publishing drug repurposing experiments, using previous experimental data, with main targeting other viruses' treatments, with *in silico* and *in vitro* experiments. On the other hand, clinical experiments with previously approved drugs against the new coronavirus lack in number of drug options, number of patients in different health conditions and control groups, as well as the time of evaluation, which is understandable, since only less than seven months have passed after the start of the SARS-CoV-2 outbreak. Furthermore, many vaccination options are still in different clinical test phases in many countries, without any guarantee to solve this global problem. Therefore, the use of clinical protocol-based scientific evidence data for SARS-CoV-2 prophylaxis, as well as for the daily routine in hospitals for its treatment, is crucial for controlling the disease spread, prognostic and patient recovery, and can indeed help save many lives.

In this review, we proposed a list of 22 approved drugs and compounds, with relevant clinical data, and *in vivo*, *in vitro* and *in silico* evidences, which can guide prophylaxis studies with large individual groups. Additionally, we included more 22 compounds between FDA approved drugs and drug-like like molecules, which were tested in large-scale screenings, as well as those repurposed approved drugs with new mechanism of actions. Furthermore, this review contributes to avoiding the concomitant use of drugs associated with polypharmacy (many times without scientific evidences), which can lead to serious health side and adverse effects, sometimes with toxic and degenerative drug interactions for humans. The integration between clinical trials data, *in silico*, *in vitro,* and *in vivo* screenings can assist in the rational use of new antiviral drugs not only for the COVID-19 prophylaxis, but also for its treatment, even in more advanced proliferation stages.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BA participated in the manuscript conceptualization, experimental design and writing, and supervision; FR writing and data analysis; NS experimental design, data analysis; AF, WS, and SS writing and data analysis; DB, AG-N, AB, and VA overall manuscript review and English review.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.590598/full#supplementary-material.

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frontiers in Pharmacology

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The Large Action of Chlorpromazine: Translational and Transdisciplinary Considerations in the Face of COVID-19

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Coronavirus disease 2019 (COVID-19) is a severe acute respiratory syndrome (SARS) in humans that is caused by SARS-associated coronavirus type 2 (SARS-CoV-2). In the context of COVID-19, several aspects of the relations between psychiatry and the pandemic due to the coronavirus have been described. Some drugs used as antiviral medication have neuropsychiatric side effects, and conversely some psychotropic drugs have antiviral properties. Chlorpromazine (CPZ, Largactil®) is a well-established antipsychotic medication that has recently been proposed to have antiviral activity against SARS-CoV-2. This review aims to 1) inform health care professionals and scientists about the history of CPZ use in psychiatry and its potential anti- SARS-CoV-2 activities 2) inform psychiatrists about its potential anti-SARS-CoV-2 activities, and 3) propose a research protocol for investigating the use of CPZ in the treatment of COVID-19 during the potential second wave. The history of CPZ's discovery and development is described in addition to the review of literature from published studies within the discipline of virology related to CPZ. The early stages of infection with coronavirus are critical events in the course of the viral cycle. In particular, viral entry is the first step in the interaction between the virus and the cell that can initiate, maintain, and spread the infection. The possible mechanism of action of CPZ is related to virus cell entry via clathrin-mediated endocytosis. Therefore, CPZ could be useful to treat COVID-19 patients provided that its efficacy is evaluated in adequate and well-conducted clinical trials. Interestingly, clinical trials of very good quality are in progress. However, more information is still needed about the appropriate dosage regimen. In short, CPZ repositioning is defined as a new use beyond the field of psychiatry.

Keywords: coronavirus, COVID-19, SARS-CoV-2, phenothiazine, antipsychotics, antiviral, chlorpromazine, clathrin

INTRODUCTION

The discovery of antidepressants has two parallel origins. On the one hand, the observation that an anti-tuberculosis drug, iproniazide chemically similar to isoniazid, improved the mood of *tuberculosis* patients and inhibited monoamine oxidase at the same time. On the other hand, a new molecule called imipramine was synthesized in order to obtain a neuroleptic like chlorpromazine, which had in fact not the expected but an antidepressant effect. Thus, two groups of antidepressants were born, MAOs and tricyclics. With the former, infectious disease medicine fueled the psychiatric practice. Could the opposite happen now? This article addresses this issue with the overall goals to: 1) educate health care professionals, including virologists, about the history of chlorpromazine (CPZ) use in psychiatry and its potential anti-SARS-CoV-2 activities, 2) inform psychiatrists about its potential anti-SARS-CoV-2 activities, and 3) propose a research protocol to investigate the use of CPZ in the treatment of COVID-19 for a potential second wave with psychiatric patients.

CHLORPROMAZINE AND CORONAVIRUS

The COVID-19 pandemic has had a major impact on mental health (Coccolini et al., 2020). The most common psychological and behavioral reactions are distress reactions which often include anxiety, insomnia, frustration, sense of insecurity, anger, increased use or avoidance of health services for fear of illness and indulging in risky maladaptive behaviors (for example, increased consumption of alcohol, illicit drugs and tobacco, change in work-life balance, social isolation, increased family conflicts and violent behavior) (Coccolini et al., 2020). Owing to these behaviors, psychiatrists have to be very involved, and at times, are obliged to adapt themselves due to the reorganization, redeployment and closing of services, following precautions for social distancing, use of telemedicine and wearing of personal protective equipment. They are not only repositioning themselves, but also offer repositioning of medication that they know very well. This is the case with the first known antipsychotic medication, CPZ (Plaze et al., 2020a; Plaze et al., 2020b; Stip, 2002; Stip, 2020). In 2020, a Canadian research letter (Stip, 2020) proposed the possibility that CPZ, a medication used in psychiatry for a long time, could potentially be used to counter COVID-19 (Nobile et al., 2020). The proposal was based on an old antimicrobial and antiviral CPZ data that had been documented when studying the history of the introduction of CPZ in North America (Stip, 2015). The first use in psychiatry of CPZ was in France and interestingly it is from there too that a concrete statement for CPZ repositioning for COVID-19 has just emerged (Plaze et al., 2020a; Plaze et al., 2020b). Since then, controlled clinical trials are underway with well-supported hypotheses and a rigorous methodology (NCT 04366739, Repurposing of Chlorpromazine in COVID-19 Treatment, reCoVery, France and NCT0434805, Egypt). CPZ is well-known to psychiatrists (Stip, 2000) and perhaps less well-known to virologists. In this article, we retrace the history of the use of CPZ in psychiatry and summarize the scientific arguments for prescribing CPZ in the context of viral infections, especially coronavirus infections due to the continuing COVID-19 pandemic.

Coronaviruses are a large group of non-segmented, (+) sense, enveloped RNA viruses with one of the largest genomes known, ranging from 27 to 33 kilo bases (kb). They cause a wide variety of diseases, ranging from respiratory, enteric, and hepatic to neurologic diseases (reviewed in Weiss and Leibowitz, 2011; Fehr and Perlman, 2015). Widely spread among the animal kingdom (mammals and birds), these viruses can be divided into four genera: α, β, γ, and δ. Coronaviruses are not new to the human species and so far, seven different strains of coronaviruses have crossed into the humans (HCoVs) belonging to either α or β coronavirus genera (**Table 1**; Ye et al., 2020). Of these, four strains cause mild upper respiratory infections of self-limiting nature, two of which were discovered in the 1960s (HCoV-OC43 and HCoV-229E). However, three other strains have entered the human species lately, causing a severe acute respiratory syndrome (SARS) in humans with high case fatality rates (CFR). The first such strain that had a CFR of 11% (Tsang et al., 2003), followed by Middle Eastern respiratory coronavirus (MERS-CoV) with a CFR of ~35% (Bermingham et al., 2012; Khan and Sheek-Hussein, 2020), and the most recent one is SARS-CoV-2 in December 2019, the etiologic agent of COVID-19, with an evolving CFR of ~2.4% (Wu F. et al., 2020; Wu A. et al., 2020; Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020; Uddin et al., 2020). Based on this species jump trajectory, further introductions of these viruses into humans are most likely; therefore, urgent efforts are needed to have effective therapies against these serious viral pathogens.

Among the three virulent strains of HCoVs, SARS-CoV-1 was the first human coronavirus that caused a global epidemic in 2003, spreading from Guangdong, China to over 12 dozen countries in several continents, causing 8,096 infections with 774 deaths (reviewed in Ye et al., 2020). It is thought to have originated in bats, followed by civet cats, and into humans. Other than fever, headaches, cough, and fatigue, patients infected with SARS-CoV-1 also displayed severe acute respiratory distress in the form of shortness of breath, atypical pneumonia, and cytokine storm. In 2012, MERS-CoV was identified (Zaki et al., 2012; Khan, 2013), again with origins in bats, but the dromedary camels were identified as the intermediate host for further transmission into humans (de Wit et al., 2016). Once in the human population, the virus spreads from person to person, primarily within the Middle East, but also leading to small outbreaks in Europe, Tunisia, and Korea (Ye et al., 2020). As of December 31, 2019, MERS-CoV had infected almost 2,500 individuals with 866 deaths, making it one of the most lethal human viruses known (Khan and Sheek-Hussein, 2020). Persons infected with MERS-CoV present with clinical symptoms resembling infection with SARS-CoV-1, except that some also show acute renal failure. Unlike SARS-CoV-1 and MERS-CoV, since its emergence in December 2019, SARS-CoV-2 has spread all over the world like wildfire, infecting >35 million people worldwide as of October 7, 2020 with over 1 million deaths across the world (COVID-19 Dashboard). Although SARS-CoV-2 is nearly 82% homologous to SARS-CoV-1 (Wu A. et al., 2020; Wu F. et al., 2020), SARS-CoV-2 is less lethal than SARS-CoV-1, but much more infectious.

TABLE 1 | Human coronaviruses (HCoVs) and their characteristics.

Human coronavirus	Genera	Entry receptor	Disease/Pathogenesis	References
HCoV-229E (1966)	α	Aminopeptidase (APN)	Upper respiratory tract infection with mild symptoms. Now endemic in human population, causing 15–30% of common colds	Hamre and Procknow (1966), Fehr and Perlman (2015)
HCoV-OC43 (1967)	β	Neu5, Ac2-containing moiety		McIntosh et al. (1967), Weiss and Navas-Martin (2005)
HCoV-NL63 (2004)	α	Angiotensin-converting enzyme 2 (ACE2)		van der Hoek et al. (2004), Fehr and Perlman (2015)
HCoV-HKU1 (2005)	β	?		Woo et al. (2005)
SARS-CoV-1 (2003)	β	ACE2	Other than symptoms of common cold and pneumonia, these viruses predominantly cause infection of lower respiratory tract causing acute respiratory distress syndrome (ARDS) and cytokine storm	Ksiazek et al. (2003), Drosten et al. (2003), Fehr and Perlman (2015)
MERS-CoV (2012)	β	Dipeptidyl peptidase 4 (DPP4)		Bermingham et al. (2012), Fehr and Perlman (2015)
SARS-CoV-2 (2019)	β	ACE2		Hoffman et al. (2020), Wu A. et al. (2020), Wu F. et al. (2020)

ARDS, acute respiratory distress syndrome; DPP4, dipeptidyl peptidase 4.

Other than symptoms of common cold and pneumonia, both viruses (SARS-CoV-1 and SARS-CoV-2) can cause acute respiratory distress syndrome, cytokine storm, and additionally diarrhea, unlike some of the other HCoVs (Ye et al., 2020). As can be seen from **Table 1**, the different HCoVs use different types of proteins as their receptors to enter cells. Interestingly, unlike MERS-CoV that uses the dipeptidyl peptidase 4 enzyme as its entry receptor, both SARS-CoV-1 and SARS-CoV-2 use the same receptor for entry into human cells, the angiotensin-converting enzyme 2 (ACE2) (**Table 1**), suggesting similar mechanism of entry into susceptible cells (Hoffman et al., 2020; Uddin et al., 2020).

Recently, Dyall and co-workers (Dyall et al., 2014) examined nearly 300 FDA approved drugs for antiviral activity against MERS-CoV and SARS-CoV-1 and found CPZ to be active against both of these coronaviruses. Similarly, de Wilde and co-workers found from a screening of 348 molecules that CPZ was one of the most promising agents for inhibiting coronaviruses in humans (de Wilde et al., 2014). Hence, CPZ merits further clinical investigation, in particular in a small-animal model for MERS-CoV infection. The fast worldwide spread of SARS-CoV-2 has created a need to find effective treatments. The repositioning of drugs already known and approved for a long time in humans is a practical and efficient approach to look for new therapeutic options in the face of this pandemic.

PHENOTHIAZINES

CPZ belongs to the phenothiazine family of drugs that are primarily used for the treatment of schizophrenia and other forms of psychosis. Few psychiatrists know that methylene blue (one of the first antimalarial drugs) is a phenothiazine, with several biomedical and biological therapeutic facets (Henry et al., 2020). In addition to their antipsychotic activity, phenothiazines also have a significant antimicrobial effect, thanks to an action on the bactericidal function of macrophages and on inhibition efflux pumps (Chakrabarty et al., 1991; Barbe et al., 1995; Dastidar et al., 2013). They also eliminate bacterial resistance plasmids and destroy bacteria due to their membrane destabilizing effect. Methylene blue was one of the first synthetic drugs in medicine, with multiple indications, such as clinical pain syndromes, malaria, and psychotic disorders, and was used over a century ago (Bodoni, 1899; Stip, 2015). Methylene blue is a cationic thiazine dye with redox cycle properties and a selective affinity for the nervous system. Although CPZ was named as the first antipsychotic, methylene blue had actually been used to treat psychotic patients half a century earlier. In addition to treating psychotic patients, the use of methylene blue has also been explored in treating the bipolar disorder (Alda et al., 2017).

DISCOVERY OF CHLORPROMAZINE

CPZ is a neuroleptic,- a class of medication primarily used to manage psychosis. The first neuroleptic medication (Stip, 2002; Stip, 2015), CPZ was the product of research on antihistamines, discovered in 1937 by the Nobel Prize winner, Daniel Bovet. In

1944, he isolated phenothiazine and diethazine (Diparcol), which Jean Sigwald used in 1946 to treat Parkinson's disease (Sigwald and Bouttier, 1953). Bernard Halpern had already introduced the use of phenothiazine antihistamines, such as promethazine (Phenergan), in medicine. In 1950, Paul Charpentier synthesized CPZ at the Rhône-Poulenc Laboratories (Stip, 2015). The drug was called 4560 RP and then Largactil in France. In 1952, Henri Laborit, Pierre Huguenard and Raymond Alluaume published the first use of 4560 RP (Stip, 2015). Afterwards, in his work on anesthesia, Laborit reported that some drowsiness was observed with 50–100 mg of intravenous CPZ, as well as, above all, a lack of interest of the patients to their surroundings. In December 1951, a clinical trial of 4560 RP was carried out by two psychiatrists, Jean Sigwald and Daniel Bouttier at the Paul Brousse Psychiatric Hospital in Paris: CPZ was effective in a series of cases of patients with hallucinations (Stip, 2015). Léon Chertok carried out what was probably the first clinical experience in a psychiatric environment with 4560 RP alone. It led to a normal subject experiment in Villejuif on a resident in psychiatry, Cornelia Quarti (Stip, 2015). In March 1952, three psychiatrists from the Val-de-Grâce Hospital in Paris, Hamon, Paraire and Velluz, published a case study of a patient suffering from manic attacks treated with CPZ, in association with a barbiturate, but the effect was insufficient (Hamon, 1952). Delay, Deniker, and Harl, published the first long-term observational study on May 26, 1952, on the occasion of the centenary of the Société Medico-Psychologique (Stip, 2015). The same team in Sainte-Anne Hospital in Paris published six articles over a period of 6 months, paving the way for the introduction of CPZ in psychiatry (Delay and Deniker, 1952; Delay et al., 1952). Initial trials outside of France, such as those in Padua with Rigotti, of Arnold in Vienna and Labhardt in Basel, Switzerland, produced similar results (Staehelin, 1954). The doses were then increased to 150–300 mg intramuscularly and 300–500 mg orally (Lemperiere and Ginestet, 2001). The first British report, by Anton-Stephens, identified indifference as the greatest effect on patients (Anton-Stephens, 1954). CPZ was introduced in 1954 in North America by two Canadian psychiatrists Roland Saucier and Heinz Lehmann (see Stip, 2015). By the end of 1955, there were also reports describing use of CPZ from Switzerland, Germany, Hungary, Latin America, Australia, Russia, and the United States (Bente and Itil, 1954; Sal et al., 1954; Staehelin, 1954; Kardos and Pertorini, 1955; Webb, 1955). CPZ was marketed in the United States as Thorazine by SmithKline, in France and England as Largactil by May and Baker (Stip, 2015), and in Japan by the name of Cotomin. The name of "Largactil" in French comes from its "broad action" because of the breadth (*largeur*) of its pharmacodynamic actions or due to its wide-ranging effects on different symptoms.

In 1957, the psychophysiological definition of neuroleptics was proposed based on five classical criteria: 1) creation of a state of psychomotor indifference, 2) reduction in states of excitement, agitation and aggressiveness, 3) progressive reduction of acute or chronic psychotic disorders, 4) neurological and neurovegetative side effects, and 5) predominantly subcortical in action. In the United States, the name of "neuroleptic class" was changed to "major tranquilizer" and "antipsychotic." It took almost another 10 years to understand the mechanism of action of neuroleptics (antipsychotics), for the identification of dopaminergic receptors and the development of the dopamine (DA) hypothesis of schizophrenia with the contributions from Arvid Carlsson (Stip, 2002; Stip, 2015). This hypothesis received additional support with the correlation between clinical doses of CPZ and its power to block dopamine D2 (DA D2) receptors. Pharmacological interventions to treat psychosis have generally focused on modifying dysfunctional neurotransmitter systems to improve symptoms. So what can we learn from the discovery of CPZ? It is that a psychotropic drug can have the indication for a psychiatric disease before one knows its real mechanism of action.

CHLORPROMAZINE AND MICROBIAL INFECTIONS

Interestingly, Jean Delay's book (Delay, 1950), published in 1950, 2 years before his articles on 4560 RP, indicates that antibiotics, such as penicillin, were among the recommended treatments for psychosis. With global use of CPZ, reports have shown that patients receiving CPZ had a lower incidence of bacterial infections (Kristiansen and Amaral, 1997). There is also growing evidence to suggest that inflammation, infection, oxidative stress, changes in the glutamatergic system, and neurotrophins are involved in schizophrenia (Hong and Bang, 2020). CPZ also has antimicrobial activity against *Staphylococcus aureus in vitro*, at concentrations greatly exceeding those achieved clinically (Ordway et al., 2002; Amaral and Molnar, 2012). The activity of CPZ against various microorganisms, including intracellular pathogens (leishmania, trypanosomes, amebae), has been studied both *in vitro* and *in vivo* (Molnar et al., 1976; Amaral and Molnar, 2012). These antimicrobial properties of CPZ reside in the side chains of the molecule and have led to the development of this phenothiazine as an anti-malarial agent (Molnar et al., 1976; Tsay, 2013). This creates a kind of loop, bringing us back to the malaria parasite, which was used to treat mental illness from the early 1920s to the late 1950s (Himmelweit, 1960; Tsay, 2013).

CHLORPROMAZINE AND VIRAL INFECTIONS

Chlorpromazine has also shown antiviral activity against a number of viruses, including adeno (Diaconu et al., 2010), Ebola (Bhattacharyya et al., 2010), influenza (Rossman et al., 2012), and coronaviruses (De Wilde et al., 2014). Anti-viral activity of CPZ is mainly explained by inhibiting clathrin-mediated endocytosis (Wang et al., 1993; Joki-Korpela et al., 2001; Nawa et al., 2003; reviewed in Glebov, 2020; Yang and Shen, 2020). A key component of their virulence is the process of viral entry into host cells using the endocytic pathway, though other non-endosomal pathways may also be employed, depending upon the cell type and virus strain used (Inoue et al., 2007; Wang et al., 2008; reviewed in Glebov, 2020; Yang and Shen, 2020).

SARS-CoV-2 specifically enters its target cells by the binding of the viral spike protein S with the ACE2 cell surface receptor (**Figure 1**; Hoffman et al., 2020). Once attached to its target receptor, the virus is enclosed by the cell membrane which begins to form a vesicle (Burkard et al., 2014; Burkard et al., 2015). It rounds and stiffens due to the agglomeration of a cage of fibrous proteins, the clathrins, then separates from the cell membrane by gradually closing: it forms a neck which shrinks and disappears under the action of the dynamin, a protein that closes the bag. Then the vesicle loses its clathrins and fuses with an endosome. Endosome has a membrane not a wall and the fusion occurs between the viral envelope and the endosomal membrane. Under the action of a membrane protease, the virus fuses with the wall of the endosome (**Figure 1**). SARS-CoV-2 entry requires priming by cell-membrane bound proteases such as TMPRSS2. It is the S1 subunit that interacts with the ACE2 receptor via the receptor binding domain. The S2 domain initiates the process of membrane fusion via further activation by lysosomal proteases, allowing release of its genetic material within the target cell cytosol (**Figure 1**). This mode of penetration, known as "clathrin and dynamin-dependent," is also observed with SARS-CoV-1, MERS-CoV, and also with the hepatitis C virus and the Influenza A virus (Zucker et al., 1990; Nawa et al., 2003; Inoue et al., 2007). Thus, infection of host cells by SARS-CoV-2 has been shown to be susceptible to lysosomotropic agents such as chloroquine that neutralize the acidic pH observed in the endosome-lysosomal compartments (Wang et al., 2008; Nobile et al., 2020). CPZ belongs to the family of cationic amphiphilic drugs and thus increase intra-vesicular pH of lysosomes. This blocks virus entry into cells by inhibiting activation of the lysosomal proteases that allow virus fusion with the endosomes and release of the viral genetic material into the cytosol. Based on these observations, the endocytic pathway comprising the endosome and the lysosome has become an important target for drug development in the fight against diseases caused by coronaviruses.

Thus, the early stages of viral infection are critical events in the course of the viral life cycle. In particular, viral entry is the first step in the interaction between a virus and a cell that can initiate, maintain and spread the infection (Joki-Korpela et al., 2001; Chu and Ng, 2004). Therefore, this stage constitutes a major target of the host's adaptive immune response. The mouse hepatitis virus (MHV) has been used as a model for study coronavirus infections. In the case of a SARS-CoV-1 infection, similar to SARS-CoV-2, viral entry also requires a low pH in the intracytoplasmic vesicles; however, little is known about how SARS-CoV-1 invades these compartments (Subtil et al., 1995). Using the MHV model, experiments have revealed that viral entry mediated by clathrin-dependent endocytosis can be significantly inhibited by treatment with CPZ. Moreover, transfection of cells with small interfering RNAs specific to clathrin heavy chain can also inhibit viral gene expression, indicating the essential role of clathrin in viral entry (PuZhang, 2008). Similarly, entry of Zika virus has also been shown to be significantly inhibited by CPZ (Li et al., 2020). CPZ slows or suppresses the replication of alphaviruses, hepatitis C virus, SARS-CoV-1, and MHV-2. In fact, phenothiazines including CPZ, have various biological activities (Deetz et al., 2003). Phenothiazines have potent anti-plasmid and anti-bacterial activity *in vitro*. The benzo phenothiazine derivatives exhibit antibacterial activity *in vivo* and stimulate the differentiation of human myeloid leukemic cell lines (Motohashi et al., 2000; Huang et al., 2018). Thus, trifluoroperazine (Stelazine, Terfluzine) and CPZ have significant inhibitory effects on the replication of arenaviruses (Junin, Tacaribe and Pichinde viruses) (Candurra et al., 1996). *In vitro* antiviral activity on strains of herpes simplex virus type 2 (HSV-2) has also been demonstrated in cell cultures (Mitchell, 1994; Mucsi et al., 2001).

CPZ has also been shown to have affinity for sigma one receptors (Ki = 146 nM (Tam and Cook, 1984) and IC50 = 200 nM (Lang, 1995). Sigma one receptors are chaperone proteins found in the endoplasmic reticulum that may be responsible for proper folding of proteins after their translation. Being an enveloped virus, SARS-CoV-2 assembly takes place intracellularly where several of its structural proteins [spike (S), membrane (M), and envelope (E)] mature through the endoplasmic reticulum-golgi intermediate compartment (ERGIC) (Krijnse-Locker et al., 1994; Risco et al., 2002). This compartment is also used for the assembly of the virus particles that are transported to the cell surface in vesicles that are released via exocytosis. Thus, it is possible that CPZ may be acting not only at the entry phase of SARS-CoV-2 replication, but also at the later stages, potentially inhibiting the later stages of virus replication such as assembly and exocytosis from the infected cells. In fact, ERGIC has been proposed as a potential target for the investigation of antiviral drugs for SARS-CoV-2 (Tozzi, 2020).

In summary, CPZ as an anti-COVID-19 candidate is a fair illustration for repositioning the molecule as a treatment for COVID-19 with the findings of the two studies demonstrating *in vitro* activity against SARS-CoV-2 (Plaze et al., 2020b; Weston et al., 2020). These two pre-print studies demonstrate the *in vitro* activity of chlorpromazine against SARS-CoV-2 are crucial for proposing a repositioning.

Recently, French researchers (Plaze et al., 2020a; Plaze et al., 2020b; Nobile et al., 2020) have also identified several other mechanisms and advantages of chlorpromazine (Zucker et al., 1990; Tarazona et al., 1995). For example, CPZ stimulates the production of IgM. Moreover, its pulmonary concentration is 20–200 times higher than its plasma concentration. It is the same for the salivary concentration (20–60 times higher than that of plasma) and, of course, encephalic (25 times higher than that of plasma). This preferential distribution therefore seems particularly suitable for use in COVID-19 (Tarazona et al., 1995; Inoue et al., 2007).

DYNAMIN INHIBITION

Dynamin inhibition is an important part of the mechanism of action of CPZ (see **Figure 1**). This can be seen by the fact that there are many dynamin inhibitors available most of which are anti-infection in cells and a few in animal models, including bacterial, toxin and viral infections. The proof of dynamin being the mediator of this inhibition is commonly demonstrated by dominant-negative dynamin mutants (Harper et al., 2013).

Among the dynamin inhibitors, only the phenothiazines are clinically approved that are used at about the same concentrations

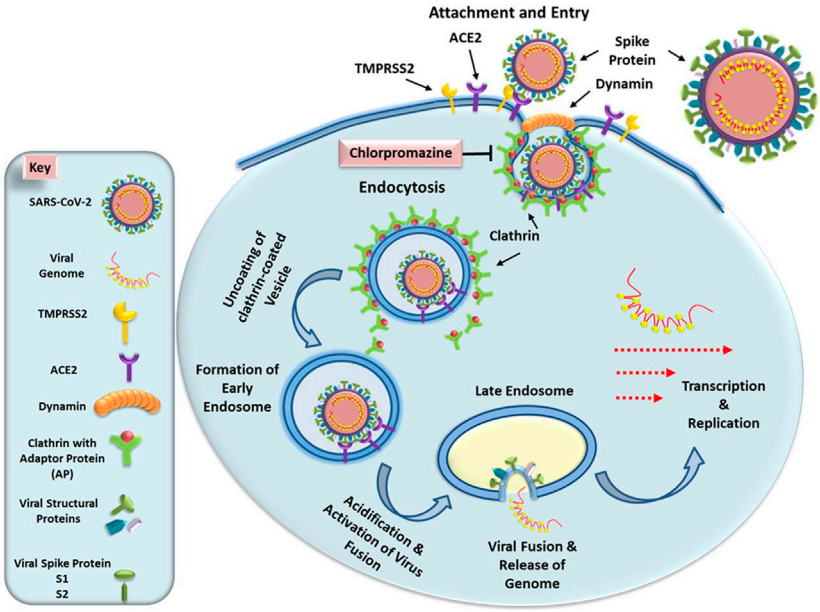

FIGURE 1 | Schematic presentation of early steps of SARS-CoV-2 life cycle. The virus enters the cells by interacting with its receptor ACE2 protein using the spike protein and priming by the cell surface protease TMPRSS2. This induces formation of a vesicle via clathrin-dynamin-mediated endocytosis. The internalized vesicle loses its clathrin coat and fuses with an endosome. As the endosome undergoes acidification, it activates host proteases that induce fusion of the virus particle with the endosomal membrane, releasing the viral genomic RNA into the cytosol. Since SARS-CoV-2 RNA is (+) sense, it can immediately undergo translation and further transcription to allow the remaining steps of virus life cycle to continue. The left panel illustrates the key explaining the different components of this pathway. See text for details.

and in clinically relevant levels. This potentially explains their mechanism of action and doses (Daniel et al., 2015). These concepts are important background that can actually be used to expand this hypothesis to other phenothiazines. Indeed, in March 2020, it was reported that the dynamin inhibitor of the phenothiazine class, prochlorperazine (prochlorperazine: Stemetil) blocks clathrin-mediated endocytosis in humans at clinical doses. This provides the evidence that the mechanism of action of phenothiazines is via inhibition of the clathrin-mediated endocytic pathway, thus strengthening the hypothesis (Chew et al., 2020). It is hard not to see that this extrapolates to all clinically-approved phenothiazines.

ADVERSE EFFECTS OF CHLORPROMAZINE

CPZ is widely used as an antipsychotic agent and is relatively safe to treat schizophrenia. Many side effects are linked to CPZ, including: drowsiness, indifference, anxious reaction, mood swings, dry mouth, constipation, blurred vision, urine retention, orthostatic hypotension, involuntary movements, tics, stiffness and difficulty coordinating movements, parkinsonism, tremor, tardive dyskinesia, dystonia, hyperprolactinemia, sexual side effects, weight gain, hyperglycemia, allergic skin reaction, photosensitization, QT prolongation and cardiac rhythm disorders, neuroleptic malignant syndrome and agranulocytosis (**Figure 2**; Solmi et al., 2017).

A recent meta-analysis from Huhn et al. (2019) compared the tolerability of 32 oral antipsychotics during the acute treatment of adults with multi-episode schizophrenia. Results for CPZ were given individually for six areas, namely: weight gain, extrapyramidal side effects, akathisia, raised prolactin, sedation, and anticholinergic side-effects compared with placebo. The results were then ranked cumulatively in comparison with all other treatments. In terms of weight gain, CPZ showed a mean difference (MD) of 2.37 (range = 1.43–3.32 kg), with a high level of confidence. This is the fourth highest out of all studied treatments (following zotepine, olanzapine, and sertindole), strongly favoring placebo treatment. The use of anti-Parkinson's medication was used as a measure of extrapyramidal side-effects. CPZ showed a risk ratio (RR) of 2.17 (1.48–2.91) and was ranked 14th of the 33 treatments in favoring placebo treatment. For akathisia, CPZ showed a RR of 2.58 (1.30–4.30) and ranked 16 out of the 31 treatments in favoring placebo treatment. CPZ showed a MD in prolactin elevation of 8.70 (−8.16–25.75 ng/ml), ranking fifth out of the 21 treatments in favoring placebo treatment. For sedation, CPZ showed a RR of 2.55 (2.16–2.90). CPZ ranked sixth out of the 33 treatments in favoring placebo treatment. For anticholinergic side-effects, CPZ showed a RR of 2.58 (1.74–3.60), making it fifth out of the 32 antipsychotics in favoring placebo treatment.

New-generation neuroleptic agents were found to cause fewer unwanted extrapyramidal side effects than the traditional antipsychotic drugs (Solmi et al., 2017). However, they are not phenothiazines.

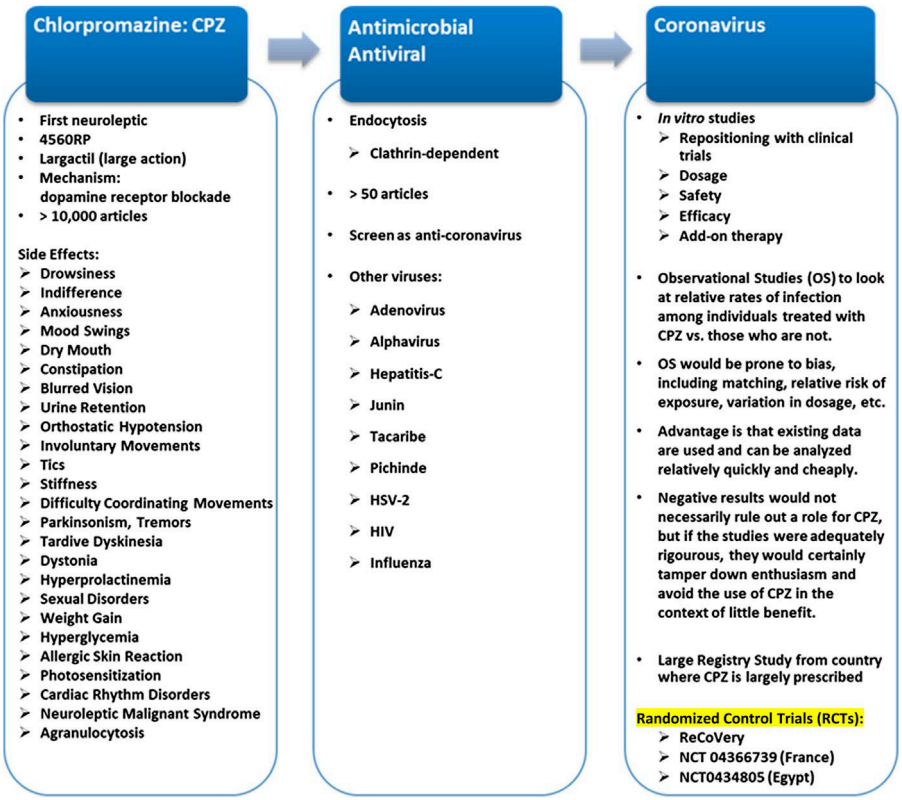

FIGURE 2 | Repositioning of CPZ*. *Chlorpromazine in the world: Ampliactil (Argentina); Aspersinal (Argentina); Bellacina (Paraguay); Cepezet (Indonesia); Chlomazine (Japan); Chloractil (England); Chlorazin (Bulgaria, Switzerland); Chlormazine (Thailand); Chlorpromanyl (Canada); Chlorpromed (Thailand); Clonazine (Ireland); Clorpromaz (Brazil); Clozine (India); Contomin (Japan); Duncan (Thailand); Esmino (Japan); Fenactil (Poland); Hibernal (Hungary, Sweden); Klorproman (Czech Republic, Finland); Klorpromazin (Finland); Laractyl (Philippines); Largactil (Austria, Belgium, Canada, Chile, Costa Rica, Czech Republic, Denmark, Dominican Republic, Ecuador, El Salvador, England, Finland, France, Greece, Guatemala, Honduras, Hong Kong, Indonesia, Iran, Israel, Italy, Jordan, Mexico, Netherlands, Norway, Panama, Peru, Portugal, Puerto Rico, Spain, Switzerland, Uruguay, Venezuela); Largactil Forte (New Zealand); Matcine (China, Malaysia, Thailand); Megatil (India); Neomazine (Korea); Plegomazine (Iraq, Puerto Rico, Syria); Promactil (Indonesia); Promexin (Japan); Propaphenin (Germany); Prozil (Denmark); Prozin (Italy); Psynor (Philippines); Taroctyl (Israel); Thorazine (Philippines); Winsumin (Taiwan); Wintermin (Japan, Taiwan).

Overall, the tolerability data for CPZ favored placebo treatment, with six side effects all favoring placebo treatment. For all-cause discontinuation, CPZ showed a RR of 0.91, ranking eighth out of the 33 treatments in favoring placebo treatment. However, the quality of the data was rated as either low or very low for several of the side effects; only the data for weight gain was ranked as high quality and the data for sedation was ranked as moderate quality. Furthermore, there was no usable data for CPZ on corrected QT prolongation. This suggests that there is an urgent need for more quality randomised controlled trials.

CHLORPROMAZINE AS AN ANTI-COVID-19 CANDIDATE

Because CPZ has been used for over 70 years, many pharmacological and safety data are easily available. Moreover, studies of the biological effects of CPZ have generated almost 20,000 published studies listed in PubMed, second only to Aspirin (over 50,000), largely owing to its abundant side effects. In Tokyo, November 1962, in a symposium on Japanese Encephalitis and other arboviral infections, the WHO reported: "*In the acute stage of the disease, the use of tepid sponging, ice packs, oxygen therapy, corticosteroids, salicylates, chlorpromazine may be effective*" [World Health Organization. Regional Office for the Western Pacific & Seminar on Japanese Encephalitis and other Arbovirus Infections (World Health Organization, 1962)]. To our knowledge, there is no epidemiological or observational study exploring the clinical status of COVID-19 patients under CPZ treatment. When faced with this alarming pandemic and given the concerns about the potentially high mortality rate, clinicians and patients will be tempted to try unproven therapies. CPZ repositioning includes a new use outside of psychiatry. The mechanism of action of CPZ is either via inhibition of clathrin-mediated endocytosis, and/or at later stages of virus assembly and egress which is not well known to psychiatrists. Conversely, virologists do not know very well its psychotropic effect. Thus, CPZ could prove efficacious in treating COVID-19 patients, provided adequate and good clinical trials are conducted and the results analyzed.

PROPOSAL FOR A RESEARCH PROTOCOL: PSYCHOVID

CPZ, in the context of the pandemic, could be helpful for the vulnerable psychiatric population. Psychotic patients are suffering much more often than the general population from comorbidities (cardiovascular pathologies, diabetes, obesity, smoking) which are risk factors for severe SARS-CoV-2 infection (Fonseca et al., 2020). Usually, without the pandemic context, patients hospitalized in psychiatry suffering from psychosis present a high-risk of pneumococcal infections (Seminog and Goldacre, 2013). In this context, we present a research protocol which can be conducted in a multisite setting for patients treated for psychosis and who develop COVID-19 (see **Supplementary Material**). The goal of this randomized controlled clinical trial would be to assess if CPZ is beneficial to limit the symptoms, and consequences of COVID-19 and maintain or improve psychotic symptoms as an add-on medication administered early at the onset of symptoms to the usual and adjusted antipsychotic treatment. It should be a randomized control trial with a comparison to treatment as usual arm without add-on CPZ (see **Supplementary Material**). Patients in the experimental arm should receive CPZ and should maintain their current treatment with an antipsychotic medication. To avoid increasing side effects due to antipsychotic medication, a down titration of their current treatment should be prescribed. The adjustment of the decreased dose of the antipsychotic patients for those who are in the experimental arm should be based on the well-known CPZ–Equivalent (CPZeq). The concept of CPZeq was derived from the potency for dopamine receptor blockade, which was determined empirically by judging the dose equivalence between different antipsychotic agents. The dosage of the add-on CPZ should be between 50 and 100 mg at night, according to the tolerability and the stability of the medical and psychiatric conditions. The outcome should be the efficacy of the symptoms related to COVID-19 and the global tolerability to CPZ (see **Supplementary Material**).

At this stage, the clinical trial framework we propose is a little simplistic in design, and it is not compared to design of the existing reCoVery trial (ClinicalTrials.gov Identifier: NCT04366739) for any new or unique elements. It does not include other phenothiazines, and is limited to psychiatric patients.

In fact, it could be an issue that this COVID-19 potential therapy would be limited to only psychiatric patients due to the drowsinees, and other features of CPZ. But why not if we consider psychotic patients very vulnerable and very often left out? While not ignored here, we leave a place for discussion to explore which patient population could be impacted or benefit by a successful trial outcome. CPZ is a better candidate against SARS-CoV-2 than some other medications. This property is its easy penetration in the brain. Indeed, more and more studies are emerging on the neurological complications (encephalitis, cognitive impairments, psychiatric disease such as depression, psychosis. . .) provoked by SARS-CoV-2 since it passes the blood brain barrier. It is thus important to find molecules that also pass the blood brain barrier in order to prevent neurological damages. The publication (Chew et al., 2020) indeed points to prochlorperazine as a potential better candidate for this proposed trial than CPZ, due to far less CNS penetration and reduced drowsiness (better side-effect profile).

Furthermore, it would be interesting to investigate the combined benefit of antipsychotic and antiviral activity of CPZ in patients with COVID-19 and psychosis. People with preexisting mental disorders may become more vulnerable during an epidemic (Chevance et al., 2020; Vigo et al., 2020) and any strategy that can simplify their lives and the appropriate follow-up will be welcome.

CONCLUSION

CPZ is one of the most widely used treatments for schizophrenia worldwide and remains low-cost and widely available. Despite its known side effects, CPZ is likely to remain a benchmark drug. This article makes a case for testing CPZ as a possible treatment for the COVID-19 infection. To engage the interest of virologists about a psychiatric drug, we presented in this review much historical information that provides a narrative perspective on what is otherwise a straightforward and rather "dry" reminder that some psychiatric medications may have useful antiviral properties. However, in the end, there is only scarce evidence that this drug will work, so this becomes a call to action for future studies. Currently, there are some limited, but encouraging clinical implications for these ideas with good quality controlled trials registered and in progress in France and Egypt (NCT 04366739, NCT0434805). The overview of the evidence, mainly *in vitro*, is informative. The various ways in which CPZ might have antiviral properties relative to COVID-19 might be helpful. In the end, the conclusion suggests that CPZ should probably be tried.

AUTHOR CONTRIBUTIONS

ES designed and directed the project, developed the theory and performed the review of literature, referred to his previous work on Chlorpromazine, and finalized the article. All other authors contributed to the article by performing review of the literature in their respective areas, writing and improving different portions of the article, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.577678/full#supplementary-material.

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COVID-19 Disease and Vitamin D: A Mini-Review

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Novel coronavirus disease (COVID-19) pandemic caused by SARS-CoV-2, for which there is no effective treatment except employing prevention strategies, has already instituted significant number of deaths. In this review, we provide a scientific view on the potential role of vitamin D in SARS-CoV-2 virus/COVID-19 disease. Vitamin D is well-known to play a significant role in maintaining the immune health of an individual. Moreover, it induces antimicrobial peptide expression that can decrease viral replication and regulate the levels of pro-inflammatory/anti-inflammatory cytokines. Therefore, supplementation of vitamin D has the potential to reduce the incidence, severity and the risk of death from pneumonia resulting from the cytokine storm of many viral infections including COVID-19. We suggest that supplementation of subjects at high risk of COVID-19 with vitamin D (1.000 to 3.000 IU) to maintain its optimum serum concentrations may be of significant benefit for both in the prevention and treatment of the COVID-19.

Keywords: SARS-CoV-2, COVID 19, respiratory tract infection, vitamin D3, vitamin D3 receptor

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INTRODUCTION

The occurrence of respiratory tract infections (RTI) is more common in winter, especially in the northern regions, than in the summer months (Hope-Simpson, 1981). This also applies to the rapidly spreading in the winter period around the world of the infectious Coronavirus disease 2019 (COVID-19) which became a pandemic, since the virus is more easily transmitted at low temperatures (Qu et al., 2020; Sajadi et al., 2020). This rises the possibility that insufficient intake of vitamin D_3 may have a role in the development and severity of COVID-19. Thus, in order to curb the current pandemic of COVID-19, it is opined that the administration of an adequate amounts of vitamin D_3 may stem the current situation till an effective therapy, chemoprophylaxis, and vaccination is developed.

Deficiency of vitamin D_3 in all age groups is a public health problem (Palacios and Gonzalez, 2014) that is well recognized. It is estimated that more than one billion people suffer from vitamin D_3 deficiency (Van Schoor and Lips, 2011). Several previous studies suggested that there is an independent association between low plasma concentrations of 25-hydroxyvitamin D_3 and susceptibility to acute respiratory infections (Cannell et al., 2006). Vitamin D_3 deficiency has been associated with many diseases including but not limited to type 2 diabetes mellitus, heart disease, stroke, autoimmune diseases, asthma and RTIs (Hollick, 2007; Hollick, 2017). The relation

between low levels of vitamin D_3 and infection with bovine diarrhea virus in calves has been well established (Nonnecke et al., 2014). It is evident that in winter due to the shorter time spent in the sun, the plasma levels of vitamin D_3 is likely to be low (Berardi and Newton, 2009; https://www.medlineplus.gov/vitamind.html). This is especially evident in countries such as the United States of America (USA), United Kingdom (UK), Switzerland, Italy, Spain, Iran, France, Turkey, etc. It is rather interesting that COVID-19 pandemic and its high mortality (Pharmacy Times, 2020; https://www.pharmacytimes.com/publications/issue/2010/february2010/otcfocusvitamind-0210) has been reported in these countries. According to the US National Center for Health Statistics, approximately 70% of the population may be deficient in vitamin D_3 and surprisingly while the United States is presently the most affected by COVID-19 (Kmiec et al., 2014). This is in line with the current proposal that severe acute respiratory syndrome due to SARS-CoV-2 and its associated high mortality rate may be as a result of vitamin D_3 deficiency. Furthermore, vitamin D_3 deficiency is known to elevate with increasing age and comorbidities that are associated with lower vitamin D_3 levels.

In the current review, we present a scientific rationale on the potential relationship between vitamin D_3 content and higher incidence of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) virus infection. Moreover, our review also summarizes the current understanding of the link among vitamin D_3, the immune system, and respiratory infections.

VITAMIN D AND IMMUNE SYSTEM

Vitamin D is a pluripotent hormone that modulates the innate and adaptive immune responses (Rezaei, 2018). Vitamin D could play a decisive role in the proliferation and immunomodulation of cells, affecting several immune pathways enhancing the protective properties of the mucous membranes of the body and inhibiting excessive inflammation (D'Ambrosio et al., 1998; Khare et al., 2013; Parlak et al., 2015). Immunocytes such as macrophages, B and T lymphocytes, neutrophils and dendritic cells express Vitamin D_3 receptors (VDRs) that is enable to the actions of vitamin D (Di Rosa et al., 2011). The active metabolite of vitamin D lead to the activation of VDRs that can form Retinoid X Receptor (RXR) heterodimer that, in turn, influences the proteins of the innate and adaptive immune system (the regulatory T cells, defensins, cytokines, pattern recognition receptors, etc.) (Chun et al., 2014).

The immune system is influenced in various ways by both vitamin D_3 and its metabolite 1,25-hydroxy-vitamin D_3. 1,25-hydroxy-vitamin D_3 rigorously regulates antimicrobial peptides such as defensin and cathelicidin (Adams et al., 2009). Cathelicidin possesses an antimicrobial function against mycobacteria, Gram-positive and Gram-negative bacteria due to its ability to destroy cell membranes. 1,25-hydroxy-vitamin D_3 has antiviral effect against adenovirus, herpes simplex virus, enveloped and non-enveloped retroviruses, and fungi (Herr et al., 2007). By damaging cell membranes, these peptides penetrate infected cells and neutralize the action of endotoxins (Agier et al., 2015). For instance, the LL-37, antimicrobe peptide, has antibacterial and antifungal properties by virtue of its ability to disrupt the integrity of the cell membrane and proton gradient (Bals and Wilson, 2003) by vitamin D_3 (Howell et al., 2004; Leikina et al., 2005; Steinstraesser et al., 2005; Bergman et al., 2007). In addition, vitamin D_3 inhibits the production of pro-inflammatory cytokines and augments that of anti-inflammatory cytokines (Gombart et al., 2020). Thus, vitamin D_3 influences the incidence and severity of viral infections by altering the production of pro-inflammatory cytokines. There is reasonable evidence to suggest that vitamin D_3 can inhibit the transcription induced by tumor-necrosis-factor-α (TNF-α) in latently infected cells by human immunodeficiency viruses (HIV) (Nunnari et al., 2016). These and other results suggest that vitamin D_3 can inhibit the production of inflammatory cytokines and chemokines such as TNF-α, interferon-β (IFN-β), interleukine (IL)-8, IL-6 and Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) (Hansdottir et al., 2010; Khare et al., 2013). Increase in mortality in those with COVID-19 is due to acute respiratory distress syndrome (ARDS) due to unantagonized production of pro-inflammatory cytokines IL-6 and TNF-α. Vitamin D_3 has a decisive role in the regulation of the innate and adaptive immune responses implying that adequate intake of vitamin D_3 may protect patients with COVID-19 at least, in part by inhibiting the excess production of IL-6 and TNF-α (Daneshkhah et al., 2020). Vitamin D_3 can also contribute to the modification of the antiviral response by enhancing the secretion of pro-inflammatory chemokines (C-X-C Motif Chemokine Ligand 8, CXCL8 and C-X-C Motif Chemokine Ligand 10, CXCL10) (Brockman-Schneider et al., 2014). Lytic phase of cytomegalovirus (CMV) replication can be induced by vitamin D_3 *in vitro* (Wu and Miller, 2015).

Vitamin D_3 promotes immunoglobulin and complement-mediated phagocytosis by stimulating the maturation of monocytes to macrophages. In addition, vitamin D_3 maintains self-tolerance by reducing a hyperactive adaptive immune system (Bowie and Unterholzner, 2008). Vitamin D_3 reduces the replication of influenza A (Barlow et al., 2011), rotavirus (Zhao et al., 2019) and dengue microbes (Martínez-Moreno et al., 2019). These results imply that excess innate immune response induced by viral and other microbial infections seen in patients with SARS-CoV-2 and associated cytokine storm can be effectively reduced by vitamin D_3 (Huang et al., 2020). The immunomodulatory effect of vitamin D_3 on viral infections appears to be temporary and at least, this in part could be attributed to its immunomodulatory role in viral infections is rather complex and depends on the nature of the pathogen and the type of immune function that is needed to resolve the disease process (Sacco et al.,2012; Gotlieb et al., 2018).

There is reasonable evidence to suggest that vitamin D_3 modulates adaptive immune responses by inhibiting the Th1 cell function that leads to a reduction in the production of TNF-α, IL-2, granulocyte macrophage colony-stimulating factor and IFN-β. 1,25-$(OH)_2$-Vitamin D_3 enhances the action of Th2 cells and production of their anti-inflammatory cytokines, IL-

TABLE 1 | Some effects of vitamin D on the immune system.

Immune cell type	Effect of vitamin D	References
Airway epithelium	Increases CD14 and cathelicidin. Dampens IFN-β and chemokine response during viral infection	Hansdottir et al. (2010)
Alveolar macrophages	Increases the antimicrobial peptide cathelicidin	Liu et al. (2007)
Dendritic cells	Inhibits dendritic cell differentiation, maturation and function, decreases IL-12 and increases IL-10, alters T cell activation	Penna and Adorini (2000); Piemonti et al. (2000); Fritsche et al. (2003); Sigmundsdottir et al. (2007)
T lymphocytes	Inhibits proliferation, modulates cytokine production - inhibits Th1 and Th17 cytokines but induces Tregs	Lemire et al. (1995); Penna and Adorini (2000); Sigmundsdottir et al. (2007); Daniel et al. (2008); Mora et al. (2008)
B lymphocytes	Inhibits proliferation of activated B cells and generation of plasma cells	Chen et al. (2007)

4, IL-5, and IL-10 (Hughes and Norton, 2009). In addition, supplementation of vitamin D_3 increases the number of regulatory T cells (Treg cells), suppresses IgG production and differentiation of dendritic cells (Kamen and Tangpricha, 2010; Aranow, 2011; Rondanelli et al., 2018). 1,25-$(OH)_2$-Vitamin D_3 inhibits the proliferation and activation of T cells and T and B lymphocytes (Martineau et al., 2017). Thus, vitamin D_3 suppresses T-cell-mediated inflammation and promote the proliferation of Treg cells that results in an increase in the production of IL-10 that leads to suppression of inappropriate inflammation (Adorini and Penna, 2009; Chun et al., 2014). Vitamin D_3 can also increase the expression of glutathione reductase and glutamate–cysteine ligase modifier subunit (Lei et al., 2017) that may lead to a decrease in oxidative stress. These results led to the proposal that (Biancatelli et al., 2019; Mousavi et al., 2019; Wimalawansa, 2020) vitamin D_3 may be of benefit to combat SARS-CoV-2 infection (Grant et al., 2020a).

Vitamin D deficiency is common in patients with HIV (Herr et al., 2007). The antiviral action of vitamin D_3 can also be attributed to its ability to increase the production of cathelicidin and defensins (Herr et al., 2007; Hughes and Norton, 2009; Beard et al., 2011). Furthermore, 1,25-dihydroxy-cholecalciferol is known to regulate more than 200 genes including those responsible for cell proliferation, differentiation, and apoptosis (Umar and Sastry, 2018) including those involved in immune homeostasis (Van Herwegen et al., 2017). Recent meta-analysis of randomized controlled trials (RCTs) showed that vitamin D deficiency increases the overall mortality (Bjelakovic et al., 2014; Keum et al., 2019; Manson et al., 2019; Scragg, 2020). All above-mentioned effects of Vitamin D_3 are presented in **Table 1**.

RELEVANCE OF VITAMIN D REGARDING TO RESPIRATORY TRACT INFECTIONS AND INFLUENZA

There is a provided evidence given by many reviewed studies to support the hypothesis that higher serum level of vitamin D_3 is associated with a low risk of microbial infections and deaths from RTIs caused by pneumonia and influenza. In addition, SARS-CoV-2 infection and decrease the severity and mortality may be avoided by a normal serum vitamin D_3 levels (Wimalawansa, 2020). Unfortunately, there are no standard recommendations regarding the dose and the desired optimal concentration of vitamin D_3 required to protect people from RTI during the winter season.

Epidemiological studies revealed that vitamin D_3 plays a critical role in viral RTIs and associated acute lung injury (Hansdottir and Monick, 2011). In a recent meta-analysis, it has been shown that a daily or weekly vitamin D_3 dose between 20 and 50 μg resulted in a significant reduction of RTIs (Martineau et al., 2017). A high-dose, isolated, or added bolus of (2.5 mg once or monthly) did not reduce the risk. One study supplemented for one-year high risk individual for ARDS with a 100 μg/daily (Bergman et al., 2012). The overall infection score was significantly reduced in the treated groups, and those with vitamin D_3 deficiency showed the greatest benefit of the supplementation.

In addition, it is observed that the degree of protection generally increases when the concentration of vitamin D_3 reaches its optimal range of 40 to 60 ng/ml. To reach this level, an individual must take between 2,000 and 5,000 IU/day of vitamin D_3 (Heaney et al., 2003). Calcitriol protects against acute lung injury by modulating the expression of the renin–angiotensin system including angiotensin-converting enzyme 2 (ACE2) in lung tissue (Xu et al., 2017). There seems to be a direct relationship between plasma 25-(OH)-Vitamin D_3 concentrations and severity of COVID-19 (Huang et al., 2020; Wang et al., 2020; Zhou et al., 2020). It is noteworthy that the expression of the DPP-4/CD26 receptor is significantly reduced as a result of vitamin D_3 deficiency (Komolmit et al., 2017). Furthermore, adequate provision of vitamin D_3 seems to attenuate immunological events that may lead to prolonged interferon-gamma response (Zdrenghea et al., 2017), and persistent interleukin six elevation that are negative prognostic value indicators in those with severe COVID-19 (Miroliaee et al., 2018).

VDRs are very widely distributed in respiratory epithelial cells and immune cells (B cells, T cells, macrophages and monocytes). VDRs are in the epithelium of the bronchi and immune cells (Pfeffer and Hawrylowicz, 2012). The enzyme, 1a-hydroxylase (CYP27B1), required for vitamin D activation, is induced by diverse stimuli, including cytokines and toll-like receptor ligands in the respiratory tract. Nevertheless, adequate serum levels of 25-(OH)-vitamin D_3 is required to increase levels of 1,25-$(OH)_2$-vitamin D_3 and to improve the immune response to respiratory virus infections (Greiller and Martineau, 2015). The development

of ARDS shows typical changes in membrane permeability of the alveolar capillary, progressive edema, severe arterial hypoxemia and pulmonary hypertension (Matthay et al., 2012). In animal studies, vitamin D_3 significantly attenuated lung damage caused by lipopolysaccharides (LPS) (Xu et al., 2017). This is noteworthy since LPS increase the pulmonary expression of renin and angiotensin 2 (Ang 2) that promotes inflammation. Vitamin D_3 reduces the increased renin and Ang 2 expression and thus significantly lowers lung injury. It has been suggested that vitamin D_3 promotes ACE2/Ang 1–7 activity. This is supported by the observation that calcitriol treatment significantly increased the expression of VDR mRNA and ACE2 mRNA that leads to a reduction in angiotensin II, ACE2 expression resulting in suppression of inflammation (Yang et al., 2016). VDRs are not only a negative regulator of renin, but also of NF-kB (Li et al., 2004), leading to an increase in Ang 2 formation, which promotes pro-inflammation (Jurewicz et al., 2007).

Down-regulation of ACE2 expression by SARS-CoV infection is associated with acute lung damage (edema, increased vascular permeability, reduced lung function) and associated RAS dysregulation leads to increased inflammation and vascular permeability as seen in COVID-19 (Imai et al., 2005). It was reported that COVID-19 is associated with release of pro-vitamin D_3 enhances the cellular immunity and reduces the cytokine storm induced by the innate immune system. Vitamin D_3 can reduce the production of pro-inflammatory cytokines such as TNF-α and IF-γ (Tjabringa et al., 2005; Baeke et al., 2010; Laaki, 2012). Several studies showed that adequate intake and plasma levels of vitamin D_3 reduces the risk of viral infections through their action on immunocytes (Carnell et al., 2006; Baeke et al., 2010; Schwalfenberg, 2011; Lang and Samaras, 2012). Hence, it is suggested that vitamin D_3 may have a significant role in COVID-19 due to its action on T cells (Zhang et al., 2015).

Type-II pneumocytes which are the primary target of coronaviruses, express high levels of ACE2 receptor (Bombardini and Picano, 2020). Metabolites of 25-(OH)-vitamin D_3 have been reported to stimulate surfactant synthesis in alveolar type-II cells (Rehan et al., 2002). Human fetal and adult alveolar type-II cells supplemented with 1,25-dihydroxy-vitamin D_3 show increased levels of VDRs and expression of surfactant associated protein B, a lipid-associated protein of the pulmonary surfactant, indicating the potential of vitamin D_3 to reduce surface tension in COVID-19 (Phokela et al., 2005).Comorbid conditions such as diabetes mellitus, hypertension and chronic obstructive pulmonary disease are commonly associated with low plasma vitamin D_3 levels (Malinovschi et al., 2014; Kim et al., 2015; Grant et al., 2020a). Hence, it is reasonable to propose that COVID-19 may be associated with low plasma vitamin D_3 levels. Hence, it is suggested that vitamin D_3 supplementation may be of significant benefit in COVID-19. Grant, in the latest report, suggest that vitamin D level checking will be conducted only in as elected category of patients that involves pregnant mothers, obese and elderly people and others suffering from certain comorbid conditions (Grant et al., 2020b). Multiple factors such as an ability of the assimilation by the gastrointestinal tract, body weight, genetic factors and the baseline 25-(OH)-vitamin D_3 concentration, control the increase in vitamin D concentrations with respect to oral vitamin D_3 supplementation. Given the degree of vitamin D_3 deficiency, taking 5,000 IU of vitamin per day, it could be essential to elevate 25-(OH)-vitamin D_3 levels to 40 ng/ml by (Veugelers et al., 2015).

A recent article indicates that vitamin D_3 value >20 ng/ml is required and this advice is adopted by several countries (Amrein et al., 2020). Another research suggests a higher dose for RTIs, indicating rates >30 ng/ml of vitamin D_3 as effective in decreasing cancer incidence, unfavorable pregnancy and birth outcomes and type 2 diabetes mellitus (Grant et al., 2020b). From another analysis it is suggested that optimal vitamin D_3 standard should be 40–60 ng/ml for prevention of breast and colorectal cancer (Garland et al., 2009).

The U.S. Institute of Medicine noted that no research observed negative consequences of supplementation of vitamin D_3 of less than 10,000 IU/daily, but set the upper consumption limit at 4,000 IU/daily, partially owing to retrospective tests that found U-shaped 25-(OH)-vitamin D_3 concentration/health outcome relationships. However, further findings indicate that most observations of J- or U-shaps relationships came from observational studies that did not test serum 25-(OH)-vitamin D_3 concentrations, and that the likely explanation for these relationships was the presence of some participants who started taking vitamin D_3 complementation shortly before registration (Grant et al., 2016). Particularly in winter, supplementation with vitamin D_3 is required for many individuals to reach concentrations of 25-(OH)-vitamin D_3 above 30 ng/ml (Pludowski et al., 2018). However, vitamin D_3 fortification of basic foods such as dairy and flour products may increase serum 25(OH)D concentrations by a few ng/ml among those members of different populations with the lowest concentrations (Pilz et al., 2018; Grant and Boucher 2019). This will contribute to a decreased risk of ARTIs for persons with a severe vitamin D_3 deficiency (Camargo et al., 2012; Martineau et al., 2017). However, regular or weekly treatment of vitamin D_3 is advised for greater benefits (Martineau et al., 2017), as is the annual evaluation of serum 25-(OH)-vitamin D_3 levels for health risks individuals (Grant et al., 2020b).

Table 2 describes the findings from meta-analyses that vitamin D_3 is protective against acute RTI, particularly in patients with vitamin D_3 deficiency.

HYPOTHESIS OF THE CORRELATION ON VITAMIN D_3 LEVELS AND CORONAVIRUS DISEASE-19 CASES/SEVERITY

Still there is a lack of a cohort studies and clinical trials in determining the role of vitamin D_3 in the prevention of COVID-19 infections and/or severity. Some retrospective studies have demonstrated the relationships between vitamin D_3 levels and COVID-19 cases and severity (**Table 3**).

For example, a preliminary information study from Philippines on 212 reported COVID-19 patients, found that the severity of the infection is a highly correlated to the

TABLE 2 | The finding on the efficacy of vitamin D in the respiratory tract infections.

Participants	Study characteristics	Vitamin D effect	References
5,660 participants (age ranging from 6 months to 75 years)	Eleven randomized placebo-controlled trials	Supplementation with vitamin D significantly decreased the risk of RTI (OR: 0.64; 95% CI: 0.49, 0.84; p = 0.0014)	Bergman et al. (2013)
1,868 participants (aged 1–83 years)	Five clinical trials	The reduction of episodes of RTI was significantly lower in vitamin D supplementation group compared to the control group (OR = 0.58; 95% CI: 0.42, 0.81; p = 0.001)	Charan et al. (2012)
10,933 participants (aged 0–95 years) from 14 different countries	Twenty five randomized controlled trials	Overall results showed that vitamin D supplementation has protective effective in decreasing the risk of suffering at least one acute RTI (OR 0.88; 95% CI: 0.81, 0.96; p = 0.003)	Martineau et al. (2017)

OR, Odds ratio; RTI, Respiratory tract infection; CI, Confidence interval.

vitamin D_3 levels (Alipio, 2020). Authors have found that 85.5% of patients with an adequate status of vitamin D_3 (>30 ng/ml) showed a moderate disease, while a 72.8% of patients with vitamin D_3 deficiency (<20 ng/ml) had the serious disease symptoms (Alipio, 2020). The correlation between vitamin D_3 and COVID-19 have extensively investigated in a group of 178 Indonesians (Raharusun et al., 2020). According to this study, the patients with vitamin D_3 levels in the categories, 20–30 and <20 ng/ml, were 12.55 times and 19.12 times more likely to die from COVID-19, respectively, as compared with COVID-19 patients with sufficient levels of vitamin D_3. The main conclusion is that, even after controlling for age, sex and comorbidities, deaths were 10.12 times more likely in patients with vitamin D_3 deficiency than in patients with normal vitamin D_3 levels (Raharusun et al., 2020). A limited cohort observational study with 43 cases in Singapore have found that a treatment of COVID-19 patients with an oral doses of vitamin D_3 (1.000 IU), Mg (150 mg), and vitamin B_{12} (500 μg) significantly reduced the application of the subsequent oxygen therapy compared to controls (3/17 vs. 16/26, p = 0.006) (Tan et al., 2020). Furthermore, such drugs combination have protected against the clinical deterioration (p = 0.041) even after adjustment of confounders (age, sex and comorbidity) (Tan et al., 2020). Severe COVID-19 patients and patients with pre-existing medical conditions were reported to have low levels of vitamin D_3 (Glicio et al., 2020; Lau et al., 2020). A retrospective observational study with 186 positive cases and 2717 negative controls in Belgium have demonstrated a low median for vitamin D_3 in the COVID-19 patients compared to the control subjects (p = 0.0016) (De Smet et al., 2020). A retrospective cohort study with 780 cases in Indonesia showed that below-normal vitamin D_3 levels and the pre-existing medical conditions in the older and male cases have higher odds of death. Moreover, the vitamin D_3 status has a strong relationship with COVID-19 mortality if it adjusted for age, sex and comorbidities (Raharusun et al., 2020). The similar retrospective study in the USA with many cases have showed that the reduced risks for both COVID-19 cases and the mortality are possibly associated with the sunlight and vitamin D_3, as well with the latitude as an indicator (Li et al., 2020).

In a new systematic review and meta-analysis with an ecological approach, they found a high percentage of COVID-19 patients who suffer from vitamin D_3 deficiency or insufficiency. Much more important its ecological investigation resulted in the substantial direct and reverse correlations between the recovery and mortality rates in COVID-19 patients with vitamin D_3 deficiency at the different countries. A small reverse correlation between vitamin D_3 status and the mortality rate have found globally. The populations with a lower levels of vitamin D_3 might be more susceptible to the novel coronavirus infection (Ghasemian et al., 2020). Recently, a cohort study of 489 patients who had a vitamin D_3 levels detected in the year before COVID-19 testing was 1.77 times greater for patients with vitamin D_3 deficiency compared to the patients with a normal vitamin D_3 status. These findings appear to support a role of vitamin D_3 status for the COVID-19 risk (Meltzer et al., 2020).

The hypothesis that supplementation with vitamin D_3 may reduce the risk of influenza and COVID-19 disease, as well the death should be examined in the trials to evaluate the correct doses, the serum 25-(OH)-vitamin D_3 concentrations and the existence of any health concerns. There are a good model from Atlanta and Georgia in which have done the RCT on vitamin D_3 supplementation for the ventilated ICU patients (Han et al., 2016).

There is a recommendation to take a vitamin D_3 at 10,000 IU/day as an acceptable dose to raise circulatory concentration of vitamin D_3 to the optimum range of 40–60 ng/ml; after 1 month this dose should be lowered to 5,000 IU/day to the sustain serum rate (Ekwaru et al., 2014; Shirvani et al., 2019). A recent study have suggested a loading doses of 200,000–300,000 IU of vitamin D_3 to reach the optimum serum range, thereby the reducing of the risk/severity for COVID-19 (Wimalawansa, 2020).

The observation that normal vitamin D_3 status is important for the immune system as well as for the regulation of SAR should lead to a correction of vitamin D_3 status if a deficiency has been detected. There is no experience with the use of vitamin D_3 in COVID-19. In addition, it should be noted that a very high doses of the upper limit of 4,000 IU (100 μg) per day of vitamin D_3 still have the risks and may be dangerous. Since such doses might result in to the improvements in the VDR competency and could have an inhibitory impact on the immune function (Mangin et al., 2014).

CONCLUSION

It is evident from the preceding discussion that vitamin D_3 may be of benefit in COVID-19. Since the higher plasma concentrations of vitamin D_3 is better for the protection from

TABLE 3 | The outcomes in recent studies about the correlation of vitamin D_3 concentrations with COVID-19 infections.

Country	Population type	n	Study design	Vitamin D_3 doses	Outcomes	Reference
Singapore (a tertiary academic hospital)	Adults, age ≥50 years	43	Cohort observational	Vitamin D_3 1,000 IU, Mg 150 mg, and vitamin B_{12} 500 μg (oral)	i) A fewer patients who received vitamin D_3, Mg and vitamin B_{12} required the subsequent oxygen therapy compared to controls (3/17 vs. 16/26, p = 0.006) ii) in multivariate analysis, the patients treatment with vitamin D_3, Mg and vitamin B_{12} have showed a significant protective effects against clinical deterioration (p = 0.041) after adjusting for age, gender and comorbidities	Tan et al., 2020
20 European countries	Adults	Cases and death/1 M population	Retrospective	NA	A significant negative correlation was observed for the serum 25-(OH)-vitamin D_3 levels with COVID-19 cases (p = 0.033) but not with a death (p = 0.123) per million of population	Present study
20 European countries	Adults	Cases and death/1 M population	Retrospective (as of 8 April 2020)	NA	A negative correlation was observed between the serum 25-(OH)-vitamin D_3 levels and COVID-19 cases (p = 0.050) and a death (p = 0.053) per million of population	Ilie et al. (2020)
Southern Asian countries	NA	222	Retrospective multicentral study	NA	i) The differences in the levels of vitamin D_3 mean were significant within the mild, ordinary, severe and critical cases of COVID-19 (p < 0.001) ii) Vitamin D_3 status showed a significant association with clinical outcomes (p < 0.001)	Alipio (2020)
USA (a single tertiary academic medical center)	Adults, mean age 65.2 years	20	Retrospective observational study	NA	A high vitamin D_3 insufficiency was observed in ICU patients (84.6%) than in the floor patients (57.1%) (p = 0.29)	Lau et al. (2020)
South Asia (two tertiary medical centers)	Adults, age ≥60 years	176	Retrospective	NA	i) Severe patients had a low level of vitamin D than mild patients ii) Subjects with the pre-existing medical conditions had a low level of vitamin D_3	Glicio et al. (2020)
UK (UK Biobank data 2006–2010 for vitamin D_3 and ethnicity)	Adults, age 37–73 years	449	Cross-sectional (16 March–14 April 2020)	NA	i) Vitamin D_3 levels showed a significant association with COVID-19 infection in an univariate analysis (p = 0.013) but not after an adjustment for confounders (p = 0.208) ii) Ethnicity showed a significant association with COVID-19 infection univariably	Hastie et al. (2020)

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TABLE 3 | (*Continued*) The outcomes in recent studies about the correlation of vitamin D_3 concentrations with COVID-19 infections.

Country	Population type	n	Study design	Vitamin D_3 doses	Outcomes	Reference
United Kingdom (UK Biobank data 2006 2010 for BMI, vitamin D_3 and ethnicity)	Adults, mean age 57.7 years	580 cases and 723 control	Retrospective	NA	i) No significant difference was observed for vitamin D_3 levels between COVID-19 cases and the control group ii) Vitamin D_3 status was significantly lower in those of Asian, Black and mixed ethnicity ($p < 0.0010$) compared with those of White ethnicity iii) Vitamin D_3 levels were significantly lower in those with obesity ($p < 0.001$). Overweight or obese person; living in London; being male and being of Asian, Black or mixed ethnicity was associated with a higher odd of positive cases iv) In the regression model, the interaction between BMI and vitamin D_3 status did not predict the test result in the available data set	Darling et al. (2020)
Mainland of United States (48 states and Columbia district)	1.609.488 cases and 91.094 deaths	-	Retrospective (22 Jan–23 May 2020)	NA	i) Latitudes were marginally associated with the cases ($p = 0.0792$) and the deaths ($p = 0.0599$) ii) Sunlight and vitamin D_3, with latitude as an indicator, possibly associated with reduced risks for both COVID-19 cases and mortality	Li et al. (2020)
Belgium (central network hospital)	Adults, median age 71 years (cases), 68 years (control)	186 cases, 2,717 controls	Retrospective observational (1 March–7 April 2020)	NA	i) Patients with COVID-19 had significantly a low median value of vitamin D_3 and higher vitamin D_3 deficiency compared to control subjects ($p = 0.0016$, $p = 0.0005$, respectively) ii) This difference were more pronounced in male COVID-19 subjects than male control subjects that increased with advancing radiological stage and were not confounded vitamin D_3-impacted comorbidities	De Smet et al. (2020)
Hospitals and clinics from different parts of the world	Age up to 80 years	5,000 cases	As on March 21, 2020	NA	About 15% reduction in the number of severe COVID-19 cases given a normal vitamin D_3 status within a population	Daneshkhah et al. (2020)
Indonesia (Government hospital)	Adults, mean age 54.5 years	780 cases	Retrospective cohort study (2 March 2–24 April 2020)	NA	i) In univariate analysis, older and male cases with the pre-existing medical condition and below normal vitamin D_3 levels were associated with the higher odds of death ii) After adjustment of confounders (age, sex and comorbidity), vitamin D_3 levels showed a strong relationship with the COVID-19 mortality	Raharusun et al. (2020)

various viral and respiratory infections, it is reasonable to suggest that regular supplementation of vitamin D_3 to those who are at high risk of developing various viral respiratory infections including COVID-19 need to considered seriously. To verify this proposal, double-blind placebo-controlled trials and large-scale intervention and prevention studies using vitamin D_3 are needed. If this proposal is true it leads to the development of a simple, easily implementable method of preventing the incidence of COVID-19 and reducing its serious complications by simple oral supplementation of vitamin D_3. Furthermore, vitamin D_3 has several other benefits in the form of preventing rickets, improving general health, and reducing mortality due to its deficiency (though the exact cause for this association is not clear) add strength to the concept that its supplementation is warranted.

AUTHOR CONTRIBUTIONS

MB, VI, RM, DB interpreted the data from the literature. MB, VI, RM, JF, DB wrote the original draft. MB, VI, RM, JF, FD, LG, UD, AE-A reviewed, edited and drafted the manuscript, and approved the final version.

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MINI REVIEW
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Hydroxychloroquine as a Chemoprophylactic Agent for COVID-19: A Clinico-Pharmacological Review

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Hydroxychloroquine has gained much attention as one of the candidate drugs that can be repurposed as a prophylactic agent against SARS-CoV-2, the agent responsible for the COVID-19 pandemic. Due to high transmissibility and presence of asymptomatic carriers and presymptomatic transmission, there is need for a chemoprophylactic agent to protect the high-risk population. In this review, we dissect the currently available evidence on hydroxychloroquine prophylaxis from a clinical and pharmacological point of view. *In vitro* studies on Vero cells show that hydroxychloroquine effectively inhibits SARS-CoV-2 by affecting viral entry and viral transport via endolysosomes. However, this efficacy has failed to replicate in *in vivo* animal models as well as in most clinical observational studies and clinical trials assessing pre-exposure prophylaxis and postexposure prophylaxis in healthcare workers. An analysis of the pharmacology of HCQ in COVID-19 reveals certain possible reasons for this failure—a *pharmacokinetic* failure due to failure to achieve adequate drug concentration at the target site and attenuation of its inhibitory effect due to the presence of TMPRSS2 in airway epithelial cells. Currently, many clinical trials on HCQ prophylaxis in HCW are ongoing; these factors should be taken into account. Using higher doses of HCQ for prophylaxis is likely to be associated with increased safety concerns; thus, it may be worthwhile to focus on other possible interventions.

Keywords: severe acute respiratory syndrome coronavirus 2, hydroxychloroquine, coronavirus disease 19, chemoprophylaxis, coronavirus

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INTRODUCTION

COVID-19, the disease caused by the coronavirus SARS-CoV-2, continues to be an immense challenge for the scientific community throughout the world. The number of cases and deaths has been on the rise, but currently, there are only a few therapeutic and no chemoprophylactic interventions in our arsenal to combat the virus.

Abbreviations: HCQ, hydroxychloroquine; CQ, chloroquine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MERS-CoV, Middle Eastern respiratory syndrome coronavirus; PPE, personal protective equipment; HCW, healthcare workers; G6PD, glucose-6-phosphate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor; TLR, toll-like receptor; USFDA, U.S. Food and Drug Administration.

Due to the constraints of time, there has been much focus on the strategy of "drug repurposing/repositioning," defined as identifying new uses of approved drugs that are outside the scope of their original medical indication (Ashburn and Thor, 2004). The 4-aminoquinoline hydroxychloroquine (HCQ) and its congener chloroquine (CQ) have been repurposed for COVID-19 due to their proposed antiviral properties (Savarino et al., 2003). Reports of preclinical evidence of efficacy led to HCQ receiving unprecedented attention by the scientific community as well as by the lay public and media. The political attention and the controversies surrounding this drug have further fueled a debate in the scientific community over its potential as a chemoprophylactic agent against SARS-CoV-2 and for treatment of COVID-19.

In this review, we aim to discuss the potential role of HCQ as a chemoprophylactic agent for COVID-19. We discuss why HCQ is a good candidate for a chemoprophylactic agent, followed by a dissection of the currently available evidence. The next section emphasizes the current caveats in knowledge and the complexities associated with HCQ prophylaxis with regard to its dosing and pharmacokinetic properties. Finally, we conclude with an overall assessment of the current evidence and recommendations for the future.

NEED FOR PROPHYLAXIS IN COVID-19

Chemoprophylaxis has been used in many diseases to protect high-risk groups from severe disease, such as malaria prophylaxis for patients with sickle cell disease (Oniyangi and Omari, 2019) and antiviral prophylaxis against influenza for immunosuppressed children and adults (Uyeki et al., 2019) and as a preventive measure against mass outbreaks, for example, mass prophylaxis against meningococcal infections (McNamara et al., 2018). For effective chemoprophylaxis, the drug should have activity against the infective agent and achieve tissue specific concentrations. In addition, adverse effects should be minimal to ensure acceptability. Further, the drug should be easily available and inexpensive.

Certain characteristics of SARS-CoV-2 and COVID-19 have fueled the ongoing pandemic, particularly its high transmissibility and low overall case fatality rates. Estimates for the basic reproduction number (R_0) of SARS-CoV-2 have ranged from 2 to 5.5 (Li et al., 2020; Read et al., 2020; Shen et al., 2020; Wu et al., 2020), higher than that of SARS-CoV (R_0 = 1.7–1.9) and MERS-CoV (R_0 = 0.7) (Petrosillo et al., 2020). More than 80% of COVID-19 cases report only mild symptoms (Wu and McGoogan, 2020). In contrast to SARS, patients with COVID-19 demonstrate high viral loads with active viral replication in the upper respiratory tract (Wölfel et al., 2020), with a peak of viral load occurring at the time of presentation (To et al., 2020). Moreover, recent evidence has suggested that presence of pre-symptomatic transmission and asymptomatic carriers may be common in COVID-19 (Arons et al., 2020; Chau et al., 2020). These characteristics render case-based detection less effective and add to the enigma of controlling the rampant spread of this pandemic. While nonpharmaceutical interventions like case-based isolation, contact tracing, closure of public places, and lockdowns have been able to reduce the spread to an extent (Davies et al., 2020; Patel et al., 2020), proper implementation of these measures is seldom possible for prolonged periods due to the socioeconomic fallout. Thus, in the absence of an effective vaccine in the near future, a chemoprophylactic agent can greatly help in mitigating the impact of COVID-19.

Such an agent should be targeted toward protecting the most susceptible and vulnerable groups within the population. Severe illness and hospitalization due to COVID-19 is known to be associated with older age and presence of comorbidities like diabetes mellitus, hypertension, cardiovascular disease, chronic lung disease, malignancy, and obesity (Cummings et al., 2020; Petrilli et al., 2020; Richardson et al., 2020). The incidence of noncommunicable diseases is increasing worldwide; all-age prevalence of diabetes is projected to rise to 4.4% by 2030, with nearly 366 million cases (Wild et al., 2004). Around one-fourth of the Indian population suffers from hypertension (Gupta et al., 2019), and the prevalence of diabetes and chronic obstructive pulmonary disease is 20.4 and 4.2%, respectively (Salvi et al., 2018; Tandon et al., 2018).Thus, a significant proportion of the population is at risk of severe COVID-19 infection. Close contacts of patients confirmed to have COVID-19 are also at significant risk of contracting it. Another susceptible group that needs to be protected is the healthcare workers (HCW). During the SARS epidemic, most outbreaks occurred in the healthcare setting (Yu et al., 2007). Reports from Italy have shown that as many as 20% HCW taking care of COVID-19 patients were infected (Lancet, 2020). HCW are at increased risk due to prolonged exposure to a large number of infected patients; this risk is compounded if they are involved in performing aerosol-generating procedures like endotracheal intubation or if they are wearing inadequate personal protective equipment (PPE). It is of paramount importance to protect frontline workers in order to prevent overburdening of a country's healthcare system. An effective chemoprophylactic agent is therefore the need of the hour.

In view of this overwhelming need of a chemoprophylactic agent, the exceptional circumstances created by the pandemic, and preliminary evidence of efficacy of HCQ, the COVID-19 National Task Force of India issued a recommendation for empiric use of HCQ as prophylaxis for all HCW, other frontline workers involved in COVID-19 activities, and asymptomatic household contacts of laboratory-confirmed cases (National Task Force for COVID-19, 2020). The dosage recommended was a loading dose of 400 mg twice a day on day 1, followed by 400 mg once weekly. There are no official guidelines for hydroxychloroquine prophylaxis in other countries, although off-label use of hydroxychloroquine has been reported in Africa, France, and the United States.

MECHANISM OF ACTION OF HYDROXYCHLOROQUINE AND PRECLINICAL EVIDENCE

SARS-CoV-2 enters host cells through binding of the S1 subunit of its spike (S) protein with the ACE2 receptor on the host cell

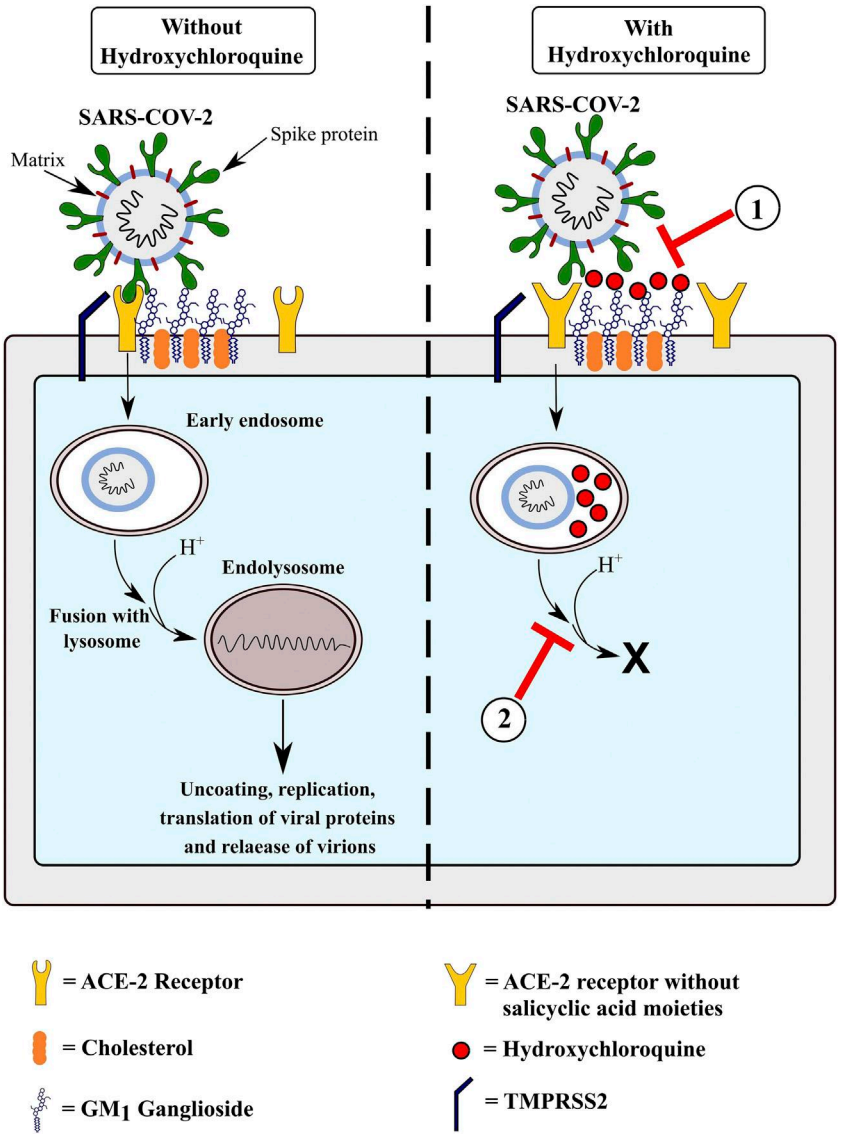

FIGURE 1 | Proposed mechanisms of action of hydroxychloroquine in preventing COVID-19 infection. 1) Hydroxychloroquine blocks entry of SARS-CoV-2 by binding to GM_1 gangliosides present on the cell membrane, preventing the interaction of the N-terminal domain of the virus' Spike protein with them. In addition, by inhibiting synthesis of sialic acid moieties on the ACE-2 receptor, it reduces binding of the virus to its target receptor. 2) Hydroxychloroquine is concentrated inside the endosomes and lysosomes in the cell. Due to their basic nature, they decrease the pH inside the endosomes, thus preventing maturation of early endosomes into endolysosomes and preventing the activity of cathepsins B and L.

(Hoffmann et al., 2020b). ACE2 binding and subsequent viral fusion requires *priming* of the S protein via proteolytic cleavage by host enzymes. Similar to SARS, S protein priming for entry into human lung epithelial cells of SARS-CoV-2 is enabled by TMPRSS2, a transmembrane serine protease (Hoffmann et al., 2020b). S protein priming can also occur via secondary pathways, such as via endolysosomal cysteine proteases cathepsins B and L; while this path is not of prime importance for viral transmission and respiratory infection. As the human airway epithelium lacks sufficient endolysosomal proteases, it is thought to contribute to invasion of extrapulmonary tissues (Park et al., 2016; Hoffmann et al., 2020b). Further, SARS-CoV-2 S protein can be *preactivated* by furin during packaging of viral particles; this has a cumulative effect on subsequent S protein activation by TMPRSS2 (Hoffmann et al., 2020a; Shang et al., 2020).

HCQ can inhibit SARS-CoV-2 by impacting viral entry and postentry steps (**Figure 1**). By inhibiting glycosylation, it affects synthesis of sialic acid moieties of ACE2 and the terminal glycosylation of the S protein, thereby reducing the interaction between ACE2 and the S protein (Savarino et al., 2006; Liu et al., 2020). *In silico* analyses have revealed that similar to other coronaviruses, the N-terminal of the S protein consists of a

ganglioside-binding domain (Fantini et al., 2020b). This domain binds to sialic acid residues linked to GM1 ganglioside cell surface receptors, facilitating binding at ACE-2. HCQ binds to these gangliosides with a high affinity, thus further inhibiting SARS-CoV-2 entry (Fantini et al., 2020b; Fantini et al., 2020a). Being a weak base, HCQ concentrates in the acidic lysosomes and endosomes. By increasing endosomal pH, it inhibits endosomal maturation and fusion of viral and endolysosomal membranes (Derendorf, 2020; Liu et al., 2020). Further, by the same mechanism, it decreases activity of endolysosomal cathepsins. The immunomodulatory action of HCQ is also believed to play a role- HCQ inhibits MHC class II expression, production of pro-inflammatory cytokines like IL-1 and TNF-alpha and inhibits TLR signaling pathways (Schrezenmeier and Dörner, 2020). This anti-inflammatory action can counter the cytokine storm responsible for severe COVID-19 and reduce severity of infection, although this remains but a hypothesis.

In vitro studies have demonstrated that HCQ effectively inhibits SARS-CoV-2. Pretreatment of Vero cells with HCQ inhibits SARS-CoV-2 replication with a half-maximal effective concentration (EC_{50}) in the range of 4.51–5.85 μM (Liu et al., 2020; Yao et al., 2020). However, this result was not replicated when a model of reconstituted human airway epithelial cells was used; HCQ did not affect apical viral titers and could not protect epithelial integrity (Maisonnasse et al., 2020). This contradiction may be explained by the role of TMPRSS2, which is not expressed in Vero cells. It has been seen that expression of TMPRSS2 attenuates the inhibition of SARS-CoV-2 by HCQ, possibly by bypassing the cathepsin B/L pathway of proteolytic cleavage of viral S protein and by facilitating the interaction between ACE2 and S protein (Ou et al., 2020).

Evidence of HCQ use in COVID-19 from *in vivo* animal models has not been encouraging. Ferrets and hamsters have been found to be permissive to SARS-CoV-2 infection, making good preclinical models. Studies in Syrian hamsters found that HCQ given in a standard dose (6.5 mg/kg) or high dose (50 mg/kg) did not prevent virus transmission and had an insignificant effect on viral replication and disease progression (Kaptein et al., 2020; Rosenke et al., 2020). Nonhuman primates such as rhesus and cynomolgus macaques have been used to develop animal models of COVID-19, resembling human disease, with rhesus macaques developing transient symptomatic disease (Munster et al., 2020; Rockx et al., 2020). Giving high-dose HCQ pre-exposure prophylaxis (30 mg/kg loading dose followed by 15 mg/kg) to cynomolgus macaques did not result in reduction of viral loads (Maisonnasse et al., 2020). Similarly, standard-dose HCQ prophylaxis was found to be ineffective in the rhesus macaque disease model (Rosenke et al., 2020). Thus, the *in vitro* efficacy of HCQ is not replicated *in in vivo* animal models, raising reasonable doubts about its efficacy as prophylaxis for COVID-19.

The dosage of HCQ for adult humans likely to be effective for prophylaxis has been estimated by deriving simulations from pharmacokinetic parameters estimated from healthy individuals and patients with malaria taking HCQ (Al-Kofahi et al., 2020). A regimen of 800 mg loading dose followed by 400 mg twice/thrice weekly for pre-exposure prophylaxis and 800 mg loading dose followed by 600 mg after 6 h and 600 mg daily for 4 days for postexposure prophylaxis has been suggested, which is much higher than required when HCQ is given as prophylaxis against malaria (400 mg once weekly). However, it should be noted that the *in vitro* EC_{50} used for estimations (0.72 μM) in this study was derived from an experiment simulating treatment; EC_{50} for *in vitro* experiments with pretreatment with HCQ, thus simulating pre-exposure prophylaxis, is in a higher range (Liu et al., 2020; Yao et al., 2020).

CRITICAL ANALYSIS OF THE CLINICAL EVIDENCE

Clinical evidence on use of HCQ as chemoprophylaxis (**Table 1**) is conflicting. HCQ is a widely prescribed drug for rheumatic diseases like systemic lupus erythematosus (SLE) and rheumatoid arthritis, and studies on this group of patients receiving chronic HCQ therapy have provided valuable insights. Initially, due to lack of reports of patients with SLE contracting COVID-19, it was thought that HCQ may have been the reason (Joob and Wiwanitkit, 2020). However, the COVID-19 Global Rheumatology Alliance, a physician-reported registry, has reported more than 600 cases of COVID-19 in patients with rheumatic diseases, including 85 with SLE. In this cohort, 130 patients (51 with SLE) were on long-term antimalarial therapy and use of antimalarials was not associated with protection against hospitalization due to COVID-19 (Gianfrancesco et al., 2020; Konig et al., 2020). In another cohort of 914 patients with 112 chronic HCQ users, HCQ use did not protect against COVID-19 infection (Favalli et al., 2020). Among almost 55,000 patients chronically exposed to antimalarials matched with thrice the number of controls, there was no significant difference in time to COVID-19 hospitalization (Sbidian et al., 2020). Thus, current evidence points that chronic HCQ use does not universally protect patients with rheumatic diseases against COVID-19. However, these results should be interpreted taking into account the limitations of these studies. They are prone to high risk of selection bias with more severe cases more likely to be reported. Other confounders known to affect outcomes such as age, presence of comorbidities, and immunosuppressive treatment may be contributory, for example, in one study, the HCQ arm had a greater proportion of patients who were on corticosteroid therapy (Favalli et al., 2020). Results from these studies cannot be applied to the general population since they only included patients with rheumatic disease.

On the other hand, few observational studies report a positive preventive effect of HCQ. In a case–control study from India involving HCW involved in care of COVID-19 patients, consumption of four or more maintenance doses of HCQ (400 mg once weekly) for prophylaxis was associated with lower risk of contracting COVID-19 after adjusting for sex, use of PPE, performance of endotracheal intubation, and COVID-19 testing date (Chatterjee et al., 2020). However, the dose–response curve noted a paradoxical increase in risk of

TABLE 1 | A summary of published clinical research regarding role of HCQ as prophylaxis against COVID-19.

S. no	Study	Methodology	Results	Limitations
1	(Gianfrancesco et al., 2020)	Cross-sectional case series from a physician-reported registry of patients with rheumatic diseases who have contracted COVID-19; 600 cases from 40 countries	No significant association found between antimalarial therapy and hospitalization after adjusting for sex, age greater than 65 years, rheumatic disease, smoking status, comorbidities, other DMARDs, NSAID use, and glucocorticoid dose	Risk of selection bias due to physician reporting. Risk of bias from unknown confounders. Results cannot be generalized. Cross-sectional analysis, thus patient end points, may have been different in reality
2	(Sbidian et al., 2020)	Retrospective matched cohort study using French national health data; 54,873 cases exposed to antimalarials and 155,689 controls	Hospitalization due to COVID-19 occurred in 128 cases and 195 controls. No significant association of exposure to antimalarial with hospitalization due to COVID-19 on multivariate conditional Cox regression	Retrospective methodology. Risk of bias from unknown confounders cannot be excluded
3	(Favalli et al., 2020)	Survey-based study to ascertain incidence of COVID-19 and its effect on treatment of patients with rheumatic diseases; 914 patients with 112 on chronic HCQ	Incidence of COVID-19 comparable among HCQ and non-HCQ group. Use of biologicals higher in non-HCQ group, and use of corticosteroids higher in HCQ group	Patient-reported data; thus, accuracy cannot be established. Lack of matching between the two groups
4	(Chatterjee et al., 2020)	Case–control study evaluating factors influencing risk of SARS-COV-2 infection in HCW; 378 cases (HCW with COVID-19) and 373 controls	On multivariate analysis, consumption of >/ = 4 maintenance doses of HCQ and use of PPE associated with decreased risk of infection. Dose–response relationship exists between frequency of exposure to HCQ and decrease in risk	Calculated sample size not achieved. Retrospective methodology. No explanation for paradoxical increase in risk with 2–3 doses. Case–controls not matched according to risk of exposure
5	(Bhattacharya et al., 2020)	Retrospective cohort study in 104 HCW (54 on HCQ prophylaxis) who had confirmed contact with a COVID-19–positive case	Distribution of age, sex, degree of exposure, type of exposure, and comorbidities similar in HCQ and non-HCQ groups. HCQ use was associated with 80.7% reduction in risk of acquiring COVID-19 on univariate analysis	Small sample size, retrospective methodology, and confounders not accounted for in univariate analysis
6	(Lee et al., 2020)	Prospective study of outbreak management at a long-term care hospital with HCQ postexposure prophylaxis (400 mg daily) for 14 days	Postexposure prophylaxis completed in >95% without adverse events; 15.6% reported adverse events. All follow-up RT-PCR at the end of 14 days negative	Lack of control group. Index cases were wearing face masks at all times, thus decreasing transmission probability
7	(Gendelman et al., 2020)	Retrospective study on a computerized healthcare database of patients screened for COVID-19	No significant difference between chronic use of HCQ between those positive for COVID-19 (0.23%) vs. those negative for COVID-19 (0.25%)	Retrospective methodology and duration of treatment unknown
8	(Boulware et al., 2020)	Double-blind RCT; 821 asymptomatic participants (719 with confirmed high-risk exposure to COVID-19 contact) randomized to receiving HCQ (414) or placebo (407) within 4 days of exposure for a total of 5 days	Incidence of new COVID-19 did not differ significantly between those taking HCQ (11.8%) vs. placebo (14.3%); 40.1% participants taking HCQ reported side effects; no serious events	Participant-reported data; thus, accuracy cannot be established. Only 18.7% of those labeled to have COVID-19 were had confirmatory RT-PCR
9	(Mitja et al., 2020)	Open-label, cluster randomized trial; 2,314 asymptomatic contacts (exposed within 7 days of enrollment) of 672 index cases randomized to HCQ (1,116) or usual care (1,198)	138 (6.0%) participants had a symptomatic PCR-confirmed COVID-19 episode with no significant difference between the HCQ group (6.2%) and usual care group (5.7%); 51.6% in the HCQ group reported side effects with no serious events	Blinding not performed; 12.2% participants had a positive baseline RT-PCR
10	(Rajasingham et al., 2020)	Double-blind RCT; 1483 HCW with ongoing COVID-19 exposure randomized 2:2:1:1 to receiving once/twice weekly HCQ or placebo for 12 weeks	97 (6.5%) participants developed COVID-19. Incidence of COVID-19 was 0.27, 0.28, and 0.38 events per person year in once-weekly HCQ, twice-weekly HCQ, and placebo group, respectively (no significant difference). Median HCQ blood concentrations did not differ among COVID and non-COVID cases. One serious AE (SVT) in the twice-weekly HCQ group	Participant-reported data; thus, accuracy cannot be established. Only 18% of those labeled to have COVID-19 had a confirmatory positive RT-PCR; 39% had negative RT-PCR during illness
11	(Abella et al., 2020) (PATCH trial)	Double-blind RCT; 132 HCW randomized to receive HCQ (600 mg daily) or placebo for 8 weeks; 125 evaluated for primary outcome	No significant difference in infection rate among participants receiving HCQ (6.3%) vs. placebo (6.6%). 45% in the HCQ group had mild side effects. Median change in QTc interval was not significantly different in both groups	Small sample size, trial terminated early. Study population comprised young HCW, thus results not generalizable
12	(Garcia-Albeniz et al., 2020)	Meta-analysis of three randomized trials on HCQ prophylaxis	Pooled risk ratio estimate with use of HCQ as prophylaxis was 0.78 (95% CI: 0.61–0.99)	End point of PCR-confirmed disease pooled with different end point of clinical disease. Results of trials with different methodologies pooled together

infection after two to three doses, which cannot be well explained and casts doubts on the actual presence of a protective effect. Another retrospective cohort study in HCW who had confirmed contact with a COVID-19 case reported HCW who took HCQ were at a lower risk of infection; however, this was only observed on univariate analysis (Bhattacharya et al., 2020). Both these studies were prone to bias due to their retrospective methodology, small sample size, and presence of confounders, such as duration/degree of exposure to COVID-19 patients. In a study from South Korea, HCQ was administered as postexposure prophylaxis to 211 individuals in a long-term care hospital after exposure to a confirmed COVID-19 case. None developed COVID-19, and acceptability was good, although this apparent protective benefit cannot be confirmed due to lack of a control group and use of facemask by the index case (Lee et al., 2020).

Several randomized trials evaluating HCQ prophylaxis in HCW are currently ongoing (Agarwal et al., 2020), with the results of a few of them now available (**Table 1**). The first trial, published in June, investigated HCQ as postexposure prophylaxis in adult HCW with high/moderate-risk exposure to a confirmed case of COVID-19. Participants were randomized to HCQ or placebo within 4 days of exposure with the dosing regimen adapted from a pharmacokinetic simulation study. The incidence of new COVID-19 did not differ between those receiving HCQ and placebo. Another trial with a similar design, investigating pre-exposure prophylaxis in HCW with once weekly or twice weekly HCQ, reported no protective benefit compared to placebo (Rajasingham et al., 2020). Both these trials had a pragmatic design, due to which certain limitations made it difficult to draw definite conclusions—only a few of the trial participants had an RT-PCR confirming their COVID-19 diagnoses, and the rest were labeled based on a symptom-based definition. Due to the trial population comprising mostly young HCW, the incidence of COVID-19 may have been underestimated due to asymptomatic infections being missed. However, other trials have also reported a similar result. A cluster randomized trial from Spain reported no benefit of HCQ postexposure prophylaxis (Mitja et al., 2020), while randomizing HCW to HCQ pre-exposure prophylaxis (600 mg daily) or placebo also did not result in any protective benefit from COVID-19 (Abella et al., 2020). Side effects were encountered in 40–50% participants taking HCQ; however, these were most commonly mild gastrointestinal adverse events like nausea, loose stools, and abdominal discomfort. One serious adverse event of syncope and supraventricular tachycardia was reported (Rajasingham et al., 2020). A recent meta-analysis pooled the results of three of these clinical trials and reported a significant risk reduction of 20% with HCQ use (Garcia-Albeniz et al., 2020), but there were glaring inaccuracies in the analysis as data with different end points (PCR-confirmed disease v/s clinically compatible disease) and trials with different methodologies, for example, results of pre-exposure and postexposure prophylaxis trials were pooled. Thus, the conclusions may not be reliable. In summary, the results from these trials indicate that HCQ in its current dosage does not seem to provide a prophylactic benefit against COVID-19.

DISCUSSION

The Conundrum of Dosing for Hydroxychloroquine Prophylaxis

One reason that may explain why the apparent *in vitro* efficacy of HCQ could not be replicated in preclinical *in vivo* studies and in clinical trials is a *pharmacokinetic* failure. 4-aminoquinolones have peculiar pharmacokinetic properties, which make it difficult to accurately estimate pharmacological parameters. HCQ is well observed orally (74% bioavailability) and has an overwhelmingly large volume of distribution, indicating extensive sequestration into tissues (Tett et al., 1988; Tett et al., 1989). Due to this reason, the volume of distribution dictates its pharmacokinetics, leading to a long half-life (~44 days), despite good clearance. Further, around 45% of HCQ in plasma is bound to plasma proteins, with >90% bound to albumin (Tett et al., 1988). It has been seen that measured plasma drug concentrations of HCQ are much more variable than measured whole blood concentrations (Gustafsson et al., 1983; Tett et al., 1988; Blanchet et al., 2020), probably due to the release of the drug from WBCs and platelets during sample processing. This has been observed even with modifications in the separation procedure like increasing centrifugation speed and decreasing time to separation. Thus, whole blood HCQ levels are a more accurate parameter for pharmacokinetic estimations.

For clinical efficacy, appropriate concentrations of the drug should be achieved at its target site. Current knowledge suggests that since HCQ blocks viral entry, its site of action would be extracellular lung tissue and intracellularly in type 1 pneumocytes. The *in vitro* EC_{50} for HCQ prophylaxis lies in the range of 4.51–5.85 μM (Liu et al., 2020; Yao et al., 2020), while the *in vivo* EC_{50} is unknown. It is important to note that this EC_{50} value has been measured in the extracellular cell culture media. Since HCQ is concentrated within tissues in the acidic intracellular compartment such as the lysosomes, endosomes, and golgi apparatus, the above EC_{50} values should be extrapolated to free extracellular tissue concentrations, which would be in turn in equilibrium with the free (unbound) plasma concentration. Correlating these values with whole lung HCQ concentrations (Yao et al., 2020) is likely to be inaccurate since they would include the high intracellular concentration (Fan et al., 2020). For example, in the case of SLE and other rheumatic diseases, HCQ is given at a maximum dose of 400 mg/day, which maintains whole blood levels in the range of 648–917 ng/ml (1.93–2.73 μM) (Blanchet et al., 2020; Mathian et al., 2020). With a blood-to-plasma ratio of HCQ concentration being 7.2 and close to 50% of plasma HCQ being protein bound (Tett et al., 1988), the free plasma HCQ concentration (which would be in equilibrium with the free extracellular tissue concentration) comes out to be in the range of 45–64 ng/ml (0.13–0.19 μM), which is considerably lower than the *in vitro* EC_{50}. This may explain why patients with rheumatological diseases on chronic HCQ do not get a protective benefit.

Currently used dosing regimens for HCQ prophylaxis (Al-Kofahi et al., 2020) have been estimated from a population pharmacokinetic model based on plasma HCQ levels in patients with malaria and healthy volunteers (Lim et al., 2009).

This estimation can lead to inaccuracies due to multiple reasons. As malaria is a bloodstream infection, the site of action of HCQ is in the blood compartment itself; but the site of action in COVID-19 is within the lung tissue. Further, the EC_{50} values used in this estimation have been derived from a treatment experiment. Indeed, whole blood levels of HCQ in human participants receiving the suggested regimen for pre-exposure prophylaxis (800 mg followed by 400 mg biweekly) were only 200 ng/ml (0.59 μM), corresponding to a free plasma concentration of 13.9 ng/ml (0.04 μM) much smaller than EC_{50} values (Rajasingham et al., 2020). Moreover, a recently published population pharmacokinetic model derived from whole blood concentrations of HCQ in treated COVID-19 patients found that current dosing regimens were inadequate for a corresponding *in vitro* EC_{50} of 4.51 μM, and body weight was a significant factor influencing HCQ clearance (Thémans et al., 2020). According to this model, much higher doses would be required for a clinical effect.

Thus, we currently do not have accurate predictions of the dosage of HCQ required for chemoprophylaxis. Measurement of HCQ blood levels and, if feasible, lung fluid levels (e.g., through bronchoalveolar lavage specimens) in participants of clinical trials of HCQ prophylaxis combined with the use of comprehensive pharmacokinetic models may provide us with answers. However, there is a flip side to this coin. Higher doses of HCQ may bring with them the risk of serious toxicity, especially cardiotoxicity in the form of QT_C prolongation and cardiac arrhythmias. Extrapolating risk of QT_C prolongation with HCQ from CQ models in children with malaria led to the conclusion that HCQ doses in the range of >/ = 800 mg BID may have significant risk (Garcia-Cremades et al., 2020). Currently used doses of HCQ are usually safe in outpatient settings (Lofgren et al., 2020), but use of HCQ to treat COVID-19 patients in inpatient and ICU settings has led to safety concerns (Bonow et al., 2020; Jeevaratnam, 2020). Many such patients are elderly and may have pre-existing cardiovascular disease. COVID-19 leads to viral myocardial injury in 7–23% patients (Pirzada et al., 2020); further patients are commonly treated with concomitant QT_C-prolonging drugs like azithromycin; this increases the risk of cardiotoxic events (Agarwal et al., 2020; Padilla et al., 2020). Indeed, observational studies have reported that HCQ for treatment of COVID-19 leads to critical QT_C prolongation (a marker for risk of Torsades de pointes) in 20–36% cases, frequently requiring drug discontinuation to avoid fatal arrhythmias (Bessière et al., 2020; Chorin et al., 2020; Mercuro et al., 2020). A recent meta-analysis noted an increased risk of cardiac arrhythmias with HCQ use compared to standard of care (Elavarasi et al., 2020). Moreover, instances of ventricular arrhythmias have occurred due to HCQ as reported by observational cohorts (Chorin et al., 2020; Mercuro et al., 2020) and pharmacovigilance data (Pharmacovigilance Memorandum. Reference ID: 4610984, 2020). Besides cardiovascular adverse effects, there have been sporadic cases of other serious adverse events including neuropsychiatric events, hepatitis, cytopenias, rhabdomyolysis, and acute kidney failure that have been attributed to HCQ (Garcia et al., 2020; Pharmacovigilance Memorandum. Reference ID: 4610984, 2020). It must be noted that most of these observations are from HCQ used as a therapy for COVID-19, but they may have implications for chemoprophylaxis as well owing to the doubtful efficacy and long half-life of HCQ (Agarwal et al., 2020). Therefore, it becomes important to balance risk with possible benefit.

Another theory that has been suggested cautioning against use of CQ/HCQ is that of *hormesis*, that is, a biphasic effect on viral replication with stimulation at lower doses and inhibition at higher doses (Calabrese et al., 2021). A paradoxical increase in viral load may occur due to a hormetic effect due to preconditioning (acquired resilience) of viral particles after exposure to certain doses of CQ/HCQ (Calabrese, 2016). This is, however, based on observations of the effect of CQ on neuroprotection and SARS-CoV-1 viral growth (Keyaerts et al., 2004); currently, there is no evidence regarding a hormetic effect of CQ/HCQ on SARS-CoV-2.

Other Caveats in Knowledge: A Role of TMPRSS2?

Several aspects of the role of HCQ in COVID-19 prophylaxis are currently incompletely understood. The translation of *in vitro* activity to *in vivo* activity is a complex process affected by a multitude of factors. As discussed above, the dosing of HCQ is likely contributory. However, other factors may also be playing a role. A recent study evaluating the *in vivo* effect of HCQ in Syrian hamster model of COVID-19 reported that HCQ did not reduce viral loads or affect viral transmission even when given at a high dose of 50 mg/kg/day (Kaptein et al., 2020). Lung tissue concentration of HCQ was derived from a mean trough plasma HCQ concentration using previously known estimates, and both cytosolic and interstitial lung tissue concentrations were found to be 5.4 μM (Kaptein et al., 2020), which is in line with the *in vitro* EC_{50}; thus, tissue concentrations were not a limiting factor. An explanation for this discrepancy is interference by TMPRSS2. Airway epithelium lacks sufficient expression of cathepsin B/L; thus, the main mechanism of SARS-CoV-2 entry is via S protein activation by TMPRSS2. HCQ has no effect on the action of TMPRSS2, and it has been seen that the inhibitory effect of HCQ on viral entry is effectively attenuated by TMPRSS2 expression (Ou et al., 2020). This also gives an explanation as to why HCQ failed to demonstrate efficacy on a reconstituted airway epithelium model (Maisonnasse et al., 2020); airway epithelium, unlike Vero cells, expresses high amounts of TMPRSS2. Further, it was seen that ablation of the furin *preactivation* site on the S protein reduced the dependence on TMPRSS2 (Ou et al., 2020). Thus, using inhibitors of TMPRSS2 and furin along with HCQ can theoretically inhibit SARS-CoV-2 entry in a comprehensive manner and will likely have additive protective benefit. TMPRSS2 is especially an attractive target since it is not required for normal homeostasis (Ts et al., 2006); its inhibitor—camostat mesilate—is approved for human use in Japan for chronic pancreatitis, and camostat has been demonstrated to protect mice models from SARS-CoV infection (Zhou et al., 2015).

Are There Alternatives to Chemoprophylaxis?

Currently, there is no direct evidence to suggest alternative drugs for chemoprophylaxis in COVID-19. Theoretically, other molecules inhibiting SARS-CoV-2 entry may be effective agents. As discussed above, the TMPRSS2 inhibitor camostat mesilate and furin inhibitors may be worth looking into. Further, molecular docking studies have identified that the S protein of SARS-CoV-2 may bind to additional molecules like heat shock protein A5 (HSPA5/GRP78) (Ibrahim et al., 2020), and certain natural compounds like phytoestrogens may inhibit this interaction (Elfiky, 2020). However, it must be reiterated that use of these agents is chemoprophylaxis is currently only a hypothesis.

CONCLUSION

Certain aspects of HCQ show promise for a chemoprophylactic agent against COVID-19; it has a plausible mechanism of action inhibiting viral entry, and it is cheap and widely available. However, current evidence, including preclinical animal models and clinical trials, suggest that HCQ in its current form is not effective for COVID-19 chemoprophylaxis. Certain questions however remain:

- Should guidelines recommending HCQ prophylaxis be revised?

During the early months of the pandemic, recommending HCQ for prophylaxis based on preclinical evidence may be justified due to the nature of the circumstances. But guidelines need to be updated in light of new evidence. Since clinical trials have shown that HCQ prophylaxis is not showing clinical benefit, current guidelines need to be revised accordingly.

- Should HCQ be tried in clinical trials using a different dosing regimen with higher doses?

In its traditional doses, HCQ has largely been a safe drug. Higher doses however have the potential to cause serious adverse events including cardiac events such as QT_C prolongation, ventricular arrhythmias, and noncardiac events. Pharmacovigilance has already detected in sporadic cases of serious adverse events with HCQ prophylaxis (National Task Force for COVID-19, 2020; Pharmacovigilance Memorandum. Reference ID: 4610984, 2020). Thus, in the setting of questionable efficacy, trying a higher dose of HCQ in clinical trials cannot be justified.

In conclusion, based on currently available research, looking beyond HCQ for COVID-19 chemoprophylaxis may prove to be a better path.

AUTHOR CONTRIBUTIONS

Authors PR and UB conceptualized the study. Authors MA and AM conducted the review of literature. Author MA prepared the primary draft of the manuscript and prepared the figure and table. Authors PR, UB, AM, and MA performed critical revision of the primary manuscript draft for intellectual content. Author PR supervised the study.

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REVIEW
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Repurposing of Biologic and Targeted Synthetic Anti-Rheumatic Drugs in COVID-19 and Hyper-Inflammation: A Comprehensive Review of Available and Emerging Evidence at the Peak of the Pandemic

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Coronavirus disease 2019 (COVID-19) is a condition caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Severe cases of COVID-19 result in acute respiratory distress syndrome and death. A detrimental, hyper-inflammatory immune response with excess release of cytokines is the main driver of disease development and of tissue damage in these patients. Thus, repurposing of biologic agents and other pharmacological inhibitors of cytokines used for the treatment of various inflammatory conditions emerged as a logical therapeutic strategy to quench inflammation and improve the clinical outcome of COVID-19 patients. Evaluated agents include the interleukin one receptor blocker anakinra, monoclonal antibodies inhibiting IL-6 tocilizumab and sarilumab, monoclonal antibodies inhibiting granulocyte-monocyte colony stimulating factor and tumor necrosis factor, and Janus kinase inhibitors. In this review, we discuss the efficacy and safety of these therapeutic options based on direct personal experience and on published evidence from observational studies and randomized clinical trials.

Keywords: Coronavirus disease 2019, severe acute respiratory syndrome coronavirus 2, disease modifying anti-rheumatic drug, DMARDs (biologic), cytokine, immunesuppressants, JAK inhibitors

INTRODUCTION

The pathogenesis of severe Coronavirus disease 2019 (COVID-19) involves an excessive, maladaptive host inflammatory response to the causative virus SARS-CoV-2 (Mehta et al., 2020; Ruan et al., 2020) (**Figure 1**). Individual predisposition to the development of excessive or inappropriate immune responses is traditionally attributed to genetic variation in the genes encoding the human leukocyte antigen (HLA) (Cavalli et al., 2016a; Hayashi et al., 2016; Klück et al., 2020). Conversely, the detrimental immune response developing in a subgroup of COVID-19 patients is mediated by the innate immune system, and is characterized by marked increases in systemic cytokines, and is paralleled by elevations in inflammatory biomarkers, such as C-reactive protein (CRP) and ferritin (Ciceri et al., 2020; Campochiaro et al., 2020a; Colafrancesco et al., 2020b;

Fominskiy et al., 2020; Mehta et al., 2020; Zangrillo et al., 2020). A similar biochemical pattern was observed in severe patients affected by pneumonia caused by previous coronaviruses SARS-CoV and MERS-CoV (Wong et al., 2004; Mahallawi et al., 2018).

Severe forms of COVID-19 pneumonia feature elevations in circulating levels of interleukin (IL) 1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor α (TNF) (Ragab et al., 2020; Rovere-Querini et al., 2020; Tang N. et al., 2020). Conversely, decreased levels of interferon I are associated with disease severity (Bastard et al., 2020; Hadjadj et al., 2020; Zhang et al., 2020).

The hyper-inflammatory response turning COVID-19 into a life-threatening disease shares conceptual and molecular resemblance with the cytokine storm developing during the macrophage activation syndrome, or with the cytokine release syndrome following chimeric antigen receptor T (CAR-T)-cell therapy (De Luca et al., 2020). Thus, pharmacological inhibition of several pro-inflammatory cytokines, along with a broader therapeutic molecular blockade (eg inhibition of Janus Kinases [JAK]), has been extensively explored during the COVID-19 pandemic (Ciceri et al., 2020; Campochiaro et al., 2020a; Campochiaro et al., 2020b; Della-Torre et al., 2020; Fominskiy et al., 2020; Mehta et al., 2020; Zangrillo et al., 2020). In this review, we discuss the biologic rationale for repurposing of available anti-cytokine therapies, as well as the available evidence on the effectiveness of different pharmacological blockers of inflammatory mediators in COVID-19 (**Table 1**).

Interleukin one

IL-1 is the prototypical pro-inflammatory cytokine. Two different gene products, IL-1α and IL-1β, can activate the IL-1 receptor. IL-1α is constitutively present as an active molecule in all mesenchymal and epithelial tissues; it is released upon cell death, and acts as an alarmin inducing local inflammation (Rider et al., 2017). IL-1β is not detectable in healthy tissues and is secreted in the extracellular space during inflammation (Dinarello, 2009; Cavalli and Cenci, 2020; Klück et al., 2020). Both IL-1α and IL-1β bind the same receptor and induce several pro-inflammatory effects (Dinarello, 2009; Rider et al., 2017; Ragab et al., 2020; Vecchié et al., 2020).

Although mechanistic insight into the host inflammatory response to COVID-19 is still limited, it is likely that both IL-1α and IL-1β play a central role in the development of the exuberant, maladaptive inflammatory response leading to life-threatening states in some patients (Ben Salem, 2017; Mehta et al., 2020; Ruan et al., 2020). Specifically, damaged epithelial and endothelial tissues release IL-1α in the lung, whereas infiltrating myeloid cells produce abundant IL-1β (Dinarello, 2011).

The main physiologic mechanism preventing runaway IL-1-mediated inflammation is the IL-1 receptor antagonist (IL-1Ra) (Gabay et al., 1997; Arena et al., 1998; Park et al., 2001). Anakinra is a recombinant form of IL-1Ra and the first-in-class IL-1 inhibitor drug (Cavalli and Dinarello, 2015; Cavalli and Dinarello, 2018). It is used for the treating rheumatoid arthritis, autoinflammatory disorder and multiple diseases characterized by excess cytokine production, including critical disease states (Abbate et al., 2015; Cavalli et al., 2015a; Cavalli et al., 2017; Tomelleri et al., 2018; Campochiaro et al., 2019). Notable therapeutic applications include adult-onset Still's disease (Cavalli et al., 2015a; Campochiaro et al., 2020b; Cavalli et al., 2020b) and macrophage activation syndrome (Grom et al., 2016; Ravelli et al., 2016; Eloseily et al., 2020), both conditions sharing similarities with COVID-19 and hyper-inflammation. In addition, re-analysis of a trial of anakinra in sepsis confirmed clinical benefits in patients with features of hyper-inflammation (Shakoory et al., 2016). A good safety profile and a short half-life of 3 h, which ensures rapid clearance from the circulation, contributes to making anakinra a suitable treatment for critically ill patients (Cavalli and Dinarello, 2015).

Based on extensively documented safety and effectiveness in quenching hyper-inflammation in multiple diseases, including cardiopulmonary insufficiencies (Cavalli et al., 2015b; Cavalli et al., 2016a; De Luca et al., 2018; Sala et al., 2020), anakinra was among the first cytokine-blocking agents evaluated for the treatment of COVID-19, as documented by multiple reports (Aouba et al., 2020; Dimopoulos et al., 2020; Pontali et al., 2020). In the first cohort study by Cavalli et al., administration of high-dose intravenous anakinra quenched hyper-inflammation and improved respiratory function in 29 severe patients with COVID-19 ARDS receiving non-invasive ventilation (NIV) (Cavalli et al., 2020). This amounted to improved survival in treated patients compared to concomitantly hospitalized patients who did not receive anakinra. Subsequent cohort studies by Huet et al. and Navarro-Millàn independently confirmed these findings in different disease severity stages (Huet et al., 2020; Navarro-Millán et al., 2020). In addition, the effectiveness of anakinra has been reported in different case series (Aouba et al., 2020; Cavalli et al., 2020; Dimopoulos et al., 2020; Huet et al., 2020; Navarro-Millán et al., 2020; Pontali et al., 2020).

The positive findings of these studies are to be interpreted with caution in view of possible biases (i.e. single-center study bias, small study bias), as well as the limited number and uncontrolled nature of the investigations. Furthermore, the dosage regimens for anakinra varied across studies, ranging from high-dose intravenous administration in the study by Cavalli et al., to relatively low dose subcutaneous administration in the study by Huet et al. (Cavalli et al., 2020a; Huet et al., 2020). The timing of administration also differed between studies due to practical reasons, although all investigators shared a conceptual attitude toward the earliest possible administration. For these limitations, no indication on which anakinra regimen is most suitable for COVID-19 can be extrapolated from these studies. However, given the safety of anakinra even at high doses, early and aggressive treatment (i.e. 10 mg/kg/day intravenously) is probably advisable, in line with current management of autoinflammatory diseases and macrophage activation syndrome (Grom et al., 2016; Ravelli et al., 2016; Eloseily et al., 2020; Vitale et al., 2020). Clinical trials of anakinra in COVID-19 are ongoing (i.e. NCT04443881 among others). If ever available, controlled evidence from these investigations will supersede currently available observational evidence.

Besides anakinra, another IL-1 antagonist was evaluated in COVID-19, that is, the anti-IL-1β monoclonal antibody canakinumab. Canakinumab is used for the treatment of adult autoinflammatory conditions such as Still's disease (Colafrancesco et al., 2017; Cavalli et al., 2019). It does not block IL-1α. Experience with canakinumab in COVID-19 is limited to a single, small case series reporting favorable responses (Ucciferri et al., 2020). Clinical trials of canakinumab are also ongoing (i.e. NCT04362813).

Interleukin six

IL-6 is a pleiotropic cytokine produced by virtually every immune cell types, which acts by engaging its receptor (IL-6R) on target cells (De Benedetti et al., 2012). IL-6 is involved in physiological hematopoiesis and response to pathogens but excessive production is associated with disorders that resemble severe COVID-19 manifestations, such as the hemophagocytic lymphohistiocytosis, and the cytokine release syndrome induced by CAR-T-cell (Henter et al., 1991; Lee et al., 2014; Tanaka et al., 2016; Chen et al., 2019; Aziz et al., 2020; Cavalli et al., 2020; Kaur et al., 2020; Ruan et al., 2020). Stemming from preliminary evidence of increased pro-inflammatory cytokines in sera and bronchoalveolar lavage of patients with COVID-19 pneumonia, IL-6 attracted remarkable attention as a possible player in the pathogenesis of SARS-CoV-2 infection and in the hyper-inflammatory response that affects patients with severe disease (Blanco-Melo et al., 2020; Conti et al., 2020; Farina et al., 2020; Giamarellos-Bourboulis et al., 2020). Indeed, elevated serum levels of IL-6 were described to be associated to poorer outcomes, coagulopathy, and increased mortality in patients with COVID-19 (Chen et al., 2020).

Based on this evidence, several IL-6 inhibitory agents such as tocilizumab and sarilumab were repurposed in the setting of severe COVID-19. Tocilizumab, a monoclonal antibody against the IL-6R, was the first biologic agent to be largely evaluated in COVID-19 patients, also based on precipitous inclusion in the Chinese guidelines for the treatment of COVID-19 patients at the beginning of the pandemic (Di Giambenedetto et al., 2020). Tocilizumab is currently approved for the treatment of multiple inflammatory diseases (Berti et al., 2015; Stone et al., 2017; Le et al., 2018), and is used off-label to treat several inflammatory conditions (Berti et al., 2017). Tocilizumab is available in America, Asia, Europe and Oceania; however, it is not universally accessible as it has been approved for use only in few African countries (Akintayo et al., 2020). The first reported experience on tocilizumab in COVID-19 was described in a Chinese cohort of 15 patients. Tocilizumab was administered intravenously, at various dosages (from 80 to 480 mg), and five patients received more than one dose. These patients were followed-up for 7 days, and three of them died. This study showed preliminary encouraging results, but it was limited by the lack of a standardized therapeutic scheme, the absence of a control arm, and the short post-treatment follow-up. Moreover, eight patients were also concomitantly treated with steroid therapy making it hard to clearly investigate the role of anti-IL-6 blockade (Luo et al., 2020). Subsequent observational retrospective series of critically ill Chinese COVID-19 patients treated with tocilizumab also reported a decrease in CRP levels, mechanical ventilation risk and mortality rate (Xu et al., 2020). Similar findings were reported irrespective of the route of administration, either intravenous or subcutaneous (Sciascia et al., 2020).

Based on these pioneering observations from China and following the westbound spread of the pandemic, a series of Italian studies evaluated off-label use of tocilizumab in COVID-19 patients. Campochiaro and colleagues studied 65 patients with hyper-inflammation and observed a non-significant decrease in mortality at 28 days in 32 tocilizumab-treated patients (16%) compared to 33 patients treated with standard of care (33%); tocilizumab was administered at a dose of 400 mg (Campochiaro et al., 2020a). In a separate cohort, Capra and colleagues evaluated 85 severe COVID-19 patients and observed a mortality at 20 days of 3% in the 62 patients treated with tocilizumab (33 patients received 400 mg intravenously, two received 800 mg intravenously, and 27 received 324 mg subcutaneously) compared to 48% in the 23 patients treated with standard of care (Capra et al., 2020). Morena and colleagues observed clinical and bioumoral improvement in 51 patients with severe COVID-19 following tocilizumab infusion (two sequential infusions at the dosage of 400 mg) (Morena et al., 2020). Reported adverse events in these three studies did not differ between patients treated with tocilizumab or standard of care only: specifically, the Authors reported hepatic enzyme elevation in 15–29% of cases; neutropenia in 14–16% of cases; and bacterial or fungal infections in 13–27% of patients.

More recently, Guaraldi et al. reported the results of a large retrospective observational cohort study evaluating the efficacy of tocilizumab in the treatment of severe COVID-19 patients. They found no difference in need for mechanical ventilation between groups (16% of the standard of care group vs. 18% of the tocilizumab group, $p = 0.41$), but reported a statistically significant reduction in mortality in the tocilizumab group (7% vs. 20%, $p < 0.001$). At multivariate analysis tocilizumab was associated with a reduced risk of invasive mechanical ventilation or death ($p = 0.020$). However, an increased rate of secondary infection was observed in tocilizumab-treated patients (13% vs. 4%, $p < 0.001$) (Guaraldi et al., 2020).

In another study, Biran and colleagues analyzed 764 COVID-19 patients in the ICU, of whom 210 received tocilizumab. At multivariable analysis with propensity matching, tocilizumab was associated with a decreased hospital-related mortality ($p = 0.004$) (Biran et al., 2020).

Despite intrinsic limitations due to their retrospective nature, the absence of adequate controls, and the low statistical power, these and other promising experiences soon prompted initiation of randomized placebo-controlled trials aimed to evaluate the safety and efficacy of tocilizumab (NCT04377750, NCT04330638, NCT04322773) (Levi, 2020). Interim updates on the first randomized trial (COVACTA) investigating tocilizumab in severe COVID-19 pneumonia yielded disappointing results. At 4 weeks, there were no differences in clinical between patients receiving tocilizumab or placebo ($p = 0.36$). Also, there were no differences either in mortality rate, ventilator-free survival, and incidence of infections between the two groups (Roche, 2020). In a

multicenter randomized trial involving 243 COVID-19 patients with signs of hyperinflammation, tocilizumab did not lead to a significant reduction of mortality or need for mechanical ventilation, nor it reduced the need for supplemental oxygen at 28 days (Stone et al., 2020). These findings were partially confirmed by another large RCT of COVID-19 patients (Hermine et al., 2020). In this trial, tocilizumab (administered at the dose of 8 mg/kg) led to a reduction in mechanical ventilation and death rate at 14 days; however, mortality at 28 days did not differ between treated patients and controls. Other parallel trials with tocilizumab in COVID-19 have been launched and results are expected by the end of the year (https://clinicaltrials.gov/; Campochiaro and Dagna, 2020).

Sarilumab is another anti IL-6R monoclonal antibody that was repurposed for the management of severe COVID-19 (https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/761037s000lbl.pdf). Sarilumab shares the mechanism of action with tocilizumab, by blocking both the membrane bound and the soluble form of IL-6R (Burmester et al., 2017). In analogy with the pioneering experiences with tocilizumab, the first uncontrolled experiences with sarilumab also created positive expectations and inspired a series of randomized, double-blind, placebo-controlled phase II/III trials worldwide (NCT04357808, NCT04386239, NCT04324073, NCT04322773). Benucci et al., for instance, treated a small series of eight hospitalized COVID-19 patients with 400 mg intravenous sarilumab and reported clinical improvements in seven patients who were discharged before day 14 (Benucci et al., 2020). In a larger study from the epidemic New York City area in the United States, Sinha and colleagues administered either intravenous tocilizumab (400 mg) or sarilumab (200 mg) to 255 critical COVID-19 patients. The mortality rate of treated patients was comparable to the overall mortality in the local area, despite the notable severity of the study population (Sinha et al., 2020). However, in an observational prospective study on 56 Italian patients with severe COVID-19, sarilumab treatment did not result in incremental survival benefit at 28 days (Della-Torre et al., 2020). Additional evidence of limited efficacy of sarilumab in COVID-19 was provided by the early termination of a randomized trial led in the US. In this RCT sarilumab treatment was not associated with statistically significant differences in clinical outcomes. There was a favorable trend of clinical improvement and mortality in patients on mechanical ventilation, but also an unfavorable trend in non-mechanically ventilated subjects (Sanofi, 2020). Based on these results, the trial was stopped, and an originally planned extension trial evaluating higher doses of sarilumab (800 mg) did not take place. A separate trial evaluating the efficacy of sarilumab (administered at the dosage of 200 or 400 mg) in 420 critical patients also suggested a positive trend without reaching statistical significance (Regeneron Pharmaceuticals, Inc., 2020).

Finally, another IL-6 antagonist that was deemed of interest for severe COVID-19 patients is siltuximab, an FDA approved chimeric monoclonal antibody used for the management of neoplastic diseases such as metastatic renal cell cancer and Castleman's disease (https://www.ema.europa.eu/en/medicines/human/EPAR/sylvant). Although there are no published experiences supporting the use of siltuximab in COVID-19 patients, similar effects to tocilizumab and sarilumab can be anticipated given the quasi-overlapping mechanism of action. A randomized trial evaluating the efficacy and safety of siltuximab (alone or in combination with anakinra) in hospitalized patients with severe COVID-19 (NCT04330638) is ongoing in Belgium, which will compare the efficacy of siltuximab to other anti-cytokine drugs (namely anakinra and tocilizumab) as well as to local standard of care.

Overall, available evidence from RCTs indicate that IL-6 inhibition is marginally or not effective for the treatment of COVID-19 (Hermine et al., 2020; Stone et al., 2020). In contrast, dexamethasone, a corticosteroid with broad anti-inflammatory properties significantly reduced mortality in a RCT of COVID-19 patients requiring supplemental oxygen or mechanical ventilation (Horby et al., 2020). IL-6 is a downstream, effector mediator of multiple inflammatory cascades. It is likely that in the massively inflamed lung of COVID-19, selective inhibition of IL-6 blocks but one of many mediators with redundant pro-inflammatory functions. This hypothesis also reconciles the negative findings of studies evaluating IL-6 inhibitors with the uncontrolled evidence suggesting that IL-1 inhibition might be effective for COVID-19: indeed, IL-1 is found more upstream in inflammatory cascades than IL-6. It is thereby likely that corticosteroids and IL-1 inhibition result in the inhibition of IL-6, as well as other mediators with a causative ole in the pathogenesis of COVID-19.

It should also be noted that observations of high circulating levels of IL-6 in COVID-19 patients can result in misled assumptions about the causal role of this cytokine in the pathogenesis of this disease. IL-6 levels are non-specifically elevated in systemic inflammation; in general, high circulating levels of any given cytokine do not indicate pathogenic causality, which is only demonstrated by the therapeutic effectiveness of selective cytokine inhibition.

Granulocyte–Macrophage Colony-Stimulating Factor

GM-CSF is a cytokine with complex biologic activity, ranging from hematopoietic to pro-inflammatory effects (Shiomi and Usui, 2015; Crotti et al., 2019). Various cell types produce GM-CSF during inflammation, including macrophages, lymphocytes and tumor cells (Hamilton and GM-CSF, 2002; Shiomi and Usui, 2015; Xu et al., 2020). GM-CSF activates several pro-inflammatory pathways and increases secretion of downstream mediators (Hamilton, 2019). Of note, GM-CSF can be placed upstream in inflammatory cascades and thus represents an appealing therapeutic target in various inflammatory conditions, including COVID-19 related cytokine storm (Favalli and Caporali, 2020). In pre-clinical studies, GM-CSF blockade reduced CAR-T-cell therapy-related toxicity by preventing cytokine release syndrome development (Sterner et al., 2019). Atypical lymphocytes expressing GM-CSF are detectable in severe COVID-19 patients (Zhou et al., 2020). Based on these observations, GM-CSF blockade was evaluated in COVID-19. Mavrilimumab is a monoclonal antibody targeting GM-CSFRα and it has been shown effective in the treatment of rheumatoid arthritis (Burmester et al., 2011). A study conducted

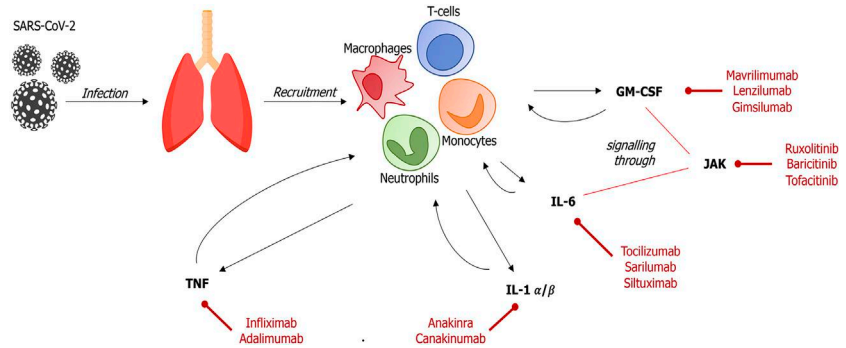

FIGURE 1 | Main pathways and treatment targets in SARS-CoV-2–induced immune response In the early stage of SARS-CoV-2 infection, infected cells and resident macrophages release signaling molecules that recruit host immune cells into the alveolar space. These cells, mainly neutrophils, T-lymphocytes and monocytes, produce and release high levels of inflammatory cytokines, leading to an uncontrolled inflammatory response (GM-CSM, granulocyte-monocyte colony-stimulating factor; IL, interleukin; JAK, Janus kinase; TNF, tumor necrosis factor).

in Milan (Italy) evaluated the efficacy of mavrilimumab in non-mechanically ventilated COVID-19 patients (De Luca et al., 2020). Specifically, 13 patients received a single intravenous dose of mavrilimumab (6 mg/kg) upon hospital admission. Outcomes at 28 days were compared to 26 patients with severe COVID-19 pneumonia and comparable baseline characteristics. Mavrilimumab was associated with a higher rate of clinical improvement ($p = 0.03$) and was well tolerated in all patients, in keeping with the good safety profile emerged in the drug development program for rheumatoid arthritis (Burmester et al., 2011). Despite clear limitations, including a small sample size and the uncontrolled nature of the investigation, this study prompted initiation of a randomized placebo-controlled trials, which is presently active in Italy (NCT04397497). Three additional monoclonal antibodies directed against GM-CSF (gimsilumab, lenzilumab, and TJ003234) are currently under investigation for the treatment of COVID-19 (NCT04351243, NCT00995449, NCT03794180).

The results of these clinical trials on GM-CSF blockade are awaited and will also address a theoretical concern related to the role of GM-CSF in the homeostasis of the alveolar surfactant. A deficit in GM-CSF has been linked to impaired differentiation of alveolar macrophages and to subsequent accumulation of surfactant components in the alveoli (Trapnell et al., 2009). Indeed, congenital deficit of GM-CSF causes the development of pulmonary proteinosis (PAP), a severe respiratory disease characterized by progressive accumulation and accumulation of exudates in the alveolar spaces. However, PAP has never been reported during the development of mavrilimumab. Similarly, PAP might not be an issue when treating COVID-19 patients, because a single intravenous dose of monoclonal antibodies typically wears off in a month, at variance with the chronic deficiency of GM-CSF observed in PAP (Bonaventura et al., 2020).

TUMOR NECROSIS FACTOR AND JANUS KINASES

TNF is a mediator of paramount importance in the development of inflammatory responses. TNF levels are increased in sera of COVID-19 patients (Wang et al., 2020). It has been suggested that this cytokine is one of the very first mediators to induce tissue damage in tissues infected by coronaviruses (Haga et al., 2008).

TNF blocking agents, such as infliximab, are the cornerstone of the therapy of chronic inflammatory diseases (Smolen et al., 2020). Previous evidence suggests the potential beneficial effect of TNF inhibitors in murine models of viral pneumonia (Hussell et al., 2001). Pharmacological TNF blockade could lead to a therapeutic effect by both reducing direct inflammatory effects of this biochemical cascade and the downregulation of ACE2 expression and shedding, which are known to be essential element of viral cell entry (Haga et al., 2008). As for other biological agents, the main safety concerns for TNF inhibitors in the setting of COVID-19 patients is a raise in bacterial and fungal superinfections rates (Feldmann et al., 2020).

Retrospective data showed that infliximab was associated with clinical improvement and reduction n inflammatory markers in severe COVID-19 patients (Stallmach et al., 2020). Despite these encouraging results, no controlled evidence is available to date. Trials evaluating the role of TNF blockade in COVID-19 are currently ongoing (ChiCTR2000030089; NCT04425538—evaluating adalimumab and infliximab, respectively).

Janus kinases (JAK) are a family of mediators involved in intracellular signaling cascades downstream the receptors of multiple cytokines, most notably IL-6, but not IL-1 or TNF (O'Shea et al., 2013). Pharmacological inhibition of JAKs is an approved strategy for the treatment of various inflammatory diseases, ranging from rheumatoid arthritis and inflammatory

TABLE 1 | Main published observational studies and randomized trials of biologic and targeted synthetic drugs for the treatment of SARS-CoV-2–induced hyperinflammation.

Agent	Ref	Study information	Sample size	Study population	Setting	Main results
Interleukin-1						
Anakinra	Cavalli et al. (2020a)	Single-centre, open-label	29 treated 16 controls	Respiratory failure, hyperinflammation	Outside ICU	Improved respiratory function, improved survival
Anakinra	Huet et al. (2020)	Single-centre, open-label	52 treateds 44 control	Respiratory failure	Outside ICU	Reduced ICU admission, improved survival
Anakinra	Navarro-Millán et al. (2020)	Single-centre, case-series	11 treated	Respiratory failure, hyperinflammation	Outside ICU	7 patients not required invasive mechanical ventilation (early-treated)
canakinumab	Ucciferri et al. (2020)	Single-centre, case-series	10 treated	Respiratory failure, hyperinflammation	Outside ICU	All patients discharged
Interleukin-6						
Tocilizumab	Campochiaro et al. (2020a)	Single-centre, open-label	52 treated 44 controls	Respiratory failure, hyperinflammation	Outside ICU	No differences in clinical improvement and survival
Tocilizumab	Capra et al. (2020)	Single-centre, open-label	62 treated 23 controls	Respiratory failure	Outside ICU	Improved respiratory function, improved survival
Tocilizumab	Morena et al. (2020)	Single-centre, case series	51 treated	Respiratory failure, hyperinflammation	ICU and non-ICU	31 patients were discharged, 17 had a worsening of the clinical status, 14 died
Tocilizumab	Guaraldi et al. (2020)	Multicentre, open-label	179 treated 365 controls	Respiratory failure	Outside ICU	Reduced ICU admission or death
Tocilizumab	Biran et al. (2020)	Multicentre, open-label	210 treated 420 controls	ARDS with mechanical support	ICU	Improved survival
Tocilizumab	Stone et al. (2020)	Multicenter RCT	161 treated 82 controls	Respiratory failure, hyperinflammation	Outside ICU	No differences in clinical improvement and survival
Tocilizumab	Hermine et al. (2020)	Multicenter RCT	64 treated 67 controls	Respiratory failure, hyperinflammation	Outside ICU	Reduced mechanical ventilation and death rate at 14 days; no differences in survival at 28 days
Tocilizumab/ sarilumab	Sinha et al. (2020)	Single-centre, case series	255 treated	Respiratory failure, hyperinflammation	Outside ICU	Mortality of severe patients was comparable to the overall COVID-19-related mortality in the local area
sarilumab	Benucci et al. (2020)	Single-centre, case series	8 treated	Respiratory failure	Outside ICU	7 patients discharged within 14 days, 1 patient died
sarilumab	Della-Torre et al. (2020)	Single-centre, open-label	28 treated 28 controls	Respiratory failure, hyperinflammation	Outside ICU	No differences in clinical improvement and survival
GM-CSF						
Mavrilimumab	De Luca et al. (2020)	Single-centre, open-label	13 treated 26 controls	Respiratory failure, hyperinflammation	Outside ICU	Greater and earlier improvement of clinical outcomes
Tumor necrosis factor						
Infliximab	Stallmach et al. (2020)	Single-centre, open-label	7 treated 17 controls	Respiratory failure, hyperinflammation	Outside ICU	Clinical improvement in 6 patients
Janus kinases						
Ruxolitinib	Cao et al. (2020)	Multicenter RCT	20 treated 21 controls	Respiratory failure	Outside ICU	Faster clinical recovery; chest CT improvement
Baricitinib	Cantini et al. (2020)	Multicentre, open-label	113 treated 78 controls	Respiratory failure	Outside ICU	Improved respiratory function, reduced ICU admission, increased discharge rate

bowel diseases to hematologic conditions (Meyer et al., 2010). In COVID-19, JAK inhibition is appealing in light of the possibility to achieve a broader modulation of inflammatory responses compared to selective blockade of individual cytokines with biologics (Seif et al., 2017; Rizk et al., 2020). Ruxolitinib is a selective inhibitor of JAK1 and JAK2 licensed for the treatment of graft-versus-host disease (Rizk et al., 2020). In a randomized clinical trial of COVID-19, treatment with ruxolitinib was associated with faster, albeit not significant, clinical improvement and a favorable safety profile (Cao et al., 2020). Ongoing trials evaluating this drug are ongoing (NCT04348071, NCT04377620, NCT04414098, NCT04362137). The JAK 1/2 inhibitor baricitinib also attracted clinical expectations, particularly following *in silico* studies postulating an inhibitory effect against viral entry into pneumocytes (Richardson et al., 2020). To date, experience with baricitinib is limited to a study evaluating combination therapy with antivirals, and reporting some degree of improvement in clinical and laboratory parameters in COVID-19 patients (Cantini et al., 2020). Ongoing trials are evaluating baricitinib or the JAK 1/3 inhibitor tofacitinib (i.e. NCT04340232, NCT04358614, NCT04345289, NCT04399798, NCT04320277). These trials will also address safety concerns related to the reported increase in thromboembolic events associated with JAK

inhibitors which may further increase the hypercoagulability risk inherent to COVID-19 (Jorgensen et al., 2020; Tang Y. et al., 2020).

CONCLUSIONS

A maladaptive, hyper-inflammatory host immune response to the virus is recognized as the main driver of disease severity in a subset of COVID-19 patients. Anti-cytokine agents with targeted anti-inflammatory effects were explored as a logical therapeutic approach in this setting. Several biotechnological drugs were repurposed for use in COVID-19, with mixed results. At present, controlled evidence indicates that IL-6 inhibition is marginally o not effective for COVID-19, whereas several uncontrolled studies evaluating IL-1 inhibition yielded overall promising results and are awaiting validation in controlled settings. Additional promising strategies include GM-CSF and JAK inhibition, although present evidence is more limited. Other theoretical options, such as TNFa inhibitor, remain relatively unexplored. Randomized clinical trials evaluating all these strategies are ongoing, but results are already available only for IL-6 inhibition. Meanwhile, as individual predisposition to the development of hyper-inflammation is revealed by COVID-19, targeted inhibition of causal cytokines is likely to confer survival benefits in some patients. Equally important, selective pharmacologic inhibition of different cytokines reveals the specific contribution of individual mediators to hyper-inflammatory responses, with translational consequences for the development of these anti-inflammatory strategies for future applications.

AUTHOR CONTRIBUTIONS

LD and CG conceived the manuscript. GC, NF, CC, GL, and ED-T drafted the manuscript: AT drafted the figure and table. All authors critically revised the manuscript and approved the final version.

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COVID-19 Incidence in Patients With Immunomediated Inflammatory Diseases: Influence of Immunosuppressant Treatments

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The effect of immunosuppressant treatments on the incidence of coronavirus disease (COVID-19) remains largely unknown. We studied the association between the pre-exposure to disease-modifying antirheumatic drugs (DMARDs) that decrease immunological responses and the incidence of COVID-19 to explore the possible effects of these treatments in early manifestations of the disease. For this purpose, we performed a cross-sectional study including 2,494 patients with immunomediated inflammatory diseases (IMIDs) recruited at the outpatient Rheumatology, Dermatology and Gastroenterology services of Hospital del Mar. The primary outcome was the clinical diagnosis of COVID-19 performed by a physician at the hospital or at the primary care center, from the March 1–29, 2020. Multivariable Poisson regression models were fitted to estimate COVID-19 relative risk (RR) adjusted by comorbidities. We revealed that biological (RR = 0.46, CI 95% = 0.31–0.67) and synthetic (RR = 0.62, CI 95% = 0.43–0.91) DMARDs used in IMIDs diminished the incidence of COVID-19. Striking sex differences were revealed with anti-TNFα compounds (RR = 0.50, CI 95% = 0.33–0.75) with higher effects in women (RR = 0.33, CI 95% = 0.17–0.647). Treatment with low glucocorticoid doses also revealed sex differences decreasing the incidence of COVID-19 predominantly in women (RR = 0.72, CI 95% = 0.42–1.22). Our results report a decreased incidence of COVID-19 in patients receiving specific DMARDs with different immunodepressor mechanisms with striking sex differences. These results underline the interest of repurposing specific DMARDs for the possibility of minimizing the severity of disease progression in the early stages of COVID-19.

Keywords: biological therapy, tumor necrosis factor inhibitor, cross-sectional study, relative risk, disease modifying antirheumatic drugs (DMARDs), gender, glucocorticoids

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INTRODUCTION

Since December 2019, cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection leading to a novel disease called COVID-19 were initially identified in China. SARS-CoV-2 infection causes respiratory symptoms that range from mild forms of presentation to more serious ones that can risk patients' lives, causing pneumonia, and damage to other organs, particularly the immune and blood system (Chen et al., 2020; Huang et al., 2020; Wang et al., 2020). This disease has rapidly expanded to multiple countries leading to a pandemic situation in March 2020 now affecting 7.360.239 individuals worldwide, with a global mortality of 416.201 deaths on June 11th. The situation has been dramatic in some European countries during the last months, such as Spain with 242.280 cases and 27.136 deaths (Dong et al., 2020). This official mortality numbers only reflect the casualties occurring in the hospitals, not in nursing homes or at home, and considering the low availability of accurate COVID-19 diagnostic tests, the current situation in Spain could unfortunately be worse. Furthermore, some patients are asymptomatic (Mizumoto et al., 2020; Nishiura et al., 2020) and the current prevalence reflects a possible underdiagnosis of the infection that has facilitated the disease expansion.

Immunomediated inflammatory diseases (IMIDs) are a group of unrelated and highly diverse conditions, such as rheumatoid arthritis and psoriasis, that share a common pathogenesis pathway, i.e., an immune dysregulation leading to an imbalance in inflammatory mediators. Treatments to relieve IMIDs are namely disease modifying antirheumatic drugs (DMARDs), subdivided into two main subgroups: synthetic (sDMARDs) and biological (bDMARDs). Both groups are aimed to decrease the hyperactivity of the immune system: bDMARDs are monoclonal antibodies presenting a much higher affinity and selectivity to their targets (mainly pro-inflammatory IL, and TNFα), while sDMARDs have a less selective immunosuppressant effect, except for Jak-inhibitors.

On the other side, evidence suggests that the hyperactivation of the immune response is of paramount relevance in COVID-19 progression. The accumulated knowledge about the pathophysiology of this disease reveals a crucial involvement of different molecules of the main inflammatory pathways, including interleukins 1, 6, and 8 (IL-1, IL-6, IL-8) and tumor necrosis factor alpha (TNFα). Drugs inhibiting some of these pathways have been used in the routine management of COVID-19, although results from clinical trials are still required to corroborate their effectiveness (Zhong et al., 2020). Clear examples are anti-IL-6 compounds for patients with severe forms of COVID-19 (Fu et al., 2020; Zhang et al., 2020; Zhou et al., 2020) and hydroxychloroquine, widely used and highly questioned (Adhanom Ghebreyesus, 2020; Mehra et al., 2020).

This similar physiopathology, as well as the mechanism of action of the drugs used for IMID management, has focused the attention on the study of patients suffering from IMID as a population of particular interest in the study of COVID-19 (Gianfrancesco et al., 2020a, Gianfrancesco et al., 2020b; Favalli et al., 2020; Michelena et al., 2020; Monti et al., 2020; Salvarani et al., 2020). Patients with an autoimmune disease might be at higher risk of developing severe infections, as these medications are immunosuppressants (Memoli et al., 2014). However, this assumption has not been confirmed for SARS-CoV-2 infection, as several studies describe that the COVID-19 incidence in IMID patients is similar to the general population (Memoli et al., 2014; Favalli et al., 2020; Michelena et al., 2020; Salvarani et al., 2020). Some studies have focused on the effect of IMID treatment on COVID-19 severity in terms of hospitalization and death. Thus, systemic glucocorticoid pretreatment was reported to represent a risk factor for severe COVID-19 (OR, 6.9; 95% CI, 2.3–20.5) in patients with inflammatory bowel disease, while anti-TNFα treatment presents no association (Brenner et al., 2020). On the other hand, the COVID-19 Global Rheumatology Alliance studied the demographic and clinical factors associated with COVID-19 hospitalization in rheumatic patients and found that a ≥10 mg/day glucocorticoid dose was associated with a higher odds of hospitalization (OR 2.05, 95% CI 1.06–3.96), whereas anti-TNFα present a decreased incidence or hospitalizations (OR 0.40, 95% CI 0.19–0.81). No association were observed neither with DMARDs nor antimalarial use (Gianfrancesco et al., 2020a; Gianfrancesco et al., 2020b). Similar results were reported in patients using immunomodulatory therapy, regardless of the underlying disease. Indeed, a trend to a higher incidence of hospitalization was observed with chronic glucocorticoid treatment <10 mg/day in these patients, while anti-TNFα use was associated with a reduced odd of hospitalization (Winthrop et al., 2020).

These studies generally use age-standardized rates, so they tackle the problem of comparing populations with different age structures. However, such populations may also differ considering their distribution of associated comorbidities and treatments for these comorbidities, which could influence the results. Furthermore, the majority of studies evaluated the effect of the treatment on developing severe symptoms, with limited data considering also mild to moderate symptoms. In that context, there is a need to study the COVID-19 incidence in IMID patients and the potential effect of immunosuppressants controlling for the influence of the different distribution of risk factors in order to evaluate the possibility of repurposing possible new drugs for COVID-19 therapy.

METHODS

Study Design and Population

This is a cross-sectional study aimed to evaluate the effect of different DMARDs on the accumulated incidence of COVID-19 during March 2020 in patients with IMIDs living in Barcelona (Spain). The studied population was composed of 1) patients with IMIDs taking bDMARDs (exposed patients) and 2) patients with IMIDs or other musculoskeletal diseases that were not taking bDMARDs (unexposed patients). All patients had been visited at the outpatient Rheumatology,

Dermatology and Gastroenterology services of Hospital del Mar (referral hospital from Barcelona) from September 2019 to March 2020.

The exclusion criteria were <18 years old, previous death not related with SARS-CoV-2 infection and patients tested negative for SARS-CoV-2 or without follow up at the primary care center during the studied period. The study was undertaken according to Good Clinical Practice guidelines and the Declaration of Helsinki. The research ethics review committee of Parc de Salut Mar approved the protocol (2020/9,246).

Data Collection

A comprehensive review of the medical history of eligible patients was carried out using the registry of the Catalan national health system (eCAP). This register of the health system of Catalonia is a computerized medical history program that collects the health status of each of the patients and all entries to the public primary care system are recorded in this register. In turn, this database is fed by other information systems of the public network so that it contains continuously updated information on all consultations to hospitals, emergency services, pharmacy, death certifiers and any other relevant clinical information. The Hospital del Mar also has its own program of computerized medical record called IMASIS. Both database platforms were consulted for reviewing the medical histories and both are interconnected online. The immediate updating of the data in these platforms avoids any type of information loss. A clinical history revision of the included patients was performed from the 1st to March 29, 2020, focusing mainly at patient's consulting disease, comorbidities and the treatments being currently followed by them (**Supplementary Tables S1, S2**). Briefly, diabetes, pulmonary disease, cardiovascular (CV) disease and chronic kidney disease were registered. In the case of arterial hypertension (AHT) and transplantation, they were only recorded if patients were receiving treatment with specific drugs for those comorbidities. Finally, cancer was recorded only if the patient had an active process or was following a treatment for a previous cancer, during the studied period.

The primary outcome was the clinical diagnosis of COVID-19 performed by a physician at the hospital or at the primary care center, from the 1st to March 29, 2020. In some patients, the diagnosis was complemented with a positive SARS-CoV-2 test, but in most of them it was based on clinical criteria following the Spanish health authorities' recommendations: fever (defined as axillary temperature >37°C) together with shortness of breath and/or cough. If only fever was present, it was also considered as COVID-19 diagnosis if it appeared together with at least two of the following symptoms: anosmia, ageusia, rhinorrhea, diarrhea of one week of evolution, pharyngitis, odynophagia or arthromyalgia.

Statistical Analysis

To evaluate the associations between different treatments and the diagnosis of COVID- 19, Poisson regression models with robust variance estimation were used to estimate relative risk (RR) and 95% confidence intervals (CI 95%). Models were adjusted by sex, age, diabetes, pulmonary disease, CV disease, chronic kidney disease, and active cancer or treatment. Model 1 aimed to estimate the association between treatments grouped by drug type 1) bDMARDs; 2) sDMARDs, 3) glucocorticoids, 4) chronic nonsteroidal anti-inflammatory drugs (NSAIDs) and 5) anti-hypertensive drugs. Then, associations between COVID-19 symptoms were estimated by each individual treatment (with >100 exposed patients; reference category = "unexposed"; Model 2). Finally, as anti-TNFα treatments were the major group of bDMARDs, the effect of each anti-TNFα drug was estimated separately in model 3. Model three also included the effect of anti-IL17 and anti-IL23 (−12), but anti-IL6 could not be analyzed as a separate group as there were not COVID-19 symptoms reported among individuals exposed to IL-6 antagonists. Interactions between different drug types were also tested (model 4). Finally, the main treatment indications for anti-TNFα, together with the studied comorbidities (sex, age, CV disease, diabetes, pulmonary disease, kidney disease and cancer) were used to create a matched dataset with propensity score matching based on the nearest neighbor method (Ho et al., 2011). Propensity score is the probability of exposure conditional upon confounders, estimated by logistic regression. Therefore, each treated individual was matched with an untreated individual whose propensity score was closest to that of the treated subject. Statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria) version 3.5.2.

RESULTS

A total of 2,544 individuals were examined for eligibility and 2,494 fulfilled inclusion/exclusion criteria and were finally included in the analysis, 902 (36.2%) men and 1,592 (63.8%) women.

Tables 1, **2** show the description of the comorbidities and treatments followed by studied population. The mean age (SD) was 58.7 (15.7) and the most prevalent underlying pathologies were spondyloarthritis (32.6%), rheumatoid arthritis (21.6%) and osteoarthritis (25.1%). Almost half of individuals had at least one of the following comorbidities: hypertension (34%), diabetes (12.1%), pulmonary disease (14%), CV disease (11%), chronic kidney disease (5%), active cancer or treatment (3%) and post-transplant (0.3%). In terms of treatments, 45% of individuals were taking bDMARDs (59% in men and 36% in women), primarily anti- TNFα (30% in total; 42% in men and 24% in women). A third of the population were exposed to sDMARDs, being methotrexate, leflunomide and chloroquine/ hydroxychloroquine the most prevalent ones (22%, 5% and 5%, respectively). Glucocorticoid consumption in women was twice that in men (26% vs 13%) but, in both cases, doses of glucocorticoids higher than 10 mg/day were unusual (<4%). NSAIDs and anti-hypertensive drugs were taken by the 20% and 27% of individuals, respectively. A 15.8% of the population (18.4% in women and 11.2% in men) did not take any of the registered treatments (**Supplementary Table S3**).

In the cohort of individuals exposed to bDMARDs, the presence of the main comorbidities (hypertension, pulmonary

TABLE 1 | Characteristics of the study population [N (%)].

Characteristic	All (N = 2,494)	Women (N = 1,592)	Men (N = 902)
Age [mean (SD)]	58.7 (15.7)	60.6 (15.5)	55.5 (15.6)
Primary diagnosis			
spondyloarthritis	812 (32.6%)	359 (22.6%)	453 (50.2%)
Rheumatoid arthritis	538 (21.6%)	424 (26.6%)	114 (12.6%)
Osteoarthritis	627 (25.1%)	480 (30.2%)	147 (16.3%)
Systemic autoimmune rheumatic diseases	165 (6.62%)	149 (9.36%)	16 (1.77%)
Vasculitis	59 (2.37%)	37 (2.32%)	22 (2.44%)
Other rheumatic diseases	38 (1.52%)	26 (1.63%)	12 (1.33%)
Juvenile arthritis	7 (0.28%)	4 (0.25%)	3 (0.33%)
Dermatological diseases	208 (8.34%)	82 (5.15%)	126 (14.0%)
Other	40 (1.60%)	31 (1.95%)	9 (1.00%)
Coexisting conditions			
Hypertension	858 (34.4%)	553 (34.7%)	305 (33.8%)
Diabetes	302 (12.1%)	174 (10.9%)	128 (14.2%)
Pulmonary disease	364 (14.6%)	241 (15.1%)	123 (13.6%)
CV Disease	290 (11.6%)	179 (11.2%)	111 (12.3%)
Chronic kidney disease	129 (5.17%)	76 (4.77%)	53 (5.88%)
Cancer or active treatment	70 (2.81%)	47 (2.95%)	23 (2.55%)
History of organ transplantation	8 (0.32%)	7 (0.44%)	1 (0.11%)
Any of these conditions	1,223 (49.0%)	797 (50.1%)	426 (47.2%)

disease and CV disease) was lower than in the cohort of individuals unexposed to bDMARDs. Also, their mean age (SD) was 52.2 (14.7) years, while in the cohort of unexposed to bDMARDs their mean age was 64 (15.4) years (see **Supplementary Table 4** for further details).

The total number of patients with COVID-19 diagnosis was 156. As shown in **Tables 3**, **4**, those presenting clinical diagnosis of COVID-19 had less spondyloarthritis, rheumatoid arthritis or dermatological diseases, and higher osteoarthritis. The proportion of diabetics in the group of individuals with COVID-19 was 20.5%, while in the group without symptoms was 11.5%. In the case of pulmonary disease, these percentages were 22.4% and 14.1%, respectively. The proportion of patients taking bDMARDS and sDMARDs was lower in the group with COVID-19 diagnosis. Among those with a clinical diagnosis of COVID-19, 32 were confirmed by a SARS-CoV-2 test and the remaining 124 had not been tested. There were 26 individuals (8 men and 18 women) hospitalized and there were 4 deaths due to COVID-19.

Adjusted associations between different exposure variables (clinical characteristics and treatments) and COVID-19 symptoms are shown in **Tables 5**, **6**. This analysis allows to control the parameters that could be playing a role in the diagnosis of COVID- 19, such as sex, age, comorbidities, or treatments. Diabetes and pulmonary disease were associated with COVID−19 diagnosis, with overall RR_{m1} of 1.64 (CI 95% 1.09, 2.47) and 1.47 (CI 95% 1.02, 2.13). Regarding treatments, all bDMARDs presented an RR of 0.46 (CI 95% 0.31, 0.67) and all sDMARDs presented an RR of 0.62 (CI 95% 0.43, 0.91). Specifically, TNF-α antagonists presented RR of 0.50 (CI 95% 0.33, 0.75) in the whole population. This effect was even higher in women (RR = 0.33; CI 95% 0.17, 0.64), while in men the RR was 0.76 (CI 95% 0.41, 1.43), and given the risk difference ranging from 0.41 to 1.43, a substantial positive association was reasonably compatible with our data. All types of TNF-α antagonists (adalimumab, certolizumab, etanercept, golimumab and infliximab) showed RR estimates <1, although the differences were only statistically significant for adalimumab (RR = 0.53, CI 95% 0.31, 0.93) and etanercept (RR = 0.37, CI 95% 0.16, 0.88). The RR of anti-IL17 was 0.20 (CI 95% 0.03–1.38) and for anti-IL23 (12) was 0.80 (CI 95% 0.39, 1.65). Methotrexate and chloroquine/hydroxychloroquine presented a RR of 0.71 (CI 95% 0.46, 1.08) and 0.76 (CI 95% 0.36, 1.62), respectively. The RR of leflunomide was 0.66 (CI 95% 0.28, 1.58) in the whole population, with higher relative risk reduction in men (RR = 0.36; CI 95% 0.07, 1.75) than in women (RR = 0.81; CI 95% 0.29, 2.87). Glucocorticoids at doses of ≤10 mg/day also showed a relative risk reduction in women (RR = 0.72, CI 95% 0.42, 1.22). **Figure 1** represents the adjusted RR for presenting COVID-19 symptoms according to the exposure to different treatments in men and women. The interactions between most prevalent combinations of treatments (bDMARDs + sDMARDs; bDMARDs + anti-hypertensive drugs; bDMARDs + chronic NSAIDs; sDMARDS + glucocorticoids) were included in Model 4 (**Supplementary Table S6**) and our results were most compatible with no important effects, except for the interaction between bDMARDs and cDMARDs (RR = 4.3; CI 95% 2.00, 9.25).

Finally, the crude RR using propensity score matching for the exposure to anti-TNFα was 0.80 (CI 95% 0.50, 1.30) and the adjusted RR (by anti-pro-inflammatory ILs, methotrexate, leflunomide, chloroquine/hydroxychloroquine, glucocorticoids, ACE inhibitors, ARBs, NSAIDs) was 0.69 (CI 95% 0.38, 1.23). A description of the matched dataset is included in **Supplementary Table S7**.

DISCUSSION

Our cross-sectional study reveals that the DMARDs treatments commonly used in IMIDs are not associated with an increase in

TABLE 2 | Characteristics of the study population [N (%)].

	All (N = 2,494)	Women (N = 1,592)	Men (N = 902)
Treatments followed			
Biologic DMARDs[1]	1,112 (44.6%)	579 (36.4%)	533 (59.1%)
Any TNFα antagonist	768 (30.8%)	388 (24.4%)	380 (42.1%)
Adalimumab	367 (14.7%)	163 (10.2%)	204 (22.6%)
Etanercept	183 (7.34%)	105 (6.60%)	78 (8.65%)
Infliximab	120 (4.81%)	60 (3.77%)	60 (6.65%)
Golimumab	65 (2.61%)	35 (2.20%)	30 (3.33%)
Certolizumab	33 (1.32%)	25 (1.57%)	8 (0.89%)
Any pro-inflammatory ILs antagonists	279 (11.2%)	136 (8.54%)	143 (15.9%)
IL-6 antagonists	52 (2.09%)	42 (2.64%)	10 (1.11%)
Tocilizumab	46 (1.84%)	37 (2.32%)	9 (1.00%)
Sarilumab	6 (0.24%)	5 (0.31%)	1 (0.11%)
IL-17 antagonists	69 (24.7%)	26 (19.1%)	43 (30.1%)
Brodalumab	2 (0.72%)	1 (0.74%)	1 (0.70%)
Secukinumab	51 (2.04%)	22 (1.38%)	29 (3.22%)
Ixekizumab	16 (5.73%)	3 (2.21%)	13 (9.09%)
IL-23 (12) antagonists	158 (56.6%)	68 (50.0%)	90 (62.9%)
Ustekinumab	155 (6.21%)	67 (4.21%)	88 (9.76%)
Guselkumab	3 (1.08%)	1 (0.74%)	2 (1.40%)
Any T lymphocyte antagonist	29 (1.16%)	22 (1.38%)	7 (0.78%)
Any B lymphocyte antagonist	42 (1.68%)	36 (2.26%)	6 (0.67%)
Vedolizumab	3 (0.12%)	2 (0.13%)	1 (0.11%)
Synthetic DMARDs[2]	850 (34.1%)	583 (36.6%)	267 (29.6%)
Methotrexate	538 (21.6%)	366 (23.0%)	172 (19.1%)
Leflunomide	116 (4.65%)	86 (5.40%)	30 (3.33%)
Chloroquine or hydroxychloroquine	115 (4.61%)	105 (6.60%)	10 (1.11%)
Azathioprine	80 (3.21%)	52 (3.27%)	28 (3.10%)
JAK inhibitors	41 (1.64%)	32 (2.01%)	9 (1.00%)
Apremilast	52 (2.09%)	20 (1.26%)	32 (3.55%)
Sulfasalazine	10 (0.40%)	7 (0.44%)	3 (0.33%)
Mycophenolate	19 (0.76%)	17 (1.07%)	2 (0.22%)
Tacrolimus	24 (0.96%)	17 (1.07%)	7 (0.78%)
Cyclosporine	3 (0.12%)	2 (0.13%)	1 (0.11%)
Dose of glucocorticoids	—	—	—
≤10 mg/d	441 (17.7%)	347 (21.8%)	94 (10.4%)
>10 mg/d	86 (3.45%)	62 (3.89%)	24 (2.66%)
Anti-hypertensive drugs[3]	684 (27.4%)	428 (26.9%)	256 (28.4%)
ACE inhibitors	397 (15.9%)	237 (14.9%)	160 (17.7%)
ARBs	293 (11.7%)	194 (12.2%)	99 (11.0%)
Chronic NSAIDs	498 (20.0%)	345 (21.7%)	153 (17.0%)

CV= cardiovascular. DMARDs = disease modifying anti-rheumatic drugs. JAK = Janus kinase. IL=interleukin. TNF=tumor necrosis factor. NSAIDs = non-steroid anti-inflammatory drugs. ACE = angiotensin-converting enzyme. ARBs = angiotensin II receptor blockers.

[1]Biologic DMARDs include TNF antagonists, pro-inflammatory ILs antagonists, vedolilzumab and T and B lymphocyte antagonists.

[2]Synthetic DMARDs include methotrexate, JAK inhibitors, sulfasalazine, mycophenolate, tacrolimus, azathioprine, cyclosporine, chloroquine or hydroxychloroquine and leflunomide and apremilast.

[3]Anti-hypertensive drugs include ACE inhibitors and ARBs.

COVID-19 incidence. All the treatments analyzed in our study were not discontinued in our cohorts of patients following the previous recommendations (Gianfrancesco et al., 2020a, Gianfrancesco et al., 2020b; Haberman et al., 2020; Michelena et al., 2020). It is important to underline that the primary outcome of our study was the manifestation of mild symptoms of COVID-19. Therefore, our results do not provide relevant information about the possible influence of these treatments in the severity of COVID-19, taking into account the low incidence of severe symptoms, hospitalizations and deaths in our cohort or early symptomatic patients. However, several studies have already reported that some IMID treatments have a protective effect on the incidence of developing severe symptoms, probably blocking the hyperactivation of the immune response occurring in the COVID-19 progression (Gianfrancesco et al., 2020a, Gianfrancesco et al., 2020b; Winthrop et al., 2020). Interestingly, in our study bDMARDs (RR = 0.46; CI 95% 0.31, 0.67) and sDMARDs (RR = 0.62; CI 95% 0.43, 0.91) treatment diminished the incidence of COVID-19, in agreement with previous preliminary observations (Haberman et al., 2020; Michelena et al., 2020). Therefore these treatments are also playing a role in the capacity to be infected by SARS-CoV-2 and/or in presenting mild symptoms of COVID-19. At these early stages of the disease, the two co-morbidities that significantly enhanced COVID-19 diagnosis

TABLE 3 | Distribution of COVID-19 across categories of study variables.

	All		Women		Men	
	No symptoms (N = 2,338)	**Symptoms (N = 156)**	**No symptoms (N = 1,484)**	**Symptoms (N = 108)**	**No symptoms (N = 854)**	**Symptoms (N = 48)**
Age [mean (SD)]	58.5 (15.7)	62.1 (16.2)	60.3 (15.5)	64.8 (15.5)	55.5 (15.5)	56.0 (16.1)
Primary diagnosis						
spondyloarthritis	770 (32.9%)	42 (26.9%)	340 (22.9%)	19 (17.6%)	430 (50.4%)	23 (47.9%)
Rheumatoid arthritis	519 (22.2%)	19 (12.2%)	408 (27.5%)	16 (14.8%)	111 (13.0%)	3 (6.25%)
Osteoarthritis	563 (24.1%)	64 (41.0%)	424 (28.6%)	56 (51.9%)	139 (16.3%)	8 (16.7%)
Systemic autoimmune rheumatic diseases	159 (6.80%)	6 (3.85%)	145 (9.77%)	4 (3.70%)	14 (1.64%)	2 (4.17%)
Vasculitis	53 (2.27%)	6 (3.85%)	35 (2.36%)	2 (1.85%)	18 (2.11%)	4 (8.33%)
Other rheumatic diseases	26 (11.1%)	12 (7.69%)	22 (1.48%)	4 (3.70%)	9 (1.05%)	3 (6.25%)
Juvenile arthritis	7 (0.30%)	0 (0.00%)	4 (0.27%)	0 (0.00%)	3 (0.35%)	0 (0.00%)
Dermatological diseases	202 (8.64%)	6 (3.85%)	80 (5.39%)	2 (1.85%)	122 (14.3%)	4 (8.33%)
Other	31 (1.33%)	9 (5.77%)	26 (1.75%)	5 (4.63%)	8 (0.94%)	1 (2.08%)
Coexisting conditions						
Hypertension	788 (33.7%)	70 (44.9%)	505 (34.0%)	48 (44.4%)	283 (33.1%)	22 (45.8%)
Diabetes	270 (11.5%)	32 (20.5%)	152 (10.2%)	22 (20.4%)	118 (13.8%)	10 (20.8%)
Pulmonary disease	329 (14.1%)	35 (22.4%)	216 (14.6%)	25 (23.1%)	113 (13.2%)	10 (20.8%)
CV Disease	265 (11.3%)	25 (16.0%)	161 (10.8%)	18 (16.7%)	104 (12.2%)	7 (14.6%)
Chronic kidney disease	117 (5.00%)	12 (7.69%)	70 (4.72%)	6 (5.56%)	47 (5.50%)	6 (12.5%)
Cancer or activetreatment	64 (2.74%)	6 (3.85%)	43 (2.90%)	4 (3.70%)	21 (2.46%)	2 (4.17%)
History of organ transplantation	7 (0.30%)	1 (0.64%)	6 (0.40%)	1 (0.93%)	1 (0.12%)	0 (0.00%)
Any of these conditions	1,122 (48.0%)	101 (64.7%)	728 (49.1%)	69 (63.9%)	394 (46.1%)	32 (66.7%)

in these group of patients were diabetes (RR = 1.64; CI 95% 1.09, 2.47) and pulmonary disease (RR = 1.47; CI 95% 1.02, 2.13). A large number of patients treated with bDMARDs (1,153) and sDMARDs (850 patients, 283 also receiving bDMARDs) has been included in our cohort. Therefore, the global decrease in the incidence of COVID-19 on patients treated with DMARDs has influenced the RR estimated for compounds that are supposed to not modify COVID-19 progression.

The protective effects of the anti-TNFα treatment on the incidence of COVID-19 symptoms reported in our study (RR = 0.50; CI 95% 0.33, 0.75) fully agree with the comments recently published about the urgent need of clinical trials of anti-TNFα therapy for COVID-19 (Feldmann et al., 2020; Robinson et al., 2020). Indeed, previous studies have reported that rheumatic patients treated with anti-TNFα present a decreased incidence of hospitalizations (OR 0.40, 95% CI 0.19–0.81) (Gianfrancesco et al., 2020a; Gianfrancesco et al., 2020b) and this protective effect was also observed in anti-TNFα treated patients regardless of the underlying disease (Winthrop et al., 2020). Our findings corroborate these protective effects considering the incidence of mild symptoms as the primary output of the study. Therefore, anti-TNFα treatment may have protective effects in the incidence of COVID-19 symptoms (our study), but also in the progression to severe manifestations of this disease (Gianfrancesco et al., 2020a, Gianfrancesco et al., 2020b; Winthrop et al., 2020). All together, these studies underlie the urgent need of clinical trials to obtain additional evidences of the possible efficacy of anti-TNFα treatment on COVID-19 (Robinson et al., 2020). Anti-TNFα therapy has been proposed to be initiated as early as is practicable in hospitalized patients with COVID-19 in order to obtain the possible optimal beneficial effects (Feldmann et al., 2020).

Although the studied population was not sex-balanced (1,592 women vs. 902 men) our analyses stratified by sex also revealed potential sex differences in the effects of several immunomodulatory compounds on the incidence of COVID-19 mild symptoms. Indeed, anti-TNFα compounds showed a decreased COVID-19 incidence that was higher in women (RR = 0.33; CI 95% 0.17, 0.64) than in men (RR = 0.76; CI 95% 0.41, 1.43). Although a possible sex influence in the therapeutic effects of anti-TNFα compounds is controversial, a positive female sex influence was already reported in the prognosis of ulcerative colitis in patients treated with infliximab, an anti-TNFα monoclonal antibody (Nasuno et al., 2017). Sex differences were also revealed in our study in the effects of glucocorticoids. Taken into account the high variability of the doses of glucocorticoids used in these patients (Ruiz-Irastorza et al., 2012) and the differential effects depending on dose exposure (Meng et al., 2020), we have stratified glucocorticoid treatment in low (≤10 mg of prednisone or equivalent) and high doses (>10 mg). Low glucocorticoids doses decreased COVID-19 incidence in women (RR = 0.72; CI 95% 0.42, 1.22), whereas high doses seemed to produce the opposite effect (RR = 1.62; CI 95% 0.75, 3.52).

Considering the high availability and the safety profile of low doses of glucocorticoids, this result could be of potential interest to further evaluate the possible benefits of using such low doses in women in early periods of SARS-CoV-2 infection to prevent progression of the disease. In contrast, the effects of leflunomide treatment were more clearly revealed in men (RR = 0.36; CI 95% 0.07, 1.75) than in women (RR = 0.81; CI 95% 0.29, 2.27). In line with our results, a significant clinical effect of leflunomide,

TABLE 4 | Distribution of COVID-19 across categories of study variables.

	All		Women		Men	
	No symptoms (N = 2,338)	**Symptoms (N = 156)**	**No symptoms (N = 1,484)**	**Symptoms (N = 108)**	**No symptoms (N = 854)**	**Symptoms (N = 48)**
Treatments followed	—	—	—	—	—	—
Biologic DMARDs[1]	1,070 (45.8%)	42 (26.9%)	560 (37.7%)	19 (17.6%)	510 (59.7%)	23 (47.9%)
Any TNFα antagonist	739 (31.6%)	29 (18.6%)	378 (25.5%)	10 (9.26%)	361 (42.3%)	19 (39.6%)
adalimumab	353 (15.1%)	14 (8.97%)	159 (10.7%)	4 (3.70%)	194 (22.7%)	10 (20.8%)
Etanercept	178 (7.61%)	5 (3.21%)	104 (7.01%)	1 (0.93%)	74 (8.67%)	4 (8.33%)
Infliximab	114 (4.88%)	6 (3.85%)	57 (3.84%)	3 (2.78%)	57 (6.67%)	3 (6.25%)
golimumab	63 (2.69%)	2 (1.28%)	34 (2.29%)	1 (0.93%)	29 (3.40%)	1 (2.08%)
certolizumab	31 (1.33%)	2 (1.28%)	24 (1.62%)	1 (0.93%)	7 (0.82%)	1 (2.08%)
All pro-inflammatory ILs antagonists	269 (11.5%)	10 (6.41%)	130 (8.76%)	6 (5.56%)	139 (16.3%)	4 (8.33%)
IL-6 antagonists	52 (2.22%)	0 (0.00%)	42 (2.83%)	0 (0.00%)	10 (1.17%)	0 (0.00%)
IL-17 antagonists	68 (2.91%)	1 (0.64%)	26 (1.75%)	0 (0.00%)	42 (4.92%)	1 (2.08%)
IL-12/23 antagonists	149 (6.37%)	9 (5.77%)	62 (4.18%)	6 (5.56%)	87 (10.2%)	3 (6.25%)
T lymphocyte antagonists	27 (1.15%)	2 (1.28%)	20 (1.35%)	2 (1.85%)	7 (0.82%)	0 (0.00%)
B lymphocyte antagonists	42 (1.80%)	0 (0.00%)	36 (2.43%)	0 (0.00%)	6 (0.70%)	0 (0.00%)
vedolizumab	2 (0.09%)	1 (0.64%)	1 (0.07%)	1 (0.93%)	1 (0.12%)	0 (0.00%)
Synthetic DMARDs[2]	807 (34.5%)	43 (27.6%)	553 (37.3%)	30 (27.8%)	254 (29.7%)	13 (27.1%)
Methotrexate	510 (21.8%)	28 (17.9%)	348 (23.5%)	18 (16.7%)	162 (19.0%)	10 (20.8%)
Leflunomide	111 (4.75%)	5 (3.21%)	82 (5.53%)	4 (3.70%)	29 (3.40%)	1 (2.08%)
Apremilast	51 (2.18%)	1 (0.64%)	19 (1.28%)	1 (0.93%)	32 (3.75%)	0 (0.00%)
Chloroquine or hydroxychloroquine	108 (4.62%)	7 (4.49%)	99 (6.67%)	6 (5.56%)	9 (1.05%)	1 (2.08%)
JAK inhibitors	39 (1.67%)	2 (1.28%)	30 (2.02%)	2 (1.85%)	9 (1.05%)	0 (0.00%)
Sulfasalazine	9 (0.38%)	1 (0.64%)	7 (0.47%)	0 (0.00%)	2 (0.23%)	1 (2.08%)
Mycophenolate	18 (0.77%)	1 (0.64%)	16 (1.08%)	1 (0.93%)	2 (0.23%)	0 (0.00%)
Tacrolimus	22 (0.94%)	2 (1.28%)	15 (1.01%)	2 (1.85%)	7 (0.82%)	0 (0.00%)
Azathioprine	77 (3.29%)	3 (1.92%)	50 (3.37%)	2 (1.85%)	27 (3.16%)	1 (2.08%)
Cyclosporine	3 (0.13%)	0 (0.00%)	2 (0.13%)	0 (0.00%)	1 (0.12%)	0 (0.00%)
Glucocorticoids	—	—	—	—	—	—
≤10 mg/d	415 (17.8%)	26 (16.7%)	330 (22.2%)	17 (15.7%)	85 (9.95%)	9 (18.8%)
>10 mg/d	77 (3.29%)	9 (5.77%)	55 (3.71%)	7 (6.48%)	22 (2.58%)	2 (4.17%)
Anti-hypertensive drugs[3]	631 (27.0%)	53 (34.0%)	391 (26.3%)	37 (34.3%)	240 (28.1%)	16 (33.3%)
ACE inhibitors	375 (16.0%)	22 (14.1%)	221 (14.9%)	16 (14.8%)	154 (18.0%)	6 (12.5%)
ARBs	260 (11.1%)	33 (21.2%)	172 (11.6%)	22 (20.4%)	88 (10.3%)	11 (22.9%)
Chronic NSAIDs	461 (19.7%)	37 (23.7%)	320 (21.6%)	25 (23.1%)	141 (16.5%)	12 (25.0%)
COVID-19 status	—	—	—	—	—	—
SARS-CoV-2 test	—	—	—	—	—	—
Not tested	0 (0.00%)	122 (78.21%)	0 (0.00%)	87 (80.56%)	0 (0.00%)	35 (72.92%)
Positive	0 (0.00%)	34 (21.79%)	0 (0.00%)	21 (19.44%)	0 (0.00%)	13 (27.08%)
Hospitalization due to COVID-19	0 (0.00%)	26 (16.67%)	0 (0.00%)	18 (16.67%)	0 (0.00%)	8 (16.67%)
Deaths due to COVID-19	0 (0.00%)	4 (2.56%)	0 (0.00%)	2 (1.85%)	0 (0.00%)	2 (4.17%)

CV = cardiovascular. DMARDs = disease modifying anti-rheumatic drugs. JAK = Janus kinase. IL = interleukin. TNF = tumor necrosis factor. NSAIDs = non-steroid anti-inflammatory drugs. ACE = angiotensin-converting enzyme. ARBs = angiotensin II receptor blockers.

[1]Biologic DMARDs include anti-TNFα, pro-inflammatory ILs antagonists, vedolizumab and T and B lymphocyte antagonists.

[2]Synthetic DMARDs include methotrexate, JAK inhibitors, sulfasalazine, mycophenolate, tacrolimus, azathioprine, cyclosporine, chloroquine or hydroxychloroquine, leflunomide and apremilast.

[3]Anti-hypertensive drugs include ACE inhibitors and ARBs.

particularly in male rheumatoid arthritis patients, has been reported. This could be explained by the synergistic effect of testosterone and leflunomide on proinflammatory cytokine production (Cutolo et al., 2009).

In the case of pre-exposure to anti-IL-17 and anti-IL-23, we observed a reduced COVID- 19 incidence (RR = 0.2; CI 95% 0.03, 1.38; and RR = 0.8; CI 95% 0.39, 1.65, respectively). It has been reported that patients infected with SARS-CoV-2 presented elevated IL-17 serum levels (Liu et al., 2020), which are significantly correlated with disease severity (Pacha et al., 2020; Schett et al., 2020). Due to its high capacity to promote the production of a vast amount of pro-inflammatory cytokines and chemokines, some authors have described that IL−17 and, therefore, the T helper 17 (TH^{17}) response, play a role in COVID-19 hyperinflammation (Wu and Yang, 2020). Taking into account that IL-23 participates in stabilization of TH^{17} cells, our results support the idea (Liu et al., 2020) that targeting this axis could have a positive effect in controlling the cytokine storm.

However, our cohort includes limited number of patients treated with two important groups of immunomodulatory compounds, IL-6 (52 patients) and B lymphocyte antagonists (42 patients). Interestingly, none of these 94 patients showed COVID-19 symptoms, which agrees with the reported efficacy of the IL-6 antagonists tocilizumab (Xu et al., 2020) and sarilumab

TABLE 5 | Adjusted Relative Risk* (aRR) with 95% confidence intervals (CI 95%) of COVID-19 according to the presence of several comorbidities and treatments, stratified by sex.

	Model 1[A]- aRR (CI 95%)			Model 2[B]- aRR (CI 95%)			Model 3[C]- aRR (CI 95%)		
	All	Women	Men	All	Women	Men	All	Women	Men
Clinical characteristics									
Women	—	—	—	—	—	—	—	—	—
Age (years-old)	1.12 (0.8, 1.57)	—	—	1.12 (0.8, 1.56)	—	—	1.12 (0.8, 1.57)	—	—
CV Disease	1 (0.99, 1.01)	1 (0.99, 1.02)	0.99 (0.97, 1.01)	1 (0.99, 1.01)	1 (0.99, 1.02)	0.99 (0.97, 1.01)	1 (0.99, 1.01)	1.01 (0.99, 1.02)	0.99 (0.97, 1.01)
Diabetes	1.12 (0.72, 1.74)	1.25 (0.75, 2.1)	0.85 (0.37, 1.99)	1.14 (0.74, 1.76)	1.23 (0.74, 2.05)	0.95 (0.42, 2.14)	1.13 (0.73, 1.74)	1.21 (0.73, 2.02)	0.95 (0.42, 2.13)
Pulmonary disease	1.64 (1.09, 2.47)	1.74 (1.08, 2.8)	1.36 (0.62, 3)	1.61 (1.08, 2.42)	1.73 (1.08, 2.76)	1.26 (0.56, 2.82)	1.58 (1.05, 2.37)	1.67 (1.04, 2.68)	1.27 (0.56, 2.85)
Kidney disease	1.47 (1.02, 2.13)	1.5 (0.97, 2.32)	1.33 (0.66, 2.72)	1.42 (0.98, 2.05)	1.47 (0.94, 2.27)	1.28 (0.62, 2.62)	1.44 (0.99, 2.08)	1.48 (0.95, 2.3)	1.25 (0.61, 2.57)
Active cancer or treatment	1.21 (0.65, 2.25)	0.87 (0.38, 1.99)	2.07 (0.76, 5.68)	1.19 (0.64, 2.21)	0.9 (0.39, 2.06)	1.83 (0.65, 5.13)	1.2 (0.65, 2.23)	0.89 (0.4, 2.01)	1.84 (0.67, 5.06)
	1.15 (0.52, 2.58)	1.05 (0.38, 2.88)	1.43 (0.39, 5.24)	1.14 (0.51, 2.57)	1.05 (0.38, 2.88)	1.44 (0.36, 5.72)	1.13 (0.5, 2.55)	1.06 (0.39, 2.87)	1.44 (0.36, 5.75)

(unpublished observations) in COVID-19 treatment. The three families of monoclonal antibodies approved to treat rheumatoid arthritis are directed against IL-6, B lymphocyte surface protein CD20 and TNFα, three targets of potential interest for further investigation in COVID-19 treatment. IL-6, TNFα and B lymphocytes have been reported to play a crucial role in the inflammatory cascade taking place days before the manifestation of the most severe forms of SARS-CoV-2 infection (Zhou et al., 2020), as well as in the physiopathological processes leading to rheumatoid arthritis (Ceribelli et al., 2020).

In spite of the decrease incidence of COVID-19 with bDMARDs and sDMARDs treatments, those patients receiving a combination of both groups of compounds (n = 298) show enhanced incidence of COVID-19 (RR = 4.3; CI 95% 2.00, 9.25). The strong immunosuppression that should result by the combination of these treatments and the severity of the diseases targeted by these drug combinations may explain this paradoxical effect. Indeed, previous studies have reported that more patients experienced infectious adverse events when increasing doses of synthetic DMARDs were combined with anti-TNFα compounds (Burmester et al., 2015; Honkila et al., 2019). In addition, the main reason for combining both treatments is related to the lack of efficacy in these particular patients (Van Vollenhoven et al., 2012), which could also have influenced our results.

Some limitations of this study must be addressed. The indications for each treatment not only depend on the underlying pathology, but also on the specific clinical manifestations of each patient, and some of the indications are risk factors of COVID-19 (Sawalha et al., 2020). Given the heterogeneity of the studied treatments and underlying pathologies, it is difficult to analyze all the factors that could cause confounding by indication. However, RR estimates of COVID-19 diagnosis after propensity score matching with some of the covariates that predict receiving anti-TNFα were not substantially different than RR estimates in the unmatched sample (**Supplementary Table S7**). The slightly different RRs found with this treatment matching the above mention covariates suggest that some of these IMID may represent an increased risk for COVID-19. Indeed, these particular comorbidities have been reported to increase COVID-19 susceptibility and severity (Sawalha et al., 2020). Furthermore, patients receiving these immunomodulatory treatments have an enhanced propensity to bacterial infection (Chiu and Chen, 2020) that could eventually provide manifestations similar to COVID-19. In spite of this possible bias that would impair the results obtained with these treatments, we have obtained promising RRs with these compounds that suggest significant protective effects on COVID-19. Furthermore, our study was focused on the early stages of COVID-19 pandemic in Spain, and the number of confirmed SARS-CoV-2 testing in our setting was limited due to the scarcity of COVID-19 tests in Spain that, for ethical reasons, were mainly reserved to patients showing more severe disease symptoms. Therefore, clinical COVID-19 diagnosis was used as the primary outcome. Consequently, the effect of the treatment

TABLE 6 | Adjusted Relative Risk* (aRR) with 95% confidence intervals (CI 95%) of COVID-19 according to the presence of several.

	Model 1[A]- aRR (CI 95%)			Model 2[B]- aRR (CI 95%)			Model 3[C]- aRR (CI 95%)		
	All	**Women**	**Men**	**All**	**Women**	**Men**	**All**	**Women**	**Men**
Treatments followed	—	—	—	—	—	—	—	—	—
Biologic DMARDs[1]	0.46 (0.31, 0.67)	0.41 (0.24, 0.69)	0.56 (0.3, 1.03)	—	—	—	—	—	—
TNFα antagonists	—	—	—	0.50 (0.33, 0.75)	0.33 (0.17, 0.64)	0.76 (0.41, 1.43)	—	—	—
Adalimumab	—	—	—	—	—	—	0.53 (0.31, 0.92)	0.32 (0.12, 0.86)	0.81 (0.38, 1.75)
Certolizumab	—	—	—	—	—	—	0.86 (0.22, 3.34)	0.58 (0.08, 4.01)	1.68 (0.34, 8.2)
Etanercept	—	—	—	—	—	—	0.37 (0.16, 0.88)	0.13 (0.02, 0.97)	0.71 (0.27, 1.9)
Golimumab	—	—	—	—	—	—	0.46 (0.12, 1.81)	0.42 (0.06, 2.94)	0.56 (0.07, 4.28)
Infliximab	—	—	—	—	—	—	0.71 (0.31, 1.64)	0.7 (0.22, 2.23)	0.81 (0.24, 2.71)
Anti- pro-inflammatory ILs (IL6/12/17/23)	—	—	—	0.47 (0.24, 0.92)	0.57 (0.24, 1.34)	0.44 (0.15, 1.27)	—	—	—
Anti-IL17	—	—	—	—	—	—	0.2 (0.03, 1.38)	NA	0.37 (0.05, 2.56)
Anti-IL23 (12)	—	—	—	—	—	—	0.8 (0.39, 1.65)	1.19 (0.5, 2.82)	0.57 (0.16, 2)
Synthetic DMARDs[2]	0.62 (0.43, 0.91)	0.68 (0.43, 1.07)	0.59 (0.31, 1.15)	—	—	—	—	—	—
Methotrexate	—	—	—	0.71 (0.46, 1.08)	0.7 (0.42, 1.19)	0.81 (0.4, 1.68)	0.74 (0.48, 1.12)	0.74 (0.44, 1.24)	0.84 (0.41, 1.72)
Leflunomide	—	—	—	0.66 (0.28, 1.58)	0.81 (0.29, 2.27)	0.36 (0.07, 1.75)	0.66 (0.27, 1.57)	0.8 (0.28, 2.23)	0.36 (0.07, 1.79)
Chloroquine/Hydroxychloroquine	—	—	—	0.76 (0.36, 1.62)	0.75 (0.32, 1.76)	1.2 (0.21, 6.79)	0.81 (0.38, 1.71)	0.79 (0.34, 1.86)	1.27 (0.23, 7.16)
Glucocorticoids	—	—	—	—	—	—	—	—	—
≤10 mg/day	0.94 (0.61, 1.43)	0.72 (0.42, 1.22)	2.06 (1.01, 4.21)	0.87 (0.57, 1.33)	0.67 (0.4, 1.12)	2.05 (0.97, 4.3)	0.84 (0.55, 1.29)	0.65 (0.39, 1.1)	1.94 (0.93, 4.04)
>10 mg/day	1.76 (0.90, 3.45)	1.62 (0.75, 3.52)	2.20 (0.53, 9.24)	1.69 (0.87, 3.27)	1.61 (0.75, 3.43)	1.78 (0.43, 7.39)	1.7 (0.88, 3.3)	1.71 (0.8, 3.68)	1.78 (0.43, 7.34)
Anti-hypertensive[3]	1.08 (0.76, 1.52)	1.04 (0.7, 1.54)	1.11 (0.56, 2.21)	—	—	—	—	—	—
ACE inhibitors	—	—	—	0.81 (0.51, 1.28)	0.85 (0.50, 1.44)	0.73 (0.31, 1.71)	0.8 (0.51, 1.27)	0.84 (0.5, 1.43)	0.72 (0.3, 1.68)
ARBs	—	—	—	1.55 (1.03, 2.33)	1.33 (0.84, 2.13)	2.07 (0.94, 4.56)	1.59 (1.06, 2.39)	1.36 (0.85, 2.18)	2.11 (0.95, 4.66)
Chronic NSAIDs	1.22 (0.85, 1.75)	1.14 (0.74, 1.74)	1.37 (0.71, 2.67)	1.2 (0.84, 1.71)	1.12 (0.73, 1.7)	1.29 (0.67, 2.49)	1.21 (0.85, 1.72)	1.13 (0.74, 1.72)	1.31 (0.67, 2.58)

**Reference categories for clinical characteristics are individuals without that comorbidity. Reference categories for treatments are unexposed individuals.*

[A]Model 1 contains the following explanatory or exposure variables: sex, age, CV disease, pulmonary disease, kidney disease, active cancer or treatment, biologic DMARDs, synthetic DMARDs, glucocorticoids, anti-hypertensive drugs and chronic NSAIDs.

[B]Model 2 contains the following explanatory or exposure variables: sex, age, CV disease, pulmonary disease, kidney disease, active cancer or treatment, TNFα antagonists, IL-6/12/17/23 antagonists, methotrexate, leflunomide, chloroquine/hydroxychloroquine, glucocorticoids, ACE inhibitors, ARBs and chronic NSAIDs.

[C]Model 3 contains the following explanatory or exposure variables: sex, age, CV disease, pulmonary disease, kidney disease, active cancer or treatment, adalimumab, certolizumab, Etanercept, golimumab, infliximab, anti- IL17, anti-IL12/23, methotrexate, leflunomide, chloroquine/hydroxychloroquine, glucocorticoids, ACE inhibitors, ARBs and chronic NSAIDs.

CV = cardiovascular. DMARDs = disease modifying anti-rheumatic drugs. JAK = Janus kinase. IL = interleukin. TNF = tumor necrosis factor. NSAIDs = non-steroid anti-inflammatory drugs. ACE = angiotensin-converting enzyme. ARBs = angiotensin II receptor blockers. N = number of observations or exposed individuals.

[1]Biologic DMARDs include TNF antagonists, pro-inflammatory ILs antagonists, vedolizumab and T and B lymphocyte antagonists.

[2]Synthetic DMARDs include methotrexate, JAK inhibitors, sulfasalazine, mycophenolate, tacrolimus, azathioprine, cyclosporine, chloroquine or hydroxychloroquine, leflunomide and apremilast.

[3]Anti-hypertensive drugs include ACE inhibitors and ARBs.

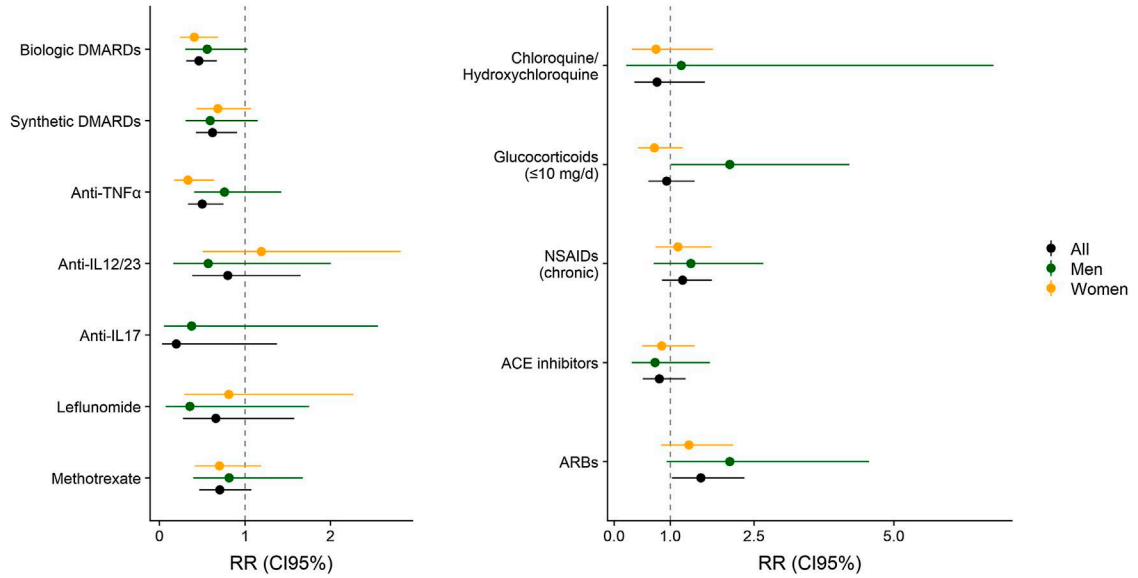

FIGURE 1 | Relative Risk (RR) with 95% Confidence Interval (CI 95%) of COVID-19 according to the exposure to different treatments, adjusted by sex, age, CV disease, diabetes, pulmonary disease, chronic kidney disease and active cancer or treatments. The aggregated effect of biologic DMARDs, syntheticDMARDs, glucocorticoids and NSAIDs are obtained from Model 1. Estimates for Anti-TNFα, Anti-IL6/12/17/24 (anti-pro-inflammatory ILs), methotrexate, ACE inhibitors, ARBs and chloroquine/hydroxychloroquine are obtained from Model 2. Model 1 and 2 are represented in **Tables 5**, **6**.

could play a role both in the risk to acquire the infection, and/or the risk of being asymptomatic. Finally, it is also important to underline that the clinical symptoms of COVID-19 were recorded from 14 days before the COVID-19 alarm was announced in Spain (March 16th) when patients could be supposed to protect themselves more if they are at risk. Therefore, this potential self-protection would not represent any important bias for the interpretation of our results considering the time schedule of our symptoms recording.

In summary, all these results suggest that bDMARDs and sDMARDs should be continued for IMIDs treatment in COVID-19 patients. The decreased incidence of COVID-19 in patients treated with anti-TNFα and anti-proinflammatory ILs compounds underline the potential interest of these medications for further studies to open novel possible therapeutic strategies to avoid serious COVID-19 manifestations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The Committee of Parc de Salut Mar approved the protocol (2020/9246); IMIM (Hospital del Mar Medical Research Institute), PRBB, c/ Dr. Aiguader, 88, 08003 Barcelona. The ethics committee waived the requirement of written informed consent for participation.

COVIDMAR STUDY GROUP MEMBERS

The Covidmar Study Group members are: Hospital del Mar, Barcelona: Selene Labrada, Miguel Mejía-Torres (Rheumatology Service) and Irene Carrión-Barberà, Carolina Pérez-García, Fabiola Ojeda, Tarek Carlos Salman-Monte, Josep Blanch-Rubió (Rheumatology Service and IMIM-Hospital del Mar Medical Research Institute) collected data and provided care for study patients; IMIM-Hospital del Mar Medical Research Institute: Luciano Polino, Laura Triginer, Anna Ribes (Cell Research on Inflammation and Cartillage Research Group, Inflammatory and Cardiovascular Processes Program) collected data; Maria-Victòria Puig (Integrative Pharmacology and Systems Neuroscience Research Group, Neurosciences Research Program and IMIM-Hospital del Mar Medical Research Institute); contributed to analysis design; Parc Sanitari Pere Virgili, Barcelona: Maria Teresa Martí Vila, Maria Luisa Perez Miras (CAP Vila Olímpica) collected data; Universitat Pompeu Fabra, Barcelona: Beltrán Álvarez-Pérez, Araceli Bergadà-Martínez, Pablo Calvé Alba Calvet-Pavón, Mireia Carcolé, Laura Domingo-Rodríguez, Alejandra Escudero-Lara, Lorena Galera-López, Jolita Jančytė, Marta Linares-López, Sara Martínez-Torres, Antonio Ortega-Álvaro, Andrés Ozaita, Sheila Piedra-Barrull, Dulce Real-Muñoz, Maria Sanchis-Ollé, Clara Seira Oriach, Miquel-Ángel Serra, Anna Vázquez-Oliver (Laboratory of Neuropharmacology,

Department of Experimental and Health Sciences and IMIM-Hospital del Mar Medical Research Institute) collected data.

AUTHOR CONTRIBUTIONS

NS-D participated in selection of statistical tests/analyses, performed the statistical analyses, computations and related computer work, and participated in writing the manuscript. LT was involved in conceptualizing the research idea, setting-up the research design, making the primary interpretation of the statistical analyses and participated in writing the manuscript. JL-O was involved in conceptualizing the research idea, setting-up the research design, making the primary interpretation of the statistical analyses and participated in writing the manuscript. EM-G contributed to the statistical analyses and revised the manuscript. PN contributed to the statistical analyses and revised the manuscript. RT contributed to the statistical analyses and revised the manuscript. AG was responsible for patient cohort data collection. RM was involved in conceptualizing the research idea, creating the research design, making the final interpretation of the statistical analysis, and writing the first draft and revision of the manuscript. JM was involved in conceptualizing the research idea, creating the research design, making the final interpretation of the statistical analysis, and writing the first draft and revision of the manuscript. Covidmar Study Group participated in collecting data and provided care for study patients.

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BRIEF RESEARCH REPORT
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Methylene Blue Inhibits the SARS-CoV-2 Spike–ACE2 Protein-Protein Interaction–a Mechanism that can Contribute to its Antiviral Activity Against COVID-19

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Due to our interest in the chemical space of organic dyes to identify potential small-molecule inhibitors (SMIs) for protein-protein interactions (PPIs), we initiated a screen of such compounds to assess their inhibitory activity against the interaction between SARS-CoV-2 spike protein and its cognate receptor ACE2, which is the first critical step initiating the viral attachment and entry of this coronavirus responsible for the ongoing COVID-19 pandemic. As part of this, we found that methylene blue, a tricyclic phenothiazine compound approved by the FDA for the treatment of methemoglobinemia and used for other medical applications (including the inactivation of viruses in blood products prior to transfusion when activated by light), inhibits this interaction. We confirmed that it does so in a concentration-dependent manner with a low micromolar half-maximal inhibitory concentration (IC_{50} = 3 μM) in our protein-based ELISA-type setup, while chloroquine, siramesine, and suramin showed no inhibitory activity in this assay. Erythrosine B, which we have shown before to be a promiscuous SMI of PPIs, also inhibited this interaction. Methylene blue inhibited the entry of a SARS-CoV-2 spike bearing pseudovirus into ACE2-expressing cells with similar IC_{50} (3.5 μM). Hence, this PPI inhibitory activity could contribute to its antiviral activity against SARS-CoV-2 even in the absence of light by blocking its attachment to ACE2-expressing cells and making this inexpensive and widely available drug potentially useful in the prevention and treatment of COVID-19 as an oral or inhaled medication.

Keywords: ACE2, antiviral, chloroquine, COVID-19, methylene blue, protein-protein interaction, SARS-CoV-2, spike protein

Abbreviations: ACE2, angiotensin converting enzyme 2; CoV, coronavirus; MeBlu, methylene blue; PPI, protein-protein interaction; SARS, severe acute respiratory syndrome; SMI, small-molecule inhibitor.

INTRODUCTION

Severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2), a novel betacoronavirus and the most recent one of the seven coronaviruses (CoVs) known to infect humans, is responsible for COVID-19, which has been declared a pandemic by the World Health Organization in March 2020 and continues to spread worldwide (Liu et al., 2020; Matheson and Lehner, 2020; Moore and June, 2020). While four CoVs (HCoV 229E, OC43, NL63, and HKU1) are responsible for about one third of the common cold cases in humans, three have caused recent epidemics associated with considerable mortality: SARS-CoV-1 (2002–2003, causing ~10% mortality), MERS-CoV (Middle East respiratory syndrome coronavirus; 2012, causing ~35% mortality), and now SARS-CoV-2 (2019–2020), which seems to be less lethal but more transmissible (Guy et al., 2020). SARS-CoV-2 is the most infectious agent in a century (Tiwari et al., 2020) and has already caused infections in the order of tens of millions and deaths that are likely to be in the order of millions worldwide. According to current early estimates, about 3% of infected individuals need hospitalization and 0.5% die, a range that is strongly age-dependent increasing from 0.001% in <20 years old to 8.3% in those >80 years old (Salje et al., 2020). Accordingly, there is considerable interest in possible preventive or therapeutic treatments. There are several possible targets in the coronavirus life cycle for therapeutic interventions including attachment and entry, uncoating, gRNA replication, translation in endoplasmic reticulum (ER) and Golgi, assembly, and virion release (Guy et al., 2020). Viral attachment and entry are particularly promising as they are the first steps in the replication cycle and take place at a relatively accessible extracellular site; hence, they have been explored for intervention purposes for several viruses (Melby and Westby, 2009). CoVs use their glycosylated spike (S) protein to bind to their cognate cell surface receptors and initiate membrane fusion and virus entry. For both SARS-CoV and SARS-CoV-2, the S protein mediates entry into cells by binding to angiotensin converting enzyme 2 (ACE2) via its receptor-binding domain (RBD) followed by proteolytic activation by human proteases (Lan et al., 2020; Matheson and Lehner, 2020; Shang et al., 2020; Sivaraman et al., 2020). Blockade of this RBD–ACE2 protein-protein interaction (PPI) can disrupt infection efficiency; for example, SARS-CoV-2 RBD protein was shown to block S protein mediated SARS-CoV-2 pseudovirus entry into ACE2 receptor-expressing target cells (Tai et al., 2020). Antibodies can be quite effective PPI inhibitors, and they are highly target-specific and relatively stable *in vivo*. However, they cannot reach intracellular targets and, as all other protein therapies, are hindered by problems such as low solubility, propensity for immunogenicity, long elimination half-lives, lack of oral bioavailability, product heterogeneity, and possible manufacturing and storage stability issues. Since they are foreign proteins, they elicit strong immune response in certain patients (Suntharalingam et al., 2006; Wadman, 2006; Leader et al., 2008), and even if approved for clinical use, they tend to have more post-market safety issues than small-molecule drugs (Downing et al., 2017). Small-molecule inhibitors (SMIs) are more challenging to identify for PPIs, but it is now well established that they can be effective against certain PPIs and can offer useful alternatives. There are now >40 PPIs targeted by SMIs that are in preclinical development, and two such SMIs are approved for clinical use (venetoclax and lifitegrast) (Arkin and Wells, 2004; Milroy et al., 2014; Scott et al., 2016; Bojadzic and Buchwald, 2018).

Due to our interest in the chemical space of organic dyes to identify potential SMIs for PPIs (Margolles-Clark et al., 2009a; Margolles-Clark et al., 2009b; Ganesan et al., 2011; Song et al., 2014; Chen et al., 2017; Bojadzic and Buchwald, 2018; Bojadzic et al., 2018), we initiated a screen of such compounds for their ability to inhibit the interaction between SARS-CoV-2 spike protein and its cognate receptor ACE2, which is the first critical step initiating the viral attachment and entry of this CoV. As part of this, we found that methylene blue, a tricyclic phenothiazine compound approved for the treatment of acquired methemoglobinemia and some other uses (Clifton and Leikin, 2003; Schirmer et al., 2011; Bistas and Sanghavi, 2020), inhibits this interaction, and we have confirmed that it does so in a concentration-dependent manner. This can contribute to the antiviral activity of this inexpensive and widely available dye-based drug against SARS-CoV-2 making it potentially useful in the prevention and treatment of COVID-19, especially in non-industrialized nations.

MATERIALS AND METHODS

Binding Assays

Methylene blue and other test compounds used here were obtained from Sigma-Aldrich (St. Louis, MO, United States) and used as such. Purities (and catalog numbers) were as follows: methylene blue >95% (M4159), chloroquine >98.5% (C6628), erythrosine B 90% (198269), siramesine >98% (SML0976), sunset yellow FCF 90% (465224), and trypan blue 60% (302643). Suramin (>99%; cat. no. 1472) was from Tocris Bioscience (Biotechne, Minneapolis, MN, United States). ACE2-Fc and SARS-CoV-2 S1 or RBD with His tag proteins used in the binding assays were obtained from Sino Biological (Wayne, PA, United States); catalog no. 10108-H05H, 40591-V08H, and 40592-V08H). Binding inhibition assays were performed in a 96-well cell-free format similar to the one described before (Margolles-Clark et al., 2009b; Ganesan et al., 2011; Song et al., 2014; Chen et al., 2017). Briefly, microtiter plates (Nunc F Maxisorp, 96-well; Thermo Fisher Scientific, Waltham, MA, United States) were coated overnight at 4°C with 100 µL/well of Fc-conjugated ACE2 receptor diluted in PBS pH 7.2. This was followed by blocking with 200 µL/well of SuperBlock (PBS) (Thermo Fisher Scientific) for 1 h at room temperature. Then, plates were washed twice using washing solution (PBS pH 7.4, 0.05% Tween-20) and tapped dry before the addition of the tagged ligand (SARS-CoV-2 S1 or RBD) and test compounds diluted in binding buffer (100 mM HEPES, pH 7.2) to give a total

volume of 100 µL/well. After 1 h incubation, three washes were conducted, and a further 1 h incubation with anti-His HRP conjugate (BioLegend; San Diego, CA, Unites States; catalog no. 652504) diluted (1:2,500) in SuperBlock (PBS) was used to detect the bound His-tagged ligand. Plates were washed four times before the addition of 100 µL/well of HRP substrate TMB (3,3′,5,5′-tetramethylbenzidine) and kept in the dark for up to 15 min. The reaction was stopped using 20 µL of 1 M H_2SO_4, and the absorbance value was read at 450 nm. The plated concentrations of ACE2 receptor were 1.0 µg/ml for SARS-CoV-2 RBD and 2.0 µg/ml for SARS-CoV-2 S1. The concentrations of the ligand used in the inhibitory assays were 0.5 µg/ml for RBD and 1.0 µg/ml for S1. These values were selected following preliminary testing to optimize response (i.e., to produce a high-enough signal at conditions close to half-maximal response, EC_{50}). Binding assessments for CD40–CD40L and TNF-R1–TNF-α were performed as previously described (Bojadzic et al., 2018). Stock solutions of compounds at 10 mM in DMSO were used.

SARS-CoV-2 Pseudovirus Assay

Assay from Montana Molecular (Bozeman, MT, United States; catalog no. C1100R and C1100G) was used per the instructions of the manufacturer with minor modifications. Briefly, HEK293T cells (ATCC, Manassas, VA, United States; catalog no. CRL-1573) were seeded onto 96-well plates at a density of 5×10^4 cells per well in 100 µL complete medium (DMEM supplemented with 10% fetal bovine serum). A transduction mixture containing ACE2 BacMam Red-Reporter virus (1.8×10^8 VG/ml) and 2 mM sodium butyrate prepared in complete medium was added (50 µL per well) and incubated for 24 h at 37°C and 5% CO_2. Medium was removed, washed once with PBS, and replaced with 100 µL fresh medium containing methylene blue at selected concentrations, pre-incubating for 30 min at 37°C and 5% CO_2. A transduction mixture containing Pseudo SARS-CoV-2 Green-Reporter pseudovirus (3.3×10^8 VG/ml) and 2 mM sodium butyrate prepared in complete medium was added (50 µL per well) and incubated for 48 h at 37°C and 5% CO_2. Medium was removed, washed once with PBS, replaced with 150 µL fresh medium, and cells incubated for additional 48 h at 37°C and 5% CO_2. Cell fluorescence was detected using an EVOS FL microscope (Life Technologies, Carlsbad, CA, United States) and was quantified in ImageJ (United States National Institutes of Health, Bethesda, MD, United States) (Schneider et al., 2012) using the Analyze Particles tool after thresholding for the corresponding colors.

Statistics and Data Fitting

All binding inhibition and cell assays were tested in at least duplicate per plates, and assays were performed as at least two independent experiments. As before (Ganesan et al., 2011; Song et al., 2014; Chen et al., 2017), binding data were converted to percent inhibition and fitted with standard log inhibitor vs. normalized response models (Buchwald, 2020) using nonlinear regression in GraphPad Prism (GraphPad, La Jolla, CA, United States) to establish half-maximal (median) effective or inhibitory concentrations (EC_{50}, IC_{50}).

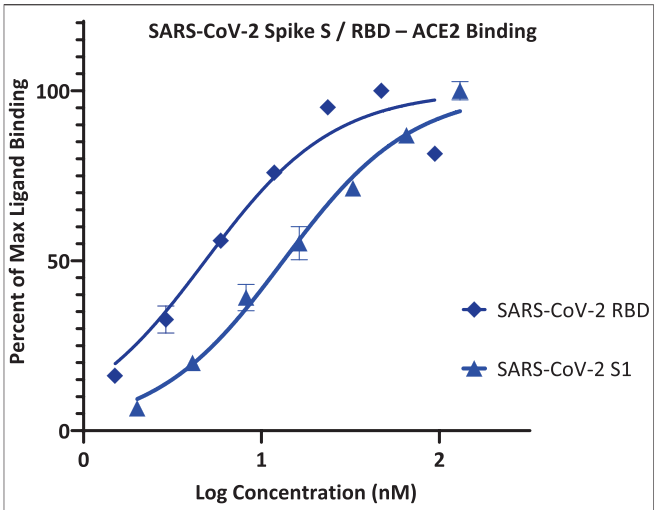

FIGURE 1 | Concentration-response curves for binding of SARS-CoV-2 spike protein S1 and RBD to ACE2 in our ELISA-based assay format. Data obtained with Fc-conjugated ACE2 coated on the plate and His-tagged S1 or RBD added in increasing amounts as shown with the amount bound detected using an anti-His–HRP conjugate (mean ± SD for two experiments in duplicates).

RESULTS

As part of our work to identify SMIs for co-signaling PPIs that are essential for the activation and control of immune cells, we discovered that the chemical space of organic dyes, which is particularly rich in strong protein binders, can offer a useful starting point. Accordingly, it seemed logical to explore it for possible inhibitors of the SARS-CoV-2 S protein–ACE2 PPI that is an essential first step for the viral entry of this novel, highly infectious coronavirus. As a first step, we explored the feasibility of setting up a screening assays using a cell-free ELISA-type 96-well format similar to those used in our previous works with Fc-conjugated receptors coated on the plate and FLAG- or His-tagged ligands in the solution (Margolles-Clark et al., 2009b; Ganesan et al., 2011; Song et al., 2014; Chen et al., 2017). To establish assay conditions, we first performed concentration-response assessments using such a format with ACE2-Fc and SARS-CoV-2 S1 or RBD with His tag, and they indicated that both bindings follow classic sigmoid patterns with a slightly stronger binding for RBD than S1 (**Figure 1**). Fitting of data gave median effective concentrations (EC_{50}s) and hence binding affinity constant (K_d) estimates of 5 and 13 nM, respectively (127 and 1,008 ng/ml)–in good agreement with the specifications of the manufacturer and published values that are also in the low nanomolar range (4–90 nM), typically based on surface plasmon resonance (SPR) studies (Sivaraman et al., 2020).

Accordingly, we can use this format for inhibitory screening, and we decided to use hACE2 with SARS-CoV-2 RBD-His, as it showed stronger binding. In fact, this assay setup is very similar to one recently shown to work as a specific and sensitive SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of this same PPI (CoV-S–ACE2) (Tan et al., 2020). With

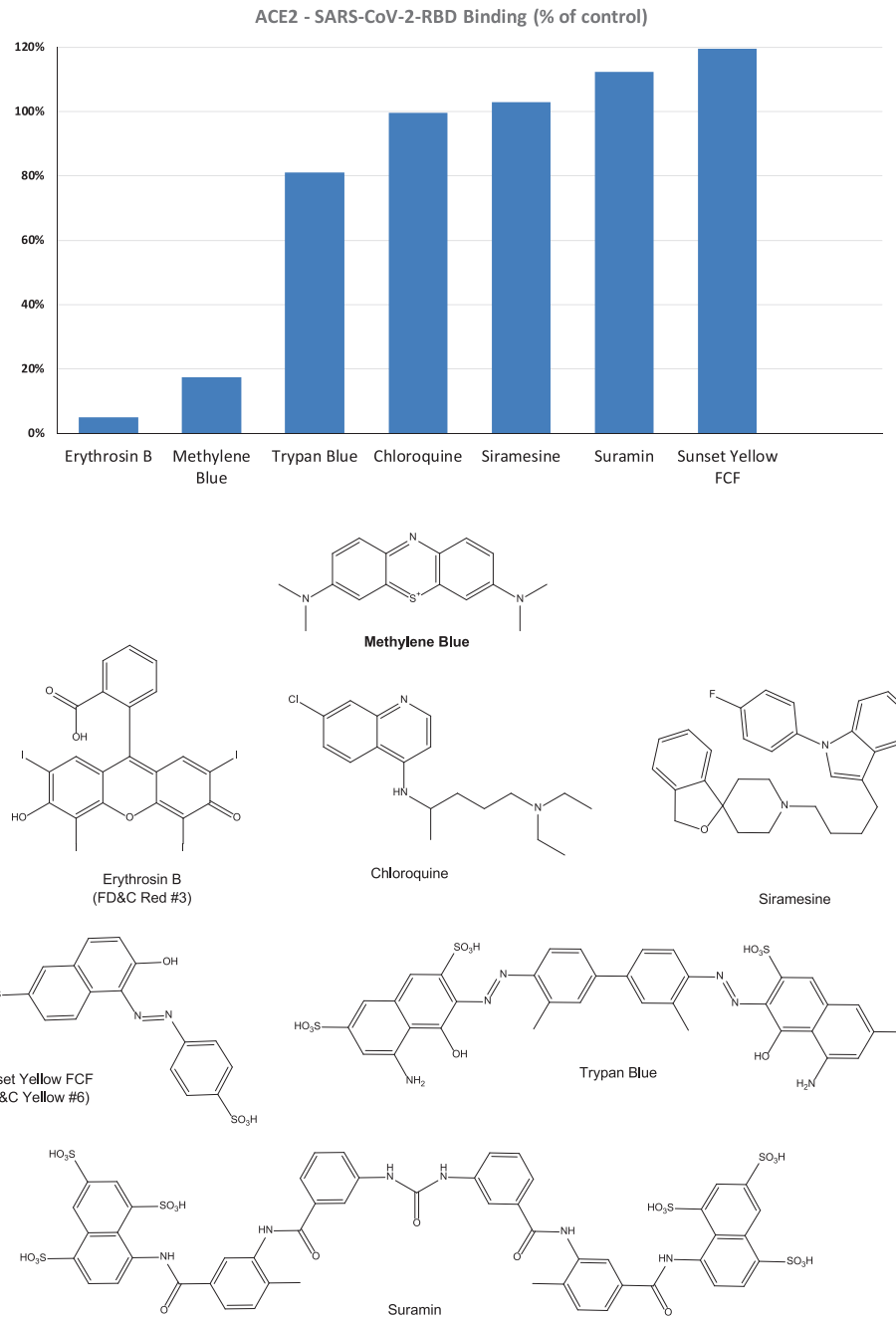

FIGURE 2 | Inhibitory effect of selected compounds on SARS-CoV-2 RBD binding to hACE2 in our screening assay. Percent inhibition values obtained at 5 μM concentration shown normalized to control (100%). Erythrosine B, a known promiscuous SMI of PPIs (Ganesan et al., 2011) and sunset yellow FCF (FD and C yellow no. 6), a food colorant likely to be inactive, were included as positive and negative controls, respectively. Chemical structures are shown for comparison purposes.

this setup in our hands, we performed a preliminary screening of representative organic dyes from our in-house library plus a few compounds that are or have been considered of possible interest in inhibiting SAR-CoV-2 by different mechanisms of action, e.g., chloroquine, siramesine, and suramin (Colson et al., 2020; Gordon et al., 2020; McKee et al., 2020; Salgado-Benvindo et al., 2020; Xiu et al., 2020). Screening at 5 μM indicated that most have no activity and, hence, are unlikely to interfere with the S-protein–ACE2 binding needed for viral attachment. Nevertheless, some showed activity; those of selected compounds of interest are shown in **Figure 2** together with corresponding chemical structures. Erythrosine B (ErB, FD and C red no.3), an FDA approved food colorant, was included as a possible positive control since

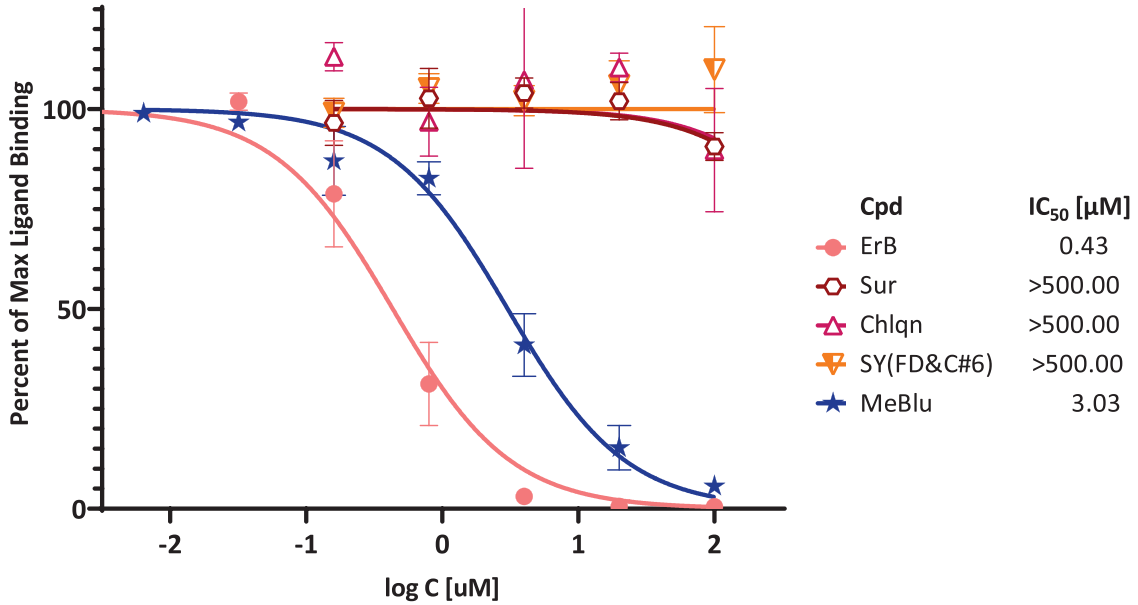

FIGURE 3 | Concentration-dependent inhibition of SARS-CoV-2 RBD binding to ACE2 by selected compounds. Concentration-response curves obtained in ELISA-type assay with Fc-conjugated ACE2 coated on the plate (1 µg/ml) and His-tagged RBD (0.5 µg/ml) added and amount bound in the presence of increasing concentrations of test compounds detected. As before, erythrosine B (ErB) and sunset yellow FCF (SY(FD&C#6)) were included as positive and negative controls, respectively. Data (mean ± SD for two experiments in duplicates) were normalized and fitted with standard inhibition curves; obtained IC_{50} values are shown at right.

we have shown it previously to be a promiscuous PPI inhibitor together with other xanthene dyes (Ganesan et al., 2011), and it indeed showed strong inhibition here. Of particular interest, methylene blue (MeBlu), which has a long history of diverse medical applications (Clifton and Leikin, 2003; Schirmer et al., 2011; Bistas and Sanghavi, 2020), also showed promising inhibitory activity.

Therefore, to confirm its activity, we performed detailed concentration-response assessments as recommended per experimental guidelines in pharmacology and experimental biology (Curtis et al., 2018; Michel et al., 2020). As shown in **Figure 3**, this confirmed that MeBlu indeed inhibited in concentration-dependent manner with an estimated IC_{50} of 3.0 µM (95% CI: 2.5–3.6 µM), whereas chloroquine and suramin showed no inhibitory activity in this assay. Chloroquine, an anti-parasitic and immunosuppressive drug primarily used to prevent and treat malaria, was included as it has potential antiviral activity against SARS-CoV-2 (subject to controversies) (Colson et al., 2020). Suramin, an antiparasitic drug approved for the prophylactic treatment of African sleeping sickness (trypanosomiasis) and river blindness (onchocerciasis), was incorporated because it was claimed to inhibit SARS-CoV-2 infection in cell culture most likely by preventing binding or entry of the virus (Salgado-Benvindo et al., 2020) (as well as because we found it earlier to inhibit the CD40–CD40L PPI (Margolles-Clark et al., 2009a)). ErB also inhibited with an IC_{50} of 0.4 µM, which is consistent with our previous observation of promiscuous PPI inhibition by this compound with a possibly slightly higher activity than found for other PPIs tested before (1–20 µM) (Ganesan et al., 2011). Sunset yellow FCF (FD and C yellow no. 6), an azo dye and an FDA approved food colorant included as a possible negative control, indeed showed no inhibitory activity.

Next, we were also able to show that MeBlu inhibits the entry of a pseudovirus bearing the SARS-CoV-2 S spike protein into ACE2-expressing HEK293T cells. This BacMam-based pseudovirus assay allows the quantification of viral entry as the pseudovirus expresses bright green fluorescent protein that is targeted to the nucleus of ACE2 and red fluorescence reporter expressing host cells, while it can be handled using biosafety level 1 containment because they do not replicate in human cells. Pseudovirus entry is indicated by expression of green fluorescence in the nucleus; if the entry is blocked, the cell nucleus remains dark. MeBlu showed clear concentration-dependent inhibition with an estimated IC_{50} of 3.5 µM (95% CI: 1.6–7.4 µM) (**Figure 4**). In this assay involving 48 h exposure, MeBlu showed signs of cytotoxicity at higher concentrations (45 µM) affecting viability even if not affecting overall ACE2-expression (red bars, **Figure 4**). Since the IC_{50} obtained for MeBlu here (3 µM) is within the range of its circulating levels following normal clinical dosage (e.g., peak blood concentration of 19 µM after 500 mg p.o. with an elimination half-life of ~14 h (Walter-Sack et al., 2009) or trough levels of 6–7 µM in healthy human volunteers following oral doses of 207 mg/day (69 mg, t.i.d.) (Baddeley et al., 2015), this inhibitory effect on viral attachment can contribute to the possible antiviral activity of MeBlu against SARS-CoV-2 and possibly other ACE2-binding CoVs such as SARS-CoV and the α-coronavirus HCoV NL63.

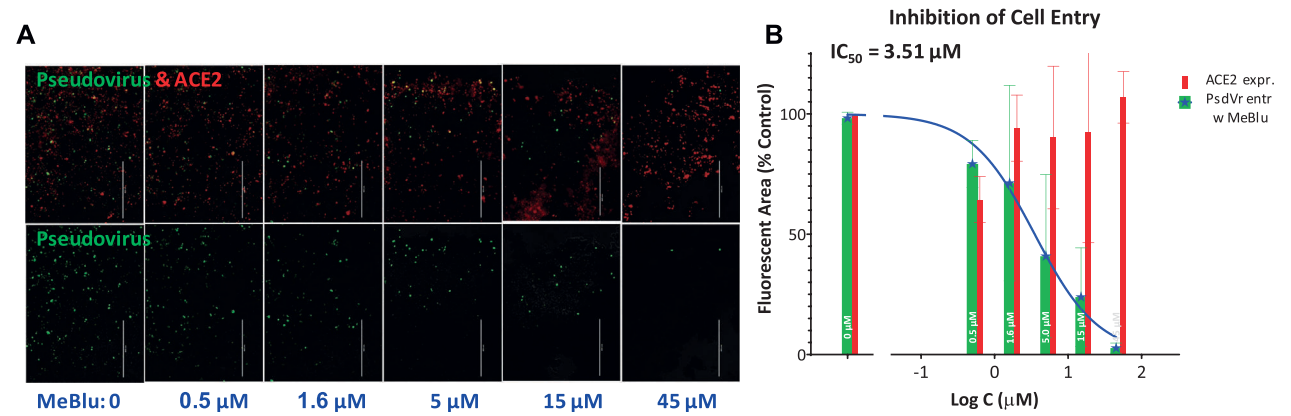

FIGURE 4 | Concentration-dependent inhibition of SARS-CoV-2 pseudovirus entry into ACE2-expressing cells by methylene blue. **(A)** Images from a serial dilution experiment showing that MeBlu inhibits the entry of a BacMam-based pseudovirus bearing the SARS-CoV-2 S protein (plus green fluorescent protein reporters) in ACE2 (plus red fluorescence) expressing host cells (HEK293T). The amount of red present **(top row)** is proportional with the number of ACE2-expressing cells; the amount of green **(bottom row)** is proportional with the number of infected cells, as green fluorescence is expressed only in pseudovirus infected cells. **(B)** Corresponding quantification for pseudovirus (green) and ACE2 expression (red) shown on semilogarithmic scale and fitted with a classic sigmoidal curve indicating an IC_{50} of 3.5 µM (average data from two independent experiments).

DISCUSSION

Results here confirm again the usefulness of our strategy to rely on the chemical space of organic dyes, known to contain strong protein binders, as a starting platform to identify SMI scaffolds for PPI inhibition. Using this strategy, we have achieved considerable progress in targeting co-signaling interactions as we have identified the first SMIs for CD40–CD40L (Margolles-Clark et al., 2009b) and OX40–OX40L PPIs (Song et al., 2014) as well as the first promiscuous SMIs of PPIs (Ganesan et al., 2011). Organic dyes contain privileged structures for protein binding (Che et al., 2006; Fletcher and Hamilton, 2006; Hershberger et al., 2007), and, contrary to usual drug-like libraries, whose chemical space does not correspond well with that of promising PPI inhibitors (Neugebauer et al., 2007; Reynès et al., 2010; Sperandio et al., 2010), they are a good starting point to identify SMIs of PPIs. Most dyes, however, are unsuitable for therapeutic development because of their strong color and, in the case of azo dyes, their quick metabolic degradation (Levine, 1991; Feng et al., 2012); hence further medicinal chemistry is needed to optimize their clinical potential (Chen et al., 2017).

More importantly, our results indicate that MeBlu, an organic dye in clinical use for some therapeutic applications in the developed world (Clifton and Leikin, 2003; Schirmer et al., 2011; Bistas and Sanghavi, 2020) and with additional potential for certain developing world applications such as malaria (Dicko et al., 2018), can inhibit the viral attachment and entry of SARS-CoV-2 by blocking the PPI of its spike protein with ACE2 on the host cell. MeBlu is a tricyclic phenothiazine dye approved by the FDA for clinical use for the treatment of methemoglobinemia, and it is also used for other applications such as prevention of urinary tract infections in elderly patients; ifosfamid-induced neurotoxicity in cancer patients; vasoplegic syndrome, a type of distributive shock that occurs during coronary procedures; and intraoperative visualization of nerves, nerve tissues, and endocrine glands (Schirmer et al., 2011; Bistas and Sanghavi, 2020). MeBlu is included in the WHO List of Essential Medicines and was, in fact, the very first fully synthetic drug used in medicine, as it was used to treat malaria since 1891 (Schirmer et al., 2011). This utilization spanned through WW2 until it was replaced by chloroquine; although, due to the blue urine it could cause, MeBlu was not well liked among the soldiers ("Even at the loo we see, we pee, navy blue") (Schirmer et al., 2011). It also served as the lead compound for the development of chlorpromazine and tricyclic antidepressants (Schirmer et al., 2011). Moreover, there is resurgent interest in its antimalarial application (Dicko et al., 2018), and it has potential for the treatment of neurodegenerative disorders such as Alzheimer's disease (AD), due to its putative inhibitory action on the aggregation of tau protein (Schirmer et al., 2011). Notably, MeBlu was also part of the first method developed for pathogen inactivation in plasma, where it has been used since 1991 to inactivate viruses in combination with light (Lozano et al., 2013). MeBlu intercalates within nucleic acid strands, and application of light causes its excitation generating highly reactive singlet oxygen that oxidizes guanosine and breaks nucleic strands (Lozano et al., 2013). Hence, in the presence of light, MeBlu has broad-spectrum virucidal activity and is used to inactivate viruses in blood products prior to transfusions.

Notably, there is also recent evidence of possible *in vitro* antiviral activity for MeBlu even in the absence of UV-induced activation. For example, one group found that MeBlu showed virucidal activity at low micromolar concentrations when incubated with Vero E6 cells and SARS-CoV-2 for 20 h in the dark (Cagno et al., 2020). Another group also found non-photoactivated MeBlu to inhibit SARS-CoV-2 replication in Vero E6 *in vitro* with an IC_{50} of 0.3 ± 0.03 µM at multiplicity of infection (MOI) of 0.25 (Gendrot et al., 2020). The ability of MeBlu to inhibit the SARS-CoV-2-S–ACE2 PPI could be a mechanism of action contributing to such activity especially as

we also showed MeBlu to inhibit the entry of SARS-CoV-2 pseudovirus into ACE2-expressing cells with low micromolar IC_{50} (**Figure 4**). If this PPI inhibitory activity of MeBlu is retained at similar levels *in vivo* as found here ($IC_{50} \approx 3$ μM), it is within a range that can be obtained in blood following typical doses (e.g., 200 mg/day) as indicated by pharmacokinetic studies in humans. For example, in one study, peak blood concentration of MeBlu was 19 μM after 500 mg p.o., and the elimination half-life was also more than adequate being around 14 h (Walter-Sack et al., 2009). In another study, trough levels of 6–7 μM were obtained following total daily oral doses of 207 mg/day (administered as 69 mg, p.o., t.i.d.) (Baddeley et al., 2015). Hence, oral administration could provide adequate concentrations (e.g., >7 μM) and inhaled applications, which have been explored in less developed countries for some respiratory treatments (Golwalkar, 2020), could be even more advantageous. MeBlu is generally safe, but it shows dose-dependent toxicity with nausea, vomiting, hemolysis, and other undesired side effects starting to occur at doses >7 mg/kg (i.e., >500 mg) (Clifton and Leikin, 2003; Bistas and Sanghavi, 2020). It also is contraindicated in certain populations, e.g., in those taking serotonin reuptake inhibitors and in persons with hereditary glucose-6-phosphate dehydrogenase deficiency (G6PD deficiency) (Schirmer et al., 2011; Bistas and Sanghavi, 2020).

It has to be noted, however, that MeBlu also inhibited the CD40–CD40L and TNF-R1–TNFα PPIs in our assays with low-to mid-micromolar potency (data not shown); hence, it is possible that MeBlu is a somewhat promiscuous PPI inhibitor limiting its usefulness. Its three-ring phenothiazine framework resembles somewhat the three-ring xanthene framework of erythrosine B (**Figure 2**), which we have shown before to act as promiscuous PPI inhibitor together with some other structural analog xanthene dyes such as rose Bengal and phloxine (Ganesan et al., 2011). MeBlu certainly shows polypharmacology and acts on a multitude of targets (Schirmer et al., 2011); many of these however can have further beneficial effects in COVID-19 patients (Scigliano and Scigliano, 2020). Its main mechanism of action is reducing the oxidized ferric form of hemoglobin (Fe^{3+}) when in a state of methemoglobinemia, which binds oxygen irreversibly, to the ferrous (Fe^{2+}) form (Bistas and Sanghavi, 2020). This increases the oxygen-binding capacity of hemoglobin and, thus, oxygen delivery to tissues–an important benefit for COVID-19 patients. COVID-19 patients often exhibiting low oxygen levels, typically incompatible with life without dyspnea–a phenomenon termed silent hypoxemia (or happy hypoxia in public media) (Tobin et al., 2020). Possibly relevant to this, MeBlu was found to improve hypoxemia and hyperdynamic circulation in patients with liver cirrhosis and severe hepatopulmonary syndrome (Schenk et al., 2000). MeBlu is being used for the treatment of pneumonia and other respiratory ailments in less developed countries with some success (Golwalkar, 2020).

Further, MeBlu was recently shown to block the PD-1–SHP2 PPI, which is downstream from the PD-1–PD-L1 co-signaling PPI, with low micromolar potency and effectively enough to counteract the suppressive activity of PD-1 on cytotoxic T lymphocytes and restore their cytotoxicity, activation, proliferation, and cytokine-secreting activity (Fan et al., 2020). This mechanism of action targeting this co-signaling pathway (PD-1) could contribute to restoring T cell homeostasis and function from exhausted state (Barber et al., 2006; Vardhana and Wolchok, 2020), which is of interest to improve viral clearance and rein-in the inflammatory immune response and the associated cytokine storm during anti-viral responses such as those causing the high mortality of COVID-19 patients (Di Cosimo et al., 2020; Liu et al., 2020; Ye et al., 2020).

As far as clinical applications, one promising indication comes from a report of a cohort of 2,500 French patients treated with MeBlu as part of their cancer care none of whom developed influenza like illness during the COVID-19 epidemics (Henry et al., 2020). MeBlu has also been explored in one Phase one clinical trial (NCT04370288) for treatment of critically ill COVID-19 patients in Iran as part of a three-drug last therapeutic option add-on cocktail (MeBlu 1 mg/kg, vitamin C 1500 mg/kg, and N-acetyl cysteine 2000 mg/kg) based on the hypothesis that this combination could rebalance NO, methemoglobin, and oxidative stress. Four of the five patients responded well to treatment (Alamdari et al., 2020).

In conclusion, screening of our organic dye-based library identified MeBlu as a low-micromolar inhibitor of the interaction between SARS-CoV-2 spike protein and its cognate receptor ACE2, a PPI that is the first critical step initiating the viral entry of this coronavirus. While MeBlu shows strong polypharmacology and might be a somewhat promiscuous PPI inhibitor, its ability to inhibit this PPI could contribute to the antiviral activity of MeBlu against SARS-CoV-2 even in the absence of light making this inexpensive and widely available drug potentially useful in the prevention and treatment of COVID-19 as an oral or inhaled medication.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

DB performed most of the experiments, OA performed the pseudovirus assay. PB originated and designed the project, provided study guidance, and wrote the draft manuscript. All authors contributed to writing and read the final manuscript.

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3-Hydroxyphthalic Anhydride-Modified Chicken Ovalbumin as a Potential Candidate Inhibits SARS-CoV-2 Infection by Disrupting the Interaction of Spike Protein With Host ACE2 Receptor

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The global spread of the novel coronavirus SARS-CoV-2 urgently requires discovery of effective therapeutics for the treatment of COVID-19. The spike (S) protein of SARS-CoV-2 plays a key role in receptor recognition, virus-cell membrane fusion and virus entry. Our previous studies have reported that 3-hydroxyphthalic anhydride-modified chicken ovalbumin (HP-OVA) serves as a viral entry inhibitor to prevent several kinds of virus infection. Here, our results reveal that HP-OVA can effectively inhibit SARS-CoV-2 replication and S protein-mediated cell-cell fusion in a dose-dependent manner without obvious cytopathic effects. Further analysis suggests that HP-OVA can bind to both the S protein of SARS-CoV-2 and host angiotensin-converting enzyme 2 (ACE2), the functional receptor of SARS-CoV-2, and disrupt the S protein-ACE2 interaction, thereby exhibiting inhibitory activity against SARS-CoV-2 infection. In summary, our findings suggest that HP-OVA can serve as a potential therapeutic agent for the treatment of deadly COVID-19.

Keywords: SARS-CoV-2, 3-hydroxyphthalic anhydride-modified chicken ovalbumin, spike, fusion inhibitor, angiotensin-converting enzyme 2

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INTRODUCTION

Novel coronavirus disease 2019 (COVID-19), a respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), continues to spread worldwide (Kuan et al., 2016; Sharma et al., 2020; Zhou et al., 2020). The World Health Organization (WHO) has characterized the epidemic situation of SARS-CoV-2 as a "Public Health Emergency of International Concern" (Song and Karako, 2020; Wang et al., 2020), which has aroused widespread concern in the world and has brought significant threats to international health and social stability, thus calling for the development of highly effective therapeutics and prophylactics (Kampf et al., 2020; Wu et al., 2020). SARS-CoV-2 is an enveloped positive-sense, single-stranded RNA virus and belongs to the

β-coronavirus genus, which shares high genetic sequence identity with severe acute respiratory syndrome coronavirus (SARS-CoV) and bat SARS-like coronavirus (SL-CoV) (Tian et al., 2020). Notably, SARS-CoV-2 has lower pathogenicity and higher transmissibility than SARS-CoV, which may explain the severity of the epidemic (Lai and Cavanagh, 1997; Peiris et al., 2004; Li et al., 2020).

Similar to other two coronavirus strains, including SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), cell entry of SARS-CoV-2 is the first step of cross-species transmission. SARS-CoV-2 contains four important structural proteins: the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The S, E, and M proteins promote virus assembly and entry into host cells, and the N protein is needed for RNA synthesis (Li, 2016; Schoeman and Fielding, 2019; Ortiz-Prado et al., 2020). The S protein on the surface of SARS-CoV-2 cells is composed of a receptor-binding unit S1 and a membrane-fusion unit S2 (Rota et al., 2003; Walls et al., 2020). First, S1 can bind to the cellular surface receptor angiotensin-converting enzyme 2 (ACE2) through its receptor-binding domain (RBD) to initiate infection (Hoffmann et al., 2020). Second, S2 helps viral genomes enter host cells by fusing the host cell and viral membranes. The interactions between the S protein and the ACE2 receptor play an important role in viral entry into host cells (Wu et al., 2012; Huo et al., 2020a; Tai et al., 2020). Therefore, it might be a potential approach to screen special antibodies or small-molecule inhibitors for blocking the RBD and ACE2 interaction and preventing virus infection (Chu et al., 2008; Du et al., 2009; Huo et al., 2020b).

Many molecules targeting the S protein have been found to be effective *in vitro*. The fusion inhibitors EK1C4 (Xia et al., 2020), IPB02 (Zhu et al., 2020) and nelfinavir mesylate (Viracept) (Musarrat et al., 2020) potently inhibit SARS-CoV-2 S protein-mediated cell-cell fusion and pseudovirus infection. SARS-CoV and SARS-CoV-2 cellular entry can be blocked by the protease inhibitor camostat mesylate and the cathepsin L inhibitor E-64d (Hoffmann et al., 2020). Apilimod, a potent inhibitor of phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), can significantly reduce the entry of SARS-CoV-2 S pseudovirus into 293/hACE2 cells *via* early endosomes in a dose-dependent manner (Hoffmann et al., 2020; Kang et al., 2020). Several SARS-CoV-specific neutralizing antibodies such as CR3022, m396 and S309 have been further demonstrated to interact with SARS-CoV-2 S protein. However, only S309, rather than CR3022 and m396, showed potent cross-neutralizing activity on SARS-CoV-2, indicating that subtle difference in the RBD of SARS-CoV-2 and SARS-CoV may limit the cross-reactivity of SARS-CoV-specific neutralizing antibodies with SARS-CoV-2 (Hussain et al., 2020; Tian et al., 2020). Until now, there are still some disadvantages to these antiviral agents. They generally produce toxic responses, have a short half-life and cause acute side effects. Therefore, these weaknesses might affect their clinical use, and there is an urgent need to find new and effective therapeutics for the treatment of COVID-19.

Our previous studies have reported that several kinds of viruses, including human immunodeficiency virus (HIV), human papillomavirus (HPV), respiratory syncytial virus (RSV), and novel human coronavirus MERS-CoV, can be inhibited at the viral entry step by anhydride-modified proteins (Li et al., 2010a; Zhao et al., 2013; Hua et al., 2019). Furthermore, one kind of anhydride-modified bovine protein, β-lactoglobulin (β-LG), was clinically applied to treat HPV infection (Hua et al., 2019). Therefore, we decided to investigate whether anhydride-modified proteins could be utilized as anti-SARS-CoV-2 antivirals. In particular, 3-hydroxyphthalic anhydride-modified OVA is convenient for anhydride modification, which is isolated from chicken eggs and less expensive than rabbit serum albumin (RSA), which is purified from animal sera. Luckily, due to the broad-spectrum antiviral effect of anhydride-modified proteins, we screened the anti-SARS-CoV-2 activity of different anhydride-modified proteins and found a potential candidate, HP-OVA, which is highly effective in inhibiting infection by blocking the RBD and ACE2 interaction. In this study, we verify the entry-inhibitory activity of HP-OVA against SARS-CoV-2, and the results suggested that HP-OVA could be developed as a novel viral entry inhibitor used to prevent and treat SARS-CoV-2 infection.

MATERIALS AND METHODS

Cell Lines and Plasmids

The human embryonic kidney cell line 293T (HEK-293T), African green monkey kidney cell line Vero E6 and human hepatoma Huh 7 cell lines were obtained from the American Type Culture Collection (ATCC). HEK-293T cells stably expressing human ACE2 (293T/ACE2) were established by our laboratory. All of these cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Capricorn Scientific, Germany), 100 units/ml penicillin, 100 μg/ml streptomycin and 2% L-glutamine (Gibco).

The envelope-expressing plasmids SARS-CoV-2-S (*pcDNA3.1-SARS-CoV-2-S* and *pAAV-IRES-EGFP-SARS-CoV-2-S*) and *pcDNA3.1-SARS-CoV-S* were kindly provided by Dr Shibo Jiang (Fudan University, China). The plasmid *pAAV-IRES-EGFP* was purchased from Hedgehogbio Science and Technology Ltd (Shanghai, China). The luciferase reporter-expressing HIV-1 backbone *pNL4-3.Luc.R$^-$E$^-$* plasmid was maintained in our laboratory.

Chemical Modification of OVA

The modified protein HP-OVA was prepared using a previously described (Li et al., 2011). Briefly, OVA (final concentration, 20 mg/ml in 0.1 M phosphate) was treated with hydroxyphthalic anhydride (HP) (1.19 M in dimethylformamide) by the addition of five aliquots at 12 min intervals, while the pH was adjusted to 8.5 with 1 M NaOH after each mixing. The mixture was kept for 1 h at room temperature and then extensively dialyzed against phosphate-buffered saline (PBS) and filtered through 0.45 μM msyringe filters (Gelman Sciences, Ann Arbor, MI). Protein concentrations were measured by a bicinchoninic acid (BCA) protein assay reagent kit (Thermo Fisher Scientific, USA). To

quantitate the lysine residues in OVA and HP-OVA, 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) treatment was applied as previously described (He et al., 2011).

Cytopathic Effect (CPE) Inhibition Assay on Live SARS-CoV-2 Infection

To assess the inhibitory activity of HP-OVA against infection by live SARS-CoV-2, 100 50% Tissue Culture Infectious Dose ($TCID_{50}$) of SARS-CoV-2 was incubated with Vero E6 cells (2×10^5/ml) at 37°C for 2 h. After 2 h post-infection, the culture supernatants were discarded and HP-OVA at graded concentrations was added to Vero E6 cells for three days. Then, the CPE was detected by fluorescence microscopy, and the 50% inhibitory concentration (IC_{50}) was calculated by the Reed-Muench method or GraphPad Prism 5.0 software. Remdesivir was used as a positive control.

Luciferase Assay on Pseudotyped SARS-CoV-2 Infection

The infectivity of pseudotyped SARS-CoV-2 and SARS-CoV on target cells was determined by a single-cycle infection assay as described previously (Yin et al., 2018). To produce pseudovirions, 293T cells were co-transfection with a plasmid expressing the S protein of SARS-CoV-2 or SARS-CoV (pcDNA3.1-SARS-CoV-2-S or pcDNA3.1-SARS-CoV-S) and a backbone plasmid (pNL4-3.Luc.R-E-) that encodes an Env-defective, luciferase reporter-expressing HIV-1 genome. The cell supernatants containing the released virions were harvested at 48 h post-transfection, passed through a 0.45 μm filter and frozen at −80°C.

To detect the inhibitory activity of HP-OVA on pseudotyped SARS-CoV-2 and SARS-CoV infection, target cells (293T/ACE2 and Vero E6) were seeded into 96-well plates at a density of 10^4 cells per well. After overnight incubation, a series of dilutions of the compound were mixed with an equal volume of pseudovirus, and the mixture was transferred to the cells. Twelve hours after infection, the culture medium was refreshed, and then, the cells were incubated for an additional 48 h, followed by washing the cells with PBS, lysing the cells with 50 μl of lysis reagent (Promega) per well on a microperforated plate oscillator for 15 min, and transferring 30 μl of the cell lysates to 96-well Costar flat-bottom luminometer plates (Corning Costar) for the detection of relative light units using a Firefly Luciferase Assay Kit (Promega, Madison, WI). The IC_{50} was calculated as the final concentration of HP-OVA that caused a 50% reduction in relative luminescence units (RLUs) compared to the level of the virus control subtracted from that of the cell control.

SARS-CoV-2 S-Mediated Cell-Cell Fusion Assay

HEK-293T cells were transfected with pAAV-IRES-EGFP or pAAV-IRES-SARS-CoV-2-S-EGFP as the effector cells by PolyJet™ DNA *in vitro* Transfection Reagent (SignaGen, USA). Huh 7 cells/Vero E6 cells (1×10^4) expressing ACE2 receptor were incubated in 96-well plates at 37°C for 5 h followed by the addition of 293T/EGFP or 293T/SARS-CoV-2-S/EGFP cells with or without compounds. After co-culture at 37°C for 12 h, three fields in each well were randomly selected to count fused and unfused cells under an inverted fluorescence microscope (Nikon Eclipse Ti-S). The percent inhibition of cell-cell fusion was calculated using the following formula, as described elsewhere $[1 - (E - N)/(P - N)] \times 100\%$. "E" represents the percentage of cell-cell fusion in the experimental group. "P" represents the percentage of cell-cell fusion in the positive control group, where 293T/SARS-CoV-2-S/EGFP cells were used as effector cells to which no compound was added. "N" represents the percentage of cell-cell fusion in the negative control group, in which 293T/EGFP cells were used as effector cells. The IC_{50} was calculated using CalcuSyn software. Samples were tested in triplicate, and all experiments were repeated twice.

Western Blot Analysis

HE-K293T cells were transfected with 2 μg of plasmids encoding the SARS-CoV-2 S protein or ACE2 using polyethylenimine (PEI, Sigma). After 48 h, the cells were collected and lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS) containing 1 × protease and phosphatase inhibitor cocktail (Merck Calbiochem, Darmstadt, Germany). Then, the cells were incubated on ice for 10 min, followed by centrifugation at 12,000 × g for 10 min at 4°C. The supernatant was collected as a whole protein extract. Total protein was quantified by a BCA Protein Assay Kit (Thermo Fisher Scientific, Carlsbad, CA). The protein extract was quantified prior to being denatured by the addition of a loading buffer (0.313 M Tris-HCl (pH 6.8), 10% SDS, 0.05% bromophenol blue and 50% glycerol), followed by denaturation at 100°C for 10 min. Then, 50 μg of total protein was electrophoresed for 1.5 h on a 10% polyacrylamide gel to separate the proteins, transferred onto PVDF membranes (Roche, Indianapolis, IN, USA), and co-incubated with an anti-SARS-CoV-2 spike antibody (40150-R007, Sino Biological, China) or ACE2 antibody (#4355, CST) at 4°C overnight and secondary antibodies conjugated to horseradish peroxidase (HRP). Protein bands were detected by chemiluminescence using an ECL kit (Millipore).

Flow Cytometric Analysis

HEK-293T cells were transfected with 2 μg of plasmids encoding the SARS-CoV-2 S protein using PEI. Forty-eight hours later, the cells were detached by using PBS with 1 mM EDTA. After washing, the cells were incubated with PBS containing 10% goat serum (PBS-GS) at 4°C for 1 h before being treated with HP-OVA or OVA. After incubation at 4°C for 1 h, cells were washed three times with PBS-GS, and then polyclonal rabbit anti-OVA antibody (1:1,000 dilution) (Sigma) was added to the cells for 1 h on ice, followed by being incubated to Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:10,000) (Abcam) for 1 h. The cells were washed and resuspended in 400 μl of PBS-GS buffer, and then analyzed by flow cytometry. Unmodified OVA was used as a negative control.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed to identify the interaction of HP-OVA and the SARS-CoV-2 S protein (RBD) or ACE2 protein. Briefly, wells of 96-well polystyrene microplates were coated with 1 μg/mL S protein (RBD) (Sino Biological, China) or ACE2 protein (Invitrogen, Carlsbad, CA) in 0.01 M Tris buffer (pH 8.8) at 4°C overnight. Here, a bovine serum albumin (BSA) was used as an irrelevant coating protein antigen control. After washing with PBS-T three times, the wells were blocked for 2 h at 37°C with 5% BSA. Various concentrations of HP-OVA were added to the wells for 2 h at 37°C. After washing with PBS-T, the samples were incubated with a goat anti-OVA antibody (Sigma) for 1 h and then incubated with an HRP-labeled goat anti-mouse antibody for 1 h at 37°C. After color development, the optical density (OD) value at 450 nm was measured with a full-wavelength microplate reader (BioTek Instruments, Inc.).

The ability of HP-OVA to compete with SARS-CoV-2 S (RBD) for ACE2 binding was assessed by a competitive inhibition ELISA as previously described (Li et al., 2010b). Briefly, 1 μg/ml ACE2 protein (Invitrogen, Carlsbad, CA, USA) in 0.01 M Tris buffer (pH 8.8) was coated onto the wells of a polystyrene microplate at 4°C overnight, followed by washing with PBS-T buffer. Then the wells were blocked for 2 h at 37°C with 5% BSA and a mixture of S (RBD) (1 μg/ml) pre-incubated HP-OVA or unmodified OVA at the indicated concentrations was added and incubated. Subsequently, the samples were incubated with an anti-ACE2 antibody (40150-R007, Sino Biological, China) and then detected with an HRP-labeled goat anti-rabbit antibody for 1 h at 37°C. 3,3',5,5'-Tetramethylbenzidine (TMB), and 1N H_2SO_4 were added sequentially. The absorbance at 450 nm was measured by a full-wavelength microplate reader (BioTek Instruments, Inc).

Cytotoxicity Assay

The cytotoxicity of HP-OVA on different target cells, including Vero E6, Huh 7 and HEK-293T/ACE2 cells, were analyzed by MTT assays (Topscience, Shanghai, China). Briefly, each tested cell lines were seeded into the wells of a 96-well microtiter plate (1×10^4 per well) and incubated at 37°C overnight. Then, HP-OVA or OVA at graded concentrations were added into those cells and incubated at 37°C for 48 h. On the third day post-incubation, 100 μl of DMEM containing MTT [3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide, Sigma Aldrich, St Quentin Fallavier, France] (0.5 mg/ml) was added to equal volumes of cells in wells of 96-well plates and incubated at 37°C for another 4 h. Then, the OD was measured at 570 nm by a full-wavelength microplate reader. Unmodified OVA was used as a negative control. The 50% cytotoxicity concentrations (CC_{50}) were calculated using CalcuSyn software.

Statistical Analysis

Statistical analysis of the experimental data was performed using a one-way ANOVA test in GraphPad Prism 5.0 (San Diego, CA) and represented as means ± SD of at least three measurements. A p value of < 0.05 was regarded as statistically significant; the probability level is indicated by single or multiple asterisks (*) ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). The percent inhibition and IC_{50} values were calculated using CalcuSyn software.

RESULTS

Antiviral Activity of HP-OVA Against SARS-CoV-2 *In Vitro*

Our previous studies have shown that OVA can be converted into potent inhibitors through chemical modification with anhydrides to prevent the infection of HIV, HSV-2 and so on (Li et al., 2010a; He et al., 2011; Li et al., 2011). Based on those researches, we try to investigate the antiviral effect of HP-OVA against infection by SARS-CoV-2. At present, pseudovirus (PsV) has become an ideal tool to analyze cell entry of SARS-CoV-2 without safety concerns and possess the morphological characteristics of replication-competent SARS-CoV-2, with the S protein on the envelope membrane. As demonstrated in previous studies (Yin et al., 2018), the pseudotyped system of the SARS-CoV-2 S protein is a classic model that mimics the process of viral entry and studies the interaction of SARS-CoV-2 and host cells. Here, we first utilized SARS-CoV-2 PsV to perform a series of transduction assays. Results showed that HP-OVA exhibited potent inhibitory activity against the entry of SARS-CoV-2 S PsV to the 293T/ACE2 cells (293T cells stably expressing hACE2) in a dose-dependent manner, with an IC_{50} of 0.70 ± 0.49 μM **(Figure 1A)**. Notably, the inhibitory activities on Vero E6 cells were consistent with those on ACE2/293T cells, with an IC_{50} of 1.21 ± 0.15 μM, while unmodified OVA had no antiviral activity (**Figure 1B**). To investigate whether HP-OVA has the same effect on SARS-CoV, which is closely related to SARS-CoV-2 and also employs ACE2 for cell entry, we conducted a pilot experimental test *in vitro* on the anti-SARS-CoV PsV activity using both 293T/ACE2 cells and Vero E6 cells. We found that HP-OVA potently inhibited SARS-CoV infection, with an IC_{50} of approximately 0.85 ± 0.26 μM and 0.49 ± 0.10 μM, respectively (**Figures 1C,D**).

We next investigated the antiviral activity of HP-OVA against live SARS-CoV-2 *in vitro*. Here, Vero E6 cells were infected with 100 $TCID_{50}$ of live virus and incubated with HP-OVA at different dilution concentrations for 72 h. As shown in **Table 1**, HP-OVA inhibited the replication of SARS-CoV-2 virus, with an IC_{50} value of 4.78 μM by CPE assay. Additionally, treatment with unmodified OVA showed no inhibitory activity against live SARS-CoV-2. The positive control, remdesivir, potently inhibited virus-induced CPE, with an IC_{50} of 0.65 μM. These antiviral activities indicated that HP-OVA has potent anti-SARS-CoV-2 activity, but the mechanism remains to be explored.

HP-OVA Displays Low Cytotoxicity on the Tested Cell Lines

To evaluate the safety of HP-OVA, target cells including 293T/ACE2, Vero E6 and Huh 7 cells were treated with different concentrations of HP-OVA and assayed by MTT. As shown in

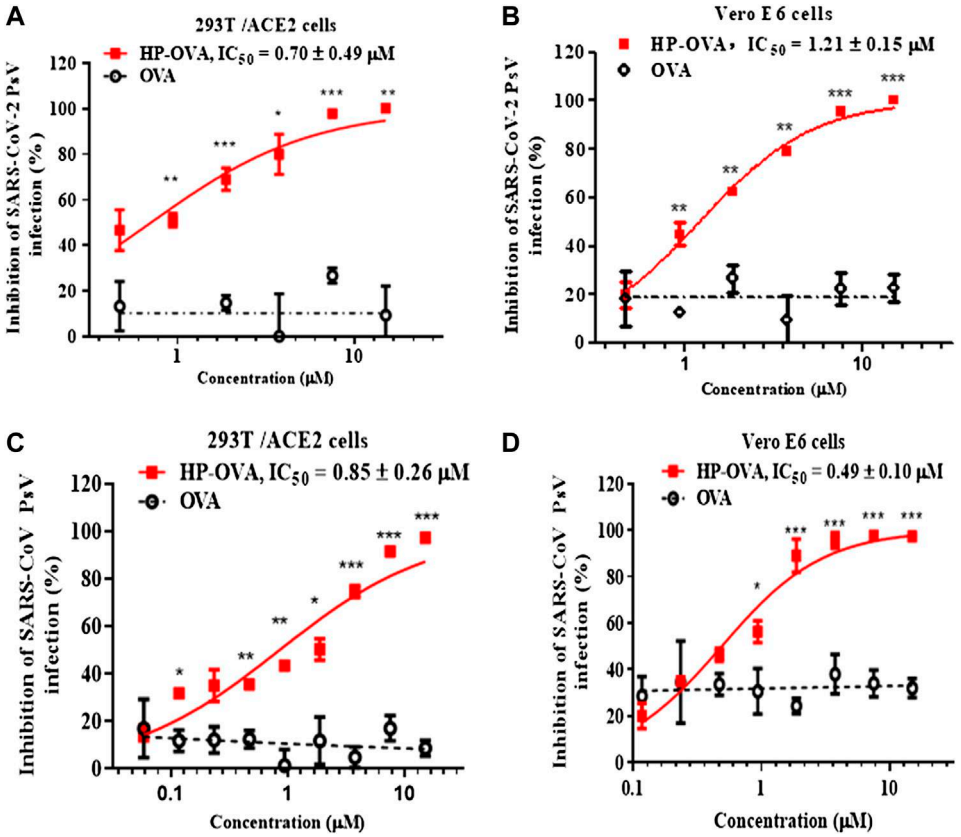

FIGURE 1 | Inhibition of HP-OVA on the infection with SARS-CoV-2 PsV and SARS-CoV PsV. Antiviral activity of HP-OVA against SARS-CoV-2 S PsV infection in 293T/ACE2 **(A)** or Vero E6 **(B)** target cells. Inhibition of single-round infection of SARS-CoV S PsV in 293T/ACE2 **(C)** and Vero E6 **(D)** cells. Data are presented as the mean ± SD of triplicate samples from a representative experiment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

TABLE 1 | Antiviral activity of HP-OVA against live SARS-CoV-2 in Vero E6 cells[a].

Compounds	IC_{50} (μM)	IC_{90} (μM)
HP-OVA	4.78 ± 1.03	12.23 ± 2.01
OVA	>50.00	>50.00
Remdesivir	0.65 ± 1.23	2.51 ± 1.41

[a]*The data are presented as mean ± SD of three independent experiments.*

TABLE 2 | Cytotoxicity of HP-OVA *in vitro*[a].

Cell lines	HP-OVA	OVA	SI[b] value of HP-OVA	
	CC_{50} (μM)	CC_{50} (μM)	SARS-CoV-2	SARS-CoV
Vero E6 cells	182.50 ± 29.00	>200.00	150.83	214.71
ACE2/293T cells	182.20 ± 59.75	>200.00	260.29	371.84
Huh 7 cells	113.50 ± 23.36	>200.00	Not done	Not done

[a]*The data are presented as mean ± SD of three independent experiments.*
[b]*SI, selectivity index = CC_{50}/IC_{50}.*

Table 2, HP-OVA displayed low cytotoxicity on all tested cell lines, with CC_{50} values ranging from 113.50 to 182.50 μM. The CC_{50} values of HP-OVA were more than 100 times higher than its IC_{50} for inhibiting authentic SARS-CoV-2 and SARS-CoV PsV infection and its selectivity index (SI = CC_{50}/IC_{50}) ranged from 150.83 to 371.84. Those results indicated that HP-OVA might be safe as an anti-SARS-CoV-2 candidate for use in patients.

HP-OVA Inhibited SARS-CoV-2 Through Inhibiting S Protein-Mediated Cell-Cell Fusion

The spike (S) glycoprotein of SARS-CoV-2 binds ACE2 cellular receptors to facilitate fusion and ultimately entry into cells. Therefore, we herein analyzed the potential role of HP-OVA on SARS-CoV-2 S-mediated cell-cell fusion. In this widely adopted cell-cell fusion system, SARS-CoV-2 S and green fluorescent protein genes were transfected into HEK-293T cells. In a syncytium-formation assay, the size of a syncytium is usually ≥ 2-fold larger than that of a normal cell, and the numbers of syncytia and fluorescence-labeled fused cells were counted under an inverted microscope. Here, we chose two kinds of cells expressing hACE2 receptor as the target cells including Vero E6 (**Figure 2A**) and Huh 7 cells (**Figure 2B**). As shown in **Figure 2A**, HP-OVA significantly inhibited S-mediated 293T/SARS-CoV-2/EGFP and Vero E6 cell-cell fusion, resulting in the

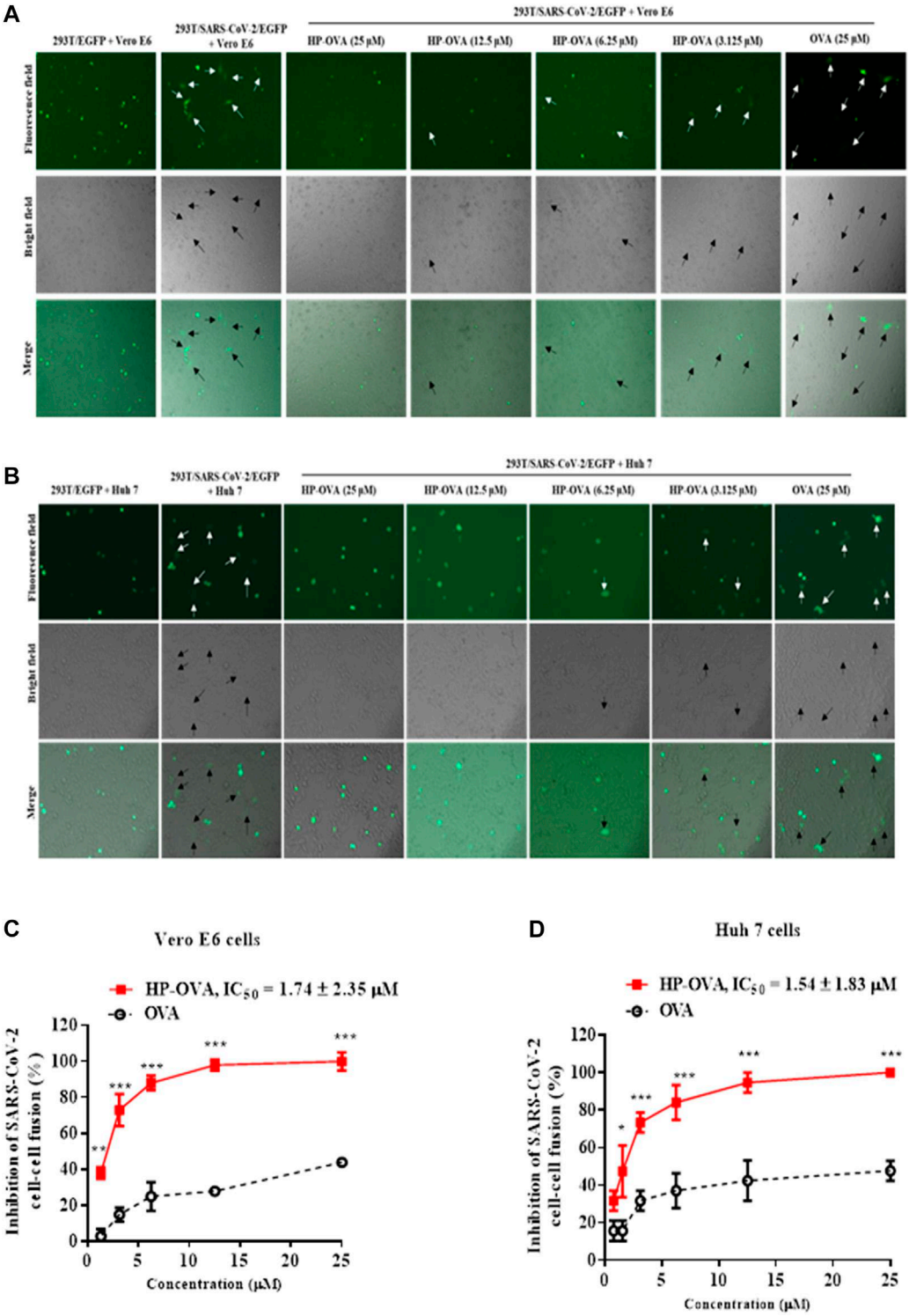

FIGURE 2 | Inhibitory activity of HP-OVA against SARS-CoV-2 S-mediated cell-cell fusion. Images were captured at 12 h after treatment with HP-OVA or OVA on SARS-CoV-2 S protein-mediated cell-cell fusion. The syncytia of Vero E6 cells **(A)** or Huh 7 cells **(B)** and HEK293T cells with SARS-CoV-2 overexpression are marked in the pictures. Representative results from three fields were selected randomly from each sample with scale bars of 50 μm **(C, D)** The number of syncytia was counted under an inverted fluorescence microscope, and the percentage of inhibition was calculated as described in the Methods. Data are presented as the mean ± SD of triplicate samples from a representative experiment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

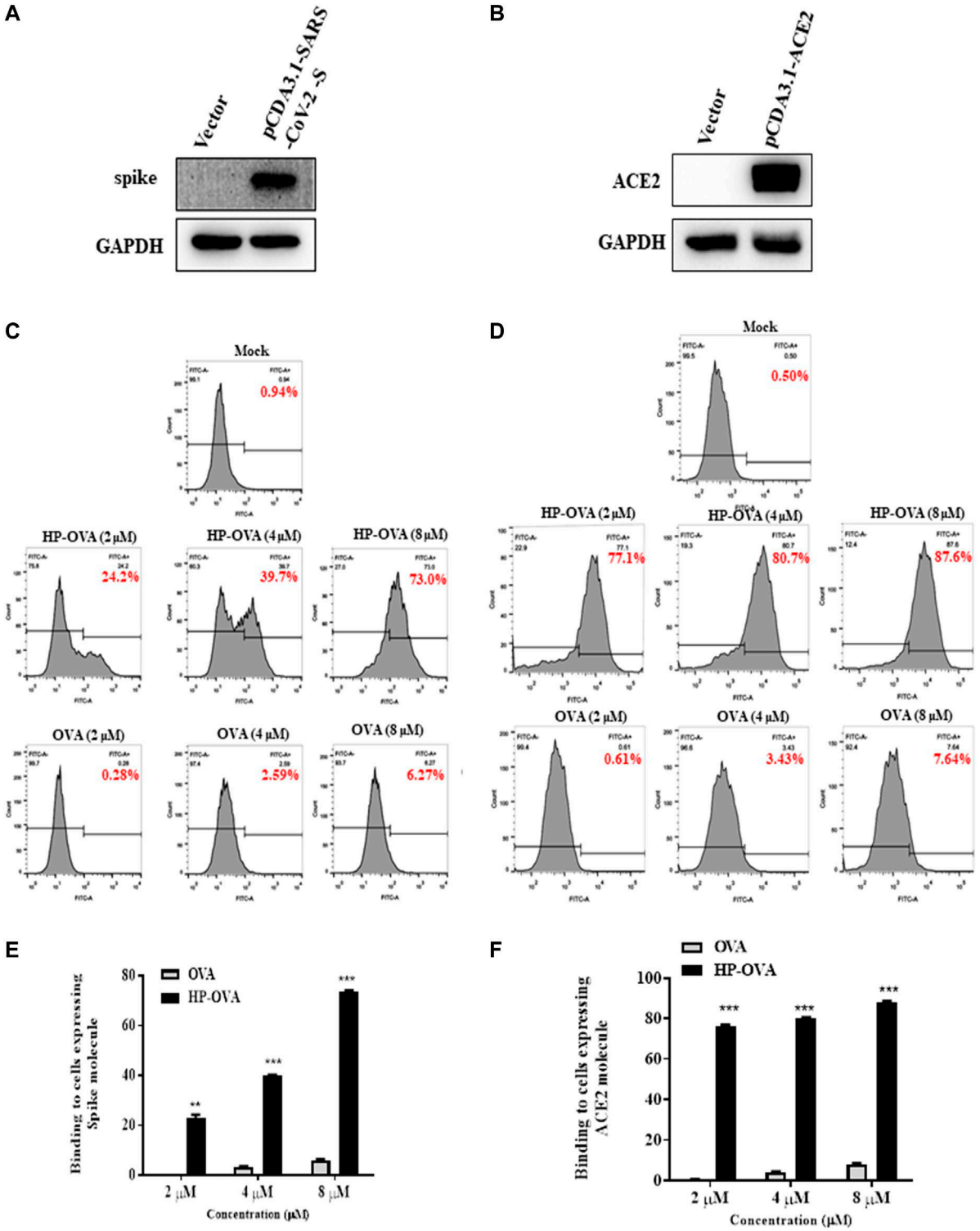

FIGURE 3 | HP-OVA binding to both SARS-CoV-2 S and ACE2 protein. Analysis of the expression of SARS-CoV-2 S **(A)** and ACE2 **(B)** in HEK-293T cells by western blot. The binding of HP-OVA to cells expressing SARS-CoV-2 S **(C)** or ACE2 **(D)** was assessed by flow cytometry. A representative flow histogram and quantification of the binding of HP-OVA to cells expressing SARS-CoV-2 S **(E)** or ACE2 **(F)** were shown. Data are presented as the mean ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

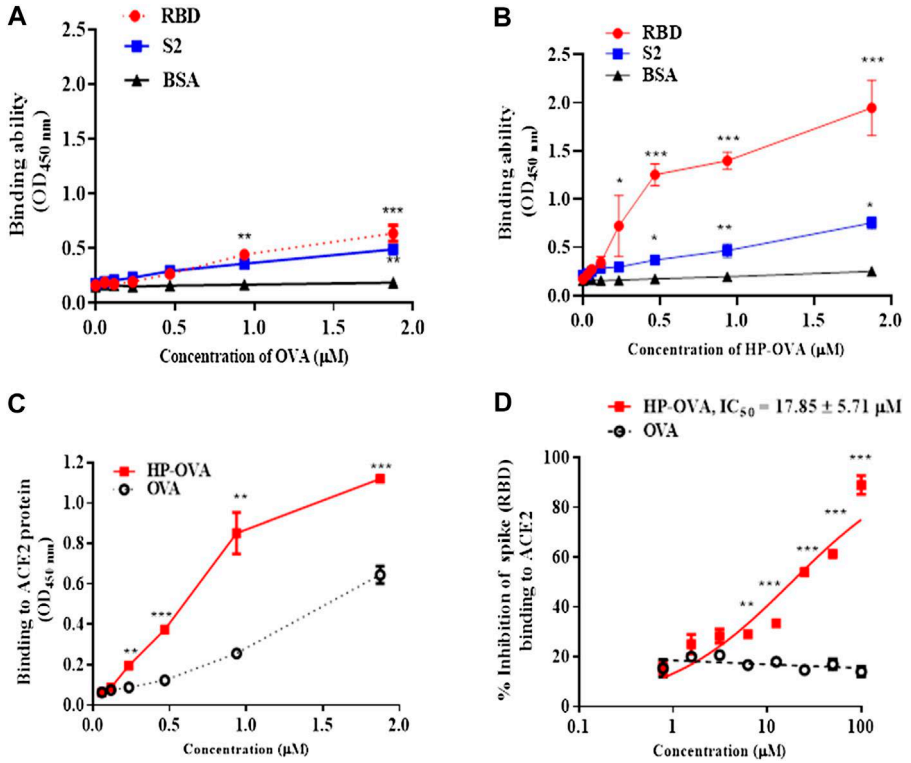

FIGURE 4 | The interaction of HP-OVA with SARS-CoV-2 S and ACE2. The binding of OVA to SARS-CoV-2 spike (RBD), S2 and BSA protein was assessed by ELISA **(A)**. The binding of HP-OVA to SARS-CoV-2 spike (RBD), S2 and a negative control BSA protein was assessed by ELISA **(B)**. The binding ability of HP-OVA to ACE2 protein was assessed by ELISA **(C)**. Inhibition of the interaction between spike (RBD) and ACE2 proteins by HP-OVA, as determined by a competitive inhibition ELISA **(D)**. Data are presented as the mean ± SD of triplicate samples from a representative experiment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

reduction in syncytium formation in a dose-dependent manner, with an IC_{50} of 1.74 μM (**Figure 2C**). Correspondingly, HP-OVA showed potent fusion inhibitory activity on SARS-CoV-2 S-mediated 293T/SARS-CoV-2/EGFP and Huh 7 cell-cell fusion, with an IC_{50} of 1.54 μM (**Figures 2B,D**). It is worth noting that unmodified OVA showed no inhibitory activity at concentrations up to 25 μM in cell-cell fusion assays. These results suggest that HP-OVA exhibits inhibitory activity against SARS-CoV-2 by blocking S-mediated cell-cell fusion.

The Antiviral Activity of HP-OVA Was Attributed to the Disruption of the S Protein-ACE2 Interaction

The SARS-CoV-2-S/ACE2 interface was found to be a key determinant of SARS-CoV-2 transmissibility. Our preliminary work have revealed that HP-OVA is highly effective against SARS-CoV-2 S-mediated cell-cell fusion and SARS-CoV-2 S PsV infection, suggesting that HP-OVA might be a viral entry inhibitor by interacting with either the S protein of coronaviruses or ACE2 receptor on the target cellular surface. To investigate this hypothesis, SARS-CoV-2 S (**Figure 3A**) or ACE2 (**Figure 3B**) was transiently overexpressed in HEK-293T cells, and flow cytometry was used to analyze the binding activity. As shown in **Figures 3C–F**, HP-OVA notably bound to HEK-293T cells overexpressing both S and ACE2 proteins in a dose-dependent manner, while unmodified OVA showed no corresponding effect. To further confirm the specific targets, the binding of HP-OVA to S or ACE2 molecules was subsequently determined by ELISA. The results also showed that HP-OVA could bind to both the S (RBD) protein (**Figure 4B**) and ACE2 protein (**Figure 4C**) in a dose-dependent manner. We further determined the binding of HP-OVA to the spike S2 protein of SARS-CoV-2 by ELISA. As shown in **Figure 4B**, HP-OVA could also bind to S2 protein, while the binding ability to S2 proteins is weaker than RBD protein. In addition, we found that HP-OVA could not bind to an irrelevant coating protein antigen control BSA, indicating to the specific binding to both S and ACE2 proteins (**Figure 4B**). These results indicated that HP-OVA inhibits SARS-CoV-2-mediated viral entry at the cell surface attachment step by directly interacting with S protein and ACE2 receptor.

To determine whether the potential effect of HP-OVA on the interaction between S protein and ACE2 receptor, a competitive inhibition assay was conducted by ELISA. As shown in **Figure 4D**, HP-OVA significantly inhibited the binding of S and ACE2 in a dose-dependent manner, with an IC_{50} of 17.85 μM. These results indicated that HP-OVA may bind to both S protein and ACE2 receptor and then interfere with their interaction, resulting in the inhibition of viral entry.

DISCUSSION

Currently, the rapid spread of COVID-19 has resulted in an urgent requirement for effective therapeutic strategies against SARS-CoV-2. Initially, without licensed vaccines or approved antiviral drugs, COVID-19 treatment was mainly based on the experience of clinicians. Nonspecific antiviral drugs, including IFN-α (recombinant human IFN-α1b, IFN-α2a), lopinavir/ritonavir (Aluvia, HIV protease inhibitors), chloroquine phosphate, favipiravir and ribavirin, have been clinically used as antiviral therapies according to the National Health Commission (NHC) of the People's Republic of China (Huang et al., 2020; Lu, 2020). To date, many potential drugs have been expected to have therapeutic potential, including inhibition of TMPRSS2 (Hoffmann et al., 2020) (i.e., camostat mesylate, nafamostat, loprazolam, and rubitecan) and antiviral drugs inhibiting viral RdRp (i.e., remdesivir, and favipiravir) (Elfiky, 2020; Lung et al., 2020) and 3CLpro (i.e., poziotinib, fostamatinib, ziprasidone, and telcagepant) (Jo et al., 2020; Ul Qamar et al., 2020) as well as virus/host cell membrane fusion (i.e., EK1C4, nelfinavir mesylate, and IBP02) (Hoffmann et al., 2020; Xia et al., 2020; Zhu et al., 2020). However, the efficacies *in vivo* still require further confirmation, and their potential use for the treatment of infection by other coronaviruses and emerging coronaviruses in the future is unclear. Therefore, drug development for treating COVID-19 is timely and important due to its rapid expansion.

Viral entry inhibitors have proven effectiveness and safety for the treatment of viral infections, and targeting viral entry may have a greater potential in the development of pan-CoV inhibitors for future coronavirus outbreaks (Chu et al., 2008; Sun et al., 2013; Xia et al., 2014; Li et al., 2017; Pu et al., 2019). Combined with our previous studies, we focused on HP-OVA because chicken OVA is the main protein in egg white, making up 60–65% of the total protein. Second, HP-OVA is convenient to synthesize by anhydride modification with OVA, which is isolated from chicken eggs and less expensive than RSA, which is purified from animal sera. Third, HP-OVA exerts a broad-spectrum effect on a series of HIV strains by blocking HIV entry. Our research demonstrated that HP-OVA could inhibit Vero E6 cell infection with live SARS-CoV-2, with an IC_{50} value of 4.78 μM and an IC_{90} value of 12.23 μM. Furthermore, HP-OVA obviously inhibited pseudotyped SARS-CoV-2 entry into two different target cells, with an IC_{50} value of 0.70 and 1.21 μM, respectively. Notably, HP-OVA also showed inhibitory activity against SARS-CoV infection of 293T/ACE2 and Vero E6 cells, with an IC_{50} value of 0.85 and 0.49 μM, respectively. Furthermore, our results showed that HP-OVA displayed low cytotoxicity on all tested cell lines, with CC_{50} values ranging from 113.50 to 182.50 μM. The CC_{50} values of HP-OVA were more than 100 times higher than its IC_{50} for inhibiting authentic SARS-CoV-2 PsV infection and its SI values ranged from 150.83 to 260.29, indicating that HP-OVA might be safe as an anti-SARS-CoV-2 candidate for use in patients. Therefore, this study suggests that HP-OVA has broad-spectrum antiviral activity by inhibiting viral entry, and it can be used for the treatment and prevention of infection by not only SARS-CoV-2 but also other human coronaviruses (HCoVs).

It is worth mentioning that OVA is a commonly used as an antigen for vaccination experiments and immunization researches. One may raise a concern about the potential of HP-OVA to induce immune responses when it is used as a nasal spray. However, several studies have reported that mucosal immunization by topical administration with soluble proteins, including OVA, without any adjuvants, are usually unable to induce strong local immune responses (Staats et al., 1994; Walker, 1994; Di Tommaso et al., 1996). Our previous studies have also certified that HP-OVA has no harmful or deleterious impact on the function of immune cells (Li et al., 2010a). Actually, anhydride-modified proteins, such as anhydride-modified bovine β-lactoglobulin, have been studied and utilized as microbicides against HIV and HPV in clinics for years, and their effectiveness and safety as drugs have been verified (Neurath et al., 1996; Guo et al., 2016; Hua et al., 2019).

Another important problem for development of HP-OVA as an antiviral agent is to confirm its *in vivo* therapeutic efficacy of HP-OVA against authentic SARS-CoV-2 infection in animal models. To date, various species have been used as animal models of SARS-CoV-2 infection, including hACE2 transgenic mice, African green monkey, Baboon, Cynomolgus macaque, and Ferret and Syrian hamster. However, there is currently no single, simple and optimal animal models for SARS-CoV-2 infection (Khoury et al., 2020; Muñoz-Fontela et al., 2020). In additional, there are several significant differences between the pathogenesis and kinetic of human infection and animal models. Furthermore, it is also not clear which is the best outcome metric to study-for example, should an intervention aim to reduce the viral titer, pathology or lethality? The most suitable animal model and outcome measure for a particular application depends on the therapeutic intention, as well as the cost, timing and availability. Taken all consideration, we have not verified the antiviral effectiveness of HP-OVA against SARS-CoV-2 infection on animal models. The next stage of assessing HP-OVA's efficacy will be typically involved animal testing, which is extremely important and will strengthen our findings.

The S protein interaction with ACE2 on the host cell cytoplasmic membrane initiates viral infection. Strategies capable of disrupting the S protein interaction with ACE2 could be of significant therapeutic value and could contribute to/favor the resolution of the pandemic that is developing worldwide because the binding affinity of the SARS-CoV-2 S protein to ACE2 is 10 to20-fold higher than that of the S protein of SARS-CoV, which may contribute to the higher contagiousness of SARS-CoV-2 than SARS-CoV (Shang et al., 2020; Walls et al., 2020). Our preliminary results indicated that HP-OVA could bind to both ACE2 and the S protein (RBD domain) directly. In addition, HP-OVA interferes to the interaction between SARS-CoV-2 S protein and ACE2 receptor on the cell surface, leading to the inhibition of SARS-CoV-2 infection and S protein-mediated cell-cell fusion. The unmodified OVA protein can not interfere to the binding of S protein and ACE2 receptor. Our previously study reported that the binding ability of HP-OVA is closely correlated with the number of the positively charged side chains of lysine and arginine residues were converted to negatively charged side

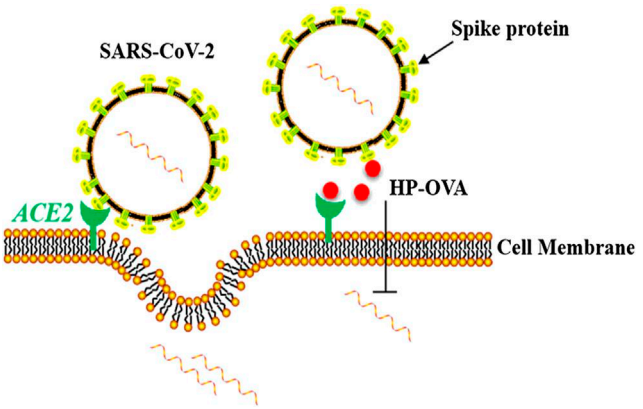

FIGURE 5 | Schematic representation of the molecular mechanisms of HP-OVA against SARS-CoV-2 infection. HP-OVA binds to both the S protein of SARS-CoV-2 and host angiotensin-converting enzyme 2 (ACE2), the functional receptor of SARS-CoV-2, and disrupts the S protein-ACE2 interaction, thereby exhibiting inhibitory activity against SARS-CoV-2 infection.

chains after modification by HP (Li et al., 2010a). Thus, the positively charged side chains of HP-OVA might account for the antiviral activity of HP-OVA since unmodified OVA did not showed either an affinity of binding to ACE2 or S protein (RBD domain), as well as the inhibitory activity against SARS-CoV-2 infection.

Our results showed HP-OVA could also bind to S2 protein, while the binding ability to S2 protein is weaker than RBD protein. Indeed, there is less enthusiasm for developing HP-OVA as a specific antiviral entry inhibitor because it can bind to a variety of viral membrane proteins. Our previous studies have certified that anhydride-modified proteins could inhibit several kinds of viruses, including HIV, HPV, RSV and MERS-CoV. It is worth mentioning that the specific antiviral inhibitors are only effective against SARS-CoV-2, whereas the non-specific antiviral agents may also be effective against other pathogens, such as SRAS or other coronavirus. The preliminary results indicated that HP-OVA was effective against SARS-CoV infection, suggesting that it has good potential to be developed as a promising active component for prevention of multiple coronavirus diseases.

Since HP-OVA can bind to ACE2 receptor and ACE2 helps modulate the many activities of angiotensin II (ANG II) that increases blood pressure and inflammation, increasing damage to blood vessel linings and various types of tissue injury. Therefore, the potential effect of HP-OVA on ACE2 is warranted. Another problem for development of chemically modified OVA as pan-CoV inhibitor-based therapeutic and prophylactic for the treatment and prevention of the current COVID-19 pandemic is the potential risk of causing side effects in people who are allergy to egg protein (Honma et al., 1996). Fortunately, egg allergy occurs seldom in adults, but mostly in young children (less than five years old) (Mine and Yang, 2008). Therefore, we expect that there will be only very few adults with egg allergy, and those people should be excluded from the clinical trials of HP-OVA-based microbicide.

Taken all consideration, HP-OVA can be more easily produced on a large scale and are more cost-effective than neutralizing antibodies and other large protein-based inhibitors, thus we believe HP-OVA is a promising candidate for optimization and development as a pan-CoV inhibitor-based therapeutic and prophylactic for the treatment and prevention of the current COVID-19 pandemic and may help in the future to prevent new viruses that have an affinity between the S protein and ACE2 receptor. The mechanism of action of HP-OVA against SARS-CoV-2 infection was shown in **Figure 5**.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the Corresponding authors.

AUTHOR CONTRIBUTIONS

TL contributed to perform all the experiments, analyze the data and draft the manuscript. JQ, XN, and QM helped with experimental design and manuscript writing. CZ, and PC performed some of the experiments. QZ and MC contributed to data analysis. ZY, SL, and LL supervised the study, edited and reviewed the manuscript. All authors read and approved the final manuscript for publication.

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TLR9 and COVID-19: A Multidisciplinary Theory of a Multifaceted Therapeutic Target

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By mapping the clinical pathophysiology of the novel coronavirus disease 2019 (COVID-19) against insights from virology, immunology, genomics, epidemiology and pharmacology, it is here proposed that the pathogen recognition receptor called toll like receptor 9 (TLR9) might have a pivotal role in the pathogenesis of COVID-19. Severe Acute Respiratory Syndrome Coronavirus 2, is causing the greatest global social and economic disruption since world war II. Lack of a vaccine, lack of successful treatment and limitations of the healthcare workforce and resources needed to safeguard patients with severe COVID-19 on the edge of life, demands radical preventive measures. It is urgently needed to identify biomarkers and drug candidates so that vulnerable individuals can be recognized early and severe multi-organ complications can be prevented or dampened. The TLR9 COVID-19 hypothesis describes a mechanism of action that could explain a wide spectrum of manifestations observed in patients with severe COVID-19. The introduced hypothesis proposes biomarkers for identification of vulnerable individuals and positions TLR9 as a promising multifaceted intervention target for prevention and/or treatment of COVID-19. TLR9 agonists might have value as prophylactic vaccine adjuvants and therapeutic immune stimulators at the early onset of disease. Additionally, in this current manuscript it is proposed for the first time that TLR9 could be considered as a target of "inhibition" aimed to dampen hyperinflammation and thrombotic complications in vulnerable patients that are at risk of developing late stages of COVID-19. The readily availability of TLR9 modulating drug candidates that have reached clinical testing for other disorders could favor a fast track development scenario, an important advantage under the current high unmet medical need circumstances regarding COVID-19.

Keywords: pathophysiology, immunology, biomarker, drug target identification, mitochondrial DNA, toll-like receptor 9, severe acute respiratory syndrome coronavirus 2, coronavirus disease 2019

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INTRODUCTION

COVID-19 Unmet Need

The COVID-19 (Coronavirus disease 2019) pandemic, caused by the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has been declared a public health emergency of international concern by the WHO Director General (WHO, January 29, 2020). The virus, first identified in Wuhan City, China, has spread worldwide, resulting in more than 65M confirmed cases and over 1,5M cases (COVID19.who.int, December 6, 2020). At the time of this writing there are no

validated specific therapies with proven effectiveness available for prevention of mortality from COVID-19. Remdesivir has been shown superior to placebo in shortening the time to recovery in adults who were hospitalized with Covid-19 but no significant benefit on mortality could be found (Beigel et al., 2020). Remdesivir is approved in certain countries for treatment of severe COVID-19, while awaiting further evidence and supply. Poor treatment options and the exceptional high burden of COVID-19 on healthcare systems still demands radical preventive measures including travel restrictions, social distancing and lockdowns, resulting in the most severe global social and economic disruption since world war II (Gossling et al., 2020; Dhama et al., 2020). Time-lines to bring a safe and efficacious vaccine for SARS-CoV-2 to market has been proposed to take 12–18 months under ideal circumstances (Billington et al., 2020). Even if intense collaboration and resource allocation can speed up vaccine development it remains a challenge to get the product to the most vulnerable individuals in time. With daily rising new cases and next waves of infections ongoing, it is urgently needed to identify and validate biomarkers and drug candidates so that vulnerable individuals can be recognized early and severe multi-organ complications can be prevented or dampened. This will help to reduce mortality rates and minimize the high pressure on the limited intensive care capacity and healthcare workforce (Adams and Walls, 2020; Dhama et al., 2020; Rolim Neto et al., 2020; Rabaan et al., 2020). Drug candidates and cell-based therapies for management of COVID-19 are being explored in ongoing clinical trials and results are eagerly awaiting (Lythgoe and Middleton, 2020; Khoury et al., 2020; Sanders et al., 2020; Schijns and Lavelle, 2020). Meanwhile, there are still pieces of the puzzle missing, which presents acute unmet medical needs. Patients with severe COVID-19 display a wide array of complications affecting multiple organs including the lungs, cardiovascular system, muscles, brains, liver and kidneys. Further unraveling the mechanisms underlying severe COVID-19 pathology is essential to uncover biomarkers and therapeutic concepts while making efficient use of resources available to allow rapid development.

TLR9 COVID-19 Hypothesis

Toll-like receptors (TLRs) are a family of 13 conserved transmembrane receptors that are at the forefront of directing innate and adaptive immune responses against invading bacteria, fungi, viruses and parasites (Akira, 2003; Takeda and Akira, 2004; Pasare and Medzhitov, 2005). When TLRs recognize structurally conserved pathogen-associated molecular patterns (PAMPs) they recruit intracytoplasmic TIR domains and specific adaptors such as MyD88, TIRAP and TRIF to control intracellular signaling pathways leading to the synthesis and secretion of appropriate cytokines and chemokines by cells of the immune system (Takeda and Akira, 2004). Among the TLR family, TLR3, TLR7, TLR8 and TLR9 are predominantly localized in intracellular compartments and form the key gatekeepers in detecting and combating viral infections (Akira and Hemmi, 2003). TLR3 is activated by viral double stranded RNA (dsRNA), whereas TLR7 and 8 recognize viral single stranded RNA (ssRNA) and bacterial RNA. TLR9 recognizes RNA and DNA motifs that are rich in unmethylated Cytosine-phosphate-Guanine (CpG) sequences. CpG-motifs are higher expressed in the bacterial and viral genome compared to the vertebrate genome (Hemmi et al., 2000). TLRs can also be activated by endogenous damage-associated molecular patterns (DAMPs) which is believed to have a function in both immune system alert and tissue homeostasis (Bianchi, 2007; Kono and Rock, 2008). Human mitochondrial DNA (mtDNA), evolutionary derived from endosymbiont bacteria, contains unmethylated CpG-motifs and is an example of a well-known DAMP that triggers inflammatory responses directly via TLR9 during injury and/or infection (Zhang et al., 2010). In the setting of COVID-19, multiple TLRs are likely relevant in viral combat and investigations of TLRs as therapeutic target are starting to emerge. Control of the cytokine storm by means of immunomodulators, including TLR7 and TLR8 antagonists and inhibitors of cellular mediators downstream of TLRs such as recombinant human IL-6 monoclonal antibody have been proposed and are currently under clinical investigation (Ye et al., 2020; Felsenstein et al., 2020; Lythgoe and Middleton, 2020; Poulas et al., 2020; Patra et al., 2020). Moreover, the TLR7 agonist, Imiquimod, is proposed as candidate to manage early stage COVID-19 patients (Angelopoulou et al., 2020). The effectiveness of TLR9 agonists for the use as vaccine adjuvants has also been suggested (Oberemok et al., 2020).In contrast to the available papers that more broadly focus on TLR3, 7 and 8, the here presented work, elaborates specifically on the role of TLR9 in defense against SARS-CoV-2 and introduces the hypothetical positioning of exaggerated TLR9 activation in severe COVID-19 pathology. The hypothesis is in line with our previously proposed synergistic disease driving effect of TLR9 agonists in the setting of COPD (Bezemer et al., 2012). TLR9 is broadly expressed on different cell types including epithelial cells in the lungs and nasal mucosa, in muscles and brains, on plasmacytoid dendritic cells and B cells, monocytes, macrophages, neutrophils, megakaryocytes and platelets, T lymphocytes, and NK cells (Hornung et al., 2002; Hayashi et al., 2003; Cognasse et al., 2005; Roda et al., 2005; Fransson et al., 2007; Kabelitz, 2007) A link between TLR9 activation and disease progression in COVID-19 is not directly obvious, since clinical investigations regarding safety and efficacy of inhaled TLR9 agonists in humans reported normal vital signs and no serious adverse effects although some “subtle” effects including moderate nature of flue like adverse events such as chills, fatigue, headache, myalgia and fever have been shown but are considered acceptable (Jackson et al., 2018). On the other hand, TLR9 activation in the airways in mice using high dose CpG-motifs, does lead to inflammation in the airways, ARDS, and sepsis (Knuefermann et al., 2007; Schwartz et al., 1997). Moreover, genetic mutations leading to TLR9 gain of function in human is associated with immune-mediated disease and with a higher incidence of ICU acquired infection (Chatzi et al., 2018; Ng et al., 2010). The TLR9 COVID-19 hypothesis proposes that in specific vulnerable patients, activation of TLR9 could be a silent but driving force explaining the worsening of hyperinflammation and thrombotic complications caused by SARS-CoV-2. Positioning TLR9 in COVID-19 pathology, could explain multi-organ complications and aligns with the fact that only a relatively small proportion of patients infected with SARS-CoV-2 develop severe

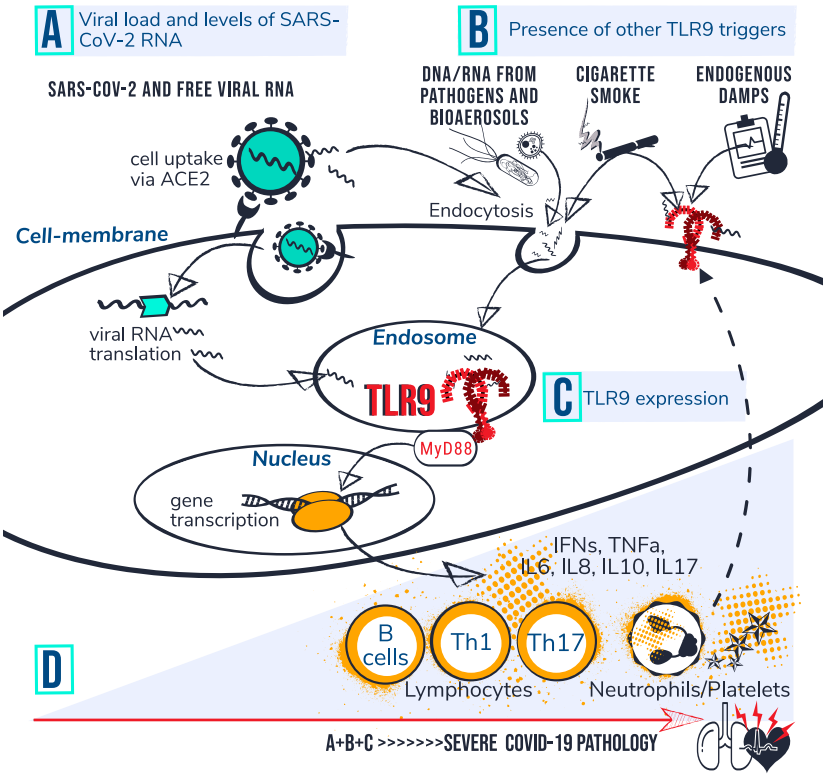

FIGURE 1 | TLR9-Covid-19 hypothesis. Set of circumstances suggested to drive COVID-19 poor outcome via TLR9 encompass; **(A)** viral load and levels of viral RNA; **(B)** presence of other TLR9 triggers, and; **(C)** TLR9 expression levels. **(D)** Individuals with high accumulated levels of A, B and C are proposed to be at risk for developing severe COVID-19 pathology. It is suggested that CpG motifs from SARS-CoV-2 reach TLR9 via ACE mediated viral uptake in the cell followed by RNA translation and transfer of viral CpG-motifs to the endosome. Circulating CpG motifs from virus and other sources could reach TLR9 via endocytosis or directly bind to cell surface at an inflamed site. Dashed line indicates that activation of platelets and neutrophils can increase TLR9 expression levels at cell surface which is suggested to drive a vicious circle of inflammation. Activated TLR9 induces downstream cascades via MyD88, leading to gene transcription, cytokine production and activation of lymphocytes, neutrophils and platelets. The Uncontrolled prolonged activation of TLR9 is suggested to contribute to severe COVID-19 pathophysiology.

symptoms requiring ICU. **Figure 1** depicts a set of circumstances and a mechanism of action of the proposed contribution of TLR9 to severe COVID-19 pathology in vulnerable patients. It should be noted that the TLR9 COVID-19 hypothesis does not rule out relevance of other TLRs in COVID-19 but rather highlights that disease caused by SARS-CoV-2, could have a worse outcome in people that are A) less well equipped to clear the virus, B) have to deal with a lot of available TLR9 stimuli over a longer period of time and, C) have high expression of functionally active TLR9. This hypothesis is relevant because it can be translated into a multifaceted window of opportunity for existing TLR9 modulating drug candidates that, depending on the disease stage, initially could stimulate, but later on preferably inhibit the TLR9 pathway in vulnerable patients. Moreover TLR9 expression levels and presence of TLR9 ligands are measurable and could potentially provide biomarkers for better identification of a group of individuals at risk for developing a more severe outcome of SARS-CoV-2 infection. High TLR9 expression levels can result from either genetic predisposition, people are simply born with it, or TLR9 expression is upregulated due to underlying health conditions, which will be explained further in the next sections. Examples of synergistically acting triggers for TLR9 include CpG-motifs from co-infecting pathogens, inhaled bioaerosols and organic dust, and cigarette smoke (Bezemer et al., 2012; Bauer et al., 2013; Martinez-Colon et al., 2019; Sun and Metzger, 2019). On top of the previously mentioned mtDNA, released from damaged host cells, also altered self-ligands, called carboxy-alkyl-pyrrole protein adducts (CAPs), that are generated during oxidative stress, are known to aggravate TLR9/MyD88 pathway activation (Zhang et al., 2010; Panigrahi et al., 2013). CAPs have been shown to promote platelet activation, granule secretion, and aggregation *in vitro* and thrombosis *in vivo* (Panigrahi et al., 2013). It is interesting to note that circulating mtDNA levels increase with age which is a familiar trait contributing to chronic inflammation, so called "inflamm-aging" in elderly people (Pinti et al., 2014). This TLR9 axis of inflamm-aging could have relevance in the context of COVID-19 where older age is associated with greater risk of development of severe complications of COVID-19. **Figure 2** provides a summarizing overview of insight from different disciplines that reason the hypothesis that TLR9 specifically

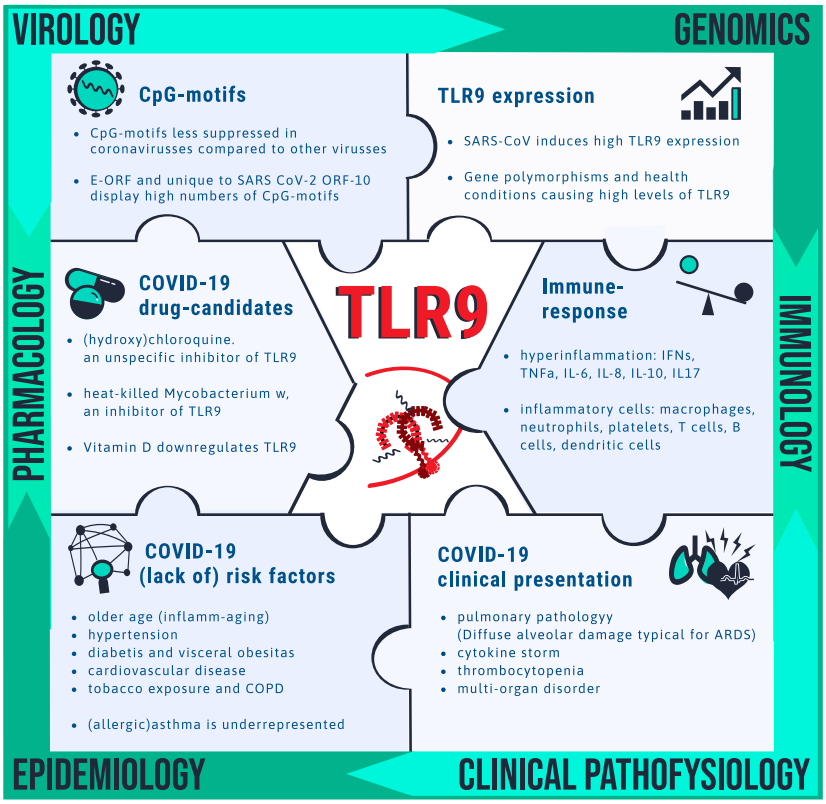

FIGURE 2 | Clues pointing toward drug target TLR9 for COVID-19. Unravelling the mechanism by which SARS-CoV-2 is causing disease is needed for identification of vulnerable patients and for drug target identification. Pieces of the complex puzzle are being filled in by insight from various disciplines including virology, genomics, immunology, clinical pathophysiology, epidemiology and pharmacology. It is proposed that TLR9 could fill in a blank spot worthwhile for further investigation. The bullet points summarize the wide spectrum of observations that can be explained via the TLR9 COVID-19 hypothesis.

could have a key role in disease caused by SARS-CoV-2. Further clarification is provided in the next sections.

MULTIDISCIPLINARY CLUES THAT REASON THE PROPOSED ROLE FOR TLR9 IN COVID-19

Virology: Presence of TLR9—Activating CpG-Motifs

In 2004, TLR9 has been linked to SARS coronavirus induced disease because of the relatively high numbers of CpG motifs in corona viral sequences (Ng et al., 2004). A paper by Ng et al., showed that human coronavirus 229E and Avian infectious bronchitis virus both contain 3 copy numbers of the CpG specific signaling motif GTCGTT, SARS-CoV viral sequence contains 7 copies number while other viruses involved in respiratory diseases have zero CpG motif copy numbers (Human rhinovirus B, Human parainfluenza virus 1, Human respiratory syncytial virus and human metapneumovirus) (Ng et al., 2004). Suppression of CpG motifs is a known mechanism of many mammalian RNA viruses, including influenza virus for adaptation to human host (Greenbaum et al., 2008). Evolving CpG suppression can help the virus to escape from the Zinc Finger Antiviral Protein (ZAP), which is a host antiviral factor that selectively binds to CG-dinucleotide-enriched RNA sequences to degrade target viral RNA (Luo et al., 2020; Gao et al., 2002; Takata et al., 2017). In the context of SARS-CoV-2, ZAP, expressed in human lung cells, has been identified as an important antiviral effector of the IFN response needed to combat SARS-CoV-2 (Nchioua et al., 2020). The authors showed that knock-down of ZAP significantly increased SARS-CoV-2 production in lung cells. The overall CpG composition of SARS-CoV-2 is lower than for other members of the betacoronavirus genus (Xia, 2020) but SARS-CoV-2 does present specific CpG "hotspots" in genomically disparate regions (Digard et al., 2020). The study of Digard et al., showed an over-representation of CpG-motifs within the Envelope (E) open reading frame (E-ORF) and ORF10 of SARS-CoV-2 which is well conserved across the sequences obtained from bat, pangolin and human (Digard et al., 2020).

Of the 4 major structural proteins of coronaviruses, the enigmatic E protein, is the smallest protein, involved in several aspects of the virus' life cycle, such as assembly, budding and envelope formation has also been implicated in the pathogenesis of coronaviruses (Schoeman and Fielding, 2019; Jimenez-Guardeno et al., 2014). During the replication cycle, E is abundantly expressed inside the infected cell, but only a small portion is incorporated into the virion envelope (Venkatagopalan et al., 2015). Across the Coronaviridae, E genes exhibit remarkably high variation in CpG composition, with those of SARS and SARS-CoV-2 having much higher CpG content than other coronaviruses isolated from humans. Moreover, E-ORF displays CpG suppression in all human-infecting viruses except SARS-CoV and SARS-CoV-2, suggesting a potential correlation between CpG presentation and disease severity in human-infecting coronaviruses (Digard et al., 2020). Notable about ORF10 is that this tiny gene, located toward the end of the viral genome, provides a short unknown protein or peptide that is unique to SARS-CoV-2 and uniformly presented in different geographical regions around the globe, and potentially a key protein responsible for SARS-CoV-2 highly contagious nature (Seema, 2020; Khailany et al., 2020; Koyama et al., 2020). The high number of CpG-motifs present in the nucleotide sequence of E-ORF and ORF10 which is unique and specific to SARS-CoV-2 warrants further investigation of a potential role of TLR9 activation in the highly severe and unique to SARS-CoV-2 disease pathogenesis.

Immunology: Inflammatory Mediators and Cellular Responses

Via the TLR pathways, including TLR9/MyD88, a plethora of inflammatory mediators and cell types can be triggered such as type 1 IFNs, TNFa, IL-6, IL-8, IL-10, IL-17 and activation of Th1 and Th17 lymphocytes, B cells, dendritic cells, neutrophils and platelets (Hemmi et al., 2000; Bezemer et al., 2012; Mortaz et al., 2010; Schwartz et al., 1997; Knuefermann et al., 2007; Greene et al., 2005; Takeda and Akira, 2005; Tasaka et al., 2009; Panigrahi et al., 2013; Hayashi et al., 2003). All these mediators and cell types have also been identified as potential contributors to the so called cytokine storm and thrombotic complications underlying the multi-organ pathological condition in patients with severe coronavirus infections (Li et al., 2020; Cheung et al., 2005; Channappanavar and Perlman, 2017; Birra et al., 2020; Huang et al., 2020; Tay et al., 2020). A clue pointing specifically toward a role for TLR9 in defense against coronaviruses, arises from a paper published in 2004 describing that in response to SARS-CoV infection, TLR9 on human PBMCs from healthy donors was surprisingly high expressed in comparison to other TLR receptors (*p*-value of 0.016) (Ng et al., 2004). The array data from the authors *in vitro* model system showed monocyte-macrophage cell activation, coagulation pathway upregulation and cytokine production together with lung trafficking chemokines such as IL8 and IL17, which were possibly activated through the TLR9 signaling pathway because of the high TLR9 expression levels and the Coronaviridae specific lack of CpG suppression in distinct regions. The TLR9 COVID-19 hypothesis, further elaborates on the idea that specific health conditions of the host that upregulate TLR9 expression contribute to TLR9 mediated inflammation which could potentially explain the differences in severity of the immune response against SARS-CoV-2 between COVID-19 patients. A pro-inflammatory status of the host for instance can drive susceptibility for TLR9 pathway activation by altering cell specific TLR9 expression levels (McKelvey et al., 2011). Life style factors such as a high fat diet and obesity are known to increase TLR9 expression in visceral adipose tissue (Nishimoto et al., 2016 MAR; Thomalla et al., 2019 FEB). Exposure to cigarette smoke, which is also a risk factor for severe COVID-19, causes increased expression of TLR4 and TLR9 on lung CD8(+) T cells of COPD patients and causes increased cytokine production (Nadigel et al., 2011 NOV 9). Upregulation of TLR expression in response to environmental stimuli has also been demonstrated in neutrophils and platelets. Study by Lindau et al. showed that primary blood neutrophils express functional TLR9 on the cell surface, a pathway that can be triggered when pathogen-derived TLR9 ligands cannot reach the endosome, offering a rescue mechanism for neutrophil activation (Lindau et al., 2013 AUG). Incubation of resting platelets with CpG motifs, showed that platelets, when primed, express TLR9 on their surface prior to signal transduction through TLR9 (Panigrahi et al., 2013).

Genomics: TLR9 Gain of Function Polymorphisms

There are many examples of genetic predisposition leading to TLR9 gain of function. One example is the single nucleotide polymorphism (SNP) of the C allele of rs5743836 (T-1237C), which is associated with immune-mediated disease and with a higher incidence of ICU acquired infection (Chatzi et al., 2018; Ng et al., 2010). T-1237C creates a loop of TLR9/IL-6 signaling amplification, leading to a deregulation in B-cell activation and proliferation upon CpG stimuli (Carvalho et al., 2011 NOV 23). Interestingly TLR9-1237T/C polymorphism is a risk factor for progression of infection to severe sepsis in patients with a male sex predisposition, which was investigated in a pediatric intensive care unit (p 0.014) (Elsherif et al., 2019). Also the SNP rs187084 (T-1486C) of the TLR9 promoter previously being associated with rheumatic disease (Hegazy et al., 2019), cancers and pulmonary tuberculosis (Bharthi et al., 2014) has been suggested to provide relevant risk estimates for the development of sepsis and multiple organ dysfunction in critically ill patients (Chen et al., 2011). A study performed among workers in swine operations furthermore showed that male workers, with polymorphisms of rs187084 in the TLR9 gene, displayed significantly lower lung function than those with wild-type (Gao et al., 2018). Sex differences in TLR9 expression has also been reported in mice, where male mice showed higher expression of TLR9 and higher activation of innate immune system with higher numbers of infiltrating neutrophils upon MCMV viral infection but similar viral load between male and female (Traub et al., 2012). Research performed in HIV patients furthermore showed that TLR9 stimulation by viral CpG DNA

contributes to HIV immunopathogenesis and the TLR9 polymorphisms 1635A/G and 1486C/T being associated with disease progression (Joshi et al., 2019). Differences in adverse outcome of Covid-19 between ethnic groups may also in part result from genetic predisposition. Recently Yuval Tal *et al.* analyzed immune factors influencing racial disparity in Covid-19 mortality rates, which revealed presence of inherent differences in the immune system, which may increase the predisposition of black Americans to a severe cytokine storm (Tal et al., 2020). The authors detected elevated expression of markers of innate immunity, including TLR7 and TLR9, and concluded therefor that black individuals would be more prone to develop a rapid and more aggressive cytokine storm.

COVID-19 Clinical Pathophysiology

Pulmonary Pathology

The airways as principal site of entry and target of SARS-CoV-2 can become severely affected in patients with COVID-19. In vulnerable patients, COVID-19 leads to the development of severe pneumonia with enhanced neutrophilia and complications including ARDS requiring mechanical ventilation (Guan et al., 2020). Postmortem examination of COVID-19 patients reveals diffuse alveolar damage with severe capillary congestion and variegated findings in lungs (Menter et al., 2020; Chen et al., 2020). Patients with preexisting lung diseases, including COPD and current smokers might be at greater risk of developing severe complications from Covid-19 (Alqahtani et al., 2020). A role for TLR9 activation in non-allergic neutrophilic airway inflammation and airway disease including COPD has been proposed previously (Greene et al., 2005; Mortaz et al., 2009; Mortaz et al., 2010; Knuefermann et al., 2007; Schwartz et al., 1997; Tasaka et al., 2009; Faust et al., 2020). Moreover, there is evidence that TLR9 can contribute to the development and worsening of ARDS and ALI (Tasaka et al., 2009; Faust et al., 2020; Huang et al., 2020). A study performed in 224 critically ill trauma patients showed that high levels of the TLR9 activator, mtDNA, are associated with ARDS and mortality which is stronger in patients with polymorphisms associated with increased expression of TLR9 (Faust et al., 2020). The prognostic value of plasma mtDNA in ARDS has also been shown in a single-center observational study in China, where higher plasma mtDNA levels at day 7 after admission indicated poor outcome of ARDS patients (Huang et al., 2020). In the airways, however the exact role of TLR9 in disease remains controversial (Bezemer et al., 2012). There is also mounting evidence for a protective role of TLR9 activation in the case of allergic asthma and rhinitis (Iwamura and Nakayama, 2008; Kline and Krieg, 2008; Gupta and Agrawal, 2010). This aligns with the interesting finding that, against odds, asthmatics, seems to be underrepresented among patients suffering from severe COVID-19 of which the current understanding is still in its early stages (Liu et al., 2020). Medication use such as inhaled corticosteroids (ICS) could potentially modify the risk of developing COVID-19 or the clinical course of COVID-19, but at present time there is no robust evidence of such conclusion (Demircan et al., 2000; Celebioglu, 2020; Maes et al., 2020). Reduced expression of ACE2 and transmembrane protease serine 2 (TMPRS2) resulting from ICS use is a potential explanation that has been put forward for understanding the individual difference in susceptibility of severe disease outcome from COVID-19 between asthma patients (Demircan et al., 2000). By other groups of researchers the question arises whether asthma is actually protective against COVID-19 and "work in progress" suggests that a Th2-skewed immunity may be protective against severe COVID-19 disease (Carli et al., 2020). Allergic asthma is a lung disease with a typical Th2 mediated eosinic inflammation whereas COVID-19 presents low level of eosinophils and it is even reported that blood eosinophils decrease during SARS-CoV-2 infections (Lu and Wang, 2020; Sun et al., 2020 Aug). Based on the TLR9 COVID-19 hypothesis, it is proposed that TLR9 mediated combat against COVID-19, as an accompanying effect could result in the sequestration of eosinophils. There is a large body of work showing that TLR9 agonists reduce eosinophilic inflammation and this approach has reached phase 2 clinical testing in human (Iwamura and Nakayama, 2008; Kline and Krieg, 2008; Gupta and Agrawal, 2010). CpG-ODNs effectiveness in the control of allergic responses can be explained by the TLR9 induced T helper 1 (Th1) response that in turn can prevent or reprogram the typical allergic Th2 polarization of the immune system (Chu et al., 1997; Krieg, 2002; Krieg, 2002; Kline et al., 2002). In this context TLR9 has been shown to induce regulatory T cells (Tregs) as well which could potentially contribute to beneficial immunosuppression in allergic asthmatic patients (Ehrlich et al., 2017; Moseman et al., 2004; Kim et al., 2016;), but also provide immune escape opportunity for SARS-CoV-2. Recent data presented by Grifoni et al. show a predominant representation of a classic Th1 response to SARS-CoV-2 with little to no Th2 cytokines (Grifoni et al., 2020).

Thrombotic Complications

Evidence is accumulating for a correlation between severe outcome of SARS-CoV-2 infection and abnormal thrombotic complications, vascular damage, dangerous blood clots, and stroke, (Tang et al., 2020; Arachchillage and Laffan, 2020; Guan et al., 2020; Menter et al., 2020; Oudkerk et al., 2020; Spiezia et al., 2020; Wang et al., 2020a; Zhou et al., 2020). COVID-19 ARDS patients compared to non-COVID-19 ARDS patients develop significantly more thrombotic complications mainly pulmonary embolisms with significantly different coagulation parameters (Helms et al., 2020). Thrombocytopenia, decreased blood platelet count, at early stage of disease is associated with poor prognosis in COVID-19 patients (Zhao et al., 2020, Yang et al., 2020). The lung-specific entry of SARS-CoV-2 could drive platelets to the lungs as one of the first lines of defense and also explains the presence of megakaryocytes in the lungs of COVID-19 patients (Thachil 2020; Lefrancais et al., 2017; Salamanna, 2020). Platelet activation can occur via multiple signaling pathways of which platelet-TLR9 has been positioned as a connector between oxidative stress, infection and platelet activation (Panigrahi et al., 2013).Of all TLRs, TLR9 is most highly expressed on platelets as analyzed in the Framingham Heart Study sample population (n = 1625) (Koupenova et al., 2015). Moreover this study showed that a high mean BMI, which

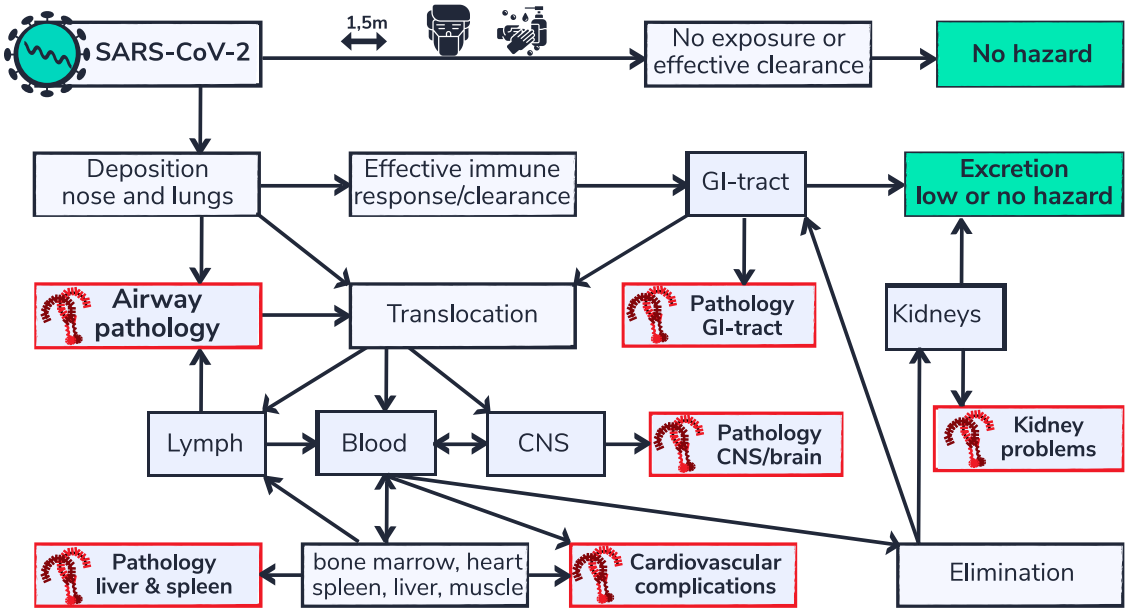

FIGURE 3 | SARS-CoV-2 respiratory deposition and multi-organ complications. Health effects of inhaled substances, including inhaled viruses are influenced by the effectiveness of clearance capability and the routes of elimination. Depicted is a flow chart (adapted from Bezemer, 2009) of body compartments that can be reached via the airways. SARS-CoV-2 is not hazardous for people that are able to avoid exposure or that are able to effectively eliminate the virus from their system. However people that are not able to eliminate the virus or that are vulnerable may develop complications. Organs for which TLR9 mediated pathology is described in literature in non-COVID-19 settings are indicated in red. Regional build-up of SARS-CoV-2 and/or viral RNA, due to inefficient clearance capability in those organs, is proposed to contribute to the typical multi-organ pathology in patients susceptible for TLR9 pathway activation.

is also a major risk factor for COVID-19, is consistently associated with higher TLR expression on platelets. A statistically significant ($p < 0.05$) association with cardiovascular disease measure and TLR9 gene expression was observed in patients that receive lipid treatment (Koupenova et al., 2015). TLR9 can shift the balance of a key initiator of coagulation, called tissue factor and tissue factor pathway inhibitor toward the procoagulant phenotype in human coronary artery endothelial cells and activated blood coagulation in mice (El Kebir et al., 2015). Also functional TLR9 signaling in neutrophils is a mechanism in early stasis experimental venous thrombogenesis (El- Sayed et al., 2016). Neutrophil extracellular traps (NETs) are part of the innate immune response to infections, can form a scaffold and stimulus for platelet adhesion and thrombus formation (Fuchs et al., 2010). NETs have been proposed to contribute to organ damage and mortality in COVID-19 (Barnes et al., 2020). mtDNA is a potent inducer of NETs that activates PMN via TLR9 and formation of mtDNA-induced NETs can completely be blocked by a TLR9 antagonist (Itagaki et al., 2015).

Multi-Organ Dysfunction

Besides lung pathology and thrombotic complications, post mortum case-series show COVID-19-related pathological changes in various organs including liver, kidney, spleen, muscles and brain (Tabary et al., 2020). SARS-CoV-2 can reach from brain to toes and uncertainty over whether it is the virus itself or the response by a person's immune system makes it hard for doctors to decide on appropriate treatment (Ledford, 2020). The hazard of inhaled substances is influenced by regional deposition sites within the respiratory tract; the effectiveness of the hosts clearance capability and translocation routes to other organs (Bezemer, 2009). The airways as primary site of SARS-CoV-2 infection, facilitates the virus and viral residue components to translocate to multiple organs within the body, which could in part explain the multi-organ complications that are seen in COVID-19 patients (**figure 3**). Translocation of intact SARS-CoV-2 to other body compartments could give rise to localized increase of viral load because ACE2, identified as key point of entrance of SARS-CoV-2 into the host cell, is widely expressed in tissues including oral and nasal mucosa, nasopharynx, lung, stomach, small intestine, colon, skin, lymph nodes, thymus, bone marrow, spleen, liver, kidney, and brain (Hamming et al., 2004). High expression of ACE2 in the human olfactory epithelium relative to upper airway epithelial cells may explain why COVID-19 is associated with loss of smell and suggest a potential entry point of SARS-CoV-2 into the central nervous system causing neurological symptoms in COVID-19 patients (Chen et al., 2020; Mao et al., 2020). The potential contribution of the nose-brain-barrier and blood-brain-barrier, to brain pathology caused by inhaled hazardous compounds has been described previously (Bezemer, 2009; Oberdörster and Utell, 2002; Tjalve et al., 1996). Dating back 1941, Bodian and Howe showed that a virus is able to move along

the axons of neurons (Bodian and Howe, 1941).When they instilled the virus of poliomyelitis in the nose of monkeys, paralytic poliomyelitis resulted only when the olfactory connections were intact. Bovine herpesvirus 5 infection, associated with fatal neurological disease in cattle, invades the CNS mainly via the olfactory pathway and has been associated with overexpression of TLR3, 7 and 9. Mann et al. found a significant increase in the expression of TLRs 3 and 7–9 in the anterior cerebral cortex during acute infection and viral reactivation. In the trigeminal ganglia, only TLR9 expression was significantly affected (Mann et al., 2014). Butchi et al. show that TLRs have differing effects in modulating viral pathogenesis and in direct toxicity in the central nervous system (Butchi et al., 2011). They show that intracerebroventricular inoculation of a TLR9 stimulant induces a more robust neuroinflammation with higher levels of proinflammatory cytokines and chemokines produced by plexus cells that did stimulation of TLR7. The TLR9 mediated increase in cytokines and chemokines correlated with breakdown of the blood-cerebrospinal fluid barrier and recruitment of peripheral cells to the CNS(Butchi et al., 2011). Based on the TLR9-COVID-19 hypothesis it is speculated that if SARS-CoV-2 and/or viral RNA could indeed translocate and accumulate in the CNS it may provoke localized immune responses via TLR9 potentially controllable via TLR9 immune modulators. TLRs, owing presence and having an immune-regulatory role within the brain are identified as attractive therapeutic target for numerous CNS disorders and infectious diseases (Hanke and Kielian, 2011). Similar to the high TLR9 expression in the brain, TLR9 is also highly expressed in skeletal muscle tissue (Nishimura and Naito, 2005). Based on the TLR9 COVID-19 hypothesis it is proposed that TLR9 could also play a role in the observed muscle weakness in COVID-19 patients. TLRs, including TLR9 also play an important role in many if not all types of renal inflammation (Anders et al., 2004). TLR9 via expression on renal infiltrating antigen presenting cells during immune injury have been reported to be involved in antigen-induced immune complex glomerulonephritis, renal vasculitis and lupus nephritis (Anders et al., 2004). Studies performed in experimental models for polymicrobial sepsis show that circulating mtDNA via activation of TLR9, contributes to cytokine production, kidney injury during and splenic apoptosis (Tsuji et al., 2016). Other experimental studies furthermore show that TLR9 is an important mediator of hepatic injury secondary to ischemic acute kidney injury (Bakker et al., 2015). Inhibition of TLR9 in mice attenuates sepsis induced mortality and provides dampening of dysregulated inflammatory markers in spleen, lung and liver (Hu et al., 2015).

Epidemiology: Riskfactors/Comorbidities Associated with Overweight and Obesity

Early epidemiological data revealed that SARS-CoV-2 is more likely to affect older males with comorbidities, and can result in severe and even fatal respiratory diseases such as ARDS and multiple organ failure (Chen et al., 2020). Reported comorbidities in infected patients that require hospital admission include cardiovascular disease/heart disease, diabetis mellitus, chronic respiratory disease, hypertension and cancer (Butchi et al., 2011; Arumugam et al.,; Huang et al., 2020; Wu and McGoogan, 2020 APR 7). Obesity, has been positioned as common denominator of impaired metabolic health, respiratory dysfunction, cardiovascular disease and diabetes mellitus in the severe course of COVID-19 (Stefan et al., 2020). Preliminary investigations show that people with obesity are at increased risk of severe COVID-19 (Goyal et al., 2020; Halasz et al., 2020; Stefan et al., 2020). The exact mechanisms through which obesity exacerbates COVID-19 infection are not fully clarified. The association of obesity with immune and metabolic derangement is one explaining suggestion for the link to adverse clinical outcomes in COVID-19 (Korakas et al., 2020). Studies in mice show that obesity induced by high fat diet or leptin deficiency result in overexpression of TLRs and related proinflammatory signaling molecules in enlarged adipose tissues, which may play an important role in the obesity-associated phenomenon of meta-inflammation (Kim et al., 2012). A high fat diet increases TLR9 expression in visceral adipose tissue in mice (Nishimoto et al., 2016 MAR). TLR9 expression is also significantly increased in visceral compared to subcutaneous adipose tissue depots in obese patients (Thomalla et al., 2019). The function of TLR9 in adipose tissue inflammation remains controversial. On the one hand it has been suggested that TLR9 may protect against obesity and the metabolic syndrome having an anti-inflammatory effect (Hong et al., 2015; Thomalla et al., 2019). On the other hand it has also been shown that obesity induced single stranded DNA (ssDNA), released from adipocytes stimulate chronic adipose tissue inflammation and insulin resistance via TLR9 (Nishimoto et al., 2016 MAR). Additionally the study from Nishimoto showed that plasma concentration of ssDNA was significantly higher in patients with visceral obesity compared to patients without visceral obesity and ssDNA was positively correlated with visceral fat area (Nishimoto et al., 2016 MAR). Ghosh et al. proposed a role for TLR9 in the activation of plasmacytoid dendritic cell fueling obesity induced chronic low-grade inflammation, so called meta-inflammation (Ghosh et al., 2016). Revelo et al. provided data on TLR9 pathway involvement in promoting obesity related inflammation of metabolic tissues including visceral adipose tissue and liver. In mice a high fat (HFD) diet induces excess of nucleic acids and related protein antigens which worsens metabolic inflammation through activation of VAT macrophages and expansion of plasmacytoid dendritic cells (pDCs) in the liver (Revelo et al., 2016). The study of Revelo furthermore confirmed that HFD-fed mice lacking TLR9, show reduced metabolic inflammation and treatment of HFD-fed mice with a TLR7/9 antagonist improved metabolic disease. A more recent study from Yuzefovych et al., showed that plasma mtDNA is elevated in obese type 2 diabetes mellitus patients and is associated with oxidative stress in skeletal muscle and correlates with insulin resistance (Yuzefovych et al., 2019). TLR9 message and protein expression levels which are higher in diabetic wounds compared to control wounds have been linked to impaired wound healing in type 2 diabetes mellitus (T2DM) cases via the induction of pro-inflammatory S100A8 and IL-8 (Singh et al, 2016). The *TLR9-1237 T/C* gene polymorphism is

considered as a molecular risk for diabetic foot among patients with T2DM (Wifi et al., 2017).

Investigational Treatment Approaches of COVID-19

Chloroquine and Hydroxychloroquine

Chloroquine and Hydroxychloroquine are medications approved for prevention and treatment of malaria with a reputation of being effective and relatively safe for treatment of systemic lupus erythematosus and mild to moderate rheumatoid arthritis because of immune suppressive properties (Rainsford et al., 2015). Chloroquine is a well-known, however not specific, inhibitor of endosomal TLRs, including TLR9 (Kuznik et al., 2011). Chloroquine and Hydroxychloroquine have been shown to inhibit SARS-CoV-2 *in vitro* and it is speculated to be effective for patients with COVID-19, although until now no single study shows any validated and proven clinical benefit (Sanders et al., 2020; Wang et al., 2020b). Also, the exact mechanism by which (Hydroxy)Chloroquine is believed to relief infection by a coronavirus remains unclear. Suggestions for (Hydroxy) Chloroquine mechanism of action include alteration of the acidic environment inside lysosomes and late endosomes, preventing endocytosis, exosome release and phagolysosomal fusion, and inhibition of the host cytokine storm (Tripathy et al., 2020). Concerns exist about using off-Label drugs for COVID-19 including Chloroquine and Hydroxychloroquine, because of the recognized side effects: QT prolongation, torsades de pointes, hepatitis, acute pancreatitis, neutropenia, anaphylaxis and increased risk of cardiac death (Kalil, 2020). Applying reverse thinking moving back from bedside to bench, it could be speculated that the TLR route, including TLR9, could have contributed to reducing overstimulation of the immune-system in the individual COVID-19 patients that experienced benefit from investigational off-label treatment with (hydroxy) chloroquine. In experimental models, TLR9 signaling is recognized as a major target for the protective actions of Chloroquine in the case of sepsis induced acute kidney injury (Yasuda et al., 2008). From this viewpoint, The specific blocking the TLR9 pathway in vulnerable critically ill COVID-19 patients, might even be a more targeted approach with potentially less side effects than investigational broad-spectrum (hydroxy) chloroquine. But keep in mind that at this point TLR9 modulation is not a treatment recommendation since more (pre)clinical research is needed to investigate the proposed hypothesis.

Mycobacterium w

Early clinical findings pointing toward a role for TLRs including TLR9 in COVID-19 disease pathology arise from a study performed with heat-killed *Mycobacterium* w (Mw) (Sehgal et al., 2020). Mw is a cost-effective immunomodulator approved in India for treatment of leprosy, and is investigated for use as vaccine and treatment option for tuberculosis and for use in autoimmune conditions such as psoriasis and optic neuritis (Sudhalkar et al., 2012). Mw received attention in drug discovery for having both TLR2 and 4 activating as well as TLR inhibiting properties, including inhibition of TLR9 (Belani et al., 2011; Sudhalkar et al., 2012; Anwar et al., 2019). A small scale study in which 4 severely ill COVID-19 patients were treated with heat-killed *Mycobacterium* w (Mw), resulted in successful management, not causing adverse events (Sehgal et al., 2015). A previously performed randomized trial in fifty patients with severe sepsis, showed that the use of Mw was associated with significant reduction in days on mechanical ventilation, ICU and hospital length of stay, lower incidence of nosocomial infection, and delta SOFA score (sequential organ failure assessment) (Sehgal et al., 2015). A randomized clinical trial to further evaluate the safety and efficacy of Mw in critically ill patients suffering from COVID-19 is currently ongoing (clinicaltrials.gov: NCT04347174). The exact mechanism by which Mw acts in sepsis remains unknown. In addition to the previously reported TLR antagonistic capability it is also suggested that Mw could enhance TLR activity, which might overcome the immune paralysis in severe sepsis (Sehgal et al., 2015).

Vitamin D

During the first wave of Covid-19, low Vitamin D levels have been found in the vulnerable aging population in Spain, Italy and Switzerland which pointed towards the potential of vitamin D in prevention of COVID-19 infection and mortality (Ilie et al., 2020). Vitamin D deficiency has indeed been found to contribute to ARDS and a narrative review on vitamin D shows accumulation of evidence that vitamin D supplementation could reduce risk of COVID-19 infections and deaths (Grant et al., 2020). Vitamin D is known to promote innate immune response against viral infection and a role for TLRs has been proposed in explaining the underlying mechanism. Martinez-Moreno et al showed that innate immune response against the dengue virus (DENV) infection, a public health problem worldwide, can be improved by vitamin D supplementation. Their study showed that an oral supplement of 4000 IU/day of vitamin D3 significantly decreased TLR9 protein levels and the mRNA abundance of TLR3, TLR7, and TLR9 in human. The lower dose of, 1000 IU/day of vitamin D only decreased the TLR9 protein level in human monocte-derived DCs infected with DENV. The finding is especially interesting because TLR9 activation, through mtDNA, contributes to DENV-induced immune activation (Martinez Moreno et al., 2020). A study performed in 2010 also showed that intracellular TLRs are differentially regulated by vitamin D3, with TLR9 being down-regulated by vitamin D3 exposure whereas TLR3 was unaffected (Dickie et al., 2010). The study by Dickie et al showed that vitamin D3 decreased TLR9 expression in monocytes and had a downstream functional effect as these cells subsequently secreted less IL-6 in response to TLR9 challenge.

MULTIFACETED POTENTIAL OF DRUG TARGET TLR9 FOR COVID-19

The novel hypothesis that TLR9 could be associated with COVID-19 pathology in vulnerable patients, positions TLR9 as a multifaceted drug target worth considering for preventing and/or treatment of critical conditions of SARS-CoV-2 infected patients. Both TLR9 activation- and inhibition could be

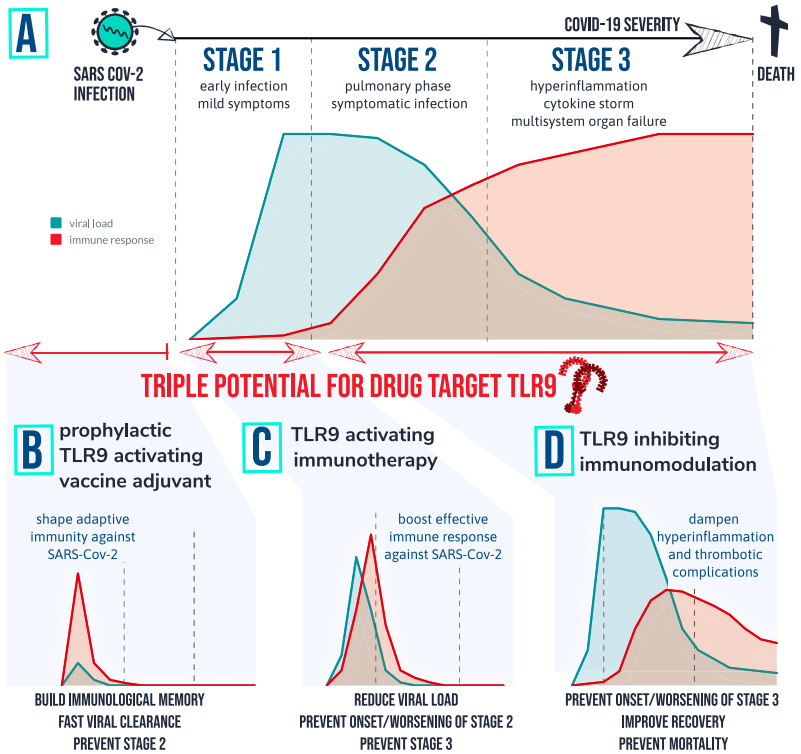

FIGURE 4 | therapeutic implications of the TLR9 COVID-19 hypothesis: Patients that develop severe symptoms of COVID-19 are tend to go through different stages of disease with different characteristics. Graph **(A)** depicts a simplified fictional scenario explaining how an inefficient viral specific immune response at start of infection (stage 1) can result in a high peak of viral load and eventually an exaggerated inflammatory response causing symptomatic infection (stage 2). When the virus remains active and/or the host immune system remains active over prolonged period of time severe complications can occur requiring ICU (stage 3) and in worst case result in death. Based on the TLR9 COVID-19 hypothesis, 3 therapeutic strategies are worthwhile investigating for following desired actions: **(B)** shape adaptive immunity against SARS-Cov-2 so that viral load remains low; **(C)** Provide a short targeted immune boost to help clearing the virus efficiently, and **(D)** Inhibit TLR9 pathway in vulnerable patients to prevent or dampen hyperinflammation and multi-organ complications.

relevant to produce opposing therapeutic effects at the different stages of disease (**Figure 4**). Prophylactic potential of TLR9 activation as vaccine adjuvant to shape adaptive immunity against SARS-Cov-2 is currently being investigated in clinical trials (Oberemok et al., 2020). This would ideally result in immunological memory to aid fast viral clearance thereby preventing severe symptomatic infection and virus induced damage. Also in the early infection stage, prior to complications it could be imagined that activation of TLR pathways including TLR9 could aid in fast and effective viral clearance especially in immunocompromised patients. In COVID-19 it seems that viral burden typically peaks early in illness, potentially even before symptoms of pneumonia and then declines as antibodies develop and antibody titers rise over the subsequent 2 to 3 weeks (Kim et al., 2020; To et al., 2020; Woelfel et al., 2020; Zou et al., 2020). Activation of TLR9 in this early window of disease would ideally result in improved viral combat thereby preventing or shortening of symptomatic infection and prevention of overwhelming viral illness and tissue damaging inflammation. The FDA approved an investigation into the efficacy of an inhalational broad acting TLR2/6/9 agonist, PUL-042 to reduce the severity of COVID-19 in adults positive for SARS-CoV-2 infection (Schijns and Lavelle, 2020). It should be noted that stimulation of other TLRs in this early window of infection could have similar therapeutic value in immunocompromised patients. Imiquimod, for instance is an activator of TLR7 and has been proposed to enhance the innate and adaptive immunity in early stage COVID-19 patients (Angelopoulou et al., 2020). Also other non-viral specific TLRs such as TLR5 which is activated by bacterial Flagellin has been proposed for vaccine or adjuvant development to generate protective innate immunity against SARS-CoV-2 (Chakraborty et al., 2020). In contrast to the numerous potential valuable TLR agonists, it is proposed that TLR9 could be considered as particular interesting target of inhibition because of the lack of CpG suppression in unique to SARS-CoV-2 regions which could be of specific concern in vulnerable patients that experience difficulties to clear the virus and that have more than normal TLR9 expression and/or more than normal synergistically TLR9 triggers present. TLR9 inhibition could thus be a strategy worth considering for treatment of the specific COVID-19 patients that are at risk for developing severe symptomatic infection and further complicated clinical course due to underlying TLR9 skewing vulnerabilities. Risk factors mentioned in this

hypothesis paper include (pre)existing thrombotic activation, chronic neutrophilic lung disease, presence of coinfections, high levels of visceral fat, high levels of circulating mtDNA levels, TLR7 loss of function gene polymorphisms and TLR9 gain of function gene polymorphisms. Taken together, the relatively high numbers of CpG-motifs in SARS-CoV2 and the upstream position of TLR9 in the inflammatory cascades and the broad expression of TLR9 on different cell types that play crucial roles in clinical COVID-19 presentation (Th1 cells, Th17 cells, B cells, neutrophils, platelets), TLR9 is positioned be a promising systemic therapeutic target to dampen or perhaps even prevent the thrombotic complications and so called cytokine storm or hyperinflammatory syndrome in certain specific patients that are suffering from severe COVID-19. Dampening of cytokine storm has evident potential for preventing the onset or worsening of ARDS and multisystem organ failure and ideally aid improved and shortened time for recovery, prevention of death and reducing post-ICU complications (Ragab et al., 2020; Ye et al., 2020). For any immunomodulating treatment concept it is however important to determine proper alignment with individual qualitative and quantitative factors of pathogen and host immune interactions. For instance immunosuppressive approaches to reduce hyperinflammation in COVID-19 may lead to unwanted impairment of anti-microbial immunity (Ritchie and Singanayagam, 2020). Moreover TLR inhibition may drive compensatory changes in other TLRs. For instance blocking of TLR7 and TLR8 which is currently being invested in a phase II trial could potentially pose risk to the specific patients that are already skewed toward TLR9 activation. Likewise blocking of TLR9 in patients that do not experience overstimulation of TLR9 may result in loss of an important innate immune signaling pathway that is needed to combat the virus. To prevent risk of viral flare up due toTLR9 antagonistic activity, the antagonist could be tested in combination with Remdesivir and other investigational antivirals. Vice versa excessive activation of a specific immune response for purposes of viral clearance via activation of TLRs, including TLR9 could contribute to hyperinflammation and thrombotic complications in susceptible patients and could therefore be followed up by immunosuppressants in patients that experience complications. This impediment thus asks for a good understanding of individual characteristics that relate to the TLR drug targets.

PREDICTIVE MARKERS FOR INDIVIDUALS VULNERABLE FOR SEVERE COVID-19

Viral load and viral RNA levels are relevant predictive parameters for disease. Viral load of SARS-CoV-2 detected from the respiratory tract of COVID-19 patients seems positively linked to biochemical indexes and disease severity (Liu et al., 2020). Studies have indicated that the highest viral load in throat swabs can be detected at the time of symptom onset (He et al., 2020). Upon resolution of symptoms, viral RNA levels may remain positive for more than 2 weeks in upper respiratory tract specimens (nasopharyngeal swab and/or an oropharyngeal swab) which is however not necessarily associated with disease severity but may result from a weaker immune response instead (Carmo et al., 2020). The underlying individual factors influencing viral combat capability and viral clearance are likely diverse, therefore challenging to encompass for early predictive purposes. An example of poor viral clearance capability due to a less robust immune response can be found in the association between older age and greater risk of development of ARDS and death from COVID-19 (Wu et al., 2020). Also very specific individual characteristics may contribute to poor viral defense. An example arises from a recent preliminary communication, in which a case series study presented that genetic variants leading to TLR7 loss of function were present in 4 young male COVID-19 patients, all previously healthy with unsuspected severe complications of COVID-19 of which 1 patients died. Besides older age and poor TLR7 function, there could be many more dysfunctional steps in the immune response that could drive high viral load, which goes beyond the scope of this hypothesis paper. Literature covering a more broad perspective of immunological aspects of COVID-19 is available (Felsenstein et al., 2020; Jensen and Thomsen, 2012; Li et al., 2020; Tay et al., 2020; Birra et al., 2020; Ragab et al., 2020). The TLR9 COVID-19 hypothesis proposes that combining measures of viral load and viral RNA with markers for TLR9 susceptibility, would provide a more precise identification of some people at risk, feed into better prevention strategies for those patients and give rationale for more targeted treatment options via modulation of TLR9. In this theory paper we discussed genetic markers including: ZAP, C allele of rs5743836 (T-1237C) in TLR9, -1486 T/C (SNP) rs187084 (T-1486C), 1635A/G and 1486C/T. Mentioned were also life style factors such as high fat diet and cigarette smoke exposure, that can increase TLR9 expression levels. Moreover we discussed the presence of measurable synergistically acting TLR9 triggers originating from other pathogen and from the host. The TLR9 COVID-19 hypothesis proposes to investigate increased levels of mtDNA and ssDN as biomarkers for COVID-19 vulnerability.

Recommendations

The TLR9 COVID-19 hypothesis is testable within the framework of current knowledge. TLR9 expression levels in response to SARS-CoV-2 can be analyzed in an *in vitro* model system such as used by Ng et al. for investigating genome-wide host response to SARS coronavirus (Ng et al., 2004). Another appropriate approach is to analyze variations in TLR9 expression levels in relevant patient samples such as sputum and/or lung lavage samples from patients with COVID-19 and in affected tissue biopsies from patients that died from severe COVID-19. Animal knockout models could give further insight in the requirement of TLR9 for SARS-Cov-2 induced pulmonary and thrombotic complications, cytokine storm and multi-organ dysfunction. An advantage under the current global emergency circumstances related to COVID-19 is that research groups and pharmaceutical companies showed long lasting interest in immunomodulating agents that engage the TLR9 pathway. There is a large body of preclinical data and early human

clinical trial results showing the safety and therapeutic potential of TLR9 modulating compounds to improve vaccines and treat cancer, infectious disease, allergy/asthma, autoimmune disorders (Anwar et al., 2019; Krieg, 2006; Bezemer et al., 2012; Gupta and Cooper, 2008). Prior art that covers safety profiles, dosing, pharmacokinetics, pharmacodynamics could help the repurposing of drug-leads and speed up the drug development process of TLR9 targeting drug candidates for COVID-19. Model systems, including TLR reporter assays and other cell- and tissue-based systems could allow fast screening of available TLR9 modulating lead compounds having the biological effects that are desired in COVID-19 as mentioned in **Figure 4**. For successful translation from bench to bedside, also a deeper understanding of the spatiotemporal kinetics of viral load and specific host factors is a recommended approach for identification of patients at risk that are most likely to benefit from treatment at defined stages of disease. Conclusions on the relevance of TLR9 as drug target and as predictive marker for identification of people at risk could be drawn from large scale, real world screening of COVID-19 disease severity in relation to the combined measures of A) viral load and SARS-CoV-2 RNA, B) Endogenous and exogenous cell free DNA including mtDNA and ssDNA from visceral fat and DNA from other pathogens, and C) TLR9 polymorphisms and TLR9 expression levels. If the TLR9 COVID-19 hypothesis can be further justified, well-controlled clinical trials to study safety and efficacy of TLR9 modulating drug leads for treatment and/or prevention of disease caused by a coronavirus are warranted. It would also be recommended to evaluate the effect of TLR9 antagonists in combination with Remdesivir or other investigational antivirals on recovery time and mortality rates in adults that are hospitalized with COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

GB formulated the TLR9 COVID-19 hypothesis, drafted the manuscript and figures, and revised the final form. JG provided key insights for manuscript revision. Both authors contributed to the article and approved the submitted version.

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OPINION
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Statins as Adjuvant Therapy for COVID-19 to Calm the Stormy Immunothrombosis and Beyond

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INTRODUCTION

Cytokine storm is a severe immune response that can be triggered by the coronavirus SARS-CoV-2 infection in susceptible patients, and, in the severe form of the COVID-19 disease, it is potentially lethal because of its systemic immunothrombogenic sequelae (Cron, 2020). The cytokine storm consists of excessive macrophage activation, haemophagocytic lymphohistiocytosis, and a syndrome characterized by excessive release of proinflammatory cytokines (Henderson et al., 2020). Clinical trials are underway to investigate, besides anti-viral agents, the use of appropriate immunosuppressive and immunomodulatory drugs, and also specific drugs to target individual pro-inflammatory cytokines (Soy et al., 2020). Since we still lack efficient means for managing the cytokine storm, there is a need for drugs that can potentially mitigate some of the downstream effects of the potentially deadly immune response. Toward this end, the widely used statins may be considered as adjuvant drugs in the treatment of severe COVID-19. The statins may ameliorate, at least partially, some components of the cytokine storm and its sequelae, which are related to poor prognosis in COVID-19 patients.

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PREDICTORS OF IN-HOSPITAL MORTALITY IN COVID-19 PATIENTS

The strongest predictors of in-hospital mortality in COVID-19 patients include elevated interleukin -6 (IL-6) and D-dimer levels at hospital admission (Cummings et al., 2020; Nadkarni et al., 2020). Indeed, a recent systematic review and meta-analysis showed that serum D-dimer concentrations in patients with severe COVID-19 are significantly higher when compared to those with non-severe forms (Paliogiannis et al., 2020). Also, increased levels of fibrinogen (Terpos et al., 2020) and IL-1 (Shakoory et al., 2016), and an elevated neutrophil-to-lymphocyte ratio (NLR) have been associated with poor prognosis in COVID-19 patients (Ciccullo et al., 2020).

The level of IL-6 has previously been used as a biomarker of viral virulence (Velazquez-Salinas et al., 2019). IL-6 possesses marked proinflammatory properties (Moore and June, 2020) and it is possible that IL-6 blockade, for example with the immunosuppressive drug tocilizumab (Rose-John et al., 2017; Quartuccio et al., 2020), may have the potential to reduce viral virulence. In a very recent study, it was found that in hospitalized patients with COVID-19 tocilizumab treatment was associated with fewer serious infections; yet, it was not effective for preventing intubation or death in the moderately ill COVID-19 patients studied (Stone et al., 2020).

High levels of circulating D-dimers have been associated with an elevated risk of thrombosis (Leonard-Lorant et al., 2020; Mucha et al., 2020). It is important to note that some COVID-19 patients have underlying diseases that may increase the risk for bleeding, and thus caution should be

exercised during anticoagulation (Wang et al., 2020). In COVID-19 patients, the pathophysiology of thrombosis is largely driven by the infectious immunoinflammatory process that occurs systemically in veins, arteries, and the microvasculature of vital organs, such as the lungs, kidneys, heart, and brain (Siddiqi et al., 2020). In the vascular system, the endothelium is the primary target of the immune-inflammatory attack, and, when attacked, the endothelial cells tend to lose their antithrombotic properties (Belen-Apak and Sarıalioğlu, 2020; Libby and Lüscher 2020). The endothelial cells can also be infected with the coronavirus. Such infectious endothelial damage, termed endotheliitis, has been observed both in the myocardial and cerebral vessels, and due to the ensuing local thrombus formation has led to severe ischemic syndromes (Crippa et al., 2020; Mosleh et al., 2020; Varga et al., 2020).

D-DIMERS, FIBRINOGEN, AND THE ENDOTHELIUM

D-dimers are fibrin degradation fragments that are generated upon fibrinolysis of a blood clot, i.e., their presence in the blood reflects coagulation activation with ensuing formation of blood clots and the accompanying clot degradation by fibrinolysis. Then, understandably, the magnitude of an increased D-dimer level discloses the extent of thrombus formation and also predicts the clinical severity of any thrombotic complication associated with a disease, such as deep vein thrombosis (Andreescu et al., 2002). In a study of patients with suspected pulmonary embolism (156 statin users and 147 antiplatelet drug users), statin use was associated with a modest 15% decrease in D-dimer levels (95% confidence interval [CI] −28 to −0.6%) whereas the use an antiplatelet drug (mainly acetylsalicylic acid and clopidogrel) had no significant effect (Schol-Gelok et al., 2018). Similarly, in a cohort study including 6,814 male and female subjects without cardiovascular disease (aged 45 to 84 years), the D-dimer levels were found to be 9% lower in statin users than in non-users (Adams et al., 2013).

Statin use does not seem to affect fibrinogen levels (Dujovne, et al., 2000; Sbarouni et al., 2000). However, treatment with statins can lead to a significant downregulation of the blood coagulation cascade as a result of decreased tissue factor expression, which, again, leads to reduced thrombin generation and attenuation of procoagulant reactions catalyzed by thrombin, such as fibrinogen cleavage (Undas et al., 2005; Undas et al., 2014). Since in the patients with COVID-19, the endothelial cells are the target of the cytokine storm and may also become infected by the virus, the dysfunctional endothelial cells lose their antithrombotic surface properties (Libby and Lüscher 2020). Indeed, the endothelial dysfunction with ensuing organ hypoxia may be the hardest challenge regarding the cardiovascular consequences in COVID-19 patients. On the other hand, the ability of statins to improve endothelial function (Masoura et al., 2011) could at least partially ameliorate the prothrombotic state of the endothelium.

INTERLEUKIN-6 AND INTERLEUKIN-1

The anti-inflammatory effects of statins have been studied using a cytokine-mediated interaction model of human vascular smooth muscle cells and mononuclear cells in culture (Loppnow et al., 2011). In this cell culture study, simvastatin, atorvastatin, fluvastatin, and pravastatin reduced IL-6 production by 53, 50, 64, and 60%, respectively. This finding suggests that, if translatable for *in vivo* applications, statins may be able to reduce the pro-inflammatory effects of IL-6 in tissues. However, no clinically significant reduction in the concentration of circulating IL-6 has been observed among statin users (Wiklund et al., 2002; Lyngdoh et al., 2011). Concerning IL-1, simvastatin use is associated with a decreased concentration of IL-1β in gingival crevicular fluid in patients with inflammatory periodontal disease (Cicek et al., 2016). The promising role of statins as inhibitors of IL-1β synthesis and release warrants further investigation (Liberale et al., 2019). Among the strategies to inhibit the effects of cytokines, blocking the IL-1 receptor has been particularly beneficial, as shown in a controlled study in sepsis patients with the macrophage activation syndrome (Shakoory et al., 2016).

NEUTROPHIL—LYMPHOCYTE RATIO

In the Danish General Suburban Population Study, inflammatory markers were analyzed in 2,922 statin users and 16,873 non-users (Sørensen et al., 2019). In this study, the neutrophil-lymphocyte ratio was reduced by 3% among statin users (95% CI 1 to 5%, $p = 0.003$). In an earlier study, initiation of statin treatment did not affect the neutrophil-lymphocyte ratio in hypercholesterolemic patients (Gungoren et al., 2016). Accordingly, we can state that, based on the available data, statins appear to have no or only a modest effect on the neutrophil-lymphocyte ratio.

OTHER VIRAL INFECTIONS

In a study of 3,043 hospitalized laboratory-confirmed influenza patients of whom one-third received statin treatment, the authors assessed the effect of statin administration before or during hospitalization using a multivariable logistic regression model (Vandermeer et al., 2012). In the cited study, an adjustment was made for age and race, as well as for cardiovascular, pulmonary, and renal disease, and influenza vaccination, and the authors found that statin consumption before or during hospitalization decreased the risk of death (adjusted odds ratio 0.59; 95% CI 0.38 to 0.92). A beneficial effect of statins has also been suggested for patients with Middle Eastern respiratory syndrome (MERS), a viral illness also caused by a coronavirus (Yuan, 2015).

COVID-19 AND STATINS

Analysis of in-hospital deaths among 8910 COVID-19 patients from Asia, Europe, and North America revealed that statin use

was associated with a favorable prognosis (Mehra et al., 2020). In a recent retrospective study among 154 COVID-19 patients in nursing homes in Belgium, De Spiegeleer et al. found a significant positive association between statin use and the absence of symptoms (OR 2.91; CI 1.27 to 6.71, $p = 0.011$), and the result remained significant after adjustment for age, sex, functional status, diabetes mellitus, and hypertension (De Spiegeleer et al., 2020). However, in this study, the effects of statin use on serious clinical outcomes did not reach statistical significance. The authors concluded that statins may be associated with a beneficial effect on COVID-19-related symptoms in old and frail persons and suggested that a potentially favorable interaction between statins and the drugs regulating the renin-angiotensin system should be further investigated. Such interaction may emerge, as in COVID-19 patients the use of either an angiotensin-converting enzyme inhibitor or a statin was associated with a lower risk of in-hospital death when compared with COVID-19 patients who did not use either class of drugs (Fedson et al., 2020; Mehra et al., 2020). To note, statin therapy has been demonstrated to associate with significant improvement in both peripheral and coronary endothelial function (Reriani et al., 2011). This seminal clinical observation helps us to understand the benefit of statin use under conditions of endothelial stress, such as occurs during infection.

Two meta-analyses on the association between statin use and COVID-19 have been published recently. In the smaller meta-analysis, association between statin use and in-hospital outcomes of COVID-19 was analyzed until August 1, 2020 by systematically searching the Google Scholar database (Hariyanto and Karniawan 2020). A total of nine studies with a total of 3,449 patients were included in the analysis. This meta-analysis showed that statin use did not improve the severity outcome (OR = 1.64; 95% CI 0.51–5.23) or the mortality rate from COVID-19 (OR = 0.78; 95% CI 0.50–1.21). Thus, no statin-dependent benefit could be demonstrated. Also, the larger meta-analysis has been carried out among hospitalized COVID-19 patients (Kow & Hasan, 2020). In this comprehensive meta-analysis, the risk of severe illness and/or mortality in COVID-19 among statin users was compared to non-statin users (total number of patients 8,990). The authors searched PubMed, Google Scholar, and medRxiv (preprint repository) databases up to July 27, 2020, and the pooled analysis revealed that among the COVID-19 patients with statin treatment, not only the severity of the illness but also the mortality was significantly reduced (HR = 0.70; 95% CI 0.53–0.94).

DISCUSSION

Increasing evidence supports the use of statins in patients with COVID-19 (Bifulco and Gazzerro, 2020; Castiglione et al., 2020; Dashti-Khavidaki and Kahlili, 2020). Accordingly, the National Institutes of Health COVID-19 Treatment Guidelines recommend that patients with COVID-19 who are prescribed statins for the treatment or prevention of cardiovascular disease should continue statin therapy (National Institutes of Health, 2020). Statins are generally safe and are cost-effective; yet, this class of drugs is underused (Chen et al., 2019). Importantly, a recent retrospective analysis of SARS-CoV-2 infection related mortality in hospitalized patients with COVID-19 not only confirmed the beneficial background of statin therapy but also revealed that maintenance of statin therapy during hospitalization correlated with an even better prognosis (Masana et al., 2020). Based on the currently available data, we consider that a patient with diagnosed COVID-19 should continue statin use as prescribed; in addition, the short- and long-term adherence to and persistence with statin therapy should be improved in patients with a low level of adherence. Moreover, if a statin-naïve adult patient with cardiovascular disease risk factors fulfills the criteria for statin therapy, the diagnosis of COVID-19 should act as an additional trigger for immediate initiation of statin therapy. Even, if the statin-naïve COVID-19 patient who may not fulfill all the criteria for statin treatment according to present guidelines (Cholesterol Treatment Trialists' (CTT) Collaborators, 2012), initiation of therapy should nevertheless be considered, as the endothelial-damaging action of the viral infection increases the risk of thrombotic complications, while statins tend to improve the function of the endothelium under stress (Reriani et al., 2011). Suitable candidates for statin therapy are middle-aged COVID-19 patients in particular, since many of them may have subclinical coronary atherosclerosis at LDL-cholesterol levels currently considered normal and even in the absence of other cardiovascular disease risk factors (Fernández-Friera et al., 2017; López-Melgar et al., 2020).

Another important reason for initiating permanent statin treatment is that the development of atherosclerotic lesions is accelerated during infection and inflammation (Mehta et al., 1998), and it is likely that COVID-19 triggers a sustained increase in the risk of cardiovascular disease, at least in patients with genetically elevated plasma cholesterol levels (Vuorio et al., 2020). It was recently estimated that approximately 5% of COVID-19 patients will experience an acute ischaemic stroke, and those with multiple-organ dysfunctions are at even higher risk of an acute stroke (Qureshi et al., 2020). It is noteworthy that the safety of statins has been shown in children with familial hypercholesterolemia aged 8 years and above (Vuorio et al., 2019). In children aged 3 to 17 years with H1N1 influenza virus infection and severe clinical manifestations of the infection, IL-1β and IL-6 plasma levels were significantly upregulated when compared to children with H1N1 and mild symptoms (Chiaretti et al., 2013).

Numerous mechanisms have been proposed to underlie the favorable effects of statins in COVID-19. One such mechanism is their mild anticoagulant effect with a potential to decrease the risk of thrombus formation in the veins, arteries, and microvessels (Undas et al., 2014), which individually or jointly are considered the primary causes of the frequently fatal respiratory and cardiovascular failures in COVID-19 patients. COVID-19 may trigger a sustained accelerated progression of atherosclerosis during the recovery phase and beyond, emphasizing the importance of the continual use of statins (Vuorio et al., 2020). There is an urgent need to collect data related to the cardiometabolic and the immunothrombotic status of hospitalized patients with COVID-19 who have received or have not received statin therapy. Analysis of such information

will enable us to test the hypothesis that the statin drugs alleviate the macrovascular cardiovascular disease and its atherothrombotic complications (acute myocardial infarction and ischemic stroke) in COVID-19 patients. Such data are necessary also for the critical evaluation of the suggested beneficial effects of statins on the immunothrombotic component of COVID-19 caused by the systemic endothelial dysfunction in the entire circulatory system in patients with the illness (Vuorio and Kovanen, 2020). Such a study of the potential beneficial effects of statin treatment before, during, and after the development of the cytokine storm should include COVID-19 patients with and without traditional cardiovascular disease risk factors, notable hypercholesterolemia. Only then will it be possible for us to learn whether the beneficial effects of statins on multiple molecular targets on their pleiotropic and/or their plasma cholesterol-lowering properties. Most importantly, however, such therapeutic strategies should disclose the real value of statins as adjuvant therapy in the prevention and treatment of the stormy immunothrombosis in COVID-19 patients.

AUTHOR CONTRIBUTIONS

AV: writing the first draft; AV and PK: reviewing and editing to produce the final draft (equal contribution).

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Drug Repurposing Screen for Compounds Inhibiting the Cytopathic Effect of SARS-CoV-2

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Drug repurposing is a rapid approach to identify therapeutics for the treatment of emerging infectious diseases such as COVID-19. To address the urgent need for treatment options, we carried out a quantitative high-throughput screen using a SARS-CoV-2 cytopathic assay with a compound collection of 8,810 approved and investigational drugs, mechanism-based bioactive compounds, and natural products. Three hundred and nineteen compounds with anti-SARS-CoV-2 activities were identified and confirmed, including 91 approved drugs and 49 investigational drugs. The anti-SARS-CoV-2 activities of 230 of these confirmed compounds, of which 38 are approved drugs, have not been previously reported. Chlorprothixene, methotrimeprazine, and piperacetazine were the three most potent FDA-approved drugs with anti-SARS-CoV-2 activities. These three compounds have not been previously reported to have anti-SARS-CoV-2 activities, although their antiviral activities against SARS-CoV and Ebola virus have been reported. These results demonstrate that this comprehensive data set is a useful resource for drug repurposing efforts, including design of new drug combinations for clinical trials for SARS-CoV-2.

Keywords: COVID-19, cytopathic effect, drug repurposing and discovery, HTS, SARS-CoV-2

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a global health crisis. As of September 17, 2020, the global case report stands at 30 million, with a death toll of 942,989 (Dong et al., 2020). Only remdesivir, an investigational drug developed for Ebola virus, has been recently approved for treatment of hospitalized COVID-19 patient, though its therapeutic efficacy is mild (Eastman et al., 2020). Since an effective vaccine is currently unavailable for COVID-19, drug repurposing has received significant attention in the rapid search to fill this unmet therapeutic need.

The requirement of biosafety level 3 (BSL-3) containment laboratories for handling SARS-CoV-2 has limited the number of high throughput screening (HTS) laboratories that are capable of carrying out large scale compound screens using live SARS-CoV-2. Despite these challenges, several drug repurposing screens have been carried out using live SARS-CoV-2, showing promising results (Dittmar et al., 2020; Ellinger et al., 2020; Riva et al., 2020; Touret et al., 2020). Here we report a screening campaign against a collection of 8,810 approved and investigational drugs, mechanism-

based bioactive compounds, and natural products, carried out in quantitative HTS (qHTS) format (Inglese et al., 2006). Compounds were screened at four concentrations in a SARS-CoV-2 cytopathic effect (CPE) assay in Vero E6 cells that were selected for high ACE2 expression, with an accompanying cytotoxicity counter-assay. The primary screen yielded 319 hits with confirmed anti-SARS-CoV-2 activity. The primary screening data have been made publicly available on the National Center for Advancing Translational Sciences (NCATS) OpenData Portal (https://opendata.ncats.nih.gov/covid19/index.html) (Brimacombe et al., 2020). We intend this manuscript as a companion to guide investigators in utilizing that data, and to present further details of qHTS with the SARS-CoV-2 CPE assay, including identification of top annotated hits.

MATERIALS AND METHODS

Compounds and Compound Libraries

All compound libraries were assembled internally at NCATS. The NCATS pharmaceutical collection (NPC) contains 2,678 compounds, covering drugs approved by US FDA and foreign health agencies in European Union, United Kingdom, Japan, Canada, and Australia, as well as some clinical trialed experimental drugs (Huang et al., 2019). The NCATS Mechanism Interrogation Plate (MIPE) 5.0 library contains 2,480 mechanism based bioactive compounds, targeting more than 860 distinct mechanisms of action (Lin et al., 2019). The NCATS Pharmacologically Active Chemical Toolbox (NPACT) is a library of mechanistically defined molecules and natural products (5,099 compounds). Other small custom NCATS collections were also screened: anti-infective (752 compounds), kinase inhibitors (977 compounds), epigenetic modulators (335 compounds). A commercially available autophagy-focused screening library (Cayman #23537) was analyzed and 29 compounds that were not already present in our collections were purchased. All compounds were dissolved in DMSO to make 10 mM stock solutions, unless solubility was limiting, and was diluted four times at 1:5 ratio for the primary screens, and at 1:3 ratio for follow up assays at eight concentrations.

CPE Assay

A SARS-CoV-2 CPE assay was conducted in the BSL3 facilities at the contract research organization Southern Research (Birmingham, AL). Briefly, compounds were titrated in DMSO and acoustically dispensed into 384-well assay plates at 60 nL/well at NCATS, and provided to Southern Research. Cell culture media (MEM, 1% Pen/Strep/GlutaMax, 1% HEPES, 2% HI FBS) was dispensed at 5 μL/well into assay plates, and incubated at room temperature to allow for compound dissolution. Vero E6 African green monkey kidney epithelial cells (selected for high ACE2 expression) were inoculated with SARS-CoV-2 (USA_WA1/2020) at a multiplicity of infection (MOI) of 0.002 in media, and quickly dispensed into assay plates as 25 μL/well. The final cell density was 4,000 cells/well. Assay plates were incubated for 72 h at 37°C, 5% CO_2, and 90% humidity. CellTiter-Glo (30 μL/well, Promega #G7573) was dispensed into the assay plates. Plates were incubated for 10 min at room temperature. Luminescence signal was measured on Perkin Elmer Envision or BMG CLARIOstar plate readers. An ATP content cytotoxicity counter-assay was conducted using the same protocol as the CPE assay, without the addition of SARS-CoV-2 virus.

Data Analysis

Results from the primary screen and confirmation screens were processed at NCATS using a software developed in-house (Wang et al., 2010). For the CPE assay, raw plate data were normalized with DMSO-only wells as 0% CPE rescue (negative signal control), and no-virus control wells as 100% CPE rescue (positive signal control). For the cytotoxicity assay, raw plate data were normalized with DMSO-only wells as 100% viability (positive signal control), and cells treated with hyamine (benzethonium chloride) control compound as 0% viability (negative signal control). The half-maximum effective values (EC_{50}) and percent efficacy were obtained by fitting the concentration-response titration data to a four-parameter Hill equation. Compounds with >55% efficacy were selected for cherry-pick confirmation. The concentration-response curves of re-tested compounds were also plotted using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA). Results in the figures are expressed as mean ± standard deviation (SD).

RESULTS

High Throughput Screening With SARS-CoV-2 CPE Assay

Our aims were two-fold in initiating this program. The first was to identify active compounds that may provide opportunities for repurposing, or identify mechanistic targets of interest. The second was to create a complete HTS reference dataset that can be shared openly with the scientific community for study of disease pathology and new therapeutics development. The CPE reduction assay format has been widely employed to screen for antiviral agents due to its ease of scalability for HTS (Heaton, 2017). In this assay, viral infection kills host cells, and the cell viability is used as a surrogate readout for viral infection and replication. In other words, compounds with anti-viral activities rescue cells from the cytopathic effect of SARS-CoV-2 (a gain-of-signal assay).

A total of 9,952 compounds were tested in the primary screen, but due to the overlapping composition of the libraries, a significant number of compounds were tested multiply. A total of 8,810 unique compounds in six compound libraries were tested in the primary screen including the NCATS Pharmaceutical Collection (NPC), NCATS Mechanism Interrogation Plate (MIPE), NCATS Pharmacologically Active Chemical Toolbox (NPACT), Epigenomic library, Autophagy library, and anti-infective library. These compounds contain 1,345 approved drugs (by the FDA, EMA, DPD), 751 compounds approved outside of those countries, 1,067 investigational drugs (tested in clinical trials), 1,057 pre-clinical compounds (tested in animals), and 4,472 bioactive compounds (tool compounds) (**Figure 1A**). By their mechanisms of action and clinical applications, these compounds are divided into diverse groups (**Figure 1B**).

The CPE assay performed well in the primary screen, with an average Z' factor of 0.83 over 133 plates, from three batched runs (**Figure 2A**). Remdesivir concentration-response was included as a

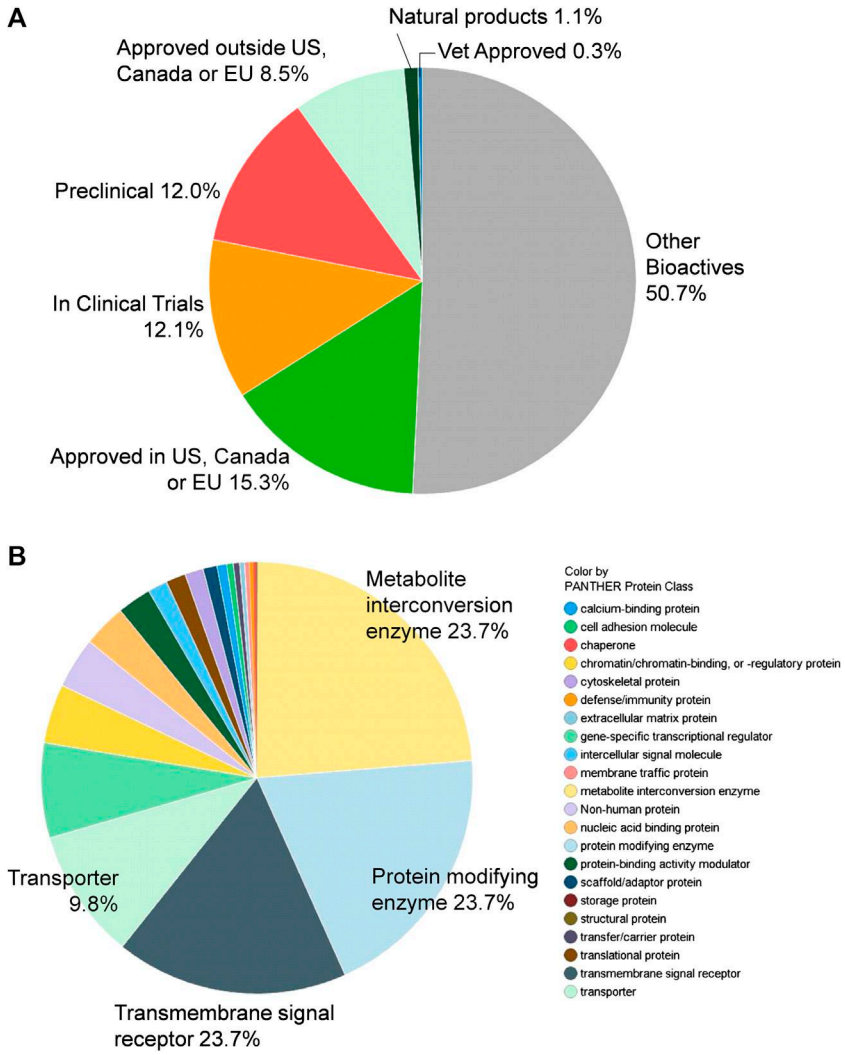

FIGURE 1 | Compound library description. **(A)** By approval status: approved drugs (FDA and others), tested in clinical trials, or preclinical. **(B)** By mechanism of action.

control for each screening run, and yielded consistent EC_{50} values of 4.56, 4.42 and 7.28 μM (**Figure 2B**). Using the criteria of >55% efficacy, 380 compounds were selected as the primary screen hits, out of which, 319 compounds were confirmed using 8-point, 1:3 titration, in duplicate. Among these primary hits, 89 of 319 had previously reported activity against SARS-CoV-2, including reports of live virus assays, enzymatic assays, or virtual screening, while 230 were novel hits from this qHTS (**Table 1**, **Supplementary Table S1**). In the following sections, these newly identified SARS-CoV-2 CPE-protective compounds are further descriped.

91 Approved Drugs and 49 Investigational Drugs Protected Against Cytopathic Effect of SARS-CoV-2 Infection

There were 56 top confirmed hits with EC_{50} values of ≤10 μM and efficacy values of greater than 80% in the CPE assay, and with greater than 10-fold selectivity index (SI) between cytotoxicity and CPE assays (**Table 1**, **Figure 3**). When grouped by mechanism of action targets, 19 compounds were GPCR modulators, eight were host protease inhibitors, five were kinase modulators, and three were autophagy modulators (**Figure 3**). Interestingly, in the 56 top hits, remdesivir is only one that has a viral target as a known primary mechanism, whereas the known mechanisms of action of the other compounds are directed against host targets.

There have been several previous drug repurposing screens reported for SARS-CoV-2 in 2D cell culture infection models (Dittmar et al., 2020; Ellinger et al., 2020; Jeon et al., 2020; Riva et al., 2020; Touret et al., 2020; Weston et al., 2020). These screens had some compound overlap with our qHTS screen, particularly for the FDA approved drugs. We performed a literature search of our confirmed compounds and previous reports were noted in **Table 1** and **Supplementary Table S1**. Three of the top 56 hits were novel and FDA approved. These hits are chlorprothixene,

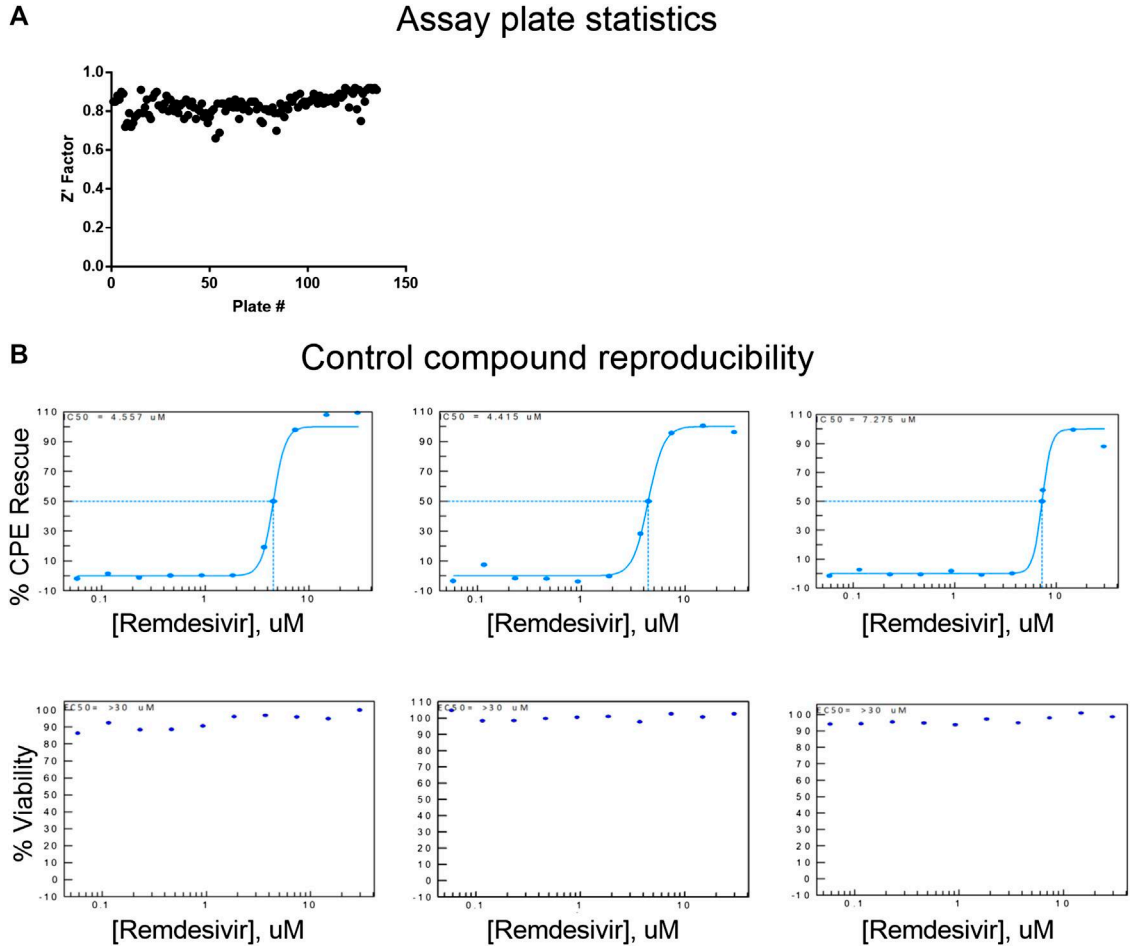

FIGURE 2 | Assay reproducibility. **(A)** Assay plate statistics showing Z′ factors across all 133 384-well plates in the primary screen. **(B)** Concentration-response curve fittings for remdesivir in four independent runs for primary screens and hit confirmation. EC_{50} values of 4.56, 4.42, 7.28, and 5.17 μM of remdesivir in the CPE assay demonstrate day-to-day reproducibility of the assay.

methotrimeprazine, and piperacetazine, which showed 10 μM potencies in the CPE assay. In order for a drug to be efficacious *in vivo*, the *in vivo* exposure at the site of infection (e.g. drug plasma concentration) would need to be higher than the *in vitro* potency (e.g. EC_{50}). To help guide compound prioritization, the reported clinical plasma pharmacokinetic values of the top confirmed hits are summarized in **Table 2**. Of the top approved drugs that are active against SARS-CoV-2 in the CPE assay, only amiodarone HCl showed lower EC_{50} value in the CPE assay than plasma C_{max}, whereas, remdesivir and imatinib showed EC_{50} values that were within 2-fold of plasma C_{max} (**Table 2**).

Four drugs approved outside of the US were also identified as novel compounds with anti-SARS-CoV-2 effects: difeterol, rescimetol, melitracen HCl, and proglumetacin. Furthermore, we identified 7 novel clinical trial drugs with anti-SARS-CoV-2 activities: N-methylspiperone HCl, Lu AE58054 HCl, balicatib, berzosertib, JTV519 hemifumarate, DMP 777, and dexanabinol. In addition to the above novel hits, four drugs, approved by the FDA and elsewhere, methdilazine, maprotiline HCl, deserpidine, and flunarizine, were previously reported in virtual screens against SARS-CoV-2 targets without supporting biological data. Here, we report their activities against SARS-CoV-2 infection. In addition, we have confirmed 53 approved drugs with anti-SARS-CoV-2 effects that were reported previously (**Table 1** and **Supplementary Table S1**). Together, our results demonstrate a comprehensive set of 91 approved drugs and 49 investigational drugs with anti-SARS-CoV-2 activity that can be considered for design of new clinical trials, especially drug combination therapies, to increase and improve treatment options for COVID-19.

DISCUSSION

In contrast to the other reported drug repurposing screens for SARS-CoV-2 using a single drug concentration in the primary screens (Dittmar et al., 2020; Ellinger et al., 2020; Jeon et al., 2020; Riva et al., 2020; Touret et al., 2020; Weston et al., 2020),

TABLE 1 | Top confirmed anti-SARS-CoV-2 compounds.

Sample ID	Sample name	CPE EC_{50} (uM)	CPE % efficacy	Cytotox CC50 (uM)	% Cytotox	Previous reports against CoVs	Approval status	MOA
Viral target								
NCGC00686694	Remdesivir	10.0	133.1	N/A	<30	Clinical (Beigel et al., 2020)	FDA	RdRP inhibitor
Autophagy modulators								
NCGC00387732	VPS34-IN1	0.63	103.0	10.0	−76.5	None	Bioactive	Autophagy modulator
NCGC00344081	STF-62247	1.1	107.1	11.2	−56.6	None	Preclinical	Autophagy modulator; Renal cell growth inhibition
NCGC00507892	VPS34 Inhibitor 1	1.4	98.3	N/A	<30	None	Preclinical	Autophagy modulator
GPCR modulators								
NCGC00346896	MCOPPB	3.5	85.6	N/A	<30	None	Preclinical	ORL1 (OP4, NOP) agonists
NCGC00370950	GW 803430	3.5	93.3	N/A	<30	None	Bioactive	Melanin-concentrating hormone receptor 1 antagonist
NCGC00017063	Amodiaquine dihydrochloride	4.0	87.2	N/A	<30	*In vitro* live virus (Ianevski et al., 2020)	FDA	Histamine receptor antagonist
NCGC00485045	N-Methylspiperone hydrochloride	4.5	80.0	N/A	<30	None	Clinical trial	Serotonin 2 (5-HT2) receptor antagonist
NCGC00016710	Clemastine fumarate	7.9	96.0	N/A	<30	Mpro assay (Vatansever et al., 2020)	FDA	Histamine receptor antagonist
NCGC00386477	GMC 2-29	7.9	117.2	N/A	<30	None	Bioactive	5-hydroxytryptamine receptor 1D antagonist
NCGC00378842	Lu AE58054 hydrochloride	10.0	97.2	N/A	<30	None	Clinical trial	Serotonin 6 (5-HT6) receptor antagonist
NCGC00013683	Chlorprothixene	10.0	104.4	N/A	<30	None	FDA	Dopamine receptor antagonist
NCGC00014482	Methdilazine hydrochloride	10.0	86.4	N/A	<30	Virtual: AI prediction (Grzybowski et al., 2020)	FDA	Antihistamine
NCGC00179370	Methotrimeprazine maleate	10.0	84.6	N/A	<30	None	FDA	Antagonist for adrenergic, dopamine, histamine, cholinergic and serotonin (5-hydroxytryptamine; 5-HT) receptors
NCGC00016642	Piperacetazine	10.0	103.7	N/A	<30	None	FDA	Dopamine receptor antagonist
NCGC00181913	Difeterol	10.0	113.4	N/A	<30	None	Approved outside of US	Antihistamine
NCGC00386484	(R)-(-)-LY 426965 dihydrochloride	10.0	110.7	N/A	<30	None	Bioactive	Serotonin 2b (5-HT2b) receptor modulator
NCGC00015608	Loperamide hydrochloride	10.0	98.6	N/A	<30	*In vitro* live virus (Jeon et al., 2020)	FDA	Opioid receptor agonist
NCGC00485321	Naltrindole isothiocyanate hydrochloride	10.0	114.7	N/A	<30	None	Bioactive	Delta opioid receptor antagonist
NCGC00165726	AM1241	10.0	97.6	N/A	<30	None	Bioactive	Cannabinoid CB2 receptor agonist
NCGC00386703	CpdD hydrochloride	10.0	96.9	N/A	<30	None	Bioactive	Ghrelin receptor antagonist
NCGC00386219	SB 271046 hydrochloride	10.0	107.5	N/A	<30	None	Bioactive	Serotonin 6 (5-HT6) receptor antagonist
NCGC00386479	GMC 2-113	10.0	129.7	N/A	<30	Virtual: RdRP (Dwivedy et al., 2020)	Bioactive	5-hydroxytryptamine receptor 1D antagonist
Host protease inhibitors								
NCGC00386330	Z-FA-FMK	0.13	104.8	N/A	<30	Mpro assay, *in vitro* live virus (Zhu et al., 2020b)	Bioactive	Cathepsin L inhibitor
NCGC00485951	VBY-825	0.14	97.8	N/A	<30	*In vitro* live virus (Riva et al., 2020)	Clinical trial	Cathepsin S inhibitor
NCGC00345807	CAA-0225	0.20	99.3	N/A	<30	None	Preclinical	Cathepsin L inhibitors
NCGC00386232	Cathepsin Inhibitor 1	0.25	114.4	N/A	<30	None	Bioactive	Cathepsin inhibitors
NCGC00163432	Calpeptin	0.50	111.7	N/A	<30	Mpro assay, *in vitro* live virus (Ma et al., 2020)	Preclinical	Calpain inhibitor
NCGC00485375	Z-Gly-Leu-Phe-chloromethyl ketone	1.3	87.2	N/A	<30	None	Bioactive	Granzyme B Inhibitor
NCGC00371151	Balicatib	2.0	100.3	N/A	<30	None	Clinical trial	Cruzipain (Trypanosoma cruzi) inhibitor

(Continued on following page)

TABLE 1 | (*Continued*) Top confirmed anti-SARS-CoV-2 compounds.

Sample ID	Sample name	CPE EC_{50} (uM)	CPE % efficacy	Cytotox CC50 (uM)	% Cytotox	Previous reports against CoVs	Approval status	MOA
NCGC00016166	Calpain Inhibitor I, ALLN	2.0	111.1	N/A	<30	None	Bioactive	Calpain inhibitor
Kinase modulators								
NCGC00263093	Apilimod	0.023	104.4	N/A	<30	*In vitro* live virus (Riva et al., 2020)	Clinical trial	IL-12 Production inhibitor; PIKfyve inhibitor
NCGC00386313	Berzosertib	0.71	87.9	11.2	-98.5	None	Clinical trial	ATR Kinase inhibitor
NCGC00347280	IKK-2 inhibitor VIII	7.1	91.7	N/A	<30	None	Preclinical	IKK-2 (IKK-beta) inhibitor
NCGC00387166	NSC 33994	8.9	107.6	N/A	<30	None	Bioactive	Jak2 inhibitor
NCGC00159456	Imatinib	10.0	119.0	N/A	<30	Clinical (Morales-Ortega et al., 2020)	FDA	Bcr-Abl kinase inhibitor; KIT inhibitor; PDGFR tyrosine kinase receptor inhibitor
Others								
NCGC00178090	Pristimerin	0.11	87.4	1.1	–93.2	SARS Mpro assay (Ryu et al., 2010)	Preclinical	Monoacylglycerol lipase (MGL) inhibitor
NCGC00385252	alpha-L-Arabinopyranose	2.4	104.0	N/A	<30	None	Bioactive	Induces Pbad promoter expression in *E. coli*
NCGC00351072	ML414	3.2	79.6	N/A	<30	None	Bioactive	Oligosaccharyltransferase inhibitor
NCGC00379165	IT1t dihydrochloride	3.5	96.3	N/A	<30	None	Bioactive	CXCR4 inhibitor
NCGC00485648	S-15176 difumarate salt	3.8	127.4	N/A	<30	None	Bioactive	Oxidative stress inhibitor
NCGC00384450	JTV519 Hemifumarate	5.5	85.7	N/A	<30	None	Clinical trial	Ryanodine receptor (RyR) inhibitor
NCGC00253604	Rescimetol	8.9	81.8	N/A	<30	None	Approved outside of US	Antihypertensive agent
NCGC00164559	Duloxetine hydrochloride	10.0	90.0	N/A	<30	Mpro assay (Vatansever et al., 2020)	FDA	Norepinephrine reuptake inhibitor; Serotonin-norepinephrine reuptake inhibitor (SNRI)
NCGC00181168	Trifluomeprazine 2-butenedioate	10.0	90.2	N/A	<30	None	Bioactive	Antipsychotic agents
NCGC00169804	Asteriscunolide D	10.0	93.3	N/A	<30	None	Bioactive	Natural product
NCGC00485925	Genz-123346 (free base)	10.0	99.4	N/A	<30	*In vitro* live virus (Vitner et al., 2020)	Bioactive	Ceramide glucosyltransferase inhibitor
NCGC00015708	Maprotiline hydrochloride	10.0	103.7	N/A	<30	Virtual: Mpro docking (Chauhan, 2020)	FDA	Norepinephrine reputake inhibitor; tricyclic antidepressant
NCGC00168786	Deserpidine	10.0	84.7	N/A	<30	Virtual: NSP16 docking (Jiang et al., 2020)	FDA	Angiotensin converting enzyme inhibitor
NCGC00015096	Amiodarone hydrochloride	10.0	100.5	N/A	<30	Clinical (Castaldo et al., 2020)	FDA	Potassium channel blocker
NCGC00181088	Melitracen hydrochloride	10.0	97.1	N/A	<30	None	Approved outside of US	Antidepressive agents, tricyclic
NCGC00015428	(+/-) -Fluoxetine	10.0	115.8	N/A	<30	*In vitro* live virus (Zimniak et al., 2020)	FDA	Selective serotonin reuptake inhibitor (SSRI)
NCGC00018102	Flunarizine	10.0	94.1	N/A	<30	Virtual: Spike docking (Chernyshev, 2020)	Approved outside of US	Calcium channel blocker
NCGC00183024	Proglumetacin	10.0	87.6	N/A	<30	None	Approved outside of US	Cyclooxygenase inhibitor
NCGC00378760	DMP 777	10.0	92.5	N/A	<30	None	Clinical trial	Leukocyte elastase inhibitor
NCGC00476094	Dexanabinol	10.0	110.8	N/A	<30	None	Clinical trial	NMDA antagonist

we have used a quantitative HTS (qHTS, concentration-response) method (Inglese et al., 2006) where four compound concentrations were used in the primary screen instead of a single compound concentration. We also assessed the cytotoxicity of each compound against Vero E6 cells (without virus infection) in parallel with the SARS-CoV-2 CPE screening. The concentration-response for each compound used in the primary screen can improve identification of positive hits, especially compounds with biphasic actions (bell-shaped curves) or screening errors. In addition, NCATS has more inclusive compound collections with drugs approved by

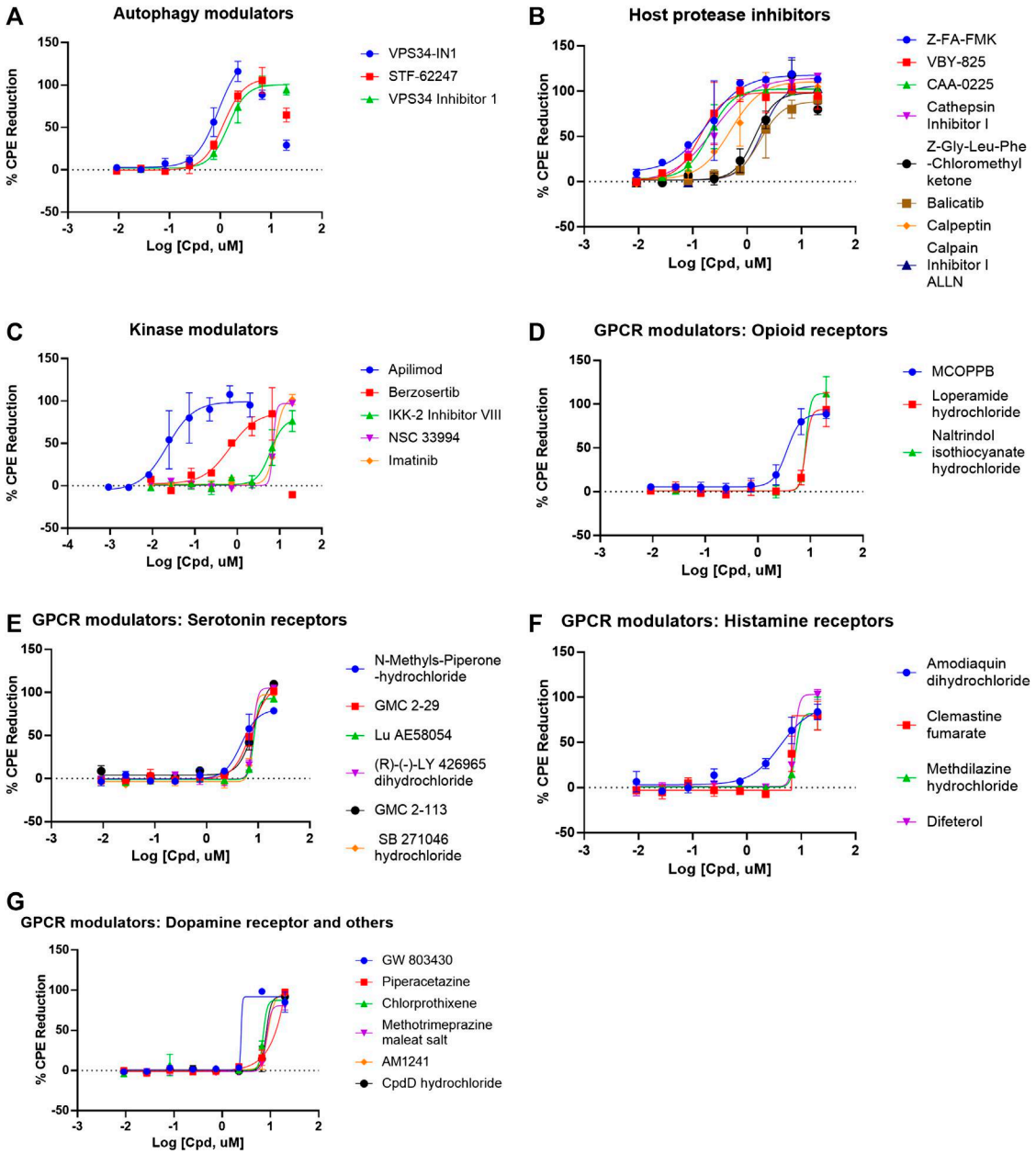

FIGURE 3 | Compounds concentration-response curves in the CPE assay. **(A)** Autophagy modulators, **(B)** host protease inhibitors, **(C)** kinase modulators, **(D)** opioid receptor modulators, **(E)** serotonin receptor modulators, **(F)** histamine receptor modulators, and **(G)** dopamine and other GPCR receptor modulators. Berzosertib, VPS34-IN1, and STF-62247 showed bell-shaped concentration-responses due to cytotoxicity. No other compounds caused any reduction in viability in the cytotoxicity assay.

regulatory agencies outside of the US, such as Canada, Europe and Japan, that were not previously screened in SARS-CoV-2 assays. We also screened a set of investigational drugs that have human clinical data for drug properties such as the mechanism(s) of action, pharmacokinetics, and drug toxicity, which could be leveraged to speed up drug development. The other bioactive compounds screened have drug targets and mechanisms of action that may be useful for further studies of disease pathophysiology and for potential drug development.

We identified 319 compounds with activity against SARS-CoV-2 CPE from a qHTS of 8,810 unique compounds. Among the top 56 hits identified with <10 μM EC_{50} values and >80% efficacies, the anti-SARS-CoV-2 activity of 37 of them has not been reported elsewhere. Of these novel top hits, three were FDA approved drugs with novel anti-SARS-CoV-2 activity. Chlorprothixene is a dopamine receptor antagonist, a classic antipsychotic agent approved for treatment of schizophrenia (Schrijver et al., 2016). Methotrimeprazine, also named as

TABLE 2 | Reported human pharmacokinetic properties of FDA-approved top hits.

Sample name	C_{max} (ng/ml)	MW (g/mol)	C_{max} (μM)	Elimination $T_{1/2}$	Dosing regimen	References
(+/-) -Fluoxetine	15–55	309.33	0.05–0.18	1–3 days	Single dose 40 mg PO	Eli Lilly and Company (1987)
Amiodarone hydrochloride	5,000–41,000	681.78	7.33–60.14	9–36 days	Single dose 5 mg/kg IV	Hospira (1995)
Amodiaquine dihydrochloride	32 ± 3	464.8	0.069	5.2 ± 1.7 h	Single dose 600 mg PO	Winstanley et al. (1987)
Chlorprothixene	430 ± 81	315.9	1.36	25.8 ± 13.6 h	Single dose 100 mg IV	Bagli et al. (1996)
Clemastine fumarate	0.577 ± 0.252	460	0.0013	21.3 ± 11.6 h	Single dose 1.34 mg PO	Schran et al. (1996)
Deserpidine	0.172	578.66	0.0003	42.9 ± 17.8 h	Single dose 0.25 mg PO	Zhang et al. (2009)
Duloxetine hydrochloride	110	333.88	0.33	6.96–14.9 h	60 mg BID PO	Knadler et al. (2011)
Imatinib	3,395 ± 2,409	493.6	6.88	10–18.9 h	Single dose 600 mg PO	Peng et al. (2005)
Loperamide hydrochloride	2	477	0.0042	9.1–14.4 h	Single dose 2 mg PO	Janssen Pharmaceutica Inc. (1998)
Maprotiline hydrochloride	25	313.87	0.080	45 h	Single dose 75 mg PO	Maguire et al. (1980)
[a]Methdilazine hydrochloride		332.9				
Methotrimeprazine maleate	3.44	444.6	0.0077	10.8 h	Single dose 25 mg PO	AA Pharma Inc. (2012)
[a]Piperacetazine		410.6				
Remdesivir	4,420	602.58	7.34	1.05 h	Single dose 225 mg IV	Humeniuk et al. (2020)

[a]*Discontinued drugs. No PK data available.*

C_{max}: maximum serum/plasma concentration; MW: molecular weight; Elimination $T_{1/2}$: elimination half life; PO: per os (oral dosing); IV: intravenous.

levomepromazine, is another tricyclic antipsychotic agent approved for psychotic disorders including schizophrenia, and manic-depressive syndromes (Sivaraman et al., 2010). Both chlorprothixene and methotrimeprazine were previously found to inhibit the SARS-CoV replication with EC_{50}s around 10 μM (Barnard et al., 2008). Piperacetazine is also an older tricyclic antipsychotic drug approved for treatment of schizophrenia (Eslami Shahrbabaki et al., 2018). The antiviral effect of piperacetazine was found previously to block the Ebola viral entry with the EC_{50} of 9.68 μM (Kouznetsova et al., 2019).

We also confirmed the anti-SARS-CoV-2 activity of five compounds that were reported as virtual screening hits but had yet to be confirmed experimentally, including methdilazine by an AI prediction algorithm (Grzybowski et al., 2020), GMC 2-113 by a virtual screen of RNA dependent RNA polymerase (RdRP) (Dwivedy et al., 2020), maprotiline by a main protease docking (Chauhan, 2020), deserpidine by a NPS-16 docking (Jiang et al., 2020), and flunarizine by a spike protein docking screen (Chernyshev, 2020). Our data supports the utility of these emerging technologies and the field of AI for advancing drug development.

For *in vitro* screens of antiviral compounds, molecular target (mechanism) based assays and phenotypic assays are two major approaches. Common targets are viral enzymes such as viral protease, DNA and RNA polymerases, reverse transcriptase, and integrase. Development of assays targeting viral enzymes rely on viral enzyme expression, purification, assay development, and validation (Shyr et al., 2020). Alternatively, phenotypic assays involving live-virus infection are readily executed once the viruses are isolated from patients and viral replication in appropriate host cells is established. A common live virus infection assay is the measurement of CPE in virus infected host cells. There are two possibilities (fates) for the host cells after viral infection, including cytopathic infection (i.e. death of host cells) and persistent infection (Heaton, 2017). The CPE effect can be readily measured by the ATP content cell viability assay, which is robust and amenable for HTS. Due to the nature of the CPE assay, compounds that suppress CPE can act against any part of the virus infection cycle, including the binding of virus to the host cell receptor, entry into host cells, virus replication, viral assembly/budding, and virus reinfection of adjacent cells.

It is worth briefly reflecting on the limitations of the drug repurposing assay approach. A number of small molecules of interest for treating COVID-19 that are currently in clinical trials were not hits in our assay. For example, the TMPRSS2 inhibitors camostat and nafamstat are protease inhibitors approved in Japan for treating pancreatitis, and known to inhibit TMPRSS2 (Shrimp et al., 2020). While TMPRSS2 is reported to be a mediator of SARS-CoV-2 cell entry, Vero E6 cells do not express TMPRSS2, so this class of compound are not active in the Vero E6 assay. The drug efflux transporter P-glycoprotein (P-gp) can reduce cellular concentrations of test agents, and as a kidney epithelial cell line, Vero E6 cells likely expresses significant P-gp concentrations, which would reduce activity of P-gp substrates (Robey et al., 2018). Remdesivir itself is a substrate of Pgp (EMA, 2020), and is weaker against SARS-CoV-2 in assays using Vero E6 cells (EC_{50} > 1 μM) compared with Calu-3 or Huh7 cell lines (EC_{50} > 50 nM) (Stanford University, 2020). These examples highlight the need for careful interpretation and critical follow-up studies after initial high-throughput screening analyses. Furthermore, the list of compounds presented here are confirmed hits in a SARS-CoV-2 CPE assay, and will require considerable follow up work to determine their feasibility for translation to clinical use. A possible pipeline for follow up could be testing in more physiologically relevant 2D human cells using orthogonal assays, and 3D human *in vitro* respiratory tissue models. These results would require confirmation in animal efficacy models, as well as evaluation of human PK and tolerability of these compounds. Additionally, the hits identified in this screen could be further tested in pair-wise matrix combinations to identify synergistic combinations for potential cocktail treatments (Shinn et al., 2019).

Importantly, the comprehensive primary screen datasets of this study for approved and investigational drugs, and mechanism-based bioactive compounds have been made publicly available in real-time on the NCATS OpenData Portal (https://opendata.ncats.nih.gov/covid19/index.html) (Brimacombe et al., 2020). These datasets provide a wealth of quality live-virus data that is freely available to the research

community for future studies and data mining with the aim of offering new therapeutics to treat COVID-19 patients efficiently and safely (Zhu et al., 2020a; Huang et al., 2020).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found in the article/**Supplementary Material**. Primary screen data can be found at http://opendata.ncats.nih.gov. Secondary screen data are uploaded in PubChem AIDs 1508605 and 1508606. All other data are available upon request.

AUTHOR CONTRIBUTIONS

PS, ZI, and RTE prepared the assay ready plates. RB and LR conducted the CPE and cytotoxicity assays. PS, CK-T, KMW, and SGM curated the compound libraries. CZC and MDH designed the experiments. CZC, BMB, and WZ wrote the manuscript. RH, MS, XH, HG, and TZ performed data analysis and data uploads. All authors provided critical reading of the manuscript.

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frontiers in Pharmacology

REVIEW
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Perspectives of Antidiabetic Drugs in Diabetes With Coronavirus Infections

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Diabetes mellitus (DM) increases the risk of viral infections especially during the period of poor glycemic controls. Emerging evidence has reported that DM is one of the most common comorbidities in the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infection, also referred to as COVID-19. Moreover, the management and therapy are complex for individuals with diabetes who are acutely unwell with suspected or confirmed COVID-19. Here, we review the role of antidiabetic agents, mainly including insulin, metformin, pioglitazone, dipeptidyl peptidase-4 (DPP4) inhibitors, sodium-glucose cotransporter 2 (SGLT2) inhibitors, and glucagon-like peptide 1 (GLP-1) receptor agonists in DM patients with coronavirus infection, addressing the clinical therapeutic choices for these subjects.

Keywords: diabetes mellitus, coronavirus infection, COVID-19, antidiabetic agents, therapeutic choices

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by a novel severe acute respiratory syndrome coronavirus (SARS-CoV2), was declared to be a pandemic by the World Health Organization on March 11 and had aroused worldwide public concerns [https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—11-march-2020 (2020)]. The global epidemic of SARS-CoV2 has direct implications for the therapy of common metabolic diseases such as diabetes mellitus (DM). Furthermore, DM is known to be associated with an increased risk of viral respiratory tract infections, including H1N1 influenza (Allard et al., 2010) and is emerging as an important comorbidity for disease severity and mortality in the context of COVID-19 (Targher et al., 2020; Yan et al., 2020). Strikingly, prevalence of DM was about twofold increase in the nonsurviving compared to the surviving COVID-19 individuals in China and Italy (Fadini et al., 2020a; Wu C. et al., 2020a), which was consistent with the independent association of this condition with fatal complications during the other two coronavirus-related respiratory infection epidemics, such as the Middle East Respiratory Syndrome (MERS) in 2012, and the Severe Acute Respiratory Syndrome (SARS) in 2002 (Zhou et al., 2020). Proposed mechanisms for these apparent associations between COVID-19 and DM may be attributed to the dysregulated immune response (Guo et al., 2020).

To date, the management of people with DM who are acutely unwell with COVID-19 is complex, and improved glycemic control should be of utmost importance in patients with COVID-19 and preexisting type 2 diabetes (Zhu et al., 2020). Although it would be wise to stick to the ongoing or intensive treatment, the choice of antidiabetic drugs needs to be reviewed. Herein, we summarize the role and perspective of antidiabetic agents, mainly including insulin, metformin, pioglitazone, dipeptidyl peptidase-4 (DPP4) inhibitors, sodium-glucose cotransporter 2 (SGLT2) inhibitors, and glucagon-like peptide 1 receptor agonists (GLP-1RAs) in DM patients with coronavirus infection.

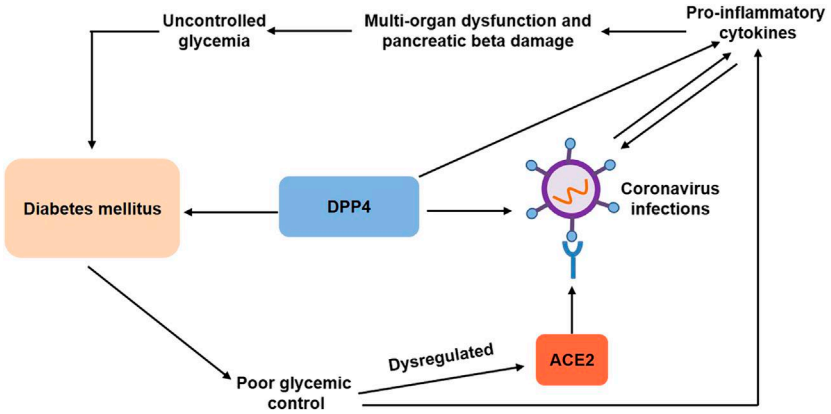

FIGURE 1 | Associations between diabetes mellitus and coronavirus infections. Diabetes mellitus contributes to poor glycemic control, which has been shown to dysregulate ACE2 glycosylation and increase proinflammatory cytokines, facilitating viral cell entry. Preexisting proinflammatory state, in turn, predisposes patients to coronavirus infections and aggravates multiorgan dysregulation and pancreatic beta damage, leading to uncontrolled glycemia and diabetes mellitus. DPP4, a common pharmacological target for type 2 diabetes, is also a functional coronavirus receptor, which increases the susceptibility to coronavirus infections. On the other hand, DPP4 exerts proinflammatory activity. DPP4, dipeptidyl peptidase-4; ACE2, angiotensin converting enzyme 2.

ASSOCIATIONS BETWEEN DM AND CORONAVIRUS INFECTIONS

DM was correlated with an increased risk of viral respiratory tract infections (Allard et al., 2010) and was considered as a major contributor to disease severity and mortality in MERS (Memish et al., 2020). A systematic review and meta-analysis described that the overall prevalence of DM in MERS cases was 3.6-fold higher than in H1N1 (Badawi and Ryoo, 2016). Moreover, both smaller and larger studies revealed that DM was strongly associated with adverse outcomes and mortality in subjects with MERS (Assiri et al., 2013; Alqahtani et al., 2018). Similarly, a retrospective study performed by Booth et al. showed that the presence of DM was independently associated with significant morbidity and mortality in 114 adults hospitalized with SARS-CoV (Booth et al., 2003). Analysis of individuals hospitalized with SARS-CoV in China demonstrated that increases in fasting glucose were involved in the increased rates of death (Yang et al., 2010).

Database from Chinese Centers for Disease Control and Prevention (CDC) showed a diabetes prevalence of approximately 5% from the 20,982 patients with COVID-19 (Epidemiology Working Group for NCIP Epidemic Response, Chinese Center for Disease Control and Prevention, 2020). A report from Italy indicated nearly 17% diabetes prevalence from the 1043 COVID-19 patients (Grasselli et al., 2020). Noteworthily, available evidence from the CDC and hospitals indicated that the risk of fatal complications from COVID-19 was up to 50% higher in patients with DM than in those without (Remuzzi and Remuzzi, 2020). Moreover, the presence of typical complications of DM (heart failure and chronic kidney disease) increased COVID-19 mortality (Barron et al., 2020; Holman et al., 2020). Among 1,590 laboratory confirmed cases of COVID-19 from China, 8.2% of patients with DM yielded poorer clinical endpoints than those without (Guan et al., 2020). Consistent with these observations, DM is one of the comorbidities associated with adverse outcomes in hospitalized patients with SARS in China and Italy (Fadini et al., 2020a).

Currently, there are mainly two specific mechanisms that might explain the link between DM and COVID-19 (**Figure 1**). First, both SARS and SARS-CoV2 coronavirus enter the body through angiotensin converting enzyme 2 (ACE2) and play a crucial role in metabolism and inflammation (Hoffmann et al., 2020). ACE2 has been identified as the receptor for the coronavirus. Poor glycemic control has been shown to dysregulate ACE2 glycosylation (Brufsky, 2020), which might facilitate viral cell entry or make the cells vulnerable to the inflammation (Hoffmann et al., 2020). Preexisting proinflammatory state accentuated the cytokine storm and was believed to contribute to multiorgan dysfunction and severity of diseases (Maddaloni and Buzzetti, 2020). In addition, the expression of ACE2 on pancreatic β cells could directly affect the β cell function (Yang et al., 2010; Roca-Ho et al., 2017), which might additionally worsen the clinical outcomes.

Second, dipeptidyl peptidase-4 (DPP4) enzyme, a common pharmacological target for type 2 diabetes, was also a functional coronavirus receptor (Raj et al., 2013), which might be another potential mechanism that explains the link between COVID-19 and DM. Transgenic mice expressing human DPP4 became susceptible to coronavirus infection with MERS-CoV (Li et al., 2016). Antibodies inhibited MERS-CoV infection of primary cells by directing against DPP4 (Raj et al., 2013). Analogously, recombinant human adenosine deaminase blocked MERS-CoV spike protein S1 binding to DPP4 and inhibited MERS-CoV infection of cells transfected with human DDP-4 (Raj et al., 2014). Moreover, human neutralizing antibodies directed against MERS-CoV spike protein blocked viral binding to DPP4, thereby inhibiting MERS-CoV infection (Tang et al., 2014). Surprisingly, transgenic mice overexpressing human DPP4 exhibited relative resistance to MERS-CoV infection and

reduced rates of mortality (Algaissi et al., 2019). Although the association of SARS-CoV-2 and DDP-4 remains unknown, the use of DDP-4 inhibitors can provide therapeutic opportunities for the treatment of diabetic patients with COVID-19 in clinical practice (Iacobellis, 2020).

PERSPECTIVES OF ANTIDIABETIC AGENTS IN DM PATIENTS WITH CORONAVIRUS INFECTION

Considering the severity and mortality, extra precautions should be taken in DM patients with coronavirus infection. Of note, specific attention should be paid to the use of antidiabetic agents in these patients.

Insulin

Insulin has been widely used for decades in critically ill hospitalized patients with DM and the usage of continuous glucose monitoring reduces the rates of hypoglycemia associated with insulin use (Lu et al., 2018). Of interest, insulin was also a preferred treatment option for critically ill patients with DM amid the COVID-19 pandemic (Drucker, 2020; Gupta et al., 2020). Additionally, selective loss of insulin action attenuated the anti-inflammatory T cell response to influenza infection in murine immune cells (Tsai et al., 2018). Furthermore, insulin played an important role in anti-inflammatory actions and reduced markers of inflammations in hospitalized patients with critical illness (Hansen et al., 2003). Intravenous insulin treatment had strong beneficial effects on inflammation and coagulation in hospitalized type 2 diabetic patients with COVID-19 over a period of 2 weeks (Sardu et al., 2020). As with other severe infection, diabetic ketoacidosis (DKA) has been reported in DM patients with COVID-19. Available evidence highlighted that subcutaneous insulin therapy was a useful strategy for uncomplicated DKA during the pandemic (Palermo et al., 2020). Particularly, Chen et al. showed that attention needed to be paid to patients with DM and COVID-19 who use insulin (Chen et al., 2020). They performed a retrospective study involving 904 patients with DM and COVID-19 and confirmed that insulin users had a greater risk of poor prognosis compared with noninsulin users (aOR 3.58 [95% CI 1.37, 9.35]; $p = 0.009$), but the study could not rule out the possible existence of truly uninfected patients among the clinically diagnosed cases (Chen et al., 2020).

Using the nonobese diabetic mice model, Heleia et al. reported that insulin downregulated ACE2 receptors (Roca-Ho et al., 2017), which might reduce the risk of viral infection. Moreover, an observational study revealed significantly higher insulin requirements among COVID-19 patients (Bornstein et al., 2020), which might be attributed to the beta-cell dysfunction induced by SARS-CoV2. Further research is required to clarify the clinical influence of insulin in the context of COVID-19.

Metformin

Metformin, a first line antidiabetic drug in the treatment of type 2 diabetes, has anticipated antiproliferative and immunomodulatory effects. Previous studies suggested prohibiting metformin in patients with DM and COVID-19, due to an anticipated DKA in the context of multiorgan dysregulation (Puig-Domingo et al., 2020; Sinclair et al., 2020; Singh et al., 2020). Emerging evidence found that treatment with metformin in DM patients with coronavirus infection is not harmful and could possibly be beneficial (Kumar Singh and Singh, 2020). A multicenter study explored the association of blood glucose control and outcomes in patients receiving different antidiabetic agents with COVID-19 and found no harm with metformin (Zhu et al., 2020). In the Coronavirus Disease and Diabetes Outcome (CORONADO) trial, Bertrand et al. showed that only metformin users had a lower rate of death among all the antidiabetic agents, but the sample size and short-term prognosis (i.e., 7 days after admission) limited the credibility of the study (Cariou et al., 2020). Consistent with this result, Luo et al. performed a retrospective study including 283 patients with COVID-19 and suggested that in-hospital mortality was significantly lower in those receiving metformin compared with those not receiving (2.9% *vs.* 12.3%; $p = 0.01$) (Luo et al., 2020), but this finding might have been driven by selection bias, as patients with severe respiratory problems could not be treated with metformin. Noteworthily, metformin was recommended to be contraindicated in patients with or at risk of acidosis (Flory et al., 2020), and it should be discontinued if the glomerular filtration rate (GFR) was less than 30 ml per minute per 1.73 m^2 [https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-fda-revises-warnings-regarding-use-diabetes-medicine-metformin-certain (2017)]. Recently, the guidelines for the management of diabetes during the COVID-19 pandemic addressed that it was recommended to stop treatment with metformin in those with fever and acute illness (body temperature >38.5 ˚C, GFR <30 ml/min/1.73 m^2) (Sinclair et al., 2020).

Mechanistically, metformin activates AMP-activated protein kinase (AMPK) by causing its phosphorylation and regulates glucose and lipid metabolism (Zhou et al., 2001). Of note, as a downstream of AMPK, PI3K/AKT/mTOR pathway played major roles in MERS-CoV infection (Kindrachuk et al., 2015). Therefore, metformin may offer benefits in DM patients with coronavirus infection by indirectly mediating the mTOR pathway.

Pioglitazone

Pioglitazone, a classical antidiabetic agent, has anti-inflammatory and antifibrotic activities (Radwan and Hasan, 2019). Studies have suggested that pioglitazone upregulated the expression of ACE2 (Zhang, et al., 2013), raising concerns about possible increased susceptibility to SARS-CoV2 infection (Pal and Bhadada, 2020). Furthermore, due to its adverse effects such as fluid retention (Alam et al., 2019), pioglitazone was recommended for discontinuation in acutely ill patients. In contrast, Mukherjee et al. considered that pioglitazone had more potential benefit than harm, and it could be continued in people with moderate COVID-19 (Jagat et al., 2020). Indeed, pioglitazone has been shown to decrease the secretion of various proinflammatory cytokines in the monocytes and macrophages (Bassaganya-Riera et al., 2010). Similarly, pioglitazone had the potential of blunting the cytokine storm by blocking caspase recruitment domain-containing protein 9 (CARD9) at the center

of the immune activation mechanism in macrophages (Erol, 2020). Interestingly, computer-simulation-based bioinformatic analysis found that pioglitazone may target 3-chymotrypsin-like protease (3CLpro) and potentially inhibited SARS-CoV2 RNA synthesis and replication (Wu C. et al., 2020b). However, pioglitazone therapy was associated with weight gain and oedema and more importantly was associated with aggravation of heart failure (Kernan et al., 2016), which did not support the use of pioglitazone in patients with COVID-19. More clinical trials are needed to optimize the risk-benefit ratio of using pioglitazone in patients with COVID-19.

DPP4 Inhibitors

DPP4, originally known as cluster of differentiation 26 (CD26), is a multifunctional soluble and cell-bound serine protease and plays critical roles in glucose homeostasis and inflammatory responses (Deacon, 2019). A previous study identified that DPP4 was a functional receptor for MERS-CoV (Raj et al., 2013) and may also participate in SARS-CoV2 infection despite not being its primary entry receptor. Targeting DPP4 has been thus considered as a pharmacologically reasonable strategy in the case of severe respiratory diseases related to coronaviruses and COVID-19 (Reinhold and Brocke, 2014; Iacobellis, 2020). It was also noteworthy that DPP4 was also involved in inflammatory and immune functions (Trzaskalski et al., 2020). Studies have proved that sitagliptin, one of the DPP4 inhibitors, was believed to reduce levels of proinflammatory markers such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Matsubara et al., 2013; Satoh-Asahara et al., 2013). In this regard, DPP4 inhibitors might prevent coronaviruses infection and exert anti-inflammatory role. In a multicenter, retrospective study of the 338 consecutive patients with type 2 diabetes and COVID-19, sitagliptin treatment was associated with reduced mortality and improved clinical outcomes (Solerte et al., 2020). However, this retrospective study has several shortcomings, including the nonrandomized uncontrolled design, a slight increase in some of the inflammatory markers detected at baseline in the standard-of-care group as compared with the sitagliptin-treated patients, and the lack of some clinical data that were not available for all patients. Current knowledge did not all support the beneficial effects of DPP4 inhibitors on patients with diabetes and COVID-19. Recently, a retrospective study involving 904 patients with DM and moderate-severe COVID-19 showed that the use of DPP4 inhibitors did not significantly affect mortality and clinical outcomes (Chen et al., 2020). Another epidemiological study including 403 hospitalized COVID-19 patients found that DPP4 inhibitors might not affect the risk of hospitalization for COVID-19 patients with type 2 diabetes (Fadini et al., 2020b). A case series involving 387 COVID-19 patients in Italy described the association between DPP4 inhibitors treatment and a statistically reduced mortality, but the result was based on only 11 patients (Mirani et al., 2020). Of note, DPP4 inhibitors treatment was associated with worse outcomes in 27 patients with type 2 diabetes treated with DPP4 inhibitors than in 49 patients treated with other glucose-lowering drugs (Dalan et al., 2020). Consequently, there are some essential issues to be addressed before claiming possible beneficial effects of DPP4 inhibitors on COVID-19, and the effects of DPP4 inhibitors in patients with type 2 diabetes and COVID-19 should be confirmed in an ongoing randomized, placebo-controlled trial.

SGLT2 Inhibitors

SGLT2 inhibitors were proposed as the second line treatment following metformin in the latest guidelines for the management of type 2 diabetes. Although several studies have discussed the potential benefits of SGLT2 inhibitors in COVID-19 patients (Chatterjee, 2020; Koufakis et al., 2020), the use of SGLT2 inhibitors was not beyond criticism. SGLT2 inhibitors were reported to increase ACE2 expression in kidney and therefore forming theoretical concern to increase susceptibility to SARS-CoV2 infection (Pal and Bhadada, 2020). Moreover, an expert panel recommended to avoid SGLT2 inhibitors among patients with DM and moderate-to-severe COVID-19 due to risk of dehydration and euglycemic DKA (Bornstein et al., 2020). Recently, Bossi et al. showed that SGLT2 inhibitors lacked efficacy in severe pneumonia related to novel coronavirus infection (Bossi et al., 2020). Conversely, SGLT2 inhibitors might exert anti-inflammatory effect in animal models (Bonnet and Scheen, 2018), which could favorably impact the dysregulated process in the context of cytokine storm of COVID-19. Intriguingly, dapagliflozin, a SGLT2 inhibitor, has been shown to decrease lactic acidosis and reverse acid-base balance inside the cells during hypoxia, thus contributing to prevent cell injury in the setting of cytokine storm of COVID-19 illness in patients with DM (Cure and Cure Cumhur, 2020). SGLT2 inhibitors have already been reported to provide a significant cardiorenal benefit, and thus they also might offer a protection to vital organs in the context of COVID-19. With these assumptions, "Dapagliflozin in Respiratory Failure in Patients with COVID-19" (DARE-19), a phase-3 multinational double-blind placebo-controlled randomized clinical trial (NCT04350593) has been initiated [https://www.clinicaltrials.gov/ct2/show/NCT04350593 (2020)]. Although SGLT2 inhibitors have been considered to provide benefits, they should be carefully reevaluated in case of body temperature >38.5°C or in case of food abstinence of insulin deficiency. Therefore, the potential benefit of SGLT2 inhibitors requires further validation.

GLP-1RAs

GLP-1RAs, known as incretin mimetics, improve glucose homeostasis through enhancing glucose-dependent insulin secretion. Researchers found that liraglutide, the first long-acting GLP-1RAs, increased the expression of ACE2 in lungs and heart, which also raised a theoretical concern in patients with COVID-19 (Pal and Bhadada, 2020). Similar to DPP4 inhibitors, GLP-1RAs exerted anti-inflammatory effects by interfering with NF-kB signaling pathways (Lee and Jun, 2016). Furthermore, GLP-1RAs were associated with significant reduction in inflammatory cytokine in the respiratory epithelium in mice infected with respiratory syncytial virus (Bloodworth et al., 2018). Given that beneficial roles of GLP-1RAs for the prevention of cardiovascular and kidney diseases have been well established (Prattichizzo et al., 2019), these drugs could be an ideal option for the treatment of patients with DM at

TABLE 1 | Potential benefits or risks of antidiabetic agents in the context of coronavirus infections.

Antidiabetic agents	Beneficial or adverse effects	References	Recommendations
Insulin	Downregulated ACE2 receptors	Roca-Ho et al. (2017)	Preferred treatment options for critically ill patients
	Reduced inflammatory markers	Hansen et al. (2003), Sardu et al. (2020)	
	Reduced uncomplicated DKA	Palermo et al. (2020)	
	Increased the risk of poor prognosis	Chen et al. (2020)	
Metformin	Lowered deaths and interleukin-6 levels	Chen et al. (2020), Cariou et al. (2020)	Continued in mild to moderate COVID-19 and avoided in critically ill
	Lowered in-hospital mortality	Luo et al. (2020)	
	Targeted PI3K/AKT/mTOR pathways and inhibited viral replication	Kindrachuk et al. (2015)	
Pioglitazone	Upregulated ACE2 receptors	Zhang, et al. (2013)	Continued in mild to moderate COVID-19 and avoided in critically ill
	Decreased various proinflammatory cytokines	Bassaganya-Riera et al. (2010), Erol (2020)	
	Targeted 3CLpro and potentially inhibited SARS-CoV2 RNA synthesis and replication	Wu C. et al. (2020b)	
DPP4 inhibitors	Suppressed MERS-CoV infection	Reinhold and Brocke (2014)	Continued in mild to moderate COVID-19. More data needed for the acutely ill patients
	Reduced levels of proinflammatory markers	Satoh-Asahara et al. (2013), Matsubara et al. (2013)	
	Reduced mortality and improved clinical outcomes	Solerte et al. (2020)	
	Did not significantly affect mortality and clinical outcomes	Chen et al. (2020)	
	Might not affect the risk of hospitalization	Fadini et al. (2020a)	
	Associated with worse outcomes	Dalan et al. (2020)	
SGLT2 inhibitors	Upregulated ACE2 expression in kidney	Pal and Bhadada (2020)	Continued in mild to moderate COVID-19 and avoided in critically ill
	Exerted anti-inflammatory action and reduced cardiovascular and renal complications	Bonnet and Scheen (2018)	
	Decreased lactic acidosis and reversed acid-base balance inside the cells during hypoxia	Cure and Cure Cumhur (2020)	
GLP-1RAs	Increased the expression of ACE2 in lungs and heart	Pal and Bhadada (2020)	Continued in mild to moderate COVID-19. More data needed for the acutely ill patients
	Exerted anti-inflammatory effects and reduced inflammatory cytokine	Lee and Jun (2016), Bloodworth et al. (2018)	
	Reduced hypoglycemia and glucose variability	Mustafa and Whyte (2019)	

ACE2, angiotensin converting enzyme 2; DKA, diabetic ketoacidosis; COVID-19, coronavirus disease 2019; 3CLpro, 3-chymotrypsin-like protease; DPP4, dipeptidyl peptidase-4; SGLT2, sodium-glucose cotransporter 2; GLP-1RAs, glucagon-like peptide 1 receptor agonists.

such risk (Ceriello et al., 2020). Of note, GLP-1RAs therapy was associated with reduction of hypoglycemia and glucose variability in the intensive care unit (ICU) setting, which could be protective in the critically ill patients (Mustafa and Whyte, 2019). However, initiating or maintaining such therapies in acute or critical situations (such as severe COVID-19) was not recommended because they will take time to become effective, due to slow uptitration, and might provoke nausea and vomiting (Nauck and Meier, 2019). There is insufficient evidence to clarify the use of GLP-1RAs in the context of the coronavirus infection. To date, no relative clinical-epidemiological studies have been carried out concerning the correlation between GLP-1RAs and COVID-19.

CONCLUSION AND FUTURE PERSPECTIVE

As available clinical evidence implicated diabetes as important risk factor impacting the severity of coronavirus infections, including SARS-CoV2, intensive monitoring and antidiabetic drug therapy should be considered in diabetic patients with COVID-19. We have attempted to highlight the potential benefits or risks of antidiabetic agents in the context of coronavirus infections (**Table 1**). Furthermore, we also addressed the clinical therapeutic choices of these agents for critically ill or moderate COVID-19 patients.

Accumulative clinical studies have confirmed that DM was associated with a higher risk of severity and fatality of COVID-19 (Wu J. et al., 2020b; Zhang, et al., 2020), but few researchers clarified the influence of COVID-19 on DM. Remarkably, recent studies pointed that there was a bidirectional relationship between DM and COVID-19 (Rubino et al., 2020). New onset diabetes and severe metabolic complications of preexisting diabetes have been observed in patients with COVID-19 (Chee et al., 2020; Li et al., 2020), which posed challenges for clinical management of DM and suggested a complex pathophysiology of COVID-19-related diabetes. Thus, it is essential to investigate the epidemiologic features and pathogenesis of COVID-19-related diabetes and to gain clues regarding appropriate use of antidiabetic agents for patients during the COVID-19 pandemic.

Although current evidence has affirmed the role of antidiabetic agents in patients with COVID-19, it is not yet fully clear that these agents have a favorable or unfavorable effect. Nonetheless, well-controlled blood glucose is particularly crucial for DM patients with COVID-19 (Critchley et al., 2018; Wu J. et al., 2020a; Zhu et al., 2020). Therefore, it is

essential to balance blood glucose control and avoid hyperglycemia or hypoglycemia during the use of antidiabetic agents. Noteworthily, a previous study indicated that insulin combined with continuous glucose monitoring (CGM) reduced hypoglycemia and proved to be safe and feasible (Breton et al., 2018). In this regard, antidiabetic agents combined with CGM might be a good treatment option for COVID-19 patients, particularly for the critical patients. On the other hand, another research showed that lixisenatide added to basal insulin significantly balanced blood glucose excursions without increasing the risk of hypoglycemia (Umpierrez et al., 2017). Thus, antidiabetic drugs combination might contribute to good blood glucose control and reduce adverse risks in moderate COVID-19 patients. Currently, there is only weak evidence to elucidate specific effects of antidiabetic drugs on COVID-19, and the retrospective analyses are subject to biases and unmeasured confounding. Further prospective randomized studies to confirm these therapeutic strategies are warranted.

Taken together, particular attention should be given to the safety concerns related to COVID-19 and the use of antidiabetic agents in patients with DM, and further clinical research in these domains will contribute to providing evidence-based therapies.

AUTHOR CONTRIBUTIONS

BS and JZ contributed to the supervision, concept, design, and revision of the article. All other authors contributed to the design and revision of the article. All authors gave final approval to submit the article for publication.

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Several FDA-Approved Drugs Effectively Inhibit SARS-CoV-2 Infection *in vitro*

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To identify drugs that are potentially used for the treatment of COVID-19, the potency of 1403 FDA-approved drugs were evaluated using a robust pseudovirus assay and the candidates were further confirmed by authentic SARS-CoV-2 assay. Four compounds, Clomiphene (citrate), Vortioxetine, Vortioxetine (hydrobromide) and Asenapine (hydrochloride), showed potent inhibitory effects in both pseudovirus and authentic virus assay. The combination of Clomiphene (citrate), Vortioxetine and Asenapine (hydrochloride) is much more potent than used alone, with IC50 of 0.34 μM.

Keywords: drug screening, SARS-CoV-2, pseudovirus assay, vesicular stomatitis virus, drug combination

INTRODUCTION

As of September 22, 2020, the COVID-19 pandemic has claimed more than 966,399 lives, but yet effective drug is not available. It is time-consuming to develop vaccines or specific drugs for a disease caused by a novel defined virus like SARS-CoV-2. Re-purposing of approved drugs may be a faster way to find treatment for COVID-19. Verification of drugs that might suppress SARS-CoV-2 by prediction, including drugs against similar virus and broad-spectrum antiviral agents (BSAAs), is time-saving for drug re-purposing at the expense of missing some potential candidates. Integrative, antiviral drug repurposing methods based on big data analysis or molecular docking and molecular dynamics are time-saving and high throughput. However, drugs identified by virtual screening still need to be verified *in vitro* and *in vivo*.

In our previous research, a robust neutralization assay was established based on SARS-CoV-2 S-bearing vesicular stomatitis virus (VSV) pseudovirus and human ACE2-expressing BHK21 cells (BHK21-hACE2) (Xiong et al., 2020). Single-cycle infectious of recombinant VSV-SARS-CoV-2-Sdel18 mimics the entry of SARS-CoV-2. The BHK21-hACE2 cells with high expression level of human angiotensin-converting enzyme 2 (hACE2) need only 6 h to proliferate one generation, which support efficiently infection of pseudovirus and infection of pseudovirus can be detected by fluorescence 12 h after infection, enabling the assay time-saving for high-throughput screening (Xiong et al., 2020). This pseudovirus based assay is suitable for screening drugs that can block the infection of SARS-CoV-2. In this study, the anti-SARS-CoV-2 potentiality of 1403 FDA approved drugs were quantitatively evaluated by the pseudovirus-based assay and the effect of candidate drugs were confirmed using authentic virus assay.

RESULTS

Screen for Compounds Could Inhibit the Infection of Vesicular Stomatitis Virus-SARS-CoV-2-Sdel18

The screening procedure was illustrated in **Figure 1A** and described in methods. The numbers of GFP-positive cells from drug treated wells were counted and divided by the number of infected cells from the well without treatment of drugs to calculate the relative value of infection rate. The results of two repetitions showed that most of drugs did not inhibit viral infection (**Figure 1B**). Forty-four drugs with relatively better inhibitory effect, whose inhibit ratio were higher than 85% (relative value below 15%) were selected for further validation.

In the second round of screening, the effect of inhibiting viral infection and cell cytotoxicity in different concentration conditions were both evaluated (**Figure 2**). Among them, 32 drugs were excluded due to cytotoxicity (cell viability were lower than 80% when treated with compounds at concentration of 40 μM or cell viability were lower than 85% when compounds were used at concentration of 20 μM). Twelve drugs were selected for analysis of specificity to VSV-SARS-CoV-2-Sdel18 and verification by authentic SARS-CoV-2 assay.

The Specificity of Selected Compounds for Vesicular Stomatitis Virus-SARS-CoV-2-Sdel18

To verify whether these selected drugs act on spike protein of SARS-CoV-2 on the pseudovirus or the VSV backbone, we evaluated the inhibitory effect of these compounds on VSV-G (The sequence of GFP was inserted into the genome of VSV, so that the infection of VSV could be indicated by green fluorescence.). Ribavirin exhibited significant inhibitor effects on VSV-G, whereas no obvious effect was noted for other compounds (**Figure 3**).

The Effect of Selected Compounds in Pseudovirus Assay and Authentic Virus Assay

The IC50 and IC90 for VSV-SARS-CoV-2-Sdel18 pseudovirus were further analyzed (**Figure 4**). Of the 12 compounds that selected in the second round of screening, seven drugs could

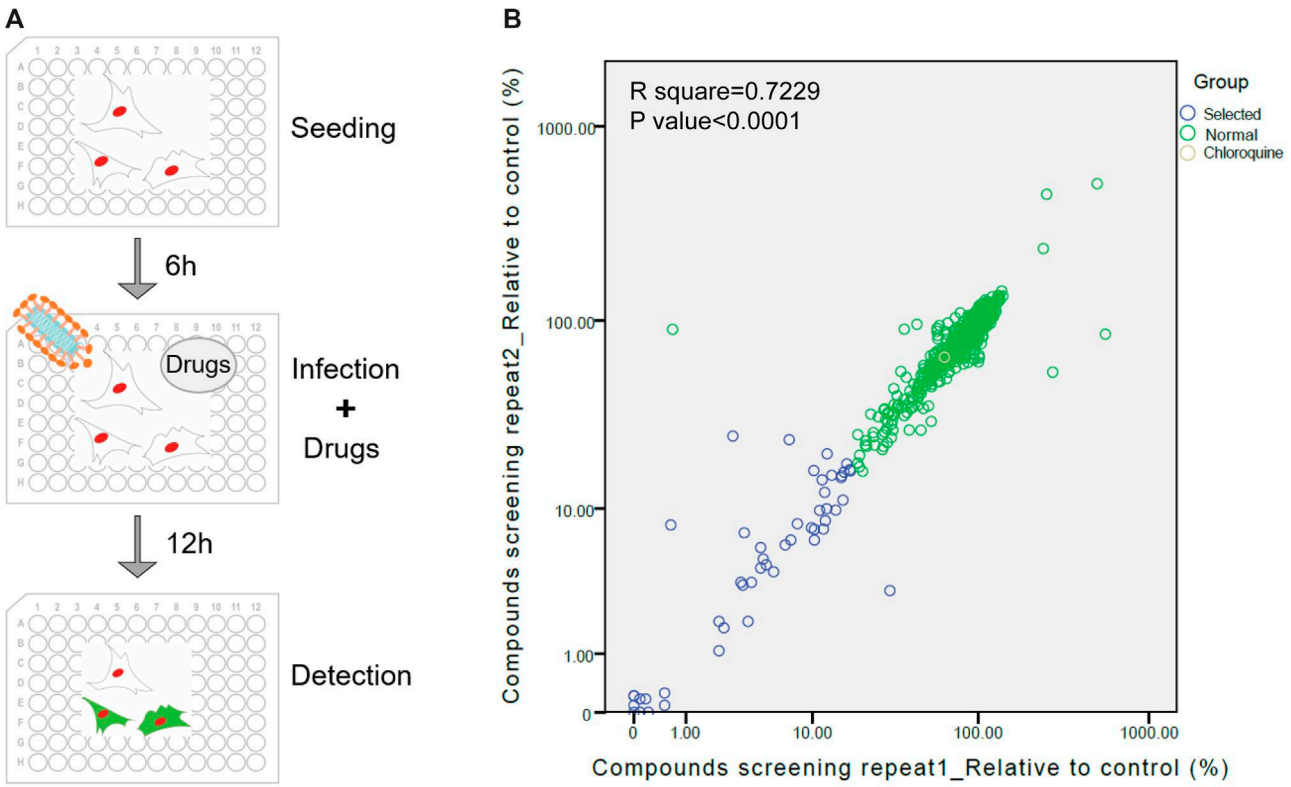

FIGURE 1 | Re-purposing of FDA-approved drugs for inhibiting SARS-CoV-2 infection. **(A)** Schematic diagram of the screening process of the pseudovirus model. **(B)** The first round of screening for compounds could inhibit the infection of VSV-SARS-CoV-2-Sdel18. The abscissa and ordinate respectively indicate two repetitions of screening. Yellow circle: chloroquine. Blue circles: 44 drugs selected for the second round of screening, Green circles: remaing FDA-approved drugs.

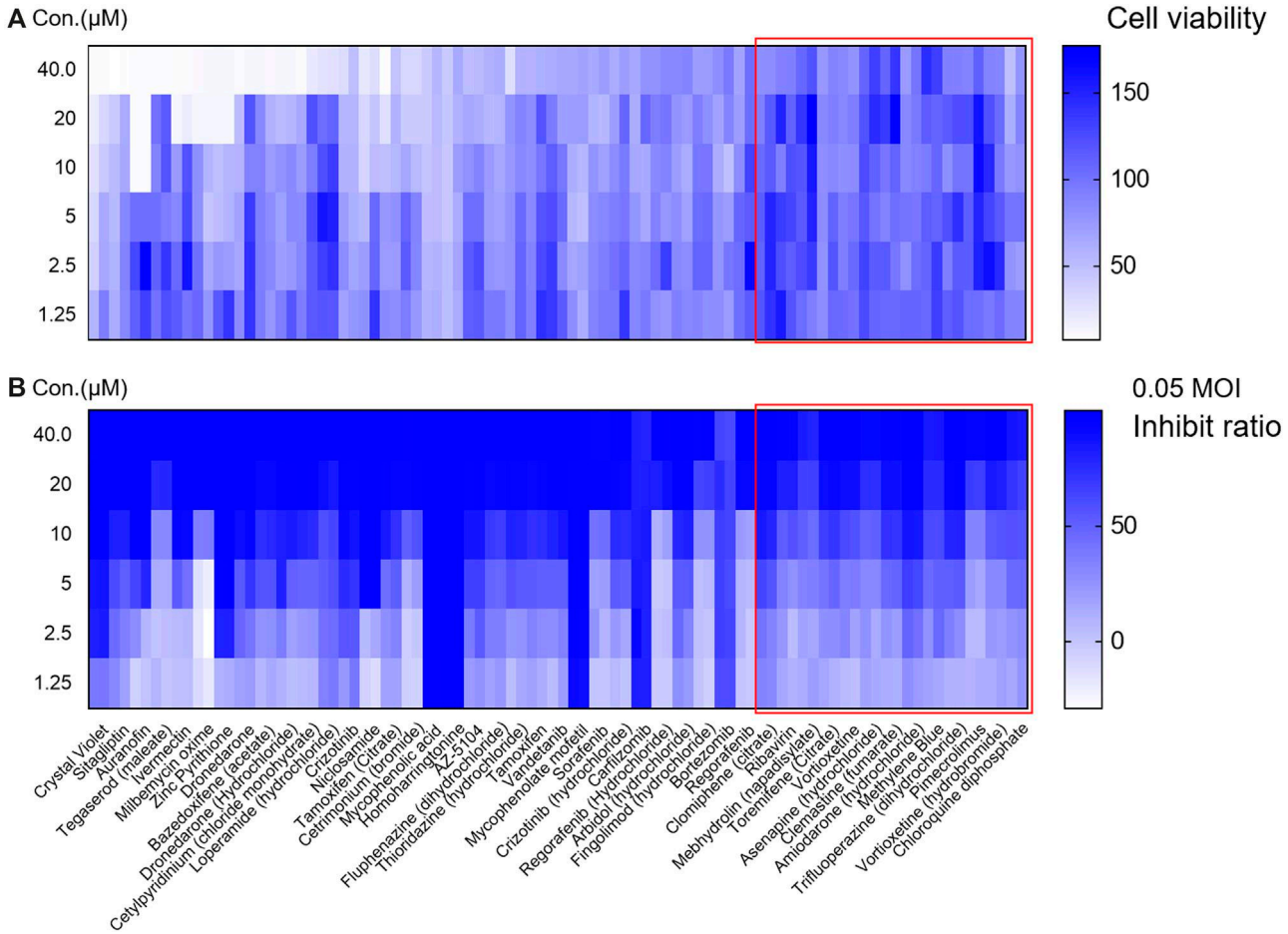

FIGURE 2 | Evaluate the effect and cytotoxicity of the 44 compounds selected in the first round of screening. The 44 compounds and control (chloroquine diphosphate) were seriously diluted to analyze the cytotoxicity **(A)** and effect in inhibiting viral infection **(B)**. Colorbar indicates cell viability **(A)** or inhibiton rate **(B)**.

inhibit viral infection with IC90 lower than 50 μM, including Amiodarone (hydrochloride), Clomiphene (citrate), Trifluoperizine (dihydrochloride), Clemastine (fumarate), Pimecrolimus, Vortioxetine (hydrobromide) and Vortioxetine. Trifluoperizine (dihydrochloride), Clemastine (fumarate) and Pimecrolimus showed more serious cytotoxic than other drugs. Although the inhibitory effect of Asenapine (hydrochloride) is not as good as the seven compounds mentioned previously, it has the lowest cytotoxicity. Even when used at the concentration of 100 μM, no obvious cell cytotoxicity was observed. Considering the inhibitory effect and cytotoxicity, five compounds inhibited the infection of VSV-SARS-CoV-2-Sdel18 pseudovirus specifically, including Clomiphene (citrate), Amiodarone (hydrochloride), Vortioxetine, Vortioxetine (hydrobromide) and Asenapine (hydrochloride), were selected and the function of these compounds was confirmed using authentic SARS-CoV-2 assay (**Figure 5**). Among them, the inhibitory effects of Clomiphene (citrate) and Vortioxetine were comparable to Chloroquine diphosphate *in vitro*, while Vortioxetine (hydrobromide) and Asenapine (hydrochloride) were slightly less effective. Whereas Amiodarone (hydrochloride) inhibited the infection of pseudovirus efficiently with IC50 around 4.44 μM, but it showed no effect on authentic SARS-CoV-2 virus infection even used at a concentration of 100 μM.

The Potential Applications in Prophylaxis and Combination Therapy

We treated the cell with pseudovirus and different drug combinations. The drug combinations were added either at the same time of pseudovirus infection or 6 h pre-infection (**Table 1**). The combination of Clomifene (citrate), Vortioxetine and Asenapine (hydrochloride) showed best effect when used both at the time of infection and pre-infection, with IC50 about 1.93 and 0.34 μM respectively. The combination of Clomifene (citrate) and Vortioxetine had a comparable effect, with IC50 about 2.36 and 0.69 μM respectively. The combination of drugs decreases the concentration of each drug required to block virus infection, which may reduce the side effects of drugs. However, it remains to be evaluated whether these drugs can be used together *in vivo*.

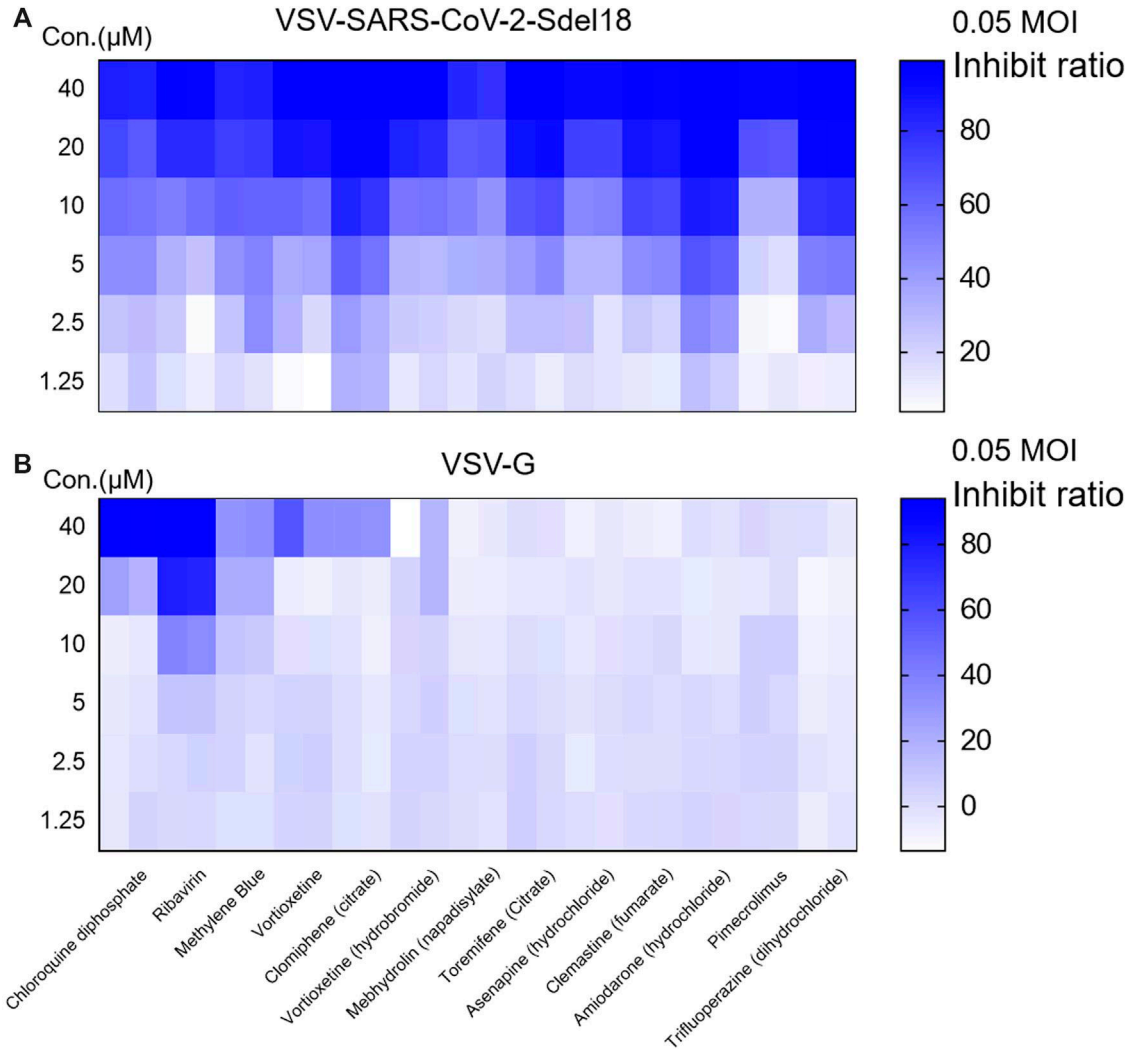

FIGURE 3 | Screening for the compounds specially block the spike protein of SARS-CoV-2 mediated viral entry. The inhibitory effect to VSV-SARS-CoV-2-Sdel18 **(A)** and VSV-G **(B)** of 13 seleted compounds was evaulated to exclude drugs inhibit infection or expression of VSV-G. Colorbar indicates inhibiton rate.

DISCUSSION

Thousands of clinical trials have been initiated to establish evidence around investigational drugs and vaccine candidates. There are currently no approved vaccines against SARS-CoV-2 for commercial use, except for two approved for early or limited use. COVID-19 vaccine candidate of CanSino and CanSino have been approved, but only for military use or medical workers. The Food and Drug Administration (FDA) has not fully approved any medication for treating people infected with SARS-CoV-2. Some drugs with good effects in clinical use are granted emergency use authorizations for certain patients hospitalized with COVID-19, such as dexamethasone and remdesivir. Dexamethasone, a cheap and widely available steroid, cut deaths by one-third among patients critically ill with COVID-19 in a large trial (Horby and Landrain, 2020). Dexamethasone can alleviate the overreaction of the immune system, which is a main cause of severe cases and fatalities (Lammers et al., 2020). Remdesivir, an investigational nucleotide analog with broad-spectrum antiviral activity by inhibiting viral replication, also showed clinical improvement. These two drugs have different mechanisms of action, the combination may be complimentary.

Both dexamethasone and remdesivir act on the steps after viral infection. The combination of drugs act on viral entry in addition may benefit further. The effect of chloroquine and hydroxychloroquine has attracted much attention. Several *in vitro* studies reported antiviral activity of chloroquine and hydroxychloroquine against SARS-CoV-2. However, this drug provided no additional benefit compared to placebo control for the treatment of COVID-19 in hospitalized patients (Frie and Gbinigie, 2020). In addition, cardiomyopathy and heart rhythm disturbances caused by treatment with chloroquine have been reported and Risambaf et al. raise concerns about the risk of

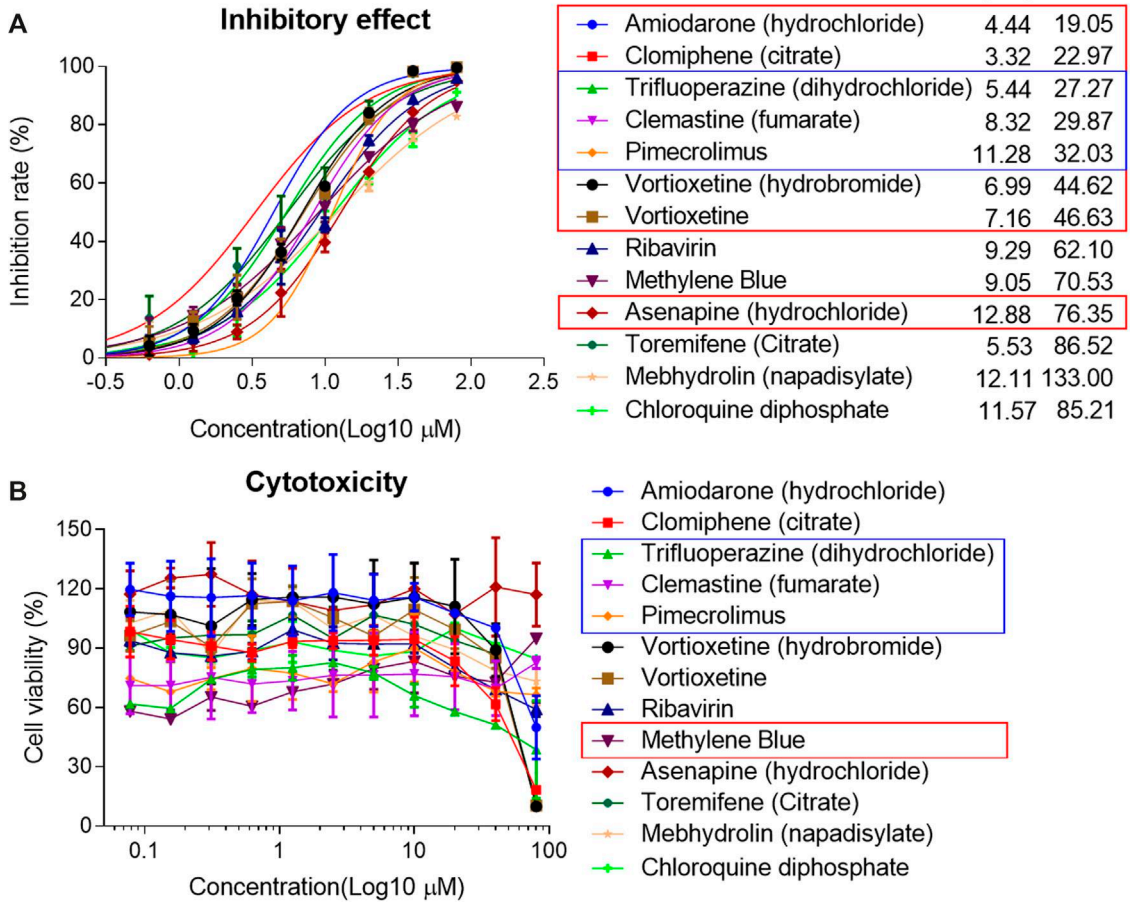

FIGURE 4 | Analyze the inhibitory effect of 12 selected compounds in VSV-SARS-CoV-2-Sdel18 pseudovirus assay. The 12 compounds and control (chloroquine diphosphate) were seriously diluted to analyze the effect in inhibiting viral infection **(A)** and cytotoxicity **(B)**. The IC50 and IC90 were calculated with non-linear regression.

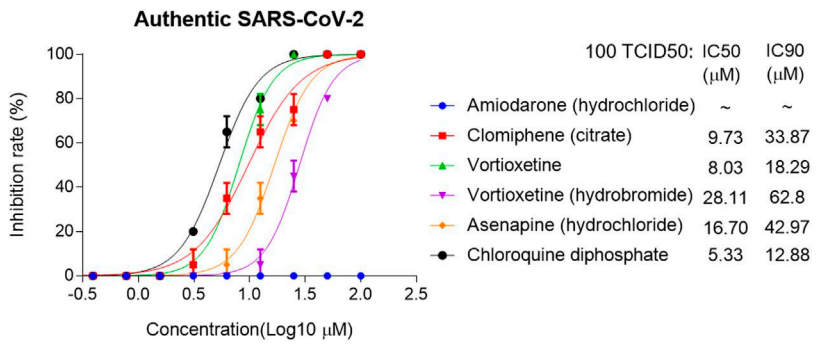

FIGURE 5 | Analyze the inhibitory effect of five selected compounds in authentic SARS-CoV-2 assay. The IC50 and IC90 were calculated with non-linear regression.

toxicity to liver and kidney caused by chloroquine and hydroxychloroquine when they are used to treat COVID-19 (Cubero et al., 1993; Costedoat-Chalumeau et al., 2007; Rismanbaf and Zarei, 2020). Drugs that inhibit the infection of SARS-CoV-2 with higher efficiency and lower side effect may be alternative for the treatment of COVID-19.

Drugs that block the infection of SARS-CoV-2 may alleviate disease progression, protect health care workers and other

TABLE 1 | The inhibitory potency of combination of drugs.

Drug	Pre-treatment			Co-treatment		
	IC50 (μM)	IC90 (μM)	CC50 (μM)	IC50 (μM)	IC90 (μM)	CC50 (μM)
Clo + Vor	0.69	4.81	14.47	2.36	11.80	23.55
Clo + Ase	1.60	10.39	21.39	3.71	20.06	36.20
Vor + Ase	2.08	11.06	14.12	3.52	17.47	28.00
Clo + Vor + Ase	0.34	5.01	14.67	1.93	9.42	16.83
Clo + CQ	2.57	12.81	28.01	7.32	17.99	37.98
Vor + CQ	3.03	13.00	~18.93	5.39	17.87	25.69
Ase + CQ	5.73	55.30	128.00	12.70	58.87	Na
Clo	2.94	10.16	24.94	9.53	19.57	54.35
Vor	3.00	13.83	28.53	6.77	22.28	27.05
Ase	17.69	127.10	Na	28.13	117.60	Na
CQ	9.27	35.22	349.10	27.60	106.20	Na

"Clo" means Clomiphene (citrate), "Vor" means Vortioxetine, "Ase" means Asenapine (hydrochloride) and "CQ" means Chloroquine diphosphate. "Pre-treatment" means cell was treated with drugs 6 h before infection, while "Co-treatment" means cells were treated with drugs at the time of infection. IC50, IC90 and CC50 were calculated using prism software (GraphPad). "na" means the value can't be calculated. MOI = 0.1.

populations at high risk of infection. In addition, drugs inhibit viral infection might be used in combination with drugs that inhibit viral replication reported previously. In this research, an efficient VSV-SARS-CoV-2-Sdel18 pseudovirus model was applied to identify candidates that can inhibit infection of SARS-CoV-2 from 1,403 approved drugs. Five drugs, which haven't been identified before, showed comparable or superior inhibitory effect to chloroquine in this model. The effect was also confirmed using authentic SARS-CoV-2 assay and four of them can also inhibit the infection of authentic SARS-CoV-2 virus.

Clomifene Citrate is a selective estrogen receptor modulator and a non-steroidal fertility medicine. It has a long history of use since 1967 and has the advantages of oral availability, good safety, and tolerability profiles. Johansen et al. identified Clomiphene as potent inhibitors of Ebola virus infection by performing an *in vitro* screen of FDA and ex-US-approved drugs. This drug showed EC50 values of 11 and 3.8 μM against the two strains EBOV-95 and EBOV-76, respectively, and a 90% of survival benefit for infected mice. It may inhibit Ebola virus through inducing accumulation of cholesterol in endosomal compartments and blocking the release of viral genome to cytoplasm (Johansen et al., 2013; Wrensch et al., 2014; Nelson et al., 2016). The viral entry of SARS-CoV-2 includes the endocytosis of enveloped viral particle, priming of spike protein by protease, fusion between viral and cellular membranes and release of viral genome, which is similar to Ebola virus (Simmons et al., 2004; Burkard et al., 2014; Hoffmann et al., 2020). Therefore, the Clomiphene may impair SARS-CoV-2 infection via the same pathway as Ebola virus.

Vortioxetine is an antidepressant drug that is used to treat major depressive disorder in adults. Vortioxetine was safe and well tolerated, it was approved in 2013 (Baldwin et al., 2016). So far, no previous study described its antiviral roles. It is reported that sever COVID-19 patients have a high probability of suffering from mental illness. Recently, another antidepressant drug fluvoxamine is evaluated for the potential to treat COVID-19 by researchers from the Washington University School of Medicine, because the drug may prevent an overreaction of the immune system called cytokine storms, which could result in life-threatening organ failure. The antiviral mechanism of Vortioxetine remains unknow. However, it may bring physical and psychological benefits for COVID-19 patients.

Asenapine is an atypical antipsychotic drug which has been approved by the US Food and Drug Administration for the treatment of schizophrenia in adults and the treatment of acute manic or mixed episodes of bipolar I in both adult and pediatric populations. Asenapine is a tetracyclic drug with antidopaminergic and antiserotonergic activity with a unique sublingual route of administration and has been approved since 2009. It showed less cytotoxicity in this study comparing to other drugs that could inhibit the infection of SARS-CoV-2. Notably, although we have evaluated the effect of these candidate drugs in two different *in vitro* model and the combination of these drugs didn't show obvious cytotoxicity *in vitro*, the effect and safety *in vivo* still remain to be confirmed.

Several drug screenings for COVID-19 have been performed before and identified some candidate drugs, for example, Yadi Zhou et al. prioritized 16 potential anti-HCoV repurposable drugs (e.g., melatonin, mercaptopurine, and sirolimus) by using network proximity analyses of drug targets and HCoV–host interactions in the human interactome, drug target proteins select by Rameez Jabeer Khan et al. were screened against an in-house library of 123 antiviral drugs, they proposed that Raltegravir, Paritaprevir, Bictegravir and Dolutegravir are excellent lead candidates for these crucial proteins and they could become potential therapeutic drugs against SARS-CoV-2, Laura Riva et al. discovered SARS-CoV-2 antiviral drugs through large-scale compound repurposing by authentic SARS-CoV-2 assay (Dyall et al., 2014; Khan et al., 2020; Riva et al., 2020; Weston et al., 2020; Zhou et al., 2020). The focus of these studies varies and shed light on the treatment of COVID-19. The hits screened out from our study were different from other studies. The combination of drug candidates obtained by different screening strategies may have synergistic effect. The screening assay based on the single-cycle infectious VSV-SARS-CoV-2-Sdel18 has its advantage from the practical perspectives-manipulation in BSL-2. However, this assay also has disadvantages. Firstly, it may not be able to screen out compounds that can specifically target the steps of SARS-CoV-2 life cycle after viral entry; secondly, it may screen out compounds that inhibit the VSV, but not SARS-CoV-2. To address the second weakness, the specificity of hits out from the pseudovirus assay were confirmed using VSV-WT and the authentic SARS-CoV-2 assay. The candidates proposed in this study mainly function on inhibiting the viral entry, they could be combined with drugs act on other pathways, for example, combined with Remdesivir that inhibit replication of virus. Another limitation of the model is that hACE2 overexpressing BHK21 cell was derived from hamster and Vero cell supporting the infection of authentic SARS-CoV-2 was from African green monkey. The effects of candidate compounds in human cells also needs to be further verified.

In summary, our study identified four FDA-approved drugs that have the potential to suppress SARS-CoV-2 infection. The robust assay based on VSV-SARS-CoV-2-Sdel18 pseudovirus screened out the potential drugs with high efficiency, then the inhibitory effect was confirmed by authentic SARS-CoV-2 assay. The inhibitory effect of Vortioxetine and Clomifene is superior and the mechanism of these drugs seems different from Chloroquine. The combination of Clomifene (citrate), Vortioxetine and Asenapine (hydrochloride) greatly decreases the IC50/IC90 of blocking virus infection. The clinical safety of these compounds has been evaluated and the availability of pharmacological data are expected to enable rapid preclinical and clinical evaluation for treatment of COVID-19. Based on the existing clinical results, it seems that it is difficult for one particular drug alone to significantly benefit COVID-19 patients, and combination therapy is more likely to make the patient recover faster. This work identified novel drugs that suppress the infection of virus and provided more candidates for post-exposure prophylaxis and combination therapies. Notice that no test *in vivo* has been conducted and the mechanism of these compounds also remains unknown. More researches are required to support the clinical application of these drugs for treatment of COVID-19.

MATERIALS AND METHODS

Cells and Samples

Vero-E6 [American Type Culture Collection (ATCC), CRL-1586], Vero (ATCC, CCL-81), BHK21-hACE2 (Xiong et al., 2020) cells were maintained in high glucose DMEM (SIGMA-ALDRICH) supplemented with 10% FBS (GIBCO), penicillin (100 IU/ml), streptomycin (100 μg/ml) in a 5% CO_2 environment at 37°C and passaged every 2 days. In addition, the culture medium of BHK21-hACE2 contains puromycin (2 μg/ml). The FDA-approved drug library, including 1,403 compounds (10 mM DMSO solutions, MCE, HY-LD-000001083), and Chloroquine diphosphate were bought from MedChemExpress (MCE, HY-17589).

Pseudovirus-Based Assay

VSV pseudovirus carrying truncated spike protein of SARS-CoV-2, named VSV-SARS-CoV-2-Sdel18 virus, was packaged as previously described (Xiong et al., 2020). VSV-G was prepared in similar way (Whitt, 2010). In the first round of screening, all compounds were diluted to 20 μM and mixed with VSV-SARS-CoV-2-Sdel18 virus, the volume of diluted compounds and virus are 80 and 20 μL respectively. Each dilution repeated twice. Added 80 μL final mixture, which containing compounds (16 μM) and pseudovirus (MOI = 0.05), to pre-seeded BHK21-hACE2. After 12 h incubation, fluorescence images were obtained by ImmunSpot@S5 UV Analyzer (Cellular Technology Limited) or Operetta CLS (PerkinElmer). For quantitative determination, the numbers of GFP-positive cell for each well were counted to represent infection performance. The reduction (%) in GFP-positive cell numbers was calculated to show the inhibitory effect of compounds. In the second round of screening, selected compounds were diluted to 50 μM, then serial two-fold dilutions are used to prepare diluted analytes. 80 μL diluted compounds were mixed with 20 μL VSV-SARS-CoV-2-Sdel18 or VSV-G and the mixture were added to pre-seeded BHK21-hACE2. The results were obtained as described previously. To analysis the IC50 of selected compounds, the compounds were diluted to 100 μM as the first work concentration and 0.098 μM as the smallest concentration. Still mixed 80 μL diluted compounds with 20 μL VSV-SARS-CoV-2-Sdel18 virus. The remaining procedures were same as previous assay. The cytotoxicity of compounds was analyzed by Cell Counting Kit-8 (CCK-8, MCE). To evaluate the effect of drug combinations, the drugs were also diluted to 100 μM (the concentration of each drugs is 100 μM in mixture) and prepared serious dilutions. To evaluate the potential of applying these drugs in prophylaxis, the cell was pre-treated with 80 μL diluted drugs, 6 h later, add 20 μL virus to the culture medium (MOI = 0.1). In combination therapy, the combos were prepared in an equal molar ratio.

Authentic SARS-CoV-2-Based Assay

Vero cells were seeded 24 h before the infection in a 96-well plate (Costar). On the day of infection, the cells were washed twice with PBS. Candidate drugs were diluted 2-fold seriously by medium supplemented with 2% FBS (GIBCO), penicillin (100 IU/ml), streptomycin (100 μg/ml). Each drug was evaluated by diluting 14 gradients, with each gradient double repeats. Aliquots (40 μL) of diluted drugs (200 μM as initial concentration) was added to 40 μL of cell culture medium containing 100 times the tissue culture infective dose (TCID50) of the BetaCoV/Shenzhen/SZTH-003/2020 strain virus (GISAID access number: EPI_ISL_406594) on a 96-well plate in duplicate and incubated at 37°C for 2 h in CO_2 5% vol/vol. After incubation, virus drugs mix was then added to cells in 96-well plates and plates were incubated at 37°C with microscopic examination for cytopathic effect after a 5-days incubation. Ten fields of view were randomly selected for each repetition and cytopathic effect was quantified by the number of fields present with CPE. For example, if CPE was observed in seven of ten fields, which mean the cytopathic effect was 70%. The complete absence of cytopathic effect in an individual culture well was defined as protection. The values of IC50 were calculated using prism software (GraphPad).

Statistic

The relative value or inhibition rate of candidate drugs were calculated according to the decrease of GFP positive cell number (for pseudovirus-based assay) or cytopathic effect (for authentic SARS-CoV-2-based assay). The IC50 (the half maximal inhibitory concentration) and IC90 (the concentration for the 90% of the maximum inhibition) values were calculated with non-linear regression, i.e. log(inhibitor) vs. normalized response—Variable slope or log (agonist) vs. response—Find EC anything using GraphPad Prism 7.00 (GraphPad Software, Inc., San Diego, CA, United States).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

HX, TZ, QY and NX had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: TZ, QY and NX. Acquisition of data: HX, JC, JM, XQ, TS. CS performed the authentic virus assay. Analysis and interpretation of data: HX, JC, TZ. Drafting of the manuscript: JC, TZ, HX. Critical revision of the manuscript for important intellectual content: SG, JZ. Study supervision: TZ, QY and NX. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Modalities and Mechanisms of Treatment for Coronavirus Disease 2019

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Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is spreading rapidly throughout the world. Although COVID-19 has a relatively low case severity rate compared to SARS and Middle East Respiratory syndrome it is a major public concern because of its rapid spread and devastating impact on the global economy. Scientists and clinicians are urgently trying to identify drugs to combat the virus with hundreds of clinical trials underway. Current treatments could be divided into two major part: anti-viral agents and host system modulatory agents. On one hand, anti-viral agents focus on virus infection process. Umifenovir blocks virus recognizing host and entry. Remdesivir inhibits virus replication. Chloroquine and hydroxychloroquine involve preventing the whole infection process, including virus transcription and release. On the other hand, host system modulatory agents are associated with regulating the imbalanced inflammatory reaction and biased immune system. Corticosteroid is believed to be commonly used for repressing hyper-inflammation, which is one of the major pathologic mechanisms of COVID-19. Convalescent plasma and neutralizing antibodies provide essential elements for host immune system and create passive immunization. Thrombotic events are at high incidence in COVID-19 patients, thus anti-platelet and anti-coagulation are crucial, as well. Here, we summarized these current or reproposed agents to better understand the mechanisms of agents and give an update of present research situation.

Keywords: SARS-CoV-2, COVID-19, mechanisms, treatment, therapeutic agents

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has now affected 210 countries and territories, with more than five million confirmed cases. COVID-19 is rapidly spreading around the world, leading to widespread public concern and a global response. SARS-CoV-2, along with severe acute respiratory

syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), belong to the beta-coronavirus family. The full-length genome sequence of SARS-CoV-2 is 79.5% similar to MERS-CoV and 50% similar to SARS-CoV (Zhou P et al., 2020). Although we have learned much about the etiology and molecular characteristics of SARS-CoV-2, the origin of this novel virus remains unclear. Many studies support the hypothesis that bats are the most likely original host, with other animals, such as snakes or minks, acting as intermediate hosts (Li et al., 2005; Guo et al., 2020; Ji et al., 2020). SARS-CoV-2 targets the respiratory tract, but the lack of specific early symptoms makes it difficult to distinguish COVID-19 from other respiratory infections. Fever, cough, fatigue, and dyspnea are the most common early symptoms of COVID-19 (Booth et al., 2003; Yang J et al., 2020). In European patients, olfactory and gustatory dysfunction may precede the onset of respiratory symptoms and can be significant (Lechien et al., 2020). Patients with severe COVID-19 are vulnerable to complications and multi-organ damage (Huang C et al., 2020; Wu T et al., 2020). Compared with SARS and MERS, COVID-19 has a lower case-fatality, but the virus has a higher basic reproduction number and higher transmissibility (Chan et al., 2003; Zumla et al., 2015). SARS-CoV-2 can be spread within communities, households, and hospitals by confirmed COVID-19 patients or by asymptomatic individuals (Zhang X et al., 2020). The predominant transmission routes are droplet transmission and close contact, although other transmission routes such as erosol and fecal-oral transmission are possible, but not confirmed or refuted. It has been suggested that each patient with COVID-19 infects approximately 2.2 close contacts (Li Q et al., 2020), which partially accounts for the global COVID-19 pandemic.

Although some potential strategies for preventing the infection are proposed (Kang et al., 2020), however, in the absence of an effective vaccine, identification of effective drugs is crucial to treatment of this novel coronavirus. Both clinical experience and exploratory studies with other coronaviruses suggest more than 20 agents that may be potentially used to treat COVID-19. Some of these drugs such as corticosteroids, Chloroquine and Hydroxychloroquine (CQ/HCQ), as well as Lopinavir and Ritonavir (LPV/r) have been widely used in clinical practice, whereas others, such as Janus Kinase (JAK) inhibitors, have been introduced only recently. In this review, we have compiled all available evidence with which to establish a framework for COVID-19 treatment as well as therapeutic optimization.

MECHANISMS OF VIRUS INFECTION

Recognition: SARS-CoV-2 is a positive-stranded ribonucleic acid (+RNA) virus, whose genes encode 16 nonstructural proteins (nsp1 to nsp16) and four structural proteins, including Membrane (M), Spike(S), Envelope (E) and Nucleocapsid (N). Among them, S protein makes contribution to homo-trimeric spikes which are responsible for the virus entry via recognizing with the host receptor angiotensin converting enzyme II (ACE2) (Chen Y et al., 2020). S proteins can be cleaved into by an appropriate protease into two functional domains (S1 and S2) (Hoffmann et al., 2020). The receptor-binding domain (RBD) within S1 subunit is a key functional component for binding with ACE2 (Lan et al., 2020). In addition, S1 can be further divided into a C-terminal domain (CTD) and N-terminal domain (NTD). In contrast with MERS-CoV and SARS-CoV, SARS-CoV-2 applies the S1 CTD to interact with ACE2 (Wang Q et al., 2020). It is reported that the combination of spikes and ACE2 promotes the dissociation of the S1 with ACE2, which results in the transition of S2 to mediate fusion with cell membrane (Gui et al., 2017). The role of ACE2 in mediating entry of the virus also is highlighted (Hoffmann et al., 2020; Zhou P et al., 2020). Binding of virus to ACE2 is an important initiation of viral infections, thus any drugs prevent the process can be identified as a treatment option for COVID-19. Convalescent plasma (CP) and immunoglobins (IG) collected from recovery COVID-19 patients contain neutralizing antibodies, which could bind to S1-RBD, inhibiting the binding of virus with receptor, thus limiting viral entry (Rojas et al., 2020). Umifenovir fights against SARS-CoV-2 effectively by blocking or hindering trimerization of S protein (Vankadari, 2020). CQ/HCQ might inhibit entry process by interfering with the glycosylation of ACE2 and CQ also possesses the ability to inhibit sialic acid, which significantly affects activity of ACE2 (Kwiek et al., 2004; Hashem et al., 2020).

Entry: Coronavirus enter the host cells through two pathways: the endocytosis or membrane fusion. During the endocytosis, the viruses engulfed into a double-membrane structure firstly enter the early endosomes, and then they are mainly delivered to the late endosome, followed by fusing with lysosome. Within lysosome, the S protein undergoes a series of modifications and enzymatic cleavages, and then viral RNA is released into cytoplasm (Yang and Shen, 2020). Notably, the process is highly pH-dependent and acidic environment is required. However, CQ/HCQ might neutralize their pH by accumulating in endosomes and lysosomes (Hashem et al., 2020; Wang M et al., 2020). Umifenovir is involved in the inhibition of membrane fusion of the viral envelope and host cell membrane (Kadam and Wilson, 2017).

RNA Replication: Coronavirus replicate the virus genomes by making use of the materials of host cells. After releasing of virus RNA into cytoplasm, the ribosomes of host cells are used to produce polyproteins, which are subsequently cleaved into smaller molecules applying for replicating new viruses by enzymes, including 3-Chymotrypsin like protease (3CLpro) and the papain-like protease (PLpro). In addition, an RNA-dependent RNA polymerase (RdRp) is expressed to generate the complementary RNA strand using the virus RNA as a template (Mullard, 2018; Huang J et al., 2020; Wrapp et al., 2020). RdRp is an essential enzyme for coronavirus replication, providing a new insight for the antiviral agents for COVID-19 treatment. Remdesivir can bind with RdRp, thus RdRp is unable to incorporate RNA subunits, resulting in prevention of virus genome replication (Tchesnokov et al., 2019; Elfiky, 2020a). In addition, Ribavirin, Favipiravir and HCQ are thought to have the ability to interact with RdRp active site (Elfiky, 2020b). Zinc salts inhibits RdRp and has been shown to against coronavirus (te Velthuis et al., 2010). LPV/r have been found to tightly bind to the

active sites of SARS-CoV-2 3CLpro, inhibiting the replication of new viruses (Nutho et al., 2020).

Transcription and release: A series of sub-genomic mRNAs are produced by discontinuous transcription and then are translated into related viral proteins. The envelope glycoproteins are newly formed and inserted into the membrane of the endoplasmic reticulum (ER) or Golgi, and the nucleocapsid consists of genomic RNA and nucleocapsid protein. Then, viral particles containing viral proteins and genome RNA can be budded into the ER-Golgi intermediate compartment. Finally, they are transported through vesicles and released out of the cell by exocytosis (Li X et al., 2020). CQ/HCQ can suppress the post-translational modification of viral proteins, which occur within the ER or *trans*-Golgi network (Savarino et al., 2004). The process of assembly and budding can be interfered by CQ/HCQ with accumulation of viral vesicles in *trans*-Golgi network (Harley et al., 2001).

Cytokine storm: Like other viral infection, cytokines play an essential role in the progression of COVID-19. Higher levels of cytokines, including granulocyte-macrophage colony stimulating factor, monocyte-chemokine protein 1, interferon-inducible protein-10 and tumor necrosis factor-α (TNF-α), were more commonly seen in patients with severe COVID-19 than in those with non-severe COVID-19, suggesting cytokine profiles are closely associated with COVID-19 severity (Huang C et al., 2020). The level of interleukin (IL)-2R, IL-1 and IL-6 in serum can be significantly predictors of the severity of patients with COVID-19 (Chen L et al., 2020). In addition, pathological examination of biopsy samples demonstrate that inflammatory cellular infiltration is common in multiple organs, including the lung, heart, kidney, and liver (Tian S et al., 2020; Xu Z et al., 2020). This suggests that viruses aggravate the indirect injury through proinflammatory function or cytokine storms. Therefore, monoclonal antibodies or agents targeting different cytokine also represent attractive therapeutic options for COVID-19. It is well-known that corticosteroid, CP and IG are supposed to inhibit cytokine storms and modulate dysfunctional immune system (Amoss and Chesney, 1917; McGuire and Redden, 1918; Winkler and Koepsell, 2015). By preventing or attenuating the cytokine storm by secreting powerful anti-inflammatory factors, mesenchymal stem cells (MSCs) could also, theoretically, suppress overreaction of the immune system (Alhazzani et al., 2020). Various pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α can be reduced by the CQ/HCQ (Schrezenmeier and Dörner, 2020). Common anti-TNF agents, such as infliximab, adalimumab, thalidomid and golimumab, are believed to combat cytokine release syndrome, since TNF is a vital intermediated factor in the cytokine storm (Mitoma et al., 2018). Anakinra, an IL-1 receptor antagonist that blocks cytokine release, is used to treat inflammation-related diseases and can be beneficial for treating severe COVID-19 patients (Pasi et al., 2015). Tocilizumab and eculizumab (Davies and Choy, 2014) are monoclonal antibodies against IL-6 and the complement protein C5 reverse the cytokine storm respectively and improve the condition of severely COVID-19 patients.

ANTI-VIRAL AGENTS

Hydroxychloroquine and Chloroquine

CQ/HCQ exerted inhibitory effects from recognition process to cytokine storm production (**Figures 1A–F**). There are many clinical trials around the world including in China (Cortegiani et al., 2020). A RCT showed that high CQ dosage should not be recommended for critically ill patients with COVID-19 because of its potential safety hazards (Borba et al., 2020). Low dose of HCQ (200 mg twice a day for 7–10 days) reduces fatality of critically ill patients with COVID-19. In France, a clinical trial showed that HCQ significantly reduced viral load in patients infected with SARS-CoV-2, especially when co-administered with azithromycin (Gautret et al., 2020a), which was supported by the conclusion of another study (Gautret et al., 2020b). Furthermore, a clinical trial showed that CQ may have a slight advantage over LPV/r in combating SARS-CoV-2 (Huang M et al., 2020). However, the results of recent studies against these promising conclusions. Though, HCQ was reported to promote viral load reduction/disappearance in COVID-19 patients and the effect was reinforced by azithromycin, it is limited by the sample size (Gautret, et al., 2020a). A study showed no significantly reduced requirement for mechanical ventilation or decreased overall mortality in patients treated with HCQ (Magagnoli et al., 2020), another research didn't support its use in patients admitted to hospital with COVID-19 who require oxygen, either (Vinetz, 2020). Treatment with HCQ, azithromycin, or both, compared with neither treatment, was not significantly associated with differences in in-hospital mortality among patients with COVID-19 (Rosenberg et al., 2020). No evidence supported the beneficial effects of application of HCQ for COVID-19 patients who require oxygen (Mahévas et al., 2020). In terms of viral RNA clearance, administration of HCQ did not result in a significantly higher probability of negative conversion than standard of care alone in patients with mainly persistent mild to moderate COVID-19 (Tang W et al., 2020). Neither a multi-center RCT nor a retrospective study demonstrated HCQ shorten viral shedding in non-severe COVID-19 patients (Chen C P et al., 2020). Even in mild, early stage outpatients, HCQ did not substantially reduce symptom severity (Skipper et al., 2020). It was not associated with either a greatly lowered or an increased risk of the composite end point of intubation or death (Geleris et al., 2020). Notably, clinicians should pay more attention to the adverse effects caused by CQ/HCQ. CQ/HCQ showed retinal toxicity after long-term use for systemic lupus erythematosus and other rheumatoid diseases but some researchers believe the likelihood of retinal damage in COVID-19 patients seems to be extremely low because the dose is 3–4-fold lower than the normal dose and the duration of treatment is much shorter (Marmor, 2020). CQ/HCQ have also been associated with QT interval prolongation (Mercuro et al., 2020) and may thus lead to cardiac arrests (Lecuit, 2020), so QT interval should be followed repeatedly in patients with COVID-19 who are treated with HCQ/AZ (Chorin et al., 2020). A study of case series revealed key limitations, which include a potential lack of generalizability beyond the ICU, because of cardiac

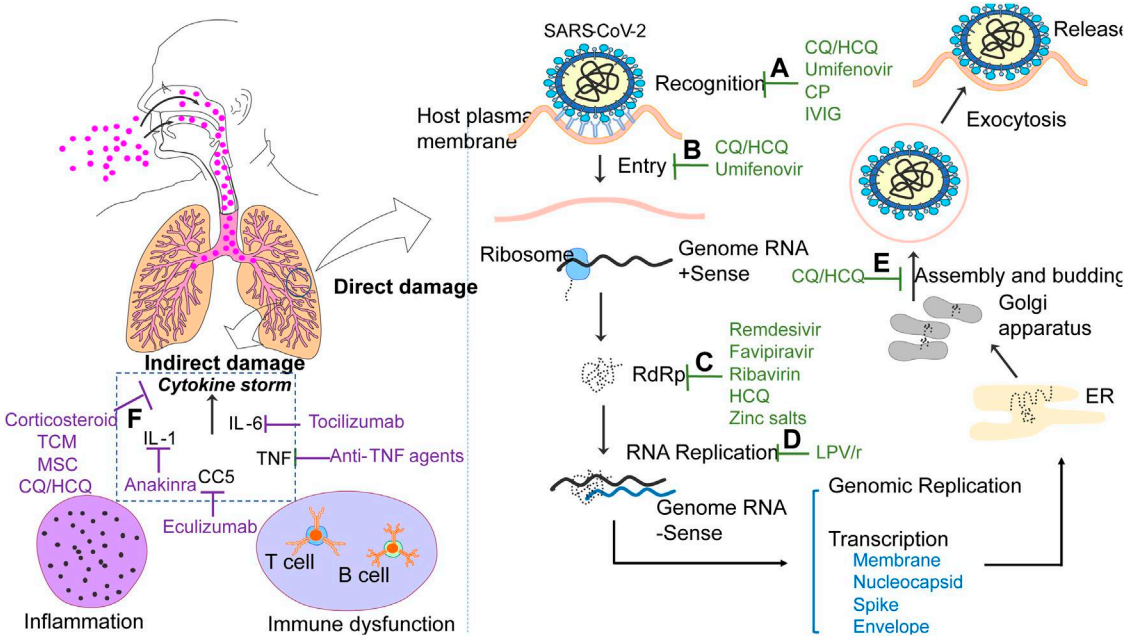

FIGURE 1 | Cellular and molecular possible sites of action of agents for COVID-19 treatment. 1. CP and IVIG inhibit the binding of virus with receptor by interacting with S1-RBD. Umifenovir interfere the recognition by blocking or hindering trimerization of S protein, while CQ/HCQ interferes with the activity of ACE2. 2. CQ/HCQ inhibit virus pH-dependent endocytosis through increasing pH. Umifenovir is also involved in the inhibition of membrane fusion. 3. Remdesivir, Ribavirin, Favipiravir, HCQ and Zinc salts can bind with RdRp, resulting in prevention of virus genome replication. 4. LPV/r inhibit the replication of new viruses by tightly binding to the active sites of virus3CLpro 5. CQ/HCQ suppress the assembly and budding of virus. 6. Treatment strategies reduce tissues damage by targeting various cytokine storms.

complication (Bessière et al., 2020). The cardiac safety profile may, however, be ameliorated by using single enantiomers of CQ/HCQ (Lentini et al., 2020). Serious cutaneous adverse reactions, fulminant hepatic failure and other side effects have also been reported (Ferner and Aronson, 2020). Because CQ has also been shown to reduce glucose-6-phosphate dehydrogenase (G6PD) activity, care should be taken when administering HCQ and CQ to G6PD-deficient patients, who may be more susceptible to SARS-CoV-2 (Kassi et al., 2020). The known and potential benefits of chloroquine and hydroxychloroquine no longer outweigh the known and potential risks, so that FDA revoked the emergency use. Recently, HCQ is further proposed as postexposure prophylaxis, unfortunately, two studies showed that it could not prevent SARS-CoV-2 infection (Boulware et al., 2020; Mitja et al., 2020). Basically, conflicting conclusion from current research, high possibility of adverse effects and lack of clinical trials in large population restrict CQ/HCQ use.

Remdesivir

Remdesivir fights against virus by interacting with RdRp active site to inhibit virus replication (**Figure 1C**). In view of it antiviral capacity, researches show significant *in vitro* activity of remdesivir against different viruses, including Ebola virus, Paramyxoviridae, Pneumoviridae (Lo et al., 2017) and many coronaviruses (Sheahan et al., 2017). Administration of remdesivir in both animal model and patients with Ebola showed amelioration of symptoms (Jacobs et al., 2016; Warren et al., 2016). Hence, the clinical potential of remdesivir is now being re-examined by clinicians as a result of the current SARS-CoV-2 pandemic (Ko et al., 2020). In the first confirmed case of SARS-Cov-2 in the United States, the patient's oropharyngeal swab tested turned negative after administration of remdesivir for 6 days (Holshue et al., 2020). Remdesivir can also benefit patients with SARS-CoV-2 pneumonia hospitalized outside ICU where clinical outcome was better and adverse events are less frequently observed (Antinori et al., 2020). Additionally, a simulated two-arm controlled study corroborated the efficacy of remdesivir, including reducing death, increasing rate of discharge (Hsu et al., 2020). Compared to placebo group, remdesivir accelerates recovery in adult patients and decreased respiratory tract infection rate (Beigel et al., 2020). Remdesivir was related to significantly greater recovery and 62% reduced odds of death vs. standard-of-care treatment in severe patients (Olender et al., 2020). A special population, pregnant and postpartum women, with severe COVID-19 receiving compassionate use remdesivir, got high recovery rates and were at a low-risk suffering from serious adverse events (Burwick et al., 2020). However, a 5-days course and a 10-days course of remdesivir did not make any difference in patients with severe Covid-19 not requiring mechanical ventilation (Goldman et al., 2020). A RCT indicated difference of clinical status between a 5-days course of remdesivir and standard care was of uncertain clinical importance (Spinner et al., 2020). More disappointingly, accordingly to a clinical trial, compared with placebo group, remdesivir neither speeded up recovery nor reduced death in COVID-19 patients, but the true effectiveness was uncovered by

lack of new enrolled patients in Wuhan (Grein et al., 2020). A randomized, double-blind, placebo-controlled, multicenter trial showed remdesivir was not associated with statistically significant clinical benefits among adult patients admitted to hospital for severe COVID-19. However, the numerical reduction in time to clinical improvement in those treated earlier requires confirmation in larger studies (Wang Y et al., 2020). By the way, one note of caution is that high doses of remdesivir may induce testicular toxicity and result in deterioration of sperm parameters in mice (Fan et al., 2020). Given the low certainty evidence for critical outcomes and promising faster clinical improvement for the treatment of SARS-CoV-2, use of remdesivir is weakly recommended. Ongoing trials, together with further randomized controlled trials following ethical approval, are needed to fully evaluate the efficacy and safety of remdesivir.

Lopinavir and Ritonavir

LPV/r inhibits viral replication by binding to the active sites of SARS-CoV-2 3CLpro (**Figure 1D**). For SARS-CoV-2, LPV/r shows promising prospect based on clinical investigations. A report of case series showed significant signs of improvement in pneumonia-associated symptoms after antiviral treatment including LPV/r (Wang Z et al., 2020). Other reported cases described decreased viral load and clinical improvement after LPV/r administration (Han et al., 2020; Li Y et al., 2020a; Lim et al., 2020; Wang Z et al., 2020). Earlier administration of LPV/r treatment could shorten viral shedding (Yan et al., 2020). Compared with adjuvant drugs alone, the combination of adjuvant drugs and LPV/r could lower the body temperature and restore normal physiological mechanisms with no evident toxic and side effects (Ye X T et al., 2020). Triple combination of interferon beta-1b, LPV/r and ribavirin were safe and superior to LPV/r alone in alleviating symptoms and shortening the duration of viral shedding and hospital stay in patients with mild to moderate COVID-19 (Hung et al., 2020). However, the 28-days mortality of severe COVID-19 patients treated with LPV/r was similar with those of patients in the standard care group (Cao B et al., 2020; Stower, 2020). A randomized trial concluded that LPV/r was not associated with hospital stay, or risk of progressing to invasive mechanical ventilation or death, which do not support use for treatment of patients admitted to hospital with COVID-19 (RECOVERY Collaborative Group, 2020). In addition, the use of LPV/r was even associated with delayed clearance of viral RNA (Chen X et al., 2020). In a study carried out in Singapore, however, four out of five patient (80%) developed nausea, vomiting, and/or diarrhea, which precluded completion of the planned 14-days treatment course (Young et al., 2020). Severe jaundice was more frequently observed in patients treated with LPV/r (Levy et al., 2020). Based on pharmacokinetics, it is difficult to recommend oral LPV/r safe dose without compromising the benefit of the antiviral strategy (Lê et al., 2020). At present, the effectiveness and safety of LPV/r have not yet been confirmed due to controversial results, thus more clinical evidence is required for further evaluation of efficiency and safety.

Favipiravir

Favipiravir, which could interfere with the action of RdRp (**Figure 1D**), was reported to be effective in reducing SARS-CoV-2 infection *in vitro* (Wang M et al., 2020). A clinical study showed that treatment with FPV was safe and had no severe adverse effects. It also improved chest CT scans and viral clearance in patients with COVID-19, with a 4-days viral clearance time for FPV vs. an 11-days time for the control group (Cai et al., 2020), although the 7-days clinical recovery rate remains controversial (Chen C et al., 2020). It was also found that FPV significantly improved treatment effects on COVID-19 in terms of disease progression and viral clearance (Cai et al., 2020). Yet, another prospective study suggested viral clearance measured by RT-PCR by day 6 was not significantly advanced but FPV reduced time to defervescence (Doi et al., 2020). Addition of FPV into existing standard treatment was not proved to be beneficial, either (Lou et al., 2020). Oral administration of FPV even delayed viral clearance according a case series (Fu et al., 2020) and the adverse effect, fever, was firstly reported in two cases (Takoi et al., 2020). Favipiravir has been approved by the National Medical Products Administration of China as the first anti-COVID-19 drug in China, as the clinical trial had demonstrated efficacy with minimal side effects.

Umifenovir

Umifenovir was involved in the prevention of recognition and the inhibition of membrane fusion to fight against virus (**Figure 1B**). It is shown *in vitro* that umifenovir reduced replication of SARS-CoV-2 compared with the control group, and the inhibition occurred efficiently at both viral entry and post-entry stages (Wang X et al., 2020). However, little benefit of umifenovir monotherapy was presented for improving the clinical outcome of mild/moderate COVID-19 patients' over supportive care. This clinical trial involving 86 patients with mild COVID-19 found that the average time for SARS-CoV-2 positive-to-negative conversion in the umifenovir group was similar to that in the control group (Li Y et al., 2020a; Li Y et al., 2020b). There were also no significant differences in symptoms, or chest CT scans between the umifenovir and favipiravir groups, suggesting that umifenovir is less suitable for first-line treatment (Chen C et al., 2020). Patients in the umifenovir group had a shorter duration of positive RNA test compared to those in the lopinavir/ritonavir group (Zhu et al., 2020). However, a recent retrospective study indicated umifenovir might not improve the prognosis or accelerate SARS-CoV-2 clearance in non-ICU patients (Lian et al., 2020). Debating of umifenovir treatment strategy needs more evidence from clinical trials.

Ivermectin

Ivermectin is a specific inhibitor of importin-α/β-dependent nuclear transport and shows antiviral potential against several RNA viruses by blocking the nuclear localization of viral proteins (Lv et al., 2018). It exerts antivirus effects toward both HIV-1 and dengue virus (DENV) with respect to the HIV-1 integrase and non-structural protein 5 (NS5) polymerase proteins, respectively (Wagstaff et al., 2012).

Ivermectin can also dissociate the preformed host importin (IMP) α/β1 heterodimer, as well as prevent its formation (Rogers T F et al., 2020). These inhibitory effects coincide with the onset of intracellular viral RNA synthesis, as expected for a molecule that specifically targets the viral helicase (Mastrangelo et al., 2012). Based on its inhibition of RNA virus, Leon et al. suggested that treatment with ivermectin reduced cell-associated SARS-CoV-2 viral RNA by 93% after 24 h and by 99.8% after 48 h, with a single treatment achieving ~5000-fold reduction in viral load (Caly et al., 2020). In patients who required higher inspired oxygen or ventilatory support, ivermectin application during treatment was associated with lower mortality (Rajter et al., 2020). As an add-on therapy, ivermectin was helpful for better effectiveness, shorter hospital-stay and relatively safe (Gorial et al., 2020). However, the approved dose of ivermectin was found to exert impact *in vitro*, which was challenged by a new research. To achieve an efficient plasma concentration, ivermectin use would be over 10 times higher than the approved dose, possibly resulting in adverse events (Schmith et al., 2020). The optimal dose and combination strategy have not been decided so far and require more evidence from clinical studies.

Galidesivir

Galidesivir, an adenosine analogue, shows broad-spectrum antiviral activity against a wide range of RNA viruses and is under clinical development for treatment of Ebola and yellow fever virus infections. It mainly inhibits viral RdRp function, acting as a non-obligate RNA chain terminator (Warren et al., 2014). Galidesivir has been shown to treat several RNA viruses, such as Zika virus, and Rift Valley Fever virus, both *in vitro* and in animal model (Julander et al., 2017; Westover et al., 2018). For SARS-CoV-2, both a molecular docking study and an in silico perspective demonstrated Galidesivir can tightly bind to the RdRp of the SARS-CoV-2 strain and thus may be applied to treat the disease (Elfiky, 2020a; Elfiky, 2020b). However, *ex vivo* or *in vivo* experiment on COVID-19 is still lacking.

Nelfinavir

Nelfinavir, a well-known HIV-1 protease inhibitor, is widely prescribed as part of triple-drug combination therapy for the treatment of HIV infection. Recently, this agent also exerted inhibition on the cytopathic effect induced by SARS-CoV infection, and suppressed replication of the SARS-CoV at the post-entry step of infection (Yamamoto et al., 2004), but nelfinavir did not exhibit activity against MERS-CoV *in-vitro* (Chan et al., 2013). Several virtual screening and mocking study indicated that nelfinavir was supposed to be a potential inhibitor of COVID-19 main protease (Duan et al., 2020a; Mittal et al., 2020; Ohashi et al., 2020). In the SARS-CoV-2 research, nelfinavir mesylate might bind inside the S trimer structure, which is proximal to the S2 amino terminus, then directly inhibit Sn and So-mediated membrane fusion. This drastically inhibited S protein-mediated cell fusion with complete inhibition (Musarrat et al., 2020).

Other Antiviral Agents

It seems likely that a variety of other antiviral agents will be shown to have some effects on SARS-CoV-2 replication. Baloxavir inhibits the cap-dependent endonuclease, an essential enzyme for the initiation of mRNA synthesis of influenza viruses, thus preventing transcription of mRNA (Fukao et al., 2019). Tilorone, a synthetic small-molecule compound with antiviral activity, is proposed to induce interferon against pathogenic infection (Zhang, et al., 2015), which has been confirmed in Chikungunya virus (CHIK) and MERS-CoV was described *in vitro* (Ekins and Madrid, 2020). Recently, the activity against SARS-CoV-2 activity was shown in a Korean study (Jeon et al., 2020). Sofobuvir, an inhibitor of RdRp, was approved for treating Zika virus and hepatitis virus C(HCV) (Sacramento et al., 2017). It was also predicted to be effective against SARS-COV-2 RdRp as well based on the molecular insight that the HCV and the coronavirus share a similar viral genome replication mechanism (Ju et al., 2020). Alovudine, tenofovir and alafenamide, as RdRp inhibitors, could also have same potential against COVID-19 (Chien et al., 2020). More agents together with agents referred above which are promising in COVID-19 treatment are summarized in **Table 1**.

HOST SYSTEM MODULATORY AGENTS

Patients infected with SARS-CoV-2 have different clinical manifestations, with a range from asymptomatic to respiratory failure, multi-organ dysfunction. Better understanding of the pathogenesis facilitates proper management of COVID-19. Patients with severe respiratory failure are more likely to present sustainable TNF-α and IL-6 produced by circulating monocyte, which is distinct from bacterial sepsis or influenza (Giamarellos-Bourboulis et al., 2020). COVID-19 patients are also characterized by lower platelet count and lymphocytes, increased prothrombin time, D-dimer, and fibrin degradation products with aggravating disease (Di Minno et al., 2020; Liao et al., 2020; Tang N et al., 2020b). These coagulation abnormalities are reported to cause consequences ranging from venous embolism to DIC, or even death (Connors and Levy, 2020; Piazza et al., 2020). Immune dysregulation is reported to cause hyporeactive neutrophil and neutrophil extracellular traps, which interact with platelet and fibrin, contributing to microvascular thrombi in lung, kidney, and heart (Nicolai et al., 2020). Severe COVID-19 has a feature of an inflammatory signature, including high levels of inflammatory cytokines, alveolar inflammatory infiltrates, and vascular microthrombi, which leads to multi-organ failure (Chakraborty et al., 2020). Frustratingly, a WHO summary including most clinical trials and meta-analyses, concluded consistently these Remdesivir, HCQ, Lopinavir and interferon regimens mentioned above, appeared to have little or no effect on hospitalized COVID-19 (Pan et al., 2020). Due to absence of specific anti-viral agents and complicated pathogenesis of COVID-19, current treatment strategies focus on managing patients' conditions.

Table 1 | Summary of potential agents used in the treatment of COVID-19.

Agents	Property	Mechanisms	References
NAbs	Antibody	Combine with surface epitopes of viral particles	Jeronimo et al. (2020)
rhACE2	Enzyme	Bind to ACE2 receptor	Zhang H et al. (2020)
Interferon antagonists	Protein	Inhibit excessive interferon	Channappanavar et al. (2016)
Baricitinib	JAK inhibitors	Restrain the JAK/STAT signaling pathway	Virtanen et al. (2019)
Ivermectin	Anti-parasitic	Inhibit importin-α/β-dependent nuclear transport	Lv et al. (2018)
Galidesivir	Adenosine analogue	Inhibiting viral RdRp	Warren et al. (2014)
Nelfinavir	Protease inhibitor	Inhibit viral main protease, Inhibit S protein-mediated membrane fusion	Yamamoto et al. (2004)
Baloxavir	Cap-dependent endonuclease inhibitor	Inhibit the cap-dependent endonuclease	Fukao et al. (2019)
Tilorone	Synthetic small-molecule compound	Induce interferon	Zhang et al. (2015)
Sofobuvir	Adenosine analogue	Inhibit viral RdRp function	Ju et al. (2020)
Natural killer cells	Innate immunity cell	Respond to viral infection without T cell help	Chen et al. (2010)
Fingolimod	S1P modulator	Prevent egress of lymphocytes from lymph nodes	Huwiler and Zangemeister-Wittke (2018)
Siponimod	S1P modulator	Prevent egress of lymphocytes from lymph nodes	Goodman et al. (2019)
Metronidazole	Antibiotic and antiprotozoal	Suppress cytokines storm	Gharebaghi et al. (2020)
Amantadine	Antiviral agent	Disrupt CTSL-mediated lysosomal pathway	Smieszek et al. (2020)
Teicoplanin	Antibiotic	Block endocytosis of virus	Baron et al. (2020)
Niclosamide	Anti-parasitic and anti-tumor	Block endocytosis and autophagy of virus	Pindiprolu and Pindiprolu (2020)
Minocycline	Antibiotics	Suppress cytokines storm	Alano et al. (2006) and Ge et al. (2020)
Triiodothyronine	Hormone	Promote the ability of natural killer cells	Pantos et al. (2020)
Melatonin	Hormone	Suppress cytokines storm	Li Y et al. (2020)

ACE2: angiotensin-converting enzyme two; CTSL: Cathepsin L; JAK/STAT: Janus kinase/signal transducer and activator of transcription; NAs: Neutralizing antibodies; RdRp: RNA-dependent RNA polymerase; rhACE2: recombinant human angiotensin-converting enzyme two; S1P: sphingosine one phosphate.

Corticosteroid

Corticosteroid, which makes contributions to inhibit cytokine storm (**Figure 1F**), is widely used in clinical practice for years and administrated during SARS and MERS epidemic, even though there are many divergences on the treatment effect and safety issues. In terms of COVID-19, the administration of corticosteroids has again been a conundrum for clinicians. On the one hand, early, low dose and short-term application of corticosteroids was associated with a faster improvement of clinical symptoms and absorption of lung foci in patients with severe COVID-19 pneumonia (Guzik et al., 2020). Also, low dose corticosteroid therapy did not delay viral clearance in patients with COVID-19 (Cao Y et al., 2020). An early short course of methylprednisolone in patients with moderate to severe COVID-19 reduced escalation of care and improved clinical outcomes (Fadel et al., 2020). A 7-days fixed-dose course of hydrocortisone or a shock-dependent dosing of hydrocortisone, favors days reduction for organ support (Angus et al., 2020). Corticosteroid treatment was associated with a lower risk of 30-days mortality, which was limited in the critically ill patients (Bartoletti et al., 2020). Despite the uncertain effect of corticosteroid therapy on overall survival, prudent dosing within effective limits may be recommended for critically ill patients under certain circumstances (Lu et al., 2020). Compared to standard use, high dose of corticosteroids (1–1.5 mg/kg/day) increased mortality exclusively in elderly patients and caused higher risk of mechanic ventilation requirement or death (Monreal et al., 2020). On the other hand, Wu et al. found that patients who received methylprednisolone treatment were much more likely to develop ARDS, probably because sicker patients were more likely to receive treatment, although methylprednisolone did appear to reduce the risk of death in patients with ARDS (Wu C et al., 2020). Corticosteroids impair the immune system, and current evidence does not support their use in lung injury (Russell et al., 2020). A meta-analysis showed that patients with severe conditions are more likely to require corticosteroids, but the use may lead to increased mortality and serious adverse reactions (Yang Z et al., 2020). Another study also showed no association between corticosteroid therapy and virus clearance time (Ding C et al., 2020), length of hospital stay or duration of symptoms (Jin et al., 2020). Short course use of methylprednisolone did not reduce mortality in the overall population with regard to a double-blind RCT (Jeronimo et al., 2020). Corticosteroid use showed no benefit in reducing in-hospital mortality for severe or critical cases, so the routine use of systemic corticosteroid among severe and critical COVID-19 patients was not recommended (Wu J et al., 2020). A RCT gives the conclusion that low-dose hydrocortisone didn't significantly decrease death and duration of persistent respiratory support (Dequin et al., 2020).

In view of the current evidence and clinical experience, among adults receiving mechanical ventilation who do not have ARDS, routine use of systematic corticosteroids is advised against (weak recommendation, LQE). In those with ARDS, use of corticosteroids is advised (weak recommendation, LQE) (Poston, et al., 2020). For adults with COVID-19 and refractory shock, low dose corticosteroid therapy ("shock-reversal"), is recommended over no corticosteroid treatment. A typical corticosteroid regimen in septic shock is intravenous hydrocortisone (200 mg per day), administered either as an infusion or as intermittent doses (Alhazzani, et al., 2020). Large, well-designed clinical trials are needed to clarify the benefits of specific administration of corticosteroids in COVID-19.

Convalescent Plasma and Immunoglobins

Neutralizing antibodies contained in CP and IVIG could bind to S1-RBD, resulting in limiting viral entry (**Figure 1A**). In COVID-19, CP is an undeniable choice for administration to patients for its specificity. Observational studies of patients in Wuhan showed that CP was an effective and specific therapy for COVID-19, which decreased viral load (Ye M et al., 2020). 80% recipients showed significant increase in antibody levels posttransfusion of CP in spite of variable titers from donors (Madariaga et al., 2020). When combined with systemic corticosteroids in severely ill patients, CP contributed to a reduction in viral load and caused no severe adverse effects (Ahn et al., 2020). As a conjunction to conventional therapy, CP speeded up being free of invasive mechanical ventilation support and elevated recovery rate (Gemici et al., 2020). Uncontrolled case series of patients, including a pregnant woman, recovered from COVID-19 after transfusion with CP (Chen X et al., 2020; Shen et al., 2020). Another study including 10 patients also got good outcomes and came up that one dose of 200 ml of CP derived from recently recovered donors with the neutralizing antibody titers above 1:640 is effective (Duan et al., 2020a). Duan et al. analyzed the feasibility of using CP in 19 patients and showed that one dose (200 ml) was well-tolerated and improved clinical outcomes (Duan et al., 2020b). A study in Texas indicated that administration of CP is a safe treatment option for those with severe COVID-19 disease, although the efficacy remained unclear (Salazar et al., 2020). Analysis of case series demonstrated CP was safe and might be efficacious as well (Pal et al., 2020). The transfusion of CP is safe in 5,000 hospitalized patients with COVID-19 based on early indicators, such as transfusion-associated circulatory overload (Joyner et al., 2020). Moreover, a multicenter retrospective cohort study in China, which recruited 325 critically ill adult patients from eight treatment centers, concluded that early administration of high dose IVIG significantly reduced mortality, decreased the inflammatory response and improved the function of some organs (Shao et al., 2020). In addition, a critically ill patient was cured successfully with plasma exchange followed by IVIG (Shi et al., 2020). Nevertheless, CP did not result in a statistically significant improvement in time to clinical improvement within 28 days (Li L et al., 2020). There was neither difference in risk of mortality or rate of hospital discharge between CP and control group (Rogers R et al., 2020), nor could progression to severe COVID-19 or all cause morality be ameliorated by CP (Agarwal et al., 2020). Recently, CP was reported to end SARS-CoV-2 shedding but not reduce the mortality rate in critically ill patients with end-stage COVID-19, which suggested treatment should be initiated earlier (Zeng et al., 2020). The optimal dose of CP or IVIG and time of administration needs further investigation in larger well-controlled trials to fully evaluate the clinical benefits.

Neutralizing Antibodies (NAbs)

NAbs, which prevent viral attachment and accumulation, reduce infectivity by combining with surface epitopes of viral particles and blocking access of the virus to cells (Klasse, 2014). The constant region of the Ab can contribute to viral clearance through opsonization or complement activation, providing a highly specific immune defense (Coughlin and Prabhakar, 2012). Because SARS-CoV and SARS-CoV-2 both use ACE2 as an entry receptor (Tian X et al., 2020) and the receptor-binding domains (RBDs) of the two viruses are similar (Wan Y et al., 2020), NAbs against SARS may be effective in COVID-19 patients. Tian et al. (Tian X et al., 2020) recently showed that CR3022, a SARS-CoV NAb, binds to the RBD of SARS-CoV-2, although with an uncertain capability of neutralization. Some of the SARS-CoV-specific neutralizing antibodies that target the ACE2 binding site of SARS-CoV failed to bind 2019-nCoV spike protein, implying that the difference in the RBD of SARS-CoV and 2019-nCoV has a critical impact for the cross-reactivity of neutralizing antibodies. Bamlanivimab, a neutralizing IgG1 monoclonal antibody (mAb) directed against the spike protein of SARS-CoV-2, has been approved by FDA for treatment of mild-to-moderate COVID-19 in adult and pediatric patients (Coronavirus, 2020). A recent research even provided 11 potent human neutralizing antibodies for COVID-19 as therapeutic candidates (Wan J et al., 2020). Among NAbs isolated of from a convalescent patient, B38 and H4 block the binding between virus S-protein RBD and cellular receptor ACE2, displaying neutralization abilities. As for feasibility issues, NAbs is not only obtained from convalescent patients but also can be engineered in the laboratory (Zhao et al., 2015a; Zhao et al., 2015b; Li et al., 2017).

Traditional Chinese Medicine

TCM has a long history and plays an indispensable role in the treatment of diseases because of its important roles in regulating immune system and inhibiting cytokine storm (**Figure 1F**). During the SARS epidemic in 2003, TCM was widely used in 58.3% of confirmed cases and achieved remarkable therapeutic effects. Based on previous experience of treating SARS with TCM, clinicians, especially in China, have encouraged the integrated use of TCM and Western medicine to treat COVID-19, and TCM has been included in the guidelines for COVID-19 treatment in China. Among 701 confirmed cases treated with *Qingfei Paidu* decoction (QPD), 130 cases were cured and discharged, clinical symptoms disappeared in 51 cases and improved in 268 cases, and symptoms remained stable, with no deterioration, in 212 cases (Publicity Department of the People's Republic of China, 2020). Another investigation reviewed the results from four provincial hospitals in China that used QPD to treat 214 COVID-19 patients, taking three days as a course of treatment, and found that the total effective rate was >90%. The symptoms and imaging results of 60% of patients improved significantly and 30% of patients had stable symptoms without exacerbation (National Administration of Traditional Chinese Medicine, 2020). Another popular candidate, *Lianhuaqingwen* (LH), was shown to significantly inhibit SARS-CoV-2 replication in Vero E6 cells and markedly reduce production of pro-inflammatory cytokines (TNF-α, IL-6, CCL-2/MCP-1 and CXCL-10/IP-10) (Runfeng et al., 2020), suggesting that it might be a potential option for COVID-19 treatment. Several observational studies suggested that LH accelerated the disappearance of clinical symptoms, shortened the time for conversion to virus-negative status, and accelerated

the improvement in chest CT scans (Cheng Deizhong et al., 2020; Lyu Ruibing and Li, 2020; Yao et al., 2020; Yi, 2020). A recent multicenter, prospective, RCT indicated LH achieved a higher recovery rate and a shorter recovery without reported adverse effects. Combination use of Lianhuaqingwen and umifenovir may accelerate recovery and improve the prognosis of patients with moderate COVID-19 (Fang et al., 2020). Another Chinese herbal extract, *Xuebijing*, also reduced the time for conversion to virus-negative status (Zhang C et al., 2020). Three cases from the same family, who received Western medicine combined with the Chinese traditional patent medicine *Shuanghuanglian* oral liquid, were reported to make a rapid recovery (Ni et al., 2020). *Tanreqing* capsule, significantly reduced the negative conversion time of fecal nucleic acid and the duration of negative conversion of pharyngeal-fecal nucleic acid (Zhang X et al., 2020). A retrospective study of four cases indicated that combination of Chinese and Western medicine improved the pneumonia-associated symptoms of COVID-19 (Wang Z et al., 2020). Both data mining of on-line databases and a core outcome set also concluded that TCM is effective for management of COVID-19 (Qiu et al., 2020; Zhou Z et al., 2020). Although high-quality evidence for the safety of some Chinese herbs is lacking (Luo et al., 2012), when used correctly based on patients' situation, it is generally believed that there are no serious adverse reactions.

Mesenchymal Stem Cells

Attention has been paid to the role of MSCs in attenuating the cytokine storm and suppressing overreaction of the immune system (**Figure 1F**). Clinical trials of different types of MSCs in COVID-19 patients are ongoing. Seven patients with SARS-CoV-2 pneumonia showed improved clinical outcomes without observed adverse effects after intravenous injection with bone marrow-derived MSCs for 14 days (Leng et al., 2020). MSC transplantation improved oxygen saturation for ARDS and increased the immune indicators, including CD4 and lymphocytes (Tang L et al., 2020). Treatment with adipose-derived stromal stem cells (ASCs) also shows promise in combating SARS-CoV-2 (Gentile and Sterodimas, 2020). 13 severe COVID-19 patients who were intravenously injected with ASC, mostly were extubated and discharged from ICU, with no significant adverse events (Sanchez-Guijo et al., 2020). Among stem cells, umbilical cord stem cells seem to be most desirable for treating SARS-CoV-2, because of noninvasive extraction procedures, fast doubling times and greater plasticity (Misra et al., 2020). Adoptive transfer therapy using human umbilical cord mesenchymal stem cells in a critically ill, 65-year-old patient with COVID-19, was well tolerated and led to a significant clinical improvement (Bing Liang et al., 2020). A phase one clinical trial revealed human umbilical cord mesenchymal stem cells was safe and well-tolerated by intravenous injection (Meng et al., 2020). Incidence of disease deterioration or severe complication of MSC treatment was rarely seen (Atluri et al., 2020). A novel technology for capturing the therapeutic properties of stem cells using nanotechnology has provided a new sight into MSC therapy (Metcalfe, 2020). Because of the complexities and ethical issues surrounding the use of MSCs, further clinical trials, with the highest standards of rational and appropriate design are needed (Khoury et al., 2020).

Tocilizumab

It is well known that tocilizumab as a monoclonal antibody improving inflammatory condition by fighting against IL-6 (**Figure 1F**). A study in COVID-19 patients showed that intravenous administration of tocilizumab (8 mg/kg every 8 h) was highly beneficial (Michot et al., 2020). An Italian study also found that a patient who received tocilizumab (8 mg/kg every 12 h) for 2 days showed progressive improvements in both clinical condition and chest CT scans (Cellina et al., 2020). A single-dose use of tocilizumab, improved survival (Rossi B et al., 2020) and reduced lethality rate at 30 days with no significant toxicity in severe COVID-19 patients, who were without mechanical ventilation (Perrone et al., 2020). Response of COVID-19 pneumonia with ARDS to tocilizumab was rapid, sustained, and associated with significant clinical improvement, reduced mortality, and no obvious adverse reactions (Sciascia et al., 2020; Toniati et al., 2020; Xu X et al., 2020). It also shows short-term survival benefit in patients with severe COVID-19 illness (Ramaswamy et al., 2020). Treatment of a sickle cell patient infected with SARS-CoV-2 with tocilizumab and hydroxychloroquine led to a significant improvement in clinical condition (De Luna et al., 2020). In a preprint study, 30 selected patients showed that tocilizumab significantly reversed the cytokine storm and improved the condition of severely ill patients. The dosage of tocilizumab for COVID-19 patients can be determined based on those used to treat rheumatoid arthritis (Instruction of Tocilizumab, 2020). Time from lung injury onset to tocilizumab administration may be critical to patient recovery (Sanchez-Montalva et al., 2020). Early use lead to a positive impact during Covid-19 pneumonia with severe respiratory syndrome in terms of increased survival and favorable clinical course (Capra et al., 2020). Early stage administration of tocilizumab subcutaneously reduced the risk of death and improves clinical parameters, for example, CRP and lymphocyte counts (Malekzadeh et al., 2020). In addition to relieve hyper-inflammatory reaction, it is also beneficial for patients with liver dysfunction (Serviddio et al., 2020). Although tocilizumab group seemed to have improved survival outcome, these positive results need to be interpreted with caution since different research types and confounding factors (Wadud et al., 2020). Transient transaminitis was found to be the most common adverse reaction in patients 21 days post tocilizumab (Sirimaturos et al., 2020). However, no significant clinical improvement in temperature or oxygen requirements in most patients were observed in a US study (Rimland et al., 2020). A recent RCT concluded tocilizumab failed to prevent intubation or death in moderately ill hospitalized patients (Stone et al., 2020). Despite IL-6 receptor inhibitors might cause hypertriglyceridemia and acute pancreatitis (Morrison et al., 2020), tocilizumab is among the candidates for anti-inflammatory treatment in COVID-19.

Other Anti-cytokines Therapeutics

Common anti-TNF agents, such as infliximab, adalimumab, thalidomid and golimumab (Mitoma et al., 2018), could be functional in inflammatory diseases. Common anti-TNF agents, such as infliximab, adalimumab, thalidomid and golimumab (Mitoma et al., 2018), could be functional in inflammatory diseases. A patient with IBD and COVID-19, was given adalimumab therapy, generated a quicker hospital discharge (Tursi et al., 2020). A patient with COVID-19 treated by thalidomide (Wang, 2020), got clinical improvements. Certainly, safety and efficiency need more support from clinical trials. Another anti-inflammation candidate, anakinra may be beneficial for treating severe COVID-19 patients with secondary HLH (Dimopoulos et al., 2020). Anakinra was used on nine consecutive severe COVID-19 pneumonia patients, and early chest CT scan showed the stopped extension of lesions. In this small open-label study, anakinra use was safe (Aouba et al., 2020). Although there is a shortage of relevant research about IL-1 receptor antagonists in COVID-19 patients, anakinra could potentially be beneficial in these patients. Additionally, Eculizumab, an agent that blocks the C5a pathway, should mitigate damage in COVID-19 patients and a 4-weeks study treatment with eculizumab did indeed remarkably improve the conditions of severe pneumonia or ARDS in COVID-19 patients, a finding that was supported by subsequent CT scans (Diurno et al., 2020). This discovery highlights a novel effective anti-inflammatory treatment, focusing on the complement system, which is worthy of further exploration as a treatment for COVID-19.

Interferon Antagonists

Interferon, a glycoprotein with broad spectrum antiviral activity produced by innate immune cells, plays an important role in coronavirus infection (Hadjadj et al., 2020; Huang L et al., 2020; Volk et al., 2020). Interferon is a double-edged sword in viral diseases. On one hand, SARS-CoV encodes several proteins, including nsp13, nsp14, nsp15 and ORF6 (Yuen et al., 2020), that modulate innate immune signaling through the potential antagonism of the induction of interferon and by avoidance of interferon stimulated gene (ISG) effector functions (Totura and Baric, 2012). Downregulation of interferon expression assists SARS-CoV-2 infection because interferon is essential to prevent entry of coronaviruses into host cells (Volk et al., 2020). In addition, Interferon alfa-2a combined with ribavirin therapy is associated with significantly improved survival in MERS (Omrani et al., 2014). On the other hand, delayed IFN-I signaling promotes the accumulation of pathogenic inflammatory monocyte-macrophages (IMMs), resulting in elevated lung cytokine/chemokine levels, vascular leakage, and impaired virus-specific T cell responses in SARS-CoV-infected mice (Channappanavar et al., 2016). Early short-term blocking IFN-I after coronavirus infection evoked a long-lasting enhancement of immunological memory, which conferred improved protection upon subsequent reinfections (Palacio et al., 2020). Because excessive interferon responses during SARS-CoV-2 infection may lead to tissue damage (Zhou F et al., 2020), late phase interferon antagonist treatment should be considered.

JAK Inhibitors

The JAK/STAT signaling pathway has been widely validated as a target for inflammation-related diseases (Virtanen et al., 2019). Moreover, Hadjadj et al. found an increase in peripheral blood of IL-6 and IL-6-induced genes, TNF-α and TNF-α pathway-related genes, as well as IL-10 (Hadjadj et al., 2020). JAK-STAT activation is also known to suppress the functions of granulocyte-macrophage colony-stimulating factor (McInnes et al., 2019). All of these indicated that JAK inhibitors could potentially be used to reduce inflammation in COVID-19 patients. JAK inhibitor curded activation of ACE2 and interferon-stimulated transcriptomes in human airway epithelium (Lee et al., 2020). Because JAK2 inhibition is reversible, transient treatment would not affect TH17 responses that are essential for innate immune responses and immunity against extracellular pathogens (Praveen et al., 2020). Baricitinib, Ruxolitinib and upadacitinib, JAK1/JAK2/JAK3 inhibitors, emerges as a potential agent (Richardson et al., 2020). Baricitinib stopped progression toward a severe/extreme form of the viral disease by restraining immune dysregulation in COVID-19 (Bronte et al., 2020). Ruxolitinib attenuated SARS-CoV-2 infection (Foss et al., 2020) and it rescued a patient who are refractory to anti-IL-6 therapy. Now, results of ongoing clinical trials will give a direction of JAK inhibitors administration.

Combination of Agents

Some research indicated good outcome when oseltamivir used with other antiviral agents, like abidol, but the effect of oseltamivir alone remains unclear (Costanzo et al., 2020; Ding Q et al., 2020; Wang D et al., 2020). Moreover, oseltamivir has not been shown to have efficacy based on all investigation summary (Sanders et al., 2020). Furthermore, a retrospective study provided the first *in vivo* evidence that zinc sulfate in combination with hydroxychloroquine may play a role in therapeutic management for COVID-19 (Carlucci et al., 2020). This combination will be tested as a prophylactic regimen in a randomized clinical trial.

Treatment with IFN-α2b with or without umifenovir significantly reduced the duration of detectable virus in the upper respiratory tract and in parallel reduced duration of elevated blood levels for the inflammatory markers IL-6 and CRP (Liu et al., 2020). In COVID-19, triple combination of interferon beta-1b, lopinavir-ritonavir and ribavirin alleviated symptoms and shortened the duration of viral shedding and hospital stay in patients with mild to moderate COVID-19 (Hung et al., 2020). Besides, type III IFNs (IFN-λ) was believed to play an important role in SARS-CoV-2 and other viral infections (Prokunina-Olsson et al., 2020). Further application of interferon and combination regimen are undergoing more clinical trials.

Anti-thrombotic Therapy

Rebalancing coagulation system, especially anti-platelet and anti-coagulant is crucial for COVID-19 coagulopathy administration.

Antiplatelet therapy might be effective in improving the ventilation/perfusion ratio in COVID-19 patients with severe respiratory failure (Viecca et al., 2020). Aspirin, a classical anti-platelet agent, is possible to decrease mechanical ventilation rate, ICU admission and in-hospital mortality, without more bleeding events compared to non-aspirin use, based on evidence from a retrospective study (Chow et al., 2020). It is recommended that person suffering from SARS-CoV-2 infection should be administered with aspirin at the earliest (Haque et al., 2020). For the severe COVID-19 patients meeting SIC criteria or with markedly elevated D-dimer, using low molecular weight heparin seems to be associated with better prognosis (Tang N et al., 2020a). Dipyridamole, prohibiting platelet from aggregating, was shown to reduce viral replication, suppress hypercoagulability and enhance immune recovery, when taken as an adjunctive therapy in COVID-19 (Liu et al., 2020). Furthermore, longer duration of anti-coagulation was associated with reduced mortality risk (Paranjpe et al., 2020). Argatroban, a direct thrombin inhibitor, decreased further thrombosis complications (Arachchillage et al., 2020). Moreover, therapeutic-strength anticoagulation performed better than prophylactic anticoagulation without contributing to bleeding events (Boonyasai et al., 2020; Ferguson et al., 2020). Therapeutic anticoagulation is associated with a survival advantage among patients with COVID-19 who require mechanical ventilation in the ICU, as well (Trinh et al., 2020). Pre-admission applying anti-thrombotic, however, showed little protective effect in severe patients (Russo et al., 2020). COVID-19 patients receiving anti-coagulation medicine chronically, were prone to a higher mortality, resulting from cardiovascular events (Rossi R et al., 2020). A high incidence of venous thrombosis and worse outcome is observed, despite the use of heparin at the therapeutic dose (Pavoni et al., 2020). Routine chemical prophylaxis is believed to be inadequate in preventing venous thromboembolism in severe COVID-19, and different pharmacologic prophylaxis regimens are not helpful for lowering incidence of deep venous thrombosis (Maatman et al., 2020). High regimen thromboprophylaxis, like subcutaneous therapeutic unfractioned heparin, decreased the occurrence of pulmonary embolism (Taccone et al., 2020). Whether therapeutic or prophylaxis anti-thrombotic to be used, monitoring D-dimer is helpful for measuring efficiency and preventing adverse events (Song et al., 2020). Specific anticoagulation regimens may vary in different disease severity and need further determination based on clinical trials.

Micronutrients Supplementation

Providing patients with sufficient nutrients through all stages of COVID-19 is also vital. Micronutrients, such as vitamin D, which is a modulator of adaptive immunity, may also be important. Serum concentrations of 25-hydroxy vitamin D tend to decrease with age (Vásárhelyi et al., 2011), and this may be associated with the severity of COVID-19 in the elderly (Novel, 2020). Moreover, vitamin D deficiency served as a predictor of high severity/mortality and poor prognosis in patients with acute respiratory failure due to COVID-19 (Carpagnano et al., 2020; Radujkovic et al., 2020), and as an indicator of high infection risk for the healthy (Merzon et al., 2020). Vitamin D is supposed to lower viral replication rates and reduce concentrations of pro-inflammatory cytokines via cathelicidins and defensins (Grant et al., 2020). Due to the underlying benefits, safety and low cost, it is rational to use it as a supplementary therapeutic in COVID-19. Vitamin C is also believed to significantly lower incidence of pneumonia based on three controlled trials with human subjects (Hemilä, 1997), which suggests it may affect susceptibility to lower respiratory tract infections under certain conditions (Hemilä, 2003). A clinical trial is undergoing to access the impact of high dose of vitamin C in patients with COVID-19 (Carr and Rowe, 2020), which is closed because of rare severe cases in Wuhan. Another micronutrient, vitamin K, its reduced level emerges as a potential risk factor of severe COVID-19 (Dofferhoff et al., 2020), suggesting supplement of vitamin K might be a necessary therapy.

CONCLUSION

The ongoing public health crisis caused by SARS-CoV-2 is receiving massive global attention. Although several vaccines are being developed to protect against SARS-CoV-2, major efforts are underway to repurpose existing drugs to treat COVID-19. Herein, we have summarized current data, from both *in vitro* experiments and clinical research to address the effectiveness and safety of all candidates, applied to COVID-19 administration. CQ/HCQ inhibits virus infection via different stages, yet more risk instead of benefit is shown based on clinical studies. Remdesivir, due to the promising efficiency and more supportive evidence, is acknowledged as one of the therapeutic of COVID-19. In general, highly efficient anti-viral agents are absent in current treatment strategy. As for host system modulatory agents, use of corticosteroid and TCM need to be measured according to patients' conditions, and the optimal dosage is uncertain. CP and MSC would theoretically provide passive immunity for patients and are relatively safe, however, they failed to achieve consistent results and are limited by the resources as well. Since it is well known that the incidence of thrombotic events is high, the strategy of therapeutic and prophylactic anti-thrombotic agents remains uncertain and needs to be taken into consideration. Lack of specific treatment for COVID-19 brings more attention to vaccine development to keep the pandemic in control and reduce severe condition. In summary, more large-scale randomized clinical trials are urgently required to provide high quality data and guide clinician to make better decision on treatment.

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A Journey From SARS-CoV-2 to COVID-19 and Beyond: A Comprehensive Insight of Epidemiology, Diagnosis, Pathogenesis, and Overview of the Progress into Its Therapeutic Management

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The 2019 novel coronavirus (2019-nCoV), commonly known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or coronavirus disease 2019 (COVID-19), was first revealed in late 2019 in Wuhan city, Hubei province, China. It was subsequently spread globally and thereby declared as a pandemic by WHO in March 2020. The disease causes severe acute respiratory illness and is highly contagious due to the fast-onward transmission. As of the mid of November 2020, the disease has affected 220 countries with more than 16 million active cases and 1.3 million deaths worldwide. Males, pregnant women, the elderly, immunosuppressed patients, and those with underlying medical conditions are more vulnerable to the disease than the general healthy population. Unfortunately, no definite treatment is available. Although remdesivir as an antiviral had been approved for use in those above 12 years of age and 40 kg weight group, it has been observed to be ineffective in large-scale SOLIDARITY trials by WHO. Moreover, dexamethasone has been found to increase the recovery rate of ventilated patients; oxygen and inhaled nitric oxide as a vasodilator have been given emergency expanded access. In addition, more than 57 clinical trials are being conducted for the development of the vaccines on various platforms. Two vaccines were found to be significantly promising in phase III results. It is concluded that till the approval of a specific treatment or development of a vaccine against this deadly disease, the preventive measures should be followed strictly to reduce the spread of the disease.

Keywords: coronavirus, SARS-CoV-2, COVID-19, remdesivir, dexamethasone, hydroxychloroquine, azithromycin, vaccines

INTRODUCTION

SARS-CoV-2 and COVID-19

Severe acute respiratory syndrome coronavirus 2, which is abbreviated as SARS-CoV-2, is a single-stranded RNA virus that belongs to the Coronaviridae family (subfamily: Coronavirinae) in the order Nidovirales. The consensus report after its phylogenetic analysis by Coronaviridae Study Group of International Committee on Taxonomy of Viruses has concluded that this virus belongs to a species group of similar coronaviruses called "Severe Acute Respiratory Syndrome-Related Viruses". This particular virus has thus been recognized as "novel" in its phylogenetic character and is far more distinct than just strain and isolate of any previously known viruses (Gorbalenya et al., 2020). On January 3, 2020, the virus was first named 2019-nCoV (2019 novel coronavirus), and the disease was called novel coronavirus-infected pneumonia (NCIP) by the National Health Commission and China CDC after the revelation and analysis of the complete viral genome of the virus (Wenjie et al., 2020; Zhu et al., 2020). WHO has termed the infectious disease caused by SARS-CoV-2 as coronavirus disease 2019 (COVID-19) (WHO, 2020d). The collective symptoms due to the infection include a range of mild symptoms such as fever, dry cough, malaise, sore throat, fatigue, pain, and loss of taste or smell to a range of moderate symptoms predominantly including dyspnea, diarrhea, and pneumonia (Tay et al., 2020a; CDC, 2020). In critical situations, the patients were found to have been affected by dysfunctional immune response clinically identified as "cytokine release syndrome" and thrombosis, which often lead to fatal consequences (Merad and Martin, 2020). Since the initial report of the outbreak of the virus in Wuhan city, Hubei province, China, in December 2019, where a cluster of infections with pneumonia-like symptoms was reported, WHO declared COVID-19 a pandemic on March 11, 2020. Currently, it affects almost 220 countries across the world, with widely varying distribution of incidence and mortality among different geographies and countries. As of November 15, 2020, the total incidence of the infection stands at more than 57 million people diagnosed, with more than 1.3 million confirmed deaths reported globally (WHO, 2020a). A graphical presentation of the COVID 19 disease is presented in **Figure 1**.

Morphology and Genetic Composition

The SARS-CoV-2 is a spherical-shaped virus with irregular crown-like projections on its surface. These crowns are surrounded by several types of functional proteins submerged and protruding from them. It is enveloped with a positive-sense single-stranded RNA genome with an approximate size of 30 kilobases (Zhao et al., 2020). In terms of its size, it has an overall large diameter in a size range of 75–160 nm (Guobao et al., 2007). The genome of SARS-CoV-2 consists of 14 open reading frames (ORFs) that encode 27 proteins, 15 nonstructural proteins that are important for viral replication, and four structural proteins named spike (S), envelope (E), membrane (M), and nucleocapsid (N) along with accessory proteins (Malik et al., 2020; Wu A. et al., 2020) (see **Figure 2**). The studies have indicated its similarity to Bat-SARS-like coronavirus, SARS-CoV, and MERS-CoV (Zhou P. et al., 2020; Chen Y. et al., 2020).

Mechanism of Cell Entry and Life Cycle of the Virus

The virus enters the host's cell through angiotensin-converting enzyme 2 (ACE2) receptors present on the cell membrane of the cells of several tissues, particularly of the lower respiratory tract (LRT), heart, kidneys, and gastrointestinal tract (GIT) (Imai et al., 2010; Hoffmann et al., 2020; Wan et al., 2020).

The entry is also facilitated by TMPRSS2 protease or endosomal cathepsin L present on host's cells. The viral S protein consists of S1 and S2 subunits. The S1 binds ACE2 receptors through the RBD region, while S2 and TMPRSS2 or cathepsin L complex promote membrane fusion between the virus and the host cell. The entry is followed by the release of viral RNA, translation of ORF, production of nonstructured proteins, and formation of viral replication transcriptase complex. The complex initiates genome replication and subgenomic transcription. The viral structural proteins (S, E, M, and N) are encoded, including certain accessory proteins. Afterward, translation proteins are assembled at the endoplasmic-reticulum-Golgi intermediate compartment (ERGIC). Here, the S protein may also be modified by furin. The viral particles are thereby released from the host's cell through exocytosis (Hoffmann et al., 2020; Tang T. et al., 2020; Shereen et al., 2020; Su and Wu, 2020).

This is the same mechanism as that observed previously for the SARS-CoV virus (Imai et al., 2010; Hoffmann et al., 2020; Wan et al., 2020). Some studies have found that the ACE2 receptor affinity of SARS-CoV-2 is more efficient than that of SARS-CoV(2003) but less efficient than its 2002 strain (Guobao et al., 2007; Wu A. et al., 2020) (see **Figure 3**). It is believed that any mutation on the receptor-binding domain (RBD) of S protein could make the virus more pathogenic. However, some mutations other than the receptor interaction sites in RBD of S protein have been discovered, but the role of such mutations in its pathogenicity is still not clear (Wu A. et al., 2020; Wan et al., 2020).

EPIDEMIOLOGY

Origin

The novel coronavirus (nCoV), which has been later named SARS-CoV-2, was first reported to spread among contacts in the Huanan seafood wholesale market in Wuhan city, Hubei province, China (Wang et al., 2020b). The isolated agent was later identified as the seventh species of the coronavirus family to have caused infectious conditions in humans (Tang X. et al., 2020). It has been strongly believed that the species originated from *Rhinolophus affinis* (horseshoe bats, 96% identical genome with RaTG13 coronavirus species found in the host) with likely zoonotic spillover in *Manis javanica* intermediary host (Malayan pangolins, identification of strong similarity in six places of the RBDs of the virus with species in the hosts). This assessment substantiates the argument of its natural selection in humans either before or after zoonotic spillovers from intermediary hosts (Andersen et al., 2020).

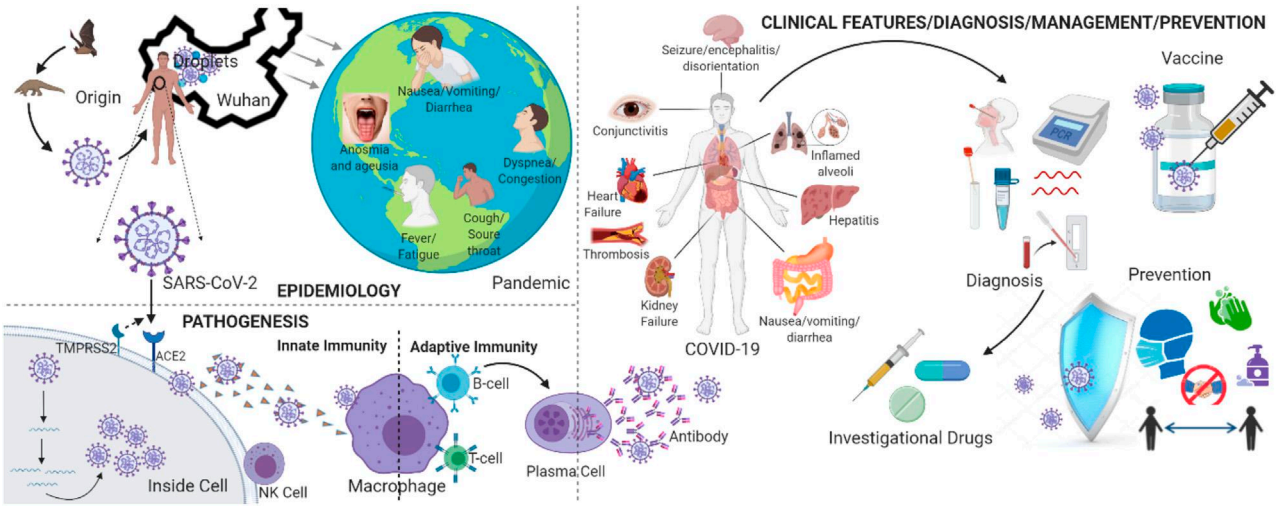

FIGURE 1 | Schematic overview of SARS-CoV-2 epidemiology, pathogenesis and clinical features, diagnosis, management, and prevention [Figure created with BioRender, www.biorender.com].

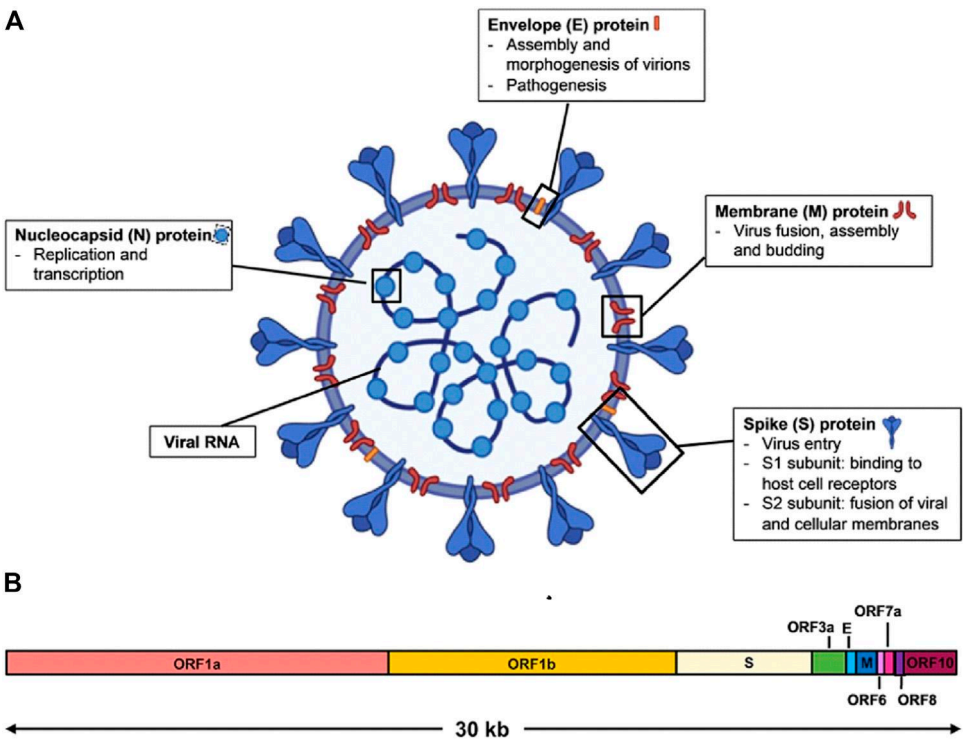

FIGURE 2 | Schematic diagram of SARS-CoV-2 virus structure and genome organization. **(A)** The viral surface proteins, spike (S), envelope (E), and membrane (M) are embedded in a lipid bilayer. The single-stranded positive-sense viral RNA is associated with the nucleocapsid (N) protein. Diagram was created with BioRender. **(B)** The genome organization of SARS-CoV-2 viral RNA, which is adapted from GenBank accession number: MN908947, is characterized by sequence alignment against two representative members of the betacoronavirus genus. The entire genome sequence is ~30 kb long [reproduced with permission] (Lee et al., 2020b).

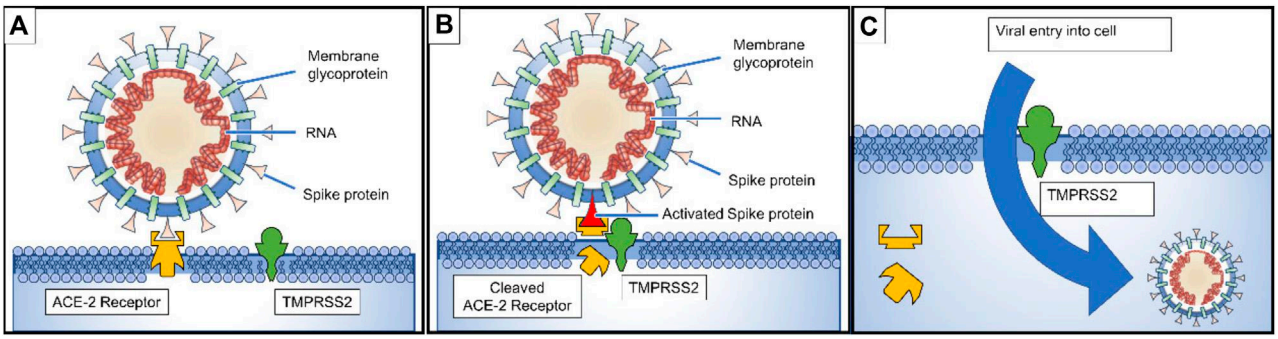

FIGURE 3 | (A) Spike proteins on the surface of the coronavirus bind to angiotensin-converting enzyme 2 (ACE2) receptors on the surface of the target cell; **(B)** The type II transmembrane serine protease (TMPRSS2) binds to and cleaves the ACE2 receptor. In the process, the spike protein is activated; **(C)** cleaved ACE2 and activated spike protein facilitate viral entry. TMPRSS2 expression increases cellular uptake of the coronavirus (Lee et al., 2020).

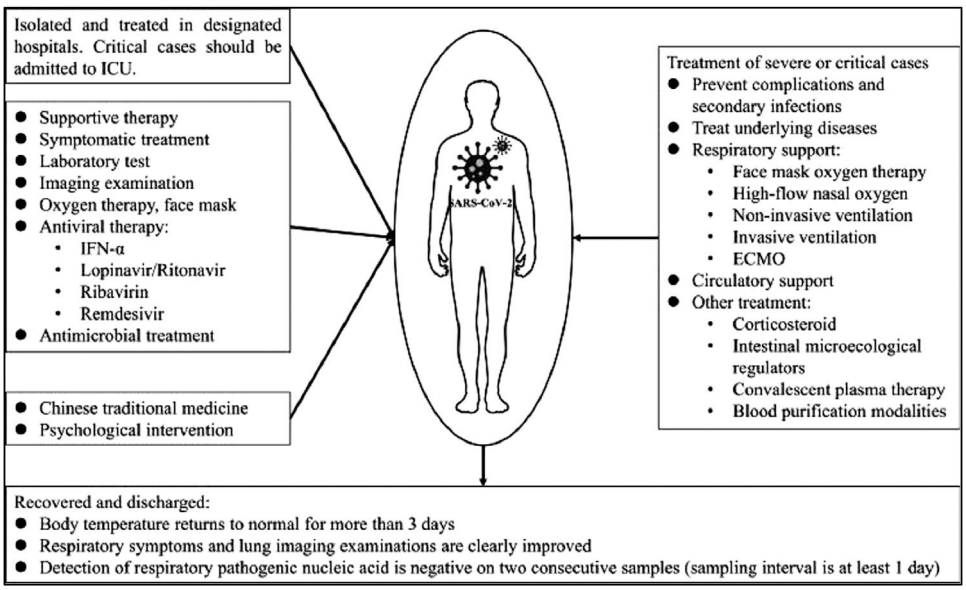

FIGURE 4 | The treatment and management option for COVID-19 patients [reproduced with permission] (Yang Y. et al., 2020).

Geographical Epidemiology, Ethnicity, and Culture

The SARS-CoV-2 virus has affected over 220 countries as of November 15, 2020. The epicenters of the pandemic are currently centered in the Americas (the United States and Central American and South American countries) and Europe, where the incidence is reported to be more than 24 million and 16 million cases, respectively (WHO, 2020a). In terms of mortality, United States, Mexico, Brazil, Columbia, and Peru are the worst affected countries in the Americas, whereas the United Kingdom, Spain, Italy, and France depict a similarly grim picture in Europe. India, Iran, and Russia are also among those countries that are badly affected globally (WHO, 2020a). In the United States, there have been approximately 11.5 million cases (highest incidence) reported with mortality of over 250,000. This is the highest mortality of any country reported so far. In terms of country-wise mortality figures, United States is followed by Brazil (~168,000), Mexico (~100,000), Argentina (~37,000), Columbia (~35,000), and Peru (~34,000). Among the various European countries that have been hit badly by the pandemic, the highest mortality is documented in the United Kingdom (~54,000), followed by Italy (~48,000), France (~47,000), and Spain (~42,000). In Asia, India leads the mortality figures with more than ~133,000 deaths attributed to COVID-19, followed by Iran with ~44,000 deaths and Russia with ~35,000 deaths (WHO, 2020e). Recently, many countries have seen a significant spike or surge in the number of new cases since the start of September 2020. This is especially true for many European countries where collectively ~270,000 cases are now being reported each day in

Europe against a modest value of ~30,000 back in late August. Similarly, these figures are also being reported for the Americas against the lowest values ~65,000 in August (WHO, 2020e). This is being referred to as the "second wave" of new infections, especially pertinent to European countries, where after significant depression in the number of new cases in recent 3–4 months (due to government measures), the recent lifting of lockdowns has led to a huge spike of new cases (Looi, 2020). Governments have therefore implemented strong measures equivalent to initial measures taken during the first wave of infections.

There is wide intercontinental variability in the spreading of COVID-19, and to assess the regional population data, individual expression of transmembrane protease serine 2 (TMPRSS2), which allows cellular uptake of the S protein, may be a determinant of SARS-CoV-2 regional infection susceptibility. Four variants of TMPRSS2 were evaluated in the local population of Africa, America, Europe, and Asia (China, Japan, and Taiwan), and the frequencies of variant alleles with high TMPRSS 2 expression in lungs were reported to be highest in the European and American population and lowest in the Asian population. Similarly, phylogenic analysis of time to the most recent common ancestor (TMRCA) was performed for SARS-CoV-2, dating to November 12, 2019, which also matched the epidemiological records of the disease. The non-Asian (Africa and Europe) outbreak of the disease has been associated with the reported reason of subhaplogroup A2 originated in Europe from Asian ancestor, where haplogroup A is regarded as an ancestral node (Gómez-Carballa et al., 2020). The emergence of COVID-19 was late in South America. The first case was reported on the 25th of February 2020 in Brazil, after which the entire country went into strict lockdown within few weeks. More than 65,000 cases were reported on the 14th of April, and Ecuador was the one found to be badly affected. The high incidence of COVID-19 was found in the state of Ceara, and most of the cases were originated from Fortaleza, the capital. The exponential rise in COVID-19 cases also led to the need for epidemiological surveillance and consistent analysis (Burki, 2020). A bibliometric study was also performed to evaluate the current trend in research on COVID-19 conducted in Latin America, and Aldeota, Cais do Porto, Centro, Edson Queiroz, and Cambeba were found to be the neighborhoods with the highest propensity for COVID-19 (Braga et al., 2020).

Ethnic factor is also a significant factor determining vulnerability among different racial groups. The susceptibility of COVID-19 among different races was reported in the following order: Black (Central and Western Africa) > Asian (Bangladesh > Indian ≥ Chinese ≥ Pakistani) > mixed ethnic groups (Epi cell, 2020). Similarly, culture plays a vital role in determining the attribution to disease, help-seeking behavior, and community acceptance to comply with measures and interventions to counter pandemic spread. Cultural norms greatly influence the failure or success of the strategies derived for the containment of the COVID-19 outbreak. They may augment the community response to volunteering efforts and making social distancing and lockdown an easier task (Mckee, 2020). For example, some Asian countries like Japan, Vietnam, and Taiwan implemented stringent lockdown at the beginning of the outbreak, overlooking their national economic damage. However, for the United States of America, the primary concern was their economy, and American authorities neglected the severity of the COVID-19 outbreak (Huynh, 2020). In addition, 1.76 million people might have been saved in the United States if strict social distancing was practiced. The Russian approach was more different, comprising penalties against the violation of the governmental instructions, whereas India and the Philippines were more stringent and were reported to arrest people not following social distancing practice (Greenstone and Nigam, 2020). Fetzer et al. (2020), studied the heterogeneous behavior of 51 countries for following social distancing and reported that Peru strictly followed the "staying at home" policy and ranked second among the countries with the highest percentage of residentials (Fetzer et al., 2020). The Asian countries applied a punitive approach to social distancing as their strict cultures, while the European countries are likely to be lenient in forcing people to stay at home. It has also been observed that countries with higher "Uncertainty Avoidance Index (UAI)," exhibited a lower proportion of public gathering. UAI shows that people in a society are in fear of unknown, uncertain, and unstructured situations; this can influence cultural perception and decision power (Huynh, 2020). The perceptions of different communities also affect the local medicinal practices; in a study performed in India, 48% of participants favored eating garlic in prophylaxis against COVID-19 (Vadivu and Annamuthu, 2014). But there can also be a sense of truculence and false hope that may drive some communities out of isolation due to a high degree of hubris. For example, in India, some organizations promoted people to take a bath in cow dung to defeat the SARS-CoV-2 (Theinterpreter, 2020).

Demographic Distribution

In terms of the distribution of incidence rates among different age and racial groups, the virus seems to be very discriminative. The data of 10 European countries on the distribution of COVID-19 cases by gender and age revealed that females of working age outnumber infected males. However, the rate in females declines at the retirement age of 60–69, which results in a crossover among males with COVID-19, so the vulnerable age group for a male is 70–79 and that of the female is 20–29, especially those bearing health and care–related occupation profiles (Sobotka et al., 2020). Moreover, it has been found that pregnant women are more vulnerable to COVID-19 infection, with a higher rate of death being associated with physiological changes during pregnancy, such as an increase in heart rate, a decrease in lung capacity, and a higher risk of thromboembolic disease (Centers for Disease Control and Prevention (CDC), 2020b). Across the globe, one pattern is very persistently observed; that is, infected males are more likely to die than females, despite all the uncertainties and dubitation (Zhou F. et al., 2020; Jordan et al., 2020; Lawton, 2020). Globally, in terms of the incidence of symptomatic incidence and mortality, elderly patients, especially with underlying conditions, are identified as the most vulnerable group (Kang and Jung, 2020). In New York state for the two oldest age groups, 65–74 years old and 75 and above, the weekly calculated infection fatality risk (IFR) was more significant than that of the younger age (0.0097% for <25 years and 0.12% for

25–44 years) group and was reported to be 6.7% and 19.1%, respectively (Yang W. et al., 2020). The cumulative infection rate across the globe is the lowest, i.e., below 1 per 1,000 among children and adolescents. The rates were found to be lowest in Spain, 0.6% (age, 0–14), and highest in Czechia, 9.2%. Comparatively stable infection rates were reported in Portugal at the ages of 20–59, but the irregular profile was exhibited in Czechia and Germany; however, a steeper rise in infection rate with age was observed in England (Sobotka et al., 2020).

Empirically, older people with comorbidities, residing in nursing homes, are at the highest risk of adverse outcomes and mortality during the running phases of a pandemic. Moreover, behavioral problems, cognitive disorders, and functional impairment may synergize the threat posed to nursing homes (Fallon et al., 2020). In Washington, about two-thirds of the residents were reported to be infected within a period of 3 weeks only at the death rate of 33%, along with 50 staff members and 16 visitors infected (Mcmichael et al., 2020). A significant number of deaths reported in Spain have been associated with nursing home residents. Many authorities are not including deaths at nursing homes from the total COVID-19 death toll (Wang et al., 2020a).

Mode of Transmission

SARS-CoV-2 has been found to transmit primarily through respiratory droplets (5–10 μm) and physical contact with contaminated matter. It is believed that the virus could also be transferred through airborne mechanisms (aerosols) where the virus gets trapped in particles (<5 μm) for an extended period of time and can be transmitted through distances of over 1 m (WHO, 2020c; Liu et al., 2020b; Morawska and Cao, 2020). Such instances are likely in closed spaces such as saturated ventilation systems or proximities where the patients with fluids of high virus loads are in contact with the susceptible individuals, such as exposure of healthcare workers and medics during intubation procedures and noninvasive positive pressure ventilation (Liu et al., 2020b). There have been reports that SARS-CoV-2 is also present in the fecal matter of the patients and can infect the gastrointestinal tract (Lamers et al., 2020). However, the scientific evidence for confirmed transmission through the fecal-oral route is missing (Xu Y. et al., 2020). Furthermore, there have been instances where the virus has been found to transfer through another intermediary host such as domesticated cats (human-cat-human), raising significant concerns of additional factors that could aid in the spread of the virus (Shi J. et al., 2020; Halfmann et al., 2020; Mallapaty, 2020).

In terms of epidemic modeling, the mean reported "reproduction number" (*R0*) for the current first wave of the pandemic has been estimated around 3.28 with a median value of 2.79, which is surprisingly very high compared to the estimates of 1.25–2.5 given by WHO at the beginning of the epidemic (Liu et al., 2020a). Moreover, this estimate is also higher than ~2 reported for SARS-CoV-1. The surprising element behind the unprecedented spread of this coronavirus is its capability of transmission presymptomatically (~48%, cases that show symptoms afterward) and asymptomatically (~10%, cases where the person does not show any symptoms). The symptomatic cases are reported to be around 38% of the total transmissions (Ferretti et al., 2020). It must also be mentioned here that citing some recent studies, WHO, in its interim guidance and public press briefing, has made claims that asymptomatic transfer is unlikely with a wide level of interest in the announcement by governments around the world in favor of reopening the economies; however, due to significant criticism from public health experts around the world, WHO has since changed the stance and maintained that the matter is not yet close to a verdict and even if there is weak evidence of asymptomatic transfer, there is still a chance of its spread. As far as the current spread is concerned, WHO has categorized the transmission within countries as either "sporadic," "cluster-based," or "community-based" transmissions and thus with the current trend of data, Saudi Arabia, Somalia, Yemen, and Magnolia are classified as "sporadic transmission" areas, whereas countries such as China, Pakistan, India, Australia, Russia, Germany, Italy and Portugal are being classified as "cluster-based transmission" areas. The remaining areas are largely classified as "community-based transmission" areas, including the Americas, Africa, and remaining countries of Europe.

Precipitating Factors Influencing the Transmission

Wide varieties of factors have been identified to influence and affect the transmission rate of SARS-CoV-2 and the severity of COVID-19 among humans. Some of these factors are related to social behavior, while others are identified as physical and environmental conditions. According to a detailed study investigating the interrelationship of various factors influencing the virulence of the COVID-19, the primary factors to limit the spread of COVID-19 are social distancing and community sense of mitigation measures recommended by WHO such as personal hygiene and mandatory wearing of face masks, especially in closed spaces (Lakshmi Priyadarsini and Suresh, 2020). Conversely, in terms of physical conditions, lower air temperature (~22°C) and lower relative humidity (40–60% RH), turbulent airflow patterns in packed areas, and closed-circuit ventilation have all been reported to participate in the spread of contaminated aerosols and thus are likely participants in the increased rate of transmission. Primary physical factors related to environmental conditions such as air temperature, relative humidity, and UV light exposure have been previously studied in detail for the SARS-CoV-1 virus, with a significant loss of virulence observed at a temperature of 38°C and >95% RH (Chan et al., 2011; Kowalski et al., 2020). Although there are few such studies on SARS-CoV-2, the same effects have been observed related to temperature and relative humidity for this virus, thereby impacting its spread (Bannister-Tyrrell et al., 2020). The virus has been found to be stable over a wide range of pH (3–10) at room temperature (Chin et al., 2020). The stability studies on different surfaces have identified that the viral titer was undetectable from printing or tissue paper after 3 h incubation and 2 h on wood and cloth but could last for 4–7 days on other surfaces. Furthermore, it has been found to be susceptible to

typical disinfectants such as hydrogen peroxide, sodium hypochlorite, and hand soaps (Chin et al., 2020).

Another significant factor related to demographics is the age bracket of exposed individuals (higher age groups are more susceptible), as it is continuously reported as a significant predisposing factor for increased mortality and morbidity due to COVID-19 (Lakshmi Priyadarsini and Suresh, 2020). The other major precipitating factor that has been noted to contribute to the severity of COVID-19 is underlying medical conditions, such as hypertension, diabetes, asthma, renal disease, and other respiratory conditions such as COPD (Lakshmi Priyadarsini and Suresh, 2020). It has been suggested that the higher baseline levels of IL-6 and other inflammatory cytokines may be the probable reason for severe infection in these conditions (Tay et al., 2020b; Lakshmi Priyadarsini and Suresh, 2020). However, the anticipated response is not observed in inflammatory arthritis patients, even with raised levels of IL-6 (Schett et al., 2020). In addition, immune-compromised patients and patients on immunomodulators are also likely to exhibit a quite grim picture of COVID-19 (Monti et al., 2020). In a study, 58 patients with multiple myeloma (MM) receiving different immunomodulators diagnosed with COVID-19 showed a mortality rate of 24%. Therefore, early intervention in immunomodulatory therapies and strict adherence to the safety measures are recommended to encounter the future outbreak of COVID-19 (Wang B. et al., 2020).

Furthermore, one of the most significant socioenvironmental factors affecting the rate of spread is the population density. This factor alone can significantly contribute to the major wave of large-scale epidemics observed in localities like New York, New Jersey, and Indian Slums Metropolitan areas (Corburn et al., 2020; Gonzalez-Reiche et al., 2020; Mishra et al., 2020). Similarly, the transfer rate and infection reproduction number (R^0) were calculated to be four times higher than those in the initial spread in Wuhan in Diamond Princess (a cruise ship affected by SARS-CoV-2 (Rocklöv and Sjödin, 2020).

Global Measures in Response to Pandemic

Since the first identification of the spread of this virus in the Wuhan city of Hubei province China, governments and policy advising agencies have been advocating for various containment and mitigation strategies to minimize the spread and flatten the pandemic curve to avoid crippling consequences on the healthcare systems due to out-of-capacity inflow of critical cases that may result in higher mortality. Four key response measures have been suggested by the OECD to the governments worldwide in light of the scientific evidence established from earlier and current pandemics. These include 1) large-scale surveillance, monitoring, and detection through centralized epidemiological and disaster management centers; 2) prevention of the spread in the community by means of social distancing measures and smart and complete lockdowns wherever necessary; 3) clinical management of cases by means of the best available scientific evidence and practices; 4) maintaining essential services to ensure the smooth running of the system and avoiding any other potential catastrophe (OECD, 2020). A mix of various containment and mitigation strategies well suited for the given country and region is advocated. In this regard, the United Nations had advised implementing contact tracing and hotspot mapping strategy to mitigate the spread for developing countries. This is especially true for developing countries such as Bangladesh, Myanmar, and India, where the economic consequences of complete lockdown are becoming disastrous (UN-DGC, 2020).

After a nearly complete global shutdown, economists are ringing alarms of the unprecedented recession of the 21st century, which is already devastating news for the developing economies, owing to their large informal sectors. The projected median decline in GDP is already threatening for many economies and may likely dip down further below the projections (Fernandes, 2020).

The measures to circumvent the spread of this virus based on manual contact tracing are insufficient and thus, along with the advocated measures of social distancing, hygiene, manual tracing of contacts, quarantine, and lockdowns, the use of digital applications, where the contacts are automatically alarmed of any potential transmission with a known case, is touted to be a major driving factor to control the spread. This strategy has been in various ways successful in countries such as South Korea if implemented with transparency and integrity to secure the public data (Ahn, 2020). Moreover, the strict implementation strategy and early timing of enforcing social distancing measures across many countries have resulted in a very contrasting and significant consequence in terms of reduction of "R" value of the spread and total fatalities and incidence of the infections (Ketchell, 2020).

DIAGNOSTIC AND MONITORING TOOLS

SARS-CoV-2 genetic material is reported to be successfully detected through throat swabs and the upper and lower respiratory tract, blood, stool, or urine samples (Pan Y. et al., 2020; Chen W. et al., 2020; Yan et al., 2020). Several methods have been introduced for the detection of SARS-CoV-2. However, the collection of various samples from different sites and the utilization of multiple techniques is usually recommended to avoid false results related to the use of a single sample or method (Wang et al., 2020b). Furthermore, the application of positive, negative, and inhibition controls is also recommended to assure quality diagnosis (Yan et al., 2020).

Nucleic Acid Amplification Tests

PCR and Real-Time PCR

PCR and RT-PCR are considered important molecular biology techniques, first introduced in the 1980s. The techniques are based on the amplification and detection of a particular gene (Panawala, 2017). The amplification of genetic material is beneficial for obtaining the satisfying quantity of specimens required for a laboratory study. Both PCR and RT-PCR involve the utilization of certain enzymes. PCR uses a DNA template, whereas RT-PCR uses RNA (Panawala, 2017). Several RT-PCR-based test kits have also been developed (Li X. et al., 2020).

Sensitivity and Specificity

The techniques are considered highly sensitive, highly specific, and reliable for the detection of CoVs (Shen M. et al., 2020). However, these methods were observed to be commonly used for SARS-CoV-2 detection (Li X. et al., 2020). Yet, the procedures are claimed to be time-consuming and expensive that require costly reagents or equipment. The absence of safe and stable EPC (external positive controls), as available for SARS-CoV-1, is another severe problem in the detection of SARS-CoV-2 (Shen M.et al., 2020). Furthermore, adequate sampling, proper handling of the sample, and sufficient genetic material are recommended for a reliable PCR-based report (Lee et al., 2020). Additionally, RT-PCR is recommended over PCR due to its superior sensitivity (Shen M. et al., 2020).

Several rapid diagnosis kits that have been developed as per WHO standards are claimed to be 95% accurate against SARS-CoV-2. An RT-PCR-based test kit has also been introduced by the Centers for Disease Control and Prevention (CDC) CDC (2020b). Rapid nucleic acid diagnostic papers have also been invented, which provide a rapid detection facility of only 3 min with unaided eye observation (Jin Y. et al., 2020). Furthermore, the kits are only limited to upper and lower respiratory tract specimens. However, FDA has recently authorized the first RT-PCR-based LabCorp COVID-19 kit with a home collection option (FDA, 2020b).

Loop-Mediated Isothermal Amplification

LAMP is known as an ultrasensitive novel isothermal nucleic acid amplification–based method. It has been claimed to be capable of detecting even a small quantity of biomaterial within an hour and without the need for expensive reagents or equipment.

Sensitivity and Specificity

Its sensitivity and detection rate against coronaviruses have been found to be similar to those of PCR-based methods. However, the technique requires a high temperature, usually 65 °C, which restricts its application (Shen M. et al., 2020).

Microarray

The microarray technique has been widely used for the detection of coronaviruses. In this method, the virus RNA is used to produce cDNA, labeled with a specific probe through reverse transcriptase followed by subsequent detection of that specific probe. The method offers a low cost with sensitivity equal to that of RT-PCR. Moreover, portable microarray chips with adequate detection limits have also been introduced (Shen M. et al., 2020).

Specific High-Sensitivity Enzymatic Reporter Unlocking

This method is based on RNA-targeting CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) related enzyme Cas13. Cas13 has been combined with LAMP to detect DNA or RNA molecules.

Sensitivity and Specificity

The method has been shown to be quick, portable, and highly sensitive for nucleic acid detection (Shen M. et al., 2020; Udugama et al., 2020).

Radiological Examination

Although the nucleic acid amplification test has been widely recommended for the detection of SARS-CoV-2, its false reports could not be overlooked that may result in a false diagnosis and other severe consequences (Li X. et al., 2020). Consequently, the CT (chest radiography) scan has become a reliable method for the diagnosis of COVID-19 in clinical practice (Jin Y. et al., 2020). The scan images of almost all COVID-19 cases indicate the same features, particularly bilateral pulmonary parenchymal ground-glass opacification and consolidative pulmonary opacities, that have been observed in nearly 60 to 77 percent of cases (Forouzesh et al., 2020). At the same time, it has been observed that patients with negative nucleic acid amplification tests may show positive chest CT scan findings. However, a repeated nucleic acid amplification test is suggested for the final remarks (Forouzesh et al., 2020). Artificial Intelligence (AI) technology has also been used for accurate and instant interpretation of CT images (Jin Y. et al., 2020; Mak, 2020).

Limitations

Some disadvantages of CT imaging have also been reported, particularly nonselectivity and hysteresis of irregular imaging (Li X. et al., 2020). Moreover, prevention from frequent exposure to radiation, especially for pregnant women and children, is strongly recommended (Forouzesh et al., 2020).

Serological Tests

Acute serological responses have been identified in COVID-19 patients (Zhou P. et al., 2020). The serological tests are considered alternative to the nucleic acid test and CT imaging. For this purpose, several colloidal gold immunochromatography assays and other related techniques, kits, and detection methods have been applied and established (Jin Y. et al., 2020; Lee et al., 2020; Li X. et al., 2020; Rashid et al., 2020). The techniques generally target coronavirus immunogenic proteins (S, N, E, and M) and RBD to detect the presence of SARS-CoV-2 related antibodies (Mcintosh et al., 2020). The IgG levels are reported to be usually increased as the IgM levels start decreasing during viral infection (Rashid et al., 2020). IgM antibodies have been detected successfully during the early phases of infection, usually within 3 days, and are claimed to be present even after a month. Similarly, SARS-CoV-2 specific IgG antibodies have been reported to be detected after 4 days of infection period with a peak level after 2 weeks. It has been shown that their levels are related to disease severity; a higher level of both antibodies indicates greater severity of the infection. Researchers have also suggested IgA detection for SARS-CoV-2 diagnosis that is related to mucosal immunity usually activated in COVID-19 patients. However, it is considered less specific than IgM and IgG (Lee et al., 2020).

A list of other serological markers has been reported for the prediction of infection severity and prognosis of the disease in patients suffering from COVID-19. Some of these include an examination of interleukins (IL) levels, particularly IL-6, IL-10, and IL-2R, ESR (Erythrocyte Sedimentation Rate), CBC (Complete Blood Count), PT (Prothrombin Time), and levels of liver, kidney, heart, and other related enzymes (Forouzesh et al., 2020).

Limitations

Although serological tests are regarded as fast, powerful, and easy to conduct, it has been noted that the antibodies' response develops after several days of infection. The CDC does not recommend these tests for the diagnosis of current COVID-19 disease. Moreover, only 70% of their sensitivity is reported even after 4–6 days of infection (Centers for Disease Control and Prevention (CDC), 2020a; Wang et al., 2020b). The antibodies, IgM, and IgG, against SARS-CoV-2, have been observed to increase progressively with infection (Lee et al., 2020). Thereby, early diagnosis of infection is not possible and may lead to false-negative reports. It has also been reported that a large population has already been exposed to other human coronaviruses, and thus the false-positive response is commonly observed due to a high level of SARS-CoV-2 similarity to other coronaviruses. Therefore, the utilization of multiple serological approaches is recommended for a true report (Lee et al., 2020).

CORONAVIRUS DISEASE 2019

Immune System

The immune system works as a defense system and plays a key role in the prevention of pathogenic attacks throughout the body; however, uncontrolled or impaired immune response may result in harmful tissue damage (Cao, 2020; Li G. et al., 2020). Overwhelming of the inflammatory response is considered to be initiated as a result of the antagonism effect of interferon by SARS-CoV-2 to promote its replication inside the cell (Tay et al., 2020a). Interferon (IFN) response is considered directly related to viral load. An increase in type 1 IFN response causes decreased viral load and vice versa. It has been observed that a decrease in total T cell count causes a declined function of these cells in COVID-19 patients (Diao et al., 2020). However, increased levels of cytokines such as interleukins (IL-6, IL-1β, IL-2, IL-8, and IL-17), granulocytes like granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM- CSF), interferon gamma-induced protein 10 (IP10), monocyte chemoattractant protein-1 (MCP1), macrophage inflammatory proteins-1 alpha (MIPα), and tumor necrosis factor (TNF) along with C-reactive proteins, D-dimers, and ferritin are reported in COVID-19 infection (Cao and Li, 2020; Xu Z. et al., 2020; Huang et al., 2020). Cytokines are responsible for shock and severe tissue damage to different organs, and slow healing of lungs is observed in patients with elevated IL-6 levels (Wang et al., 2020b). Another unique characteristic of hypercoagulation has also been commonly noticed in serious COVID-19 patients (Tang et al., 2020b; Merad and Martin, 2020). The cytokine storm and sepsis are considered the primary cause of death in about 28% of severe cases of COVID-19 (Zhang B. et al., 2020). But these immunological changes are often restored, particularly in mild to medium cases. Simultaneously, individuals with robust immunity and without comorbidities may successfully eliminate the virus before the exacerbation of immune overreaction (Cao and Li, 2020).

Organs Involvement

The organs that have been confirmed clinically to be involved in the COVID-19 infection include the eye, nervous, digestive, respiratory, circulatory, and urinary systems (Wang L.-S. et al., 2020). Although the lungs are the primary target of COVID-19 infection, it can attack or damage almost all body organs, particularly the heart, blood vessels, kidneys, intestines, and brain. The cells of these organs are rich in ACE2 receptors that are essentially required for the virus entry into the cells (Cao and Li, 2020; Cyranoski, 2020; Ky and Mann, 2020; Wadman et al., 2020; Chris Baraniuk, 2020, April 29).

Nasal Passage

The cells of the nose and throat are rich in ACE2 receptors providing an adequate environment for the virus where it starts replication. This is an asymptomatic phase, but a person could be the carrier of this deadly virus to another person (Peiris et al., 2003; Zou et al., 2020; Chris Baraniuk, 2020). The viral load of SARS-CoV-2 has been found higher in the nose than throat, unlike SARS-CoV-1, which is the probable reason for its rapid spread through respiratory droplets on close person-to-person contact (Chavez et al., 2020; Zou et al., 2020). The other sources of its spread could be the air contaminated with viral load from cough or sneeze of an infected person, touching or shaking hands, touching the mouth, nose, or eye after viral exposure, or less frequently through the fecal-oral route(Joseph and Ashkan, 2020).

It has been observed that in some cases, viruses may bypass the throat cells and enter into the lungs directly and may cause acute pneumonia without developing mild symptoms related to the throat, including cough and low-grade fever. (Cyranoski, 2020; Wadman et al., 2020).

Lungs

The lungs are considered as the main battle area. The alveoli of the lungs are rich in ACE2 receptors (Wadman et al., 2020; Chris Baraniuk, 2020, April 29). The virus attacks epithelial cells of the lungs and causes Diffuse Alveolar Damage (DAD), resulting in respiratory failure in some patients (Gu and Korteweg, 2007; Schaefer et al., 2020). The WBCs (White Blood Cells), dead cells, mucous, and pus or fluid together in alveoli after the virus attack causes ARDS (Acute Respiratory Distress Syndrome) with parallel symptoms of pneumonia-like fever, cough, and difficulty in breathing, resulting in hypoxemia (Tay et al., 2020a; Xu Z. et al., 2020; Huang et al., 2020). Fortunately, some cases are resolved by just oxygen supply, but many individuals could not survive or require intensive care and end up on ventilation commonly (Xu Z. et al., 2020; Huang et al., 2020; Wadman et al., 2020). Furthermore, the development of pulmonary lymphopenia and increased neutrophil-lymphocyte ratio in almost 80 percent of infected patients is considered due to immune cells' stimulation toward the infection site (Tay et al., 2020a; Jamilloux et al., 2020). Lymphopenia is described as a result of either T cells' death due to direct viral attack, cytokine-induced apoptosis of T cells, or immune cell redistribution (Jamilloux et al., 2020).

The clinicians believe that releasing a high amount of chemical signaling molecules or cytokine storm by the immune system may overreact or start attacking healthy cells and responsible for severe infection (Tay et al., 2020a; Wang et al., 2020b).

Cardiovascular System

Heart and blood vessels are rich in ACE2 receptors (Zheng et al., 2020; Chris Baraniuk, 2020). The studies conducted in China reveal that almost 20 to 44 COVID-19 percent of patients develop cardiac symptoms. These symptoms include arrhythmia, cardiac muscle damage, cardiac swelling and scarring, decreased heart function, and heart attack. Moreover, cardiac symptoms may develop secondary to pneumonia (Zhou F. et al., 2020; Ky and Mann, 2020; Wadman et al., 2020; Zheng et al., 2020). The cardiac symptoms may result in clotting defects, which are the additive COVID-19 severity and mortality. The hypercoagulation state is characterized by extended prothrombin time, high D-dimer levels, and fibrinogen with almost satisfactory partial thromboplastin time (Tang et al., 2020b; Merad and Martin, 2020). It is considered that cytokines, particularly IL-6, and endothelial cell injury are responsible for the activation of the coagulation system and suppression of the fibrinolytic system (Merad and Martin, 2020; Tang et al., 2020b; Wang et al., 2020b). It has been reported that almost 71.4 percent of nonsurvivors developed disseminated intravascular coagulation (DIC) (Tang et al., 2020b). The clots may progress to thrombosis formation, which may cause pulmonary embolism or stroke. It is considered to be a major cause of death due to COVID-19 infection (Zheng et al., 2020; Merad and Martin, 2020; Wang et al., 2020b). The data from the United States show that almost one-third of hospitalized patients had preexisting cardiovascular symptoms or diabetes. The studies suggest that the lack of oxygen and cytokine storm after the viral attack on the lungs is also responsible for blood vessels and heart damage (Tay et al., 2020a; Ky and Mann, 2020).

Renal System

Like other organs, kidneys are also abundant in ACE2 receptors (Chris Baraniuk, 2020, April 29). The studies show that about 59 percent of hospitalized patients develop proteinuria, whereas hematuria, increased blood urea nitrogen, and high levels of creatinine have been observed in 44 percent, 14 percent, and 10 percent of patients, respectively. Acute kidney injury (AKI) and kidney failure are seen as common (Li Z. et al., 2020). Reduced blood flow to the kidney is observed due to cytokine storms, resulting in kidney injury (Cheng et al., 2020). AKI is considered to be serious organ damage caused by COVID-19. A critical high serum creatinine (SCr) level and low urine output are reported during kidney injury (Xu D. et al., 2020). On the contrary, a study conducted in China by Wang et al. indicated fewer associations between AKI and COVID-19 (Wang et al., 2020a). Moreover, ventilators and some antivirals suggested for the treatment of COVID-19 may damage kidneys extensively, particularly in patients with preexisting conditions like diabetes, hypertension, and kidney diseases, respectively (Wadman et al., 2020) (Li Z. et al., 2020).

Central Nervous System

A large number of ACE2 receptors are present in the neural cortex and brain stem (Wadman et al., 2020; Chris Baraniuk, 2020, April 29). The SARS-CoV-2 has been detected in the cerebrospinal fluid. Neurological symptoms are seen in almost 5–10 percent of hospitalized patients (Wadman et al., 2020). However, the brain and nervous system damage should not be underestimated in patients on ventilators (Stevens et al., 2008). Hyperactivity of the nervous system, unconsciousness, loss of sense of smell and taste, meningitis, encephalitis, stroke, brain injury and seizure have been reported. Moreover, it is revealed that the cytokine storm and thrombosis are also responsible for brain swelling, stroke, and severe brain injury (Wu Y. et al., 2020).

Gastrointestinal Tract

The SARS-CoV-2 attacks the lining of the lower digestive tract that is rich in ACE2 receptors (Wadman et al., 2020; Chris Baraniuk, 2020). The virus has been detected in the stool samples of almost 53 percent of patients suffering from COVID-19. Additionally, the viral protein shell is found in the intestines biopsy indicating its replication in the gut linings (Zhou F. et al., 2020). Likewise, viral RNA has been detected on rectal swabs even after negative nasopharyngeal testing (Wang W. et al., 2020). The gastrointestinal symptoms include diarrhea, vomiting, and abdominal pain and have been observed in almost 20 percent of infected patients (Huang et al., 2020).

Liver

The injury to the liver and bile is found common in hospitalized patients, but the direct invasion of SARS-CoV-2 to the liver is not confirmed (Gu et al., 2020; Wadman et al., 2020). However, multiple events during the infection and administration of several drugs are considered responsible for elevated levels of liver enzymes and liver damage (Bangash et al., 2020). In a study conducted in China, 58% of 148 COVID-19 patients had an abnormal liver function. Higher levels of procalcitonin and C-reactive proteins have also been observed in these patients (Fan et al., 2020). Liver dysfunction is observed dominantly in severe cases (Zhang C. et al., 2020).

Eyes

Symptoms like conjunctivitis or pink eyes and watery eyes have been observed in almost one-third of hospitalized patients (Wadman et al., 2020). Chemosis, conjunctival hyperemia, epiphora, and increased secretions are reported in patients in addition to conjunctivitis. Patients with ocular symptoms have demonstrated extraordinary WBCs, neutrophil counts, procalcitonin, C-reactive protein, and lactate dehydrogenase levels compared to those without any ocular symptoms. Furthermore, RT-PCR assay of 90% of infected ocular patients showed positive results for SARS-CoV-2 from a conjunctival swab in addition to a nasopharyngeal swab (Wu P. et al., 2020).

Skin

The skin-related symptoms associated with COVID-19 were first reported in China, followed by Italy and in Spain; when a study

was conducted on 375 patients with skin lesions and positive SARS-CoV-2 test, a relationship between skin lesions and COVID-19 was established (Diotallevi et al., 2020). It has been observed that the viral attacks on the ACE2 receptors, present in arterial and venous endothelial cells and arterial smooth muscle cells, trigger the host's inflammatory response, including activation of mast cells and basophils, which may cause multiple skin conditions like rashes, diffuse or disseminated erythema, urticaria, livedo racemosa, blue toe syndrome, retiform purpura, vesicle trunk, purpuric exanthema, atopic dermatitis, and neutrophilic dermatoses, as well as less frequent cases of chilblains affecting fingers or toes (acral rash). It has been suggested that the skin manifestations may be the result of minor thrombotic events or damage to the endothelial walls of small distal vessels. These conditions have been observed in COVID-19 patients of all ages, but the rashes may be paraviral due to cytokines or drug-related during the treatment of any disease (Criado et al., 2020). Although these conditions may not be accompanied by pain or itching or other systemic symptoms, identifying rashes is important in earlier COVID-19 cases, and therefore attention to skin involvement during COVID-19 is also suggested (Bataille et al., 2020).

DRUGS BEING INVESTIGATED FOR THE TREATMENT OF COVID-19 AND ITS MANAGEMENT

Supportive Therapy

Antipyretics or NSAIDs for reducing fever and pain (Wu A. et al., 2020), oxygen therapy to maintain oxygen saturation (Røsjø et al., 2011), antibiotics as an empiric therapy (Rhodes et al., 2017), intravenous fluid resuscitation or vasopressor for regulating persistent shock (Schultz et al., 2017), early blood purification for reduction of renal workload and renal function recovery (Wang D. et al., 2020), beta-agonists such as dobutamine for the management of cardiac shock or failure, and systemic steroids against COPD exacerbation are commonly suggested as supportive therapy in COVID-19 infection (Alhazzani et al., 2020; World Health Organization, 2020a; Mcintosh, 2020). Furthermore, the use of vitamins as an immunity booster and some Chinese medicines against inflammatory responses has also been reported for the management of COVID-19 (People's daily of China, 2020; Runfeng et al., 2020; Wang L.-S. et al., 2020). A description of such medications is as follows.

Antibacterials

Azithromycin

Clinical trials are currently perceiving its effectiveness against SARS-CoV-2 (Akram et al., 2020; Hinks et al., 2020; Sivapalan et al., 2020). In a trial conducted in France, it revealed that the group of COVID-19 patients who were receiving hydroxychloroquine along with azithromycin showed significant response in comparison with the group receiving hydroxychloroquine alone. Azithromycin was used in a dose of 500 mg per day on day one, followed by 250 mg per day for 5 days along with 600 mg hydroxychloroquine per day, respectively (Gautret et al., 2020). Another study showed its combination with other drugs; especially, hydroxychloroquine was beneficial on laboratory-confirmed COVID-19 patients (Sekhavati et al., 2020). However, Molina et al. and Remo H M Furtado et al. observed contrary results in their trials and did not support this combination therapy especially in patients with severe COVID-19 infection (Furtado et al., 2020; Molina et al., 2020). Similarly, the cardiac toxicity related to these drugs is also considered a major weakness of the regimen (Juurlink, 2020).

Teicoplanin

Teicoplanin has been shown to be active against the Ebola virus, SARS-CoV-1, and MERS-CoV and suggested for the treatment of COVID-19. It targets viral S protein and has been observed to be useful during the early phases of COVID-19 infection (Zhang J. et al., 2020). The recommended dose is 100–400 mg twice daily for 10 days (Parente and Laplante, 2017).

Immunomodulators and Immunosuppressants

Interferon-1

Clinical trials are being conducted to investigate its effectiveness against COVID-19 infection (Alavi Darazam et al., 2020). It has also been found to be effective previously against SARS-CoV-1 and MERS-CoV and suggested presently for the treatment of SARS-CoV-2 (Arabi et al., 2020; Jamilloux et al., 2020; Sallard et al., 2020). Researchers believed that SARS-CoV-2 could be more sensitive to IFN (Sallard et al., 2020). However, it has been advised for the treatment of COVID-19 patients who are suffering from hyperinflammation and ARDS (Jamilloux et al., 2020). The recently published interim results of the WHO SOLIDARITY trial consortium has downplayed the role of interferon alone or in combination with lopinavir (initially) to reduce the overall mortality of hospitalized moderate and severe COVID-19 patients. A total of over 1,412 patients reportedly enrolled in the study were compared with 4,088 patients with no study drug. The study has thus concluded that no difference has been observed in the 28-day survival rate among hospitalized patients receiving 44 μg subcutaneous injection thrice weekly or 10 μg daily for 6 days in patients on high oxygen or ECMO (Pan H. et al., 2020).

Systemic Corticosteroids

Methylprednisolone has been studied on COVID-19 patients with COPD and a dose of 1–2 mg per kg per day intravenously for 5–7 days has been found effective in reducing the mortality rate reported (Wu C. et al., 2020). Other studies reported 40–80 mg dose for a period of 3–6 days for the treatment of COVID-19 (Wu R. et al., 2020). Similarly, reduction in the disease course and improvement in symptoms have been observed in patients while administering corticosteroids (Wang Y. et al., 2020). However, the IDSA (Infectious Disease Society of American) recommends using the therapy only in the treatment of ARDS (Bhimraj et al., 2020).

A comprehensive trial in the United Kingdom named "Randomized Evaluation of COVID-19 Therapy" (RECOVERY), which is studying lopinavir-ritonavir, low-dose dexamethasone, hydroxychloroquine, azithromycin, tocilizumab,

and convalescent plasma as potential treatments for the ongoing pandemic patients, has announced that low-dose dexamethasone (6 mg once daily po/iv) is found to have substantially (41%, patients on ventilation; 25%, patients of oxygen; 13%, no respiratory intervention) reduced the 28-day mortality of patients. The trial includes over 2,104 patients receiving low-dose dexamethasone and 4,321 patients on randomized to usual care (Peter Horby, 2020). FDA has also included it in the list of drugs for temporary compounding by outsourcing facilities and pharmacy compounders (FDA, 2020a). Some clinical trials are also being conducted to evaluate its effectiveness against COVID-19 (Maskin et al., 2020; Tomazini et al., 2020).

Tocilizumab/Sarilumab

Clinical trials are being conducted using these antibodies against SARS-CoV-2 (ClinicalTrials.gov, 2020; Farias et al., 2020). A study conducted in China revealed recovery of 20 out of 21 patients while using tocilizumab (NIH - U.S. National Library of Medicine, 2020, April 28). It has been found to be more effective in COVID-19 patients without the need for ventilator support and almost no toxicity is reported (Perrone et al., 2020). Furthermore, its efficacy has also been studied in the combination of high-dose methylprednisolone in COVID-19 patients and a decrease in mortality rate and mechanical ventilation support and increase in recovery have been observed (Ramiro et al., 2020). Similar significant results against COVID-19 infection and a reduction in fever at the first dose have also been reported in studies (Xu X. et al., 2020; Fu et al., 2020).

Sirolimus

It has been used for the treatment of viral infections, including infections caused by coronaviruses (Wang et al., 2014; Kindrachuk et al., 2015), and has been proposed as a potential candidate for the treatment of COVID-19 (Zhou Y. et al., 2020). Clinical trials are scheduled to be conducted using sirolimus in COVID-19 patients (NIH - U.S. National Library of Medicine, 2020, May).

Miscellaneous

NSAIDs

NSAIDs, especially acetaminophen, are considered important agents for the suppression of fever during an infection. These drugs also play a key role in reducing severe immune responses and preventing viral shedding. However, the side-effects such as GI bleeding, fluid retention, and kidney dysfunction related to these agents are of great concern (Little, 2020). Moreover, it has been claimed that NSAIDs, especially ibuprofen, upregulate ACE2 receptors and could exacerbate the factors for the COVID-19 infection (Day, 2020). Studies reported that no sufficient evidence had been found against the use of ibuprofen in patients with COVID-19 (Sodhi and Etminan, 2020).

Thiazolidinediones, ARBs, and ACE2 Receptor Blocker

It is believed that thiazolidinediones might be responsible for increased ACE2 receptor expression and could result in severe or deadly COVID-19 infection (Bauer et al., 2010; Fang et al., 2020). Researchers hypothesized that ARB's (Angiotensin Receptor Blocker) long-term use and ACE2 receptor blockers could result in overexpression of their receptors. It has been further concluded that high mortality in patients with a history of preexisting cardiovascular diseases and diabetes might be related to the long-term use of these drugs (Fang et al., 2020). On the contrary, a group of researchers claimed that no such evidence is available against thiazolidinediones, ARB, ACE inhibitors, or other related drugs (Gracia-Ramos, 2020).

Vasodilators (Nitric Oxide and Epoprostenol)

Since hypoxemia is a major risk of death in severe cases of COVID-19, vasodilators are considered to be useful. Unfortunately, no major study is reported on the treatment of vasodilators against SARS-CoV-2. However, the agents were used effectively against SARS-CoV-1 and MERS-CoV (Chen et al., 2004; Åkerström et al., 2005), and trials are also being proceeded or planned for the prevention and treatment of COVID-19 (Begun et al., 2020). In addition, FDA granted emergency expanded access to a biotherapeutics company, Bellerophon Therapeutics, for its inhaled nitric oxide (iNO plus) (GlobeNewswire, 2020, March 20). The company recently announced the first successful treatment of the COVID-19 patients (Bellerophon Therapeutics, 2020).

Vitamins

It is observed that the vitamin D levels decrease in several healthy individuals, especially during winter and additionally who get less exposure to sunlight, housebound, or work at night (Enwemeka et al., 2020). An adequate vitamin D level during summer strengthens the immune system and thereby decreases viruses' attack (News Scientist, 2020, April 1). Clinicians claimed that the low level of vitamin D in the body could be the cause of the COVID-19 outbreak during the winter season (Enwemeka et al., 2020). Vitamin C, along with vitamin D and vitamin E, is also recommended in some studies. These vitamins have been beneficial in preventing respiratory infections and enhancing body resistance toward nCoV (Wang L.-S. et al., 2020).

Anticoagulants (Low-Molecular-Weight Heparin)

A decrease in mortality has been observed in patients while administering anticoagulants (Tang et al., 2020a). Low-molecular-weight heparin is suggested for the treatment of hypercoagulation and thrombosis-associated vascular damage in COVID-19 patients (Tang et al., 2020b). In addition, heparin has also been claimed to have anti-inflammatory and antiviral activities against SARS-CoV-2 (Shi C. et al., 2020; Mycroft-West et al., 2020). The European Society of Cardiology has recently schemed an anticoagulation protocol for coagulopathy management in patients suffering from COVID-19. Patients with respiratory rate >24 bpm, dyspnea, oxygen saturation <90%, rising D-dimer levels, elevated C-reactive protein, and elevated fibrinogen levels are characterized in high thrombotic risk group and various anticoagulation strategies are therefore suggested for them. A target of 60–85 prothrombin time (aPTT) range is considered and

parenteral drip of heparin for patients admitted to Intensive Care Unit (ICU) or subcutaneous enoxaparin at a dose of 1 mg/kg two times a day for patients who do not require intensive care is suggested. Furthermore, Point-of-Care Ultrasound (POCUS) is recommended for deciding the continuation of the anticoagulation therapy (Atallah et al., 2020).

Traditional Herbal Medicines

Traditional herbal medicines have been found useful for the treatment of epidemic outbreaks, including influenza and coronaviruses (Chen et al., 2011; Xiaoyan et al., 2018; Redeploying plant defences, 2020; Xiaoyan et al., 2020). Traditional medicine treatment guidelines have also been issued by China and Korea on the treatment of COVID-19 (Ang et al., 2020). Chinese medicines, particularly Shuanghuanglian oral liquid and LianhuaQingwen capsule, have been extensively used for the treatment of COVID-19 disease (People's daily of China, 2020; Runfeng et al., 2020). The other traditional herbal medicines commonly used for the treatment of COVID-19 include *Astragalus membranaceus, Saposhnikoviae divaricata*, *Glycyrrhiza uralensis*, *Rhizoma atractylodis macrocephalae*, *Fructus forsythia, Lonicerae Japonicae Flos*, *Atractylodis Rhizoma*, *Radix platycodonis*, and *Agastache rugosa* (Luo et al., 2020). It is believed that these medicines could play a key role in the reduction of inflammatory responses developed in the human body as a result of viruses and bacteria (People's daily of China, 2020; Runfeng et al., 2020)

Specific Therapy

It is believed that the drugs targeting the SARS-CoV-2 main protease (M^{pro}), an enzyme that is important to viral replication and transcription, could play a key role in COVID-19 treatment (Jin Z. et al., 2020). A list of drugs is described as follows.

Antiprotozoals

Chloroquine (CQ) and Hydroxychloroquine (HCQ)

Researchers analyzed that antiprotozoals reduce cytokine storm, the main cause of severe infection and death in COVID-19 (Cao, 2020). The therapy is claimed to be responsible for the inhibition of ACE2 receptors glycosylation. The drugs also bind with viral S protein, resulting in the prevention of SARS-CoV-2 entry into the cells (Savarino et al., 2006; Liu J. et al., 2020; Wang M. et al., 2020). *In vitro* studies have shown supportive results in reducing viral replication and, thus, symptoms duration (Gao et al., 2020; Singh et al., 2020). A study conducted on patients with positive SARS-CoV-2 indicated that the viral RNA became undetectable on day 6 after administering 200 mg HCQ three times a day (Frie and Gbinigie, 2020).

On March 28, both were granted EUA (Emergency Use Authorization) for the treatment of COVID-19 by FDA (FDA, 2020, March 28). Later FDA, on April 24, warned the use of these agents, alone or in combination with other drugs, particularly azithromycin, outside a hospital setting because of the reported evidence of abnormal heart rhythm in a clinical trial (FDA, 2020, April 24) (Borba et al., 2020). Furthermore, their use against COVID-19 was not successful and therefore not supported by WHO (WHO, 2020, March 11). HCQ is considered less toxic due to the hydroxyl group present in its structure, which helps in easy clearance from the body (Schultz and Gilman, 1997; Singh et al., 2020).

In rather recent developments, the large-scale trials have either stopped or paused the study on HCQ, citing that the treatment does not offer any improvement in mortality; moreover, initial data showed significant adverse effects associated. This is true for RECOVERY and SOLIDARITY trials as well, which are funded by UKRI and WHO, respectively. In the case of the RECOVERY trial, 1,542 patients treated with HCQ had higher (25.7%) fatality in 28 days compared to 3,132 patients (23.5%) with standard care. Similarly, SOLIDARITY trials by WHO were also suspended on May 24, 2020, owing to reports of toxicity and nonsuperiority of the treatment. The development has also led to the retraction of a major article published in Lancet where the journal could not verify the data due to confidentiality issues of the patients amid the widespread skepticism of the drug (Kupferschmidt, 2020). Similar results have also been observed recently by Lyngbakken et al., and no improvement was found for using 400 mg HCQ two times a day for 7 days in hospitalized patients suffering from COVID-19 infection (Lyngbakken et al., 2020).

Nitazoxanide

Nitazoxanide is suggested to have strong antiviral activity against SARS-CoV-2 (Srivatsan Padmanabhan and Tech, 2020). Additionally, it has also been proposed in combination with hydroxychloroquine for the treatment of COVID-19 infection and suggested to help eradicate viral load and control overwhelming the immune system, particularly in severe cases (Srivatsan Padmanabhan, 2020). The optimal doses against SARS-CoV-2 are predicted to be 1,200 mg four times, 1,600 mg three times, and 2,900 mg two times a day in a fasted state and 700 mg four times, 900 mg three times, and 1,400 mg two times a day in fed state, respectively (Rajoli et al., 2020). In a recent clinical trial conducted on ambulatory, hospitalized, and pregnant women suffering from COVID-19 infection, nitazoxanide has been observed to be a safe therapy and found effective against SARS-CoV-2 (Meneses Calderón et al., 2020).

Antivirals

Remdesivir

It has been widely used against the Ebola virus and has shown effective against other single-stranded RNA viruses such as Marburg virus, Nipah virus, parainfluenza type 3 virus, and human coronaviruses (Warren et al., 2016; Lo et al., 2017). Clinical trials are being carried out currently to evaluate its safety and efficacy against SARS-CoV-2 (Al-Tawfiq et al., 2020; NIH - U.S. National Library of Medicine, 2020a; NIH - U.S. National Library of Medicine, 2020b). Its intravenous administration to COVID-19 patients resulted in notable recovery from pneumonia (Holshue et al., 2020). A study in USA, Japan, and Europe or Canada showed clinical improvements in 36 out of the 53 hospitalized severe COVID-19 patients (68%) with remdesivir at a dose of 200 mg on day 1, followed by 100 mg per day for 9 days (total: 10 days of therapy).

TABLE 1 | Number and types of SARS-Cov-2 vaccines with respect to clinical development phase [sources: ClinicalTrials.gov, WHO COVID-19 (DRAFT landscape of COVID-19 candidate vaccines), and Biorender.com (COVID-19 vaccine tracker)].

Types of vaccines	Clinical phase of development*	No. of vaccines
Repurposed	III/IV	3
	III	1
	II	1
RNA-based vaccines	I/II/III	2
	I/II	3
	I	2
Nonreplicating viral vector vaccines	I/II/III	4
	I/II	Nil
	I	3
DNA/plasmid vaccines	I/II/III	Nil
	I/II	4
	I	2
SARS-CoV-2 inactivated viral vaccine	I/II/III	3
	I/II	4
	I	Nil
Protein subunit/peptide vaccines	I/II/III	1
	I/II	4
	I	8
Modified antigen-presenting cells–based immunization vaccine	I/II	2
	I	1
Replicating viral vector vaccine	I/II	1
	I	4
Others (virus-like particles)	I	2

**Objective of clinical phase of vaccine development: Phase I (safety and immunogenicity), Phase II (safety, immunogenicity, and potential efficacy), Phase III (large-scale efficacy and evaluation of toxicity and immunogenicity).*

In terms of therapeutic goals, in a large randomized, double-blind, placebo-controlled clinical trial conducted in over 1,000 patients, remdesivir was found to have shortened the duration of therapy from 15 to 11 days and improvements in mortality rates have been observed (Beigel et al., 2020). In May 2020, FDA issued EUA to this drug for the treatment of severe COVID-19 cases in both adults and children. The FDA further defined severe conditions as individuals with low blood oxygen saturation levels or requiring mechanical oxygen support (FDA, 2020, May 1). Later, in October 2020, it became the first antiviral drug approved by the FDA for use in COVID-19 patients above 11 years of age and 40 kg individuals (FDA, 2020 ; U.S.F.D.A., 2020). However, very recently, the use of remdesivir has been rejected by WHO after the interim results of the SOLIDARITY trial are released. A total of 2750 COVID-19 patients were given 200 mg loading dose followed by 100 mg of drug once daily till the 9th day. The data showed that the treatment group did not show any improvement over the no-drug-of-study group comprising 4,088 patients. The drug failed to improve overall mortality or prolonged the initiation of ventilation of moderately ill patients. (Pan H. et al., 2020). Though the trial is a multicenter global study, its case-by-case recommendation remains largely weak against low-risk vs. high-risk patients as described in randomized double-blind placebo-controlled clinical study "adaptive COVID-19 treatment trial" (ACTT-1), which favors the treatment with the drug and was subsequently used by FDA before giving approval. The final results, however, are awaited before any conclusion is made on its (SOLIDARITY trial) effectiveness in any subgroups of patients (NIAID, 2020; Pan H. et al., 2020).

Lopinavir/Ritonavir

Clinical trials have been investigated using a combination of a dose of 400 mg for lopinavir and 100 mg for ritonavir two times a day for the treatment of COVID-19. The clinicians claimed that no remarkable benefits were observed. The studies further found nausea, diarrhea, and asthenia as common side-effects (Cao et al., 2020). However, a study conducted in China using 400 mg lopinavir per day with or without IFN- α2b has claimed that it is effective against COVID-19. But, the consideration of gastrointestinal side-effects and hypokalemia was also suggested (Liu F. et al., 2020). Similarly, improved COVID-19 related clinical symptoms such as fever and no reduction in SARS-CoV-2 titers have been reported in a study conducted in Korea (Lim et al., 2020). Another clinical trial has also been announced recently by Prasan Kumar Panda et al. for investigating the efficacy of lopinavir-ritonavir or hydroxychloroquine along with ribavirin in hospitalized COVID-19 patients (Panda et al., 2020). Similar to the case of remdesivir, lopinavir and ritonavir have also failed to demonstrate any considerable superiority to other treatment options used for patients with moderately ill patients in the SOLIDARITY trials (Pan H. et al., 2020).

Ribavirin

Ribavirin has been observed to be effective, particularly in combination with IFN, against viruses, including coronaviruses (Scott and Perry, 2002; Khalili et al., 2020). However, a decrease in hemoglobin concentration is reported in patients with COVID-19, while administering this drug

TABLE 2 | Description of vaccines under the clinical phase of development for SARS-CoV-2 (repurposed and RNA vaccines) [sources: ClinicalTrials.gov, WHO COVID-19 (DRAFT landscape of COVID-19 candidate vaccines), and Biorender.com (COVID-19 vaccine tracker)].

Type	Name of vaccine	Description	Primary developer/sponsor	Purpose	Phase of clinical development (no. of trials) [no. of participants]
Repurposed vaccine	BCG	Live-attenuated bacterial *Bacillus* Calmette-Guerin (BCG) vaccine (nonspecific immunity)	Multisite trials with multiple sponsors[a]	Treatment and prevention	Phase IV (4) [1,000 + 900 + 1,800 + 5,200] Phase III (15) [1,500 + 1,900 + 2,100 + 2,175 + 908 + 3,626 + 2,038 + 59 + 1,120 + 1,500 + 1,000 + 500 + 900 + 10,078 + 1,200]
	OPV	Oral polio vaccine (nonspecific immunity)	Bandim Health Project NeuroActiva, Ltd. Biomed Industries, Ltd.	Prevention	Phase IV (1) [3,400] Phase III (1) [3,600]
	MMR	Live-attenuated viral measles-mumps-rubella (MMR) vaccine (nonspecific immunity)	Kasr El Aini Hospital, Egypt	Prevention	Phase III (2) [200 + 60]
	IMM-101	Heat-killed *Mycobacterium obuense* (nonspecific immunity)	Canadian Cancer Trial Groups, Immodulon Therapeutics, Ltd., BioCan Rx	Treatment	Phase III (1) [1,500]
RNA-based vaccines	nCoV mRNA-1273	Lipid nanoparticles dispersion containing mRNA encoding for SARS-CoV-2 spike protein	Moderna TX, Inc./National Institute of Allergy and Infectious Diseases (NIAID)	Prevention	Phase III (1) [30,000] Phase II (1) [600] Phase I (1) [155]
	BNT162 (a1, b1, b2, c2)	Four lipid nanoparticles encapsulated RNA-based vaccines: 2 nucleoside modified RNA (modRNA), 1 uridine containing mRNA (uRNA), and 1 self-amplifying mRNA (saRNA)	BioNTech RNA Pharmaceuticals GmbH and Pfizer, Inc. Fosun Pharma	Prevention	Phase II/III (1) [BNT162b1, b2: 43,998] Phase I/II (1) [BNT162b2: 160] Phase I/II (1) [BNT162b3: 120] Phase I/II (1) [BNT162b1: 144] Phase I/II (1) [BNT162a1, b1, b2, c2: 456]

[a]*University of Campinas, Brazil/UMC Utrecht/Radboud University, Netherlands/Universidad de Antioquia, Colombia/TASK Applied Science/Ain Shams University, Egypt/Murdoch Children's Research Institute/Royal Children's Hospital, Australia/Bandim Health Project/University of Southern Denmark/Vakzine Projekt Management GmbH/FGK Clinical Research GmbH, Germany/Assistance Publique- Hôpitaux de Paris, France/ Conselho Nacional de Desenvolvimento Científico e Tecnológico, Instituto de Infectologia Emílio Ribas, Universidade Estadual de Campinas, Unicamp, Pontifícia Universidade Católica de Campinas, PUC-Campinas, Faculdade de Medicina de Ribeirão Preto/USP, Faculdade de Medicina de Botucatu, Unesp, Federal University of São Paulo, State Hospital Dr. Leandro Franceschini, Sumaré, Unicamp, Paulinia Municipal Hospital.*

(Khalili et al., 2020). Its efficacy in hospitalized COVID-19 patients has been recently investigated alone and in combination with sofosbuvir/daclatasvir and no significant improvement was observed. However, clinicians suggested further investigation on large clinical trials (Abbaspour Kasgari et al., 2020; Eslami et al., 2020).

Umifenovir

It has been reported effective against both SARS-CoV-1 and SARS-CoV-2 (Blaising et al., 2014; Dong et al., 2020). A retrospective study found that an increase in efficacy of lopinavir and ritonavir was observed against SARS-CoV-2 when augmented with umifenovir (Deng et al., 2020).

Favipiravir

It has been proposed as an experimental drug for the treatment of COVID-19 infection (Li and De Clercq, 2020). The clinical trial indicated that patients suffering from moderate COVID-19 infection when treated with favipiravir showed remarkable recovery within a week. It has also been found clinically superior to umifenovir against SARS-CoV-2. The former showed about 71% recovery rate, and the latter 55% only (Chen C. et al., 2020). Several clinical trials are being conducted to examine its safety and efficacy against SARS-CoV-2 (Seneviratne et al., 2020; WHO, 2020, April 11). It has been observed to be effective against SARS-CoV-2 at a dose of 1600 mg twice a day first, followed by 600 mg two times a day for 13 days (Cai et al., 2020). However, low blood concentration of this drug in severe COVID-19 patients compared to healthy individuals has been observed in a recent clinical trial and therefore, further investigation for the development of optimal treatment strategy in critically ill COVID-19 patients has been suggested (Irie et al., 2020).

Oseltamivir

Clinical trials are being conducted using this drug alone and in combination with other antivirals and antiprotozoals against COVID-19 infection (Rosa and Santos, 2020). However, scientists claim that no satisfactory results have been observed while administering oseltamivir to COVID-19 patients (Wu A. et al., 2020). Similarly, it has been suggested that as SARS-CoV-2 does not contain neuraminidase enzyme, oseltamivir and other related drugs are not expected to be effective against COVID-19 (Orders, 2020).

Convalescent Plasma Therapy

Convalescent plasma therapy has been successfully used previously against SARS-CoV and MERS-CoV coronaviruses (Zhang et al., 2005; Mair-Jenkins et al.,

TABLE 3 | Description of vaccines under clinical phase development for SARS-CoV-2 (viral vector, attenuated vaccines, and protein subunit vaccines) [sources: ClinicalTrials.gov, WHO COVID-19 (DRAFT landscape of COVID-19 candidate vaccines), and Biorender.com (COVID-19 vaccine tracker)].

Type	Name of vaccine	Description	Primary developer/sponsor	Purpose	Phase of clinical development (no. of trials) [no. of participants]
Nonreplicating viral vector vaccines	Ad26.COV2.S	Nonreplicating adenovirus type 26 expressing SARS-CoV-2 spike protein	Janssen Vaccines and Prevention Beth Israel Deaconess Medical Center Johnson and Johnson	Prevention	Phase III (2) [30,000 + 60,000] Phase I/II (1) [1,045] Phase I (1) [250]
	AZD1222 (ChAdOx1 nCoV-19)	Nonreplicating adenovirus type 5 expressing SARS-CoV-2 spike protein	Jenner Institute University of Oxford/ AstraZeneca	Prevention	Phase III (3) [100 + 5,000 + 40,051] Phase II/III (1) [12,390] Phase I/II (3) [1,090 + 256 + 2,000]
	Ad5-nCoV	Nonreplicating adenoviral type 5 vector expressing SARS-CoV-2 spike protein	CanSino Biologics Inc./Canadian Center for Vaccinology/Institute of Biotechnology, PLA of China	Prevention	Phase III (3) [508 + 40,000 + 500] Phase II/III (2) [696 + 481] Phase I (2) [108 + 144]
	Gam-COVID-Vac-Lyo	Composite vaccine with two adenoviruses (Ad5 and Ad26) containing SARS-CoV-2 genes for spike protein	Gamaleya Research Institute of Epidemiology and Microbiology, Russia	Prevention	Phase III (2) [40,000 + 100] Phase I/II (2) [38 + 110]
SARS-CoV-2 inactivated viral vaccine	CoronaVac	Chemically inactivated SARS-CoV-2 viral vaccine	Sinovac R&D Co., Ltd., China Health Institutes of Turkey Butantan Institute PT Biopharma Faculty of Medicine Universitas Padjadjaran	Prevention	Phase III (4) [13,060 + 13,000 + 1,620 + 1,040] Phase I/II (2) [744 + 552] Phase I (1) [422]
	SARS-CoV-2 vaccine unnamed	Chemically inactivated SARS-CoV-2 viral vaccine	Wuhan Institute of Biological Products, Sinopharm Universidad Peruana Cayetano Heredia	Prevention	Phase III (1) [6,000] Phase I (1) [288] Phase II (1) [1,168]
	BBIBP-CoV	Chemically inactivated SARS-CoV-2 viral vaccine	Beijing Institute of Biological Products and Sinopharm	Prevention	Phase III (3) [45,000 + 3,000 + 15,000] Phase II (1) [1,648] Phase I (1) [480]
Protein/peptide vaccines	SARS-CoV-2 rS NVX-CoV2373	Adjuvant nanoparticles with conjugated spike proteins	Novavax Inc. Coalition for Epidemic Preparedness (CEPI) Department of Health and Human Services (US)	Prevention	Phase III (2) [9,000 + 30,000] Phase II (2) [4,400 + 1,419] Phase I (2) [131 + 1,419]

2015) and is now being explored against COVID-19 (Shen C. et al., 2020; Chen L. et al., 2020). The technique has also been recommended as an emergency investigational new drug application by the FDA for the treatment of fatal or deadly COVID-19 infections (Food and Administration, 2020). A number of clinical trials exploring the safety of the therapy have concluded that the use of convalescent plasma is safe with or without other treatment options used. However, the studies have largely failed to confirm its effectiveness in a variety of settings, especially in a large randomized clinical trial with over 100 enrolled patients; the effectiveness of the therapy has been largely inconclusive in improving the condition of moderately and severely ill patients (Agarwal et al., 2020; Li L. et al., 2020; Olivares-Gazca et al., 2020). Moreover, in one study, the convalescent plasma transfusion failed to increase the neutralizing antibody titers in recipients (Bradfute et al., 2020). The treatment and management option is also described by Yang et al in 2020 (see **Figure 4**)

Landscape of Vaccine Development for SARS-CoV-2

In the wake of the current pandemic, an unprecedented response has been observed globally on the front for vaccine development against SARS-CoV-2 to protect the large masses of the global population from getting infected with long-term immunity against the virus. There are largely three ways due to which this development response is unprecedented in the history of pandemics ever. The first reason is that there are over 120 vaccine research groups and teams participating in the development, comprising majorly large and small commercial sector biotech companies followed by academic institutions and public sector research organizations (Shang et al., 2020; WHO, 2020b). A large fraction of these teams are working on already developed and tested platforms such as live-attenuated virus and chemically inactivated viruses that have been successful for a wide range of viral diseases (Shang et al., 2020). This also ensures that if one

method becomes successful, the challenges to develop and scale things up would not be cumbersome. Moreover, novel strategies like DNA plasmids and mRNA-based vaccines are also being touted as a potential breakthrough in the field as these platforms offer high production and quality control during manufacturing and can be replicated to suffice the global demand (Callaway, 2020b). On the other hand, some leading researchers are skeptical of the timeline prediction of 18 months for the vaccine citing various technical challenges and regulatory oversight reasons (GeneScript, 2020). The fastest time for a vaccine development that has been recorded is of 7 months for the Ebola, Swine flu, and Zika virus. However, in the case of SARS-CoV-2, it took only 10 weeks for RNA-based Moderna vaccine candidate to enter clinical trials (Callaway, 2020a; GeneScript, 2020). Concise information about a variety of vaccine platforms currently under various stages of clinical development (regulatory approval for human use) is given in **Table 1**. Moreover, the detailed landscape of vaccines that successfully demonstrated a safety profile and immunogenicity in phase I and II studies has been given in **Tables 2** and **3**. A variety of platforms are being explored for the development of a vaccine. These include mRNA-based vaccines, DNA- and plasmid-based vaccines, nonreplicating viral vectors, and attenuated viral vaccines, protein subunits. Apart from that, some already existing vaccines are also being explored for likely cross-/nonspecific immunity against SARS-CoV-2 infection. These vaccines include BCG, polio, and MMR vaccines, which are also part of many national child-immunization programs in the developing countries. Among all the vaccines being developed, ten vaccines have entered phase III clinical trials and have shown promising results in terms of safety and immunogenicity (WHO, 2020b).

PREVENTIVE MEASURES

As no specific treatment is available for this nCoV, it is recommended to obtain preventive measures and reduce the spread of the virus. It is advised to maintain personal hygiene, proper ventilation, a healthy lifestyle, and adequate nutritional consumption to boost immunity and enhance self-resistance (Yoshikawa and High, 2001; Simpson et al., 2015). Handwashing with soap and water or alcohol-based sanitizer after contact with any contaminated surface is directed (World Health Organization, 2020b). Protective equipment such as face mask, gown, gloves, face shield or goggles, and N95 respirator is suggested, especially in hospital settings (World Health Organization, 2020b). A study conducted in a hospital in Wuhan, China, on the association between the use of face masks and spread of COVID-19 has revealed that the infection rate in the departments using face masks, disinfectants, and handwashing was lower than that in the departments not using or frequently using the preventive measures (Wenjie et al., 2020). Close interaction is the cause of viral transmission (World Health Organization, 2020b). The infected people are recommended to be isolated or use airborne infection isolation (AIIR)/negative pressure isolation (NPI) room (Wax and Christian, 2020). Social distancing by reducing mass gatherings, social events, and group meetings is considered the best preventive measure (CDC, 2020, March 27).

CONCLUSION

COVID-19 disease is an enormous challenge to the global community. This once-in-a-century pandemic has catastrophically affected our daily lives and is an unprecedented threat to the economies of many leading nations across the globe. Besides plethoric technological advancement and awareness, the world was not prepared to face the sudden outbreak of this disease. The pace of disease progression in different regions of the world has made it imperative that quick and honest preventive measures should be implemented at macro and micro levels to limit the spread till the development and approval of effective vaccine and evidence-based management at a global scale. The virus demands respect, so keeping self-hygiene, wearing masks, sanitizing, and maintaining social distancing seem to be the only effective means of mitigating the disease. Cultural diversity among different regions should be considered in developing robust strategies and communication. Many vaccines are under various phases of clinical trials, their efficacy, safety, scale-up production, supply chain management, and cold chain maintenance are another set of challenges, especially for developing and underdeveloped nations.

AUTHOR CONTRIBUTIONS

MSH conceptualized the theme of this review article and helped in the write-up and arranging the sequence. FA helped in the write-up, collection of data, and tables. MSI has done extensive literature study and helped in writing the epidemiology section. RY helped in writing therapeutic management and MSA helped in organizing figures and formatting work.

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frontiers in Pharmacology

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Anti-IL5 Drugs in COVID-19 Patients: Role of Eosinophils in SARS-CoV-2-Induced Immunopathology

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SARS-CoV-2 infection stimulates a complex activation of the immune system. Eosinophils belong to the host's defense equipment against respiratory viruses. In the first phase of the infection, eosinophils contribution is probably appropriate and beneficial, as they facilitate the suppression of the viral replication. However, in severe COVID-19 patients, during the second and third phases of the disease, eosinophils may participate in a maladaptive immune response and directly contribute to immunopathology. In fact, in severe patients, the immune response is prevalently T helper 1 type, but T helper 2 is also present. Eosinophils' expansion and activation are stimulated by Type 2 cytokines, especially IL-5. Moreover, bronchial asthma, in which eosinophils play a central role, seems not to be a major risk factor for severe COVID-19. Among possible explanations, asthmatic patients are often treated with corticosteroids, which have been demonstrated to reduce the progression to critical COVID-19 in hospitalized patients. In addition to steroids, severe asthmatic patients are currently treated with biological drugs that target Type 2 immune response. Because IL-5 is necessary for the growth, survival, and activation of eosinophils, IL-5 inhibitors, such as mepolizumab, decrease the peripheral blood count of eosinophils, but do not influence eosinophils activation in the airway. In severe COVID-19 patients, the blockade of eosinophils' activation might contrast harmful immunity.

Keywords: COVID-19, asthma, type 2 response, eosinophils, interleukin-5, anti-IL5 drugs

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INTRODUCTION

COVID-19 pandemic is a grave challenge for every health care professional worldwide. A better understanding of disease pathogenesis might boost a more effective and targeted therapy, with the hope of saving as many lives as possible. Here, a rationale for anti-IL5 drug use in severe COVID-19 patients is discussed. Since the beginning of the Health Emergency, several drugs, mostly antiviral and anti-inflammatory, have been repurposed and a vast number of clinical trials started worldwide to assess their efficacy and safety with an unprecedented speed. However, to date, only systemic corticosteroids have been demonstrated to prevent deaths in COVID-19 most severe patients and received formal approval from Regulatory Agencies (Sterne et al., 2020).

One of the most relevant findings regarding COVID-19's natural history highlights three different clinical stages, each requiring different types of therapies. In the first stage, the principal feature is

viral replication, which can be contrasted by antiviral drugs, such as remdesivir. In the second stage, the pulmonary one, clinical symptoms become more prominent because of the beginning of the host's immune response. The third stage is characterized by an immunopathologic response, which can result in a cytokine storm in the most severe cases (Siddiqi and Mehra, 2020). In the second and third stages, immunotherapies can be indicated (Sandkovsky et al., 2020).

Mounting evidence shows that COVID-19's immunopathology has peculiar features. It has been highlighted that the classical T helper 1 (Th1) response is defective during acute infection, but it is prevalent among memory T cells in convalescent individuals (Sekine et al., 2020). Patients with moderate COVID-19 experience lower signs of inflammatory activation in comparison with severe COVID-19 patients. The inflammation follows an initial increment of cytokines, and subsequently a decrease of type 1 and type 3 responses (Lucas et al., 2020). The peripheral blood count of $CD4^+$ and $CD8^+$ T cells in terms of absolute number and frequency display a significant reduction in patients with either moderate or severe infection (Sekine et al., 2020). Nevertheless, Th1 $CD4^+$, Th1 $CD8^+$, and Natural Killer T cells are activated to promote antiviral activity and drive the disease recovery in moderate infection (Zhang J. Y. et al., 2020). Finally, in convalescent individuals, other authors analyzed SARS-CoV-2-specific memory T cells and found that $CD4^+$ T cells mainly produced Interferon-γ (IFN- γ), Interleukin-2 (IL-2), and Tumor Necrosis Factor- α (TNF-α), whereas $CD8^+$ T cells mainly produced IFN-γ (Sekine et al., 2020).

TH2 IMMUNE RESPONSE IN SEVERE PATIENTS

The first reports showed a significant difference in T helper 2 (Th2) cytokines in severe COVID-19 patients hospitalized into Intensive Care Units, especially IL-4 and IL-10, providing initial evidence in favor of Th2 activation, but significant differences in IL-5 levels were not found (Huang C. et al., 2020).

In severe patients, the immune system activation is characterized by a distorted interferon production and a disordered T cellular response that lead to profound immune exhaustion and broad T cell expansion (Zhang J. Y. et al., 2020). Patients with severe COVID-19 produce elevated levels of cytokines during the clinical course of the disease. These patients showed a pattern of Th1 activation, but also showed a Type 2 immune response, characterized by an increase of IL-5, IL-13, eotaxin-2, immunoglobulin E (IgE), and eosinophils. Type 2 biomarkers remain elevated in patients with severe COVID-19 and correlate with the worst course of the disease. Levels of eosinophils were significantly higher in patients with severe COVID-19 than those with a moderate disease or healthy controls (Lucas et al., 2020).

Similarly, levels of IL-5 were higher in severe patients than in those with a moderate disease or healthy control. IL-13 differs in severe COVID-19 compared to controls. In patients with severe disease, IgE immunoglobulins slightly increased during the disease course (Lucas et al., 2020).

Conversely, IL-4 did not diverge between the two groups (Lucas et al., 2020). The last result is confirmed by another observational study (Mann et al., 2020). Nevertheless, IL-4, IL-5, and IL-13 displayed a trend toward an increase in the clinical scenario of severe COVID-19, and a reason for the interest is the importance of IL-5 to predict mortality, with a predictive value of around 0.73 (Lucas et al., 2020).

Elsewhere, eotaxins, a group of chemokines involved in the chemotaxis of eosinophils, revealed conflicting results: in the previous study, eotaxin-1 and eotaxin-3 were reduced in COVID-19 (Lucas et al., 2020) similarly to another immunophenotyping study in which eotaxin-1 was reduced (Mathew et al., 2020). Differently, eotaxin-2 was increased as compared to controls (Lucas et al., 2020). The eosinophil count was similar and within the normal values range in the two studies: a mean count of 100 cells/μL with a maximum of 250 cells/μL in the first (Lucas et al., 2020) and count below 100 cells/μL with a maximum at 400 cells/μL, in the second (Mathew et al., 2020). However, the two studies agree with the increase of fundamentals type 2 cytokines, like IL-5 and IL-13 in severe COVID-19 patients, at least in a subset of them (Lucas et al., 2020; Mathew et al., 2020). Another immunophenotyping study shows decreasing participation of eosinophils from mild to severe groups (Mann et al., 2020).

The specific activation of the type 2 immunity has been confirmed by different groups. Roncati et al. (2020) show that in all the 15 peripheral blood samples from intensive care COVID-19 patients, cytological signals of Th2 immune response were found, namely eosinophilia, basophilia, degranulated eosinophils, and plasma cells (Roncati et al., 2020). In general, stimulation of SARS-CoV-2-specific T cells from peripheral blood of severe COVID-19 patients drives a prevalent production of Th1 cytokines (IFN-γ, TNF-α, IL-2), but also Th2 (IL-5, IL-13, IL-9, IL-10) and Th17 (IL-17A, IL-17F, and IL-22) cytokines were detected (Weiskopf et al., 2020). The specific T-cell response against SARS-CoV-2 was assessed by stimulating peripheral blood cells with the Spike protein and other viral peptides. Mononuclear blood cells from COVID-19 vs. non-COVID-19 cells produced a significant higher number of cytokines such as IL-2 (50.08 vs. 0), IFN-γ (90.16 vs. 0), IL-4 (0.52 vs. 0), IL-13 (0.84 vs. 0) and MCP-1 (4,602 vs 359.2), among which IL-4 and IL-13 are key Th2 mediators (Petrone et al., 2020). The involvement of IL-4 and IL-13 was confirmed by others, who found a relative gene expression upregulation in $CD4^+$ T-cells from COVID-19 patients by using a single cell transcriptomics approach (Kalfaoglu et al., 2020).

In a single-cell analysis, six subtypes of $CD4^+$ T cell clusters have been characterized. In particular, 2 T $CD4^+$ effector subtypes, CD4+-GZMK (granzyme) and CD4+-GNLY (granulysin) have been found. CD4+-GNLY cells displayed a high production of TBX21; consequently, they were Th1-like cells. Conversely, CD4+-GZMK and CD4+memory cells revealed Th2-like features with high production of GATA3 (Zhang J. Y. et al., 2020).

To evaluate the differences between peripheral blood and bronchoalveolar lavage fluid (BALF) in COVID-19 patients, mononuclear cells from both compartments were compared. A clonal increase of Th1, Th2, and Th17 cells was found in severe cases. A significant difference has been observed between matched BALF and plasma samples for IL-5, IL-8, IL-17, and INF-α, with higher levels of these cytokines in the BALF (Xu et al., 2020).

The timing analysis is a critical point to consider when observing the relative increase or decrease of a single cytokine or a cell subset. In a longitudinal analysis of a fatal case, IL-5 was found increased between 1- and 2-times on the 14th day since the infection, but showed a decrease between day 16 and day 22 and a further increase on day 24 (Bouadma et al., 2020). This pattern is in line with observations from a large-scale study that show an increase of IL-5 levels within days 6–11 from symptom onset, to which a subsequent increase of eosinophils follows on days 11–15, and a simultaneous slowdown of IL-5 rise on days 11–15. The last phase is characterised by a further increase of IL-5 on days 16–20 and a relative slowdown of blood eosinophil count on days 16–20 (Lucas et al., 2020). Finally, deceased patients had higher levels of IL-5 than patients with moderate or severe disease (Liu et al., 2020).

Probably, there are many explanations for these observations. In the first and early second phases of SARS-CoV-2 infection, eosinophils can contribute to the elimination of the virus, thanks to the antiviral activity of their enzymes. Later, during the advanced second phase of COVID-19, when the immune system starts slowing down viral replication, their antiviral properties are not requested, so IL-5 production is moderately reduced (Bouadma et al., 2020; Lucas et al., 2020). Nevertheless, eosinophils enrollment during the second phase may contribute to harm target tissues and progress the pathology. In the last phase of a severe course, the immune system undertakes a pathologic pathway characterized by a broad and uncontrolled cytokines storm, with a new pathological increase of IL-5 (Lucas et al., 2020; Bouadma et al., 2020; Liu et al., 2020).

ROLE OF EOSINOPHILS IN PATHOLOGY

Eosinophils play an important role in protecting the host against viral infections. They recognize viruses through Toll-like receptors (Flores-Torres et al., 2019). Eosinophils participate in the antiviral immune response because of their preformed granules, which contain cytotoxic proteins, such as eosinophil peroxidase, major basic protein, and 2 RNases (eosinophil neurotoxin and eosinophil cationic protein) (Ramirez et al., 2018; Flores-Torres et al., 2019). They produce reactive nitrogen species with antiviral activity (Flores-Torres et al., 2019).

Eosinophils protect the host from respiratory viruses, such as respiratory syncytial virus, rhinovirus, parainfluenza, and influenza virus (Ramirez et al., 2018). Eosinophils can rapidly internalize and inactivate respiratory syncytial virus and influenza virus, an ability that is compromised in asthma, with a close correlation with asthma exacerbation (Sabogal Piñeros et al., 2019a). Influenza A virus stimulates pulmonary lymphoid cells to generate large amounts of IL-5, which attracts eosinophils in the respiratory tissues (Gorski et al., 2019). In response to the influenza A virus, eosinophils activate, undergo degranulation, and act as antigen-presenting cells, then induce $CD8^+$ T cell effector functions (Samarasinghe et al., 2017). A comprehensive review of eosinophils and viral infections was made by Flores-Torres et al. (2019).

Toxic proteins and mediators released from activated eosinophils participate in the pathogenesis of asthma and other allergic and immune-mediated diseases (Ramirez et al., 2018). Asthma exacerbations can usually be triggered by viral infections (Flores-Torres et al., 2019). During asthma exacerbations, eosinophils are activated to release free eosinophil granules and undergo lysis (Muniz-Junqueira et al., 2013). In theory, two principal ways of dying exist for eosinophils: primary lysis and apoptosis. Surprisingly, some signals that induce eosinophils apoptosis, lead to cell lysis (Persson and Uller, 2013). Activated eosinophils from asthma and allergic diseases express on surface sialic acid-binding immunoglobulin-like lectin (Siglec)-8. Siglec-8 normally causes cell death, but in presence of IL-5, it induces ROS-dependent cell death, characterized by necrotic features and granules release (Kano et al., 2013). The characteristic of undergoing primary lysis clarify because apoptotic eosinophils have not been found yet in affected tissues from different eosinophilic diseases (Persson and Uller, 2013; Persson and Uller, 2014). Corticosteroids reduce eosinophilic granules in the sputum of asthma exacerbation and probably do anti-IL-5 drugs (Persson and Uller, 2014).

Primary lysis of eosinophils is characterised by cell membrane rupture, a subsequent release of free eosinophilic granules content, and damage-associated molecular patterns (DAMPs) from cytoplasm and nucleus (Persson and Uller, 2014). DAMPs, like ATP, High mobility group box 1 protein (HMGB1), RNAs, DNAs, and IL-1β stimulate a potent activation of inflammation. Through DAMPs signaling, a dying cell recruits phagocytes, like macrophages, dendritic cells, and epithelial cells. In turn, phagocytes, which have pattern recognition receptors (PRRs), start eating irreversibly damaged cells through a process called efferocytosis. Activated phagocytes by a necrotic dying cell produce pro-inflammatory cytokines such as IL-1, IL-6, and IL-12 (Kolb et al., 2017).

Interestingly, regarding COVID-19 pathology, phagocytes and related cytokines (especially IL-1 and IL-6) have been recognised to play a central role (Bonaventura et al., 2020). A similarity between COVID-19 and secondary Hemophagocytic lymphohistiocytosis (HLH) syndrome has been proposed (Mehta et al., 2020). HLH is characterized by ungoverned growth and activation of phagocytes. HLH is often triggered by viral infections, like herpes viruses (Epstein-Barr virus and cytomegalovirus mainly), H1N1 influenza virus, parvovirus B19, HIV, or other viruses. From a haematological point of view, HLH is characterized by leukopenia (Ramos-Casals et al., 2014).

A suggestive hypothesis is that, in COVID-19, eosinophils stimulated by SARS-CoV-2 infection would migrate to the lungs and undergo primary lysis, which in turn recruit phagocytes. In a severe patient, uncontrolled phagocyte activation causes hyper inflammation and a cytokine storm. The interplay between lung

eosinophils and SARS-CoV-2 needs more in-depth analysis, considering potential therapeutic implications.

EOSINOPHILS INVOLVEMENT IN COVID-19

Since the first laboratory reports of COVID-19 severe patients, the peripheral blood count of circulating eosinophils is mostly found below the normal value range (Zhang Z. L. et al., 2020), and there is a significant difference between moderate vs. critical disease (Liao et al., 2020). These observations have been confirmed by many authors and by different meta-analyses (Danwang et al., 2020; Ghahramani et al., 2020).

Since the first phases of the infection, patients may undergo an active migration of circulating eosinophils from the peripheral blood to target tissues, because of their antiviral functions (Flores-Torres et al., 2019). In the subsequent phases, peripheral eosinophils start declining. An explanation of the observed eosinopenia in the last phases of COVID-19 disease would consider the nearly concomitant increase of eosinophils-stimulating cytokines, such as IL-5 and GM-CSF, at least in a subset of patients (Lucas et al., 2020; Mathew et al., 2020), and the complex interactions with other actors of the immune system. A possible explanation is the induction of eosinophils apoptosis caused by endogenous or therapeutic glucocorticoids (Ilmarinen et al., 2014). Also, cytokines such as IFN-α and IFN-γ (type 1 IFNs) can induce eosinophils apoptosis (Morita et al., 1996). Nevertheless, in COVID-19 type 1 IFNs production is limited (Acharya et al., 2020). Another explanation contemplates the induction of cell primary lysis (Persson and Uller, 2013). The last hypothesis has much more therapeutic implications because of the stimulation of efferocytosis and inflammation from primary lytic eosinophils (Persson and Uller, 2014).

During hospitalization, eosinophils', lymphocytes', and platelets' count showed a different pattern in the survivors' peripheral blood compared to the non-survivors: in survivors, the cell count increased progressively, whereas, in the non-survivor, it maintained low levels and finally declined. The laboratory eosinophils count is a negative prognostic factor for non-survivors, specifically eosinophils on hospital admission less than 0.03×10^9/L (HR, 2.12; 95% CI, 0.91–4.98), whereas eosinophils ($x10^9$/L) > 0.05 vs ≤ 0.05 was a protective factor for fatal outcome (HR, 0.38; 95% CI, 0.17–0.83). In Kaplan-Meier analysis, the survival was significantly higher in patients with eosinophils >0.05 ($x10^9$/L) compared to those with eosinophils ≤0.05 ($x10^9$/L) (Chen et al., 2020). Blood eosinophils showed a positive correlation with lymphocytes in severe and non-severe patients after admission (Zhang J. J. et al., 2020).

The laboratory monitoring of peripheral eosinophil count has been proposed as a precision tool to monitor the clinical course of the disease and predict the admission to Intensive Care Unit (ICU) (Huang J. et al., 2020). The eosinophils count = 0 ($\times 10^9$ per L) predicts the admission to ICU with a mean sensitivity of 48.15 (95% CI: 28.7–68.1) and a mean specificity of 98.88 (95% CI: 93.9–100.0), a positive predictive value of 92.9 (95% CI: 66.1–99.8) and a negative predictive value of 86.3 (95% CI: 78.0–92.3). In the ROC analysis, the AUC of eosinophils is 0.763 (95%CI: 0.641–0.886) (Sun et al., 2020). The very high value of specificity indicates that eosinophil might be a real target of the immune derangement like the decreased lymphocytes (Sun et al., 2020). Differently, the modest value of sensitivity might be explained by a certain degree of heterogeneity between severe COVID-19 patients, as demonstrated by immunophenotyping studies that identified different clusters of the cytokine storm signature (Lucas et al., 2020; Mathew et al., 2020). There is a statistically significant difference between peripheral eosinophils' blood count in non-severe and severe patients, pointing out that this population undergoes conditioning during the acute phase of severe infection (Sun et al., 2020; Xie et al., 2020).

Nevertheless, basophils and eosinophils should contribute to the antiviral response and could complicate the immunopathology. These cells undergo a dynamic change during severe disease: they increase from acute to recovery phases (Rodriguez et al., 2020). Other authors analyzed in more detail eosinophil cell populations. These populations exhibit a temporary growth of CD62L + eosinophils from day 2 to day 6 after hospital admission (Rodriguez et al., 2020). Expansion of CD62L + eosinophils seems to be attributed to IFN-γ, one of the most relevant cytokines in severe COVID-19, and the IFN-γ levels show an increase together with the increment of CD62L + eosinophils (Rodriguez et al., 2020). The specific phenotype of such eosinophils apparently belongs to a population of lung-resident eosinophils rather than to circulating eosinophils induced by the inflammation response, and CD62L + pulmonary-resident eosinophils have an important role in the organization of inflammatory responses in the lung (Mesnil et al., 2016). Rodriguez et al. (2020) think that the clonal growth of CD62L + eosinophils, which occurs after the development of a severe pulmonary immunopathology (one week after hospital admission) is correlated to the hyper-inflammation of the lungs in COVID-19 patients (Rodriguez et al., 2020).

In a recent post-mortem series of SARS-CoV-2 deceased patients, eosinophils were found in the alveolar *interstitium* (Damiani et al., 2020). In a case report, pulmonary eosinophilic vasculitis (with transmural eosinophilic infiltrate) was found in a severe COVID-19 patient that underwent bronchopulmonary lavage and lung biopsy on day 32 after intubation. No allergic disorder was previously known. BALF showed 36% eosinophils and 2.4 pg/ml IL-5. After two weeks of corticosteroid treatment, a subsequent bronchoalveolar lavage was made that showed 3% eosinophils and 2.3 pg/ml IL-5 (Luecke et al., 2021). Another case report described a clinical picture of eosinophilic pneumonia in a COVID-19 patient, diagnosed by increased eosinophils in BALF, which responded well to steroid treatment (Murao et al., 2020). However, it must be pointed out that the findings of eosinophils in severe COVID-19 lungs do not directly demonstrate that they are responsible for the damage. The role of eosinophils in pneumonia's immunopathology still needs to be fully understood. Another point favoring eosinophils' involvement is that skin dermatoses have been described in COVID-19 patients, in which increased eosinophils were found (Gianotti et al., 2020). The preferential expansion of lung-resident eosinophil is not in contrast with the

observation that, in the most severe COVID-19 patients, peripheral blood count of eosinophils is generally decreased. Noteworthy, eosinopenia might depend on the migration of circulating eosinophils from the peripheral blood to the infected organs (Azkur et al., 2020).

SEVERE ASTHMA IN COVID-19 PATIENTS: A CASE-STUDY

Bronchial asthma is divided into two major phenotypes, which are characterized by Th2-high (eosinophilic) and Th2-low (non-eosinophilic) immune responses (Kuruvilla et al., 2019). There is still a debate in the scientific literature if patients with bronchial asthma would be at increased risk of developing a severe COVID-19 form and relative admission to the intensive care unit (Avdeev et al., 2020; Williamson et al., 2020; Choi H. G. et al., 2020). Until now, there are limited data about the effective risk of severe COVID-19 course in the population of asthmatic patients (Kow et al., 2020). A possible explanation because asthma does not appear to be a relevant risk factor for COVID-19 has been reported by Jackson et al. (2020) (Jackson et al., 2020). They hypothesized that atopic patients express lower levels of the *ACE2* gene in their airways. In fact, SARS-CoV-2 uses the ACE2 receptor to infect the host's cells. Asthmatic children with allergen sensitization showed a progressive ACE2 decrease in the nasal epithelium. Similar results were reported in adults with mild asthma that received allergen provocation (Jackson et al., 2020).

Furthermore, a position paper from European Allergologists and Clinical Immunologists' leading societies highlights that there is currently no evidence for an increased risk of a severe COVID-19 course in allergic patients (Klimek et al., 2020). This statement is particularly surprising as asthma exacerbations can usually be triggered by respiratory infections (Flores-Torres et al., 2019). This interesting fact has been confirmed in different countries such as China, the USA, South Korea, and Italy (Klimek et al., 2020; Zhu et al., 2020). In detail, in Wuhan, the percentage of seriously ill or deceased COVID-19 patients with known bronchial asthma was far below the prevalence of asthma (Li et al., 2020). In a real-world observational study performed using administrative data from Korea, 7,590 confirmed SARS-CoV-2 infection were identified. Among them, 218 (2.9%) had asthma. The mortality rate was higher in asthmatic patients than non-asthmatic controls (7.8 vs. 2.8%), but after adjusting for age, sex, and underlying conditions, asthma reveals not to be a significant risk factor for mortality (OR, 1.317; 95% CI, 0.708–2.451). Indeed, none of the asthma treatments (including ICS, inhaled corticosteroids; LABA, long-acting β2-agonists; LAMA, long-acting muscarinic antagonists; LTRA, leukotriene receptor antagonists; SABA, short-acting β2-agonists) influences the mortality rate or admission to ICU in multivariate analysis and even asthma's severity was not associated with higher mortality (Choi Y. J. et al., 2020). These results were similar to those reported by another study from Daegu, Korea (Kim et al., 2020). Other authors demonstrated that asthma diagnosis was not associated with worse outcomes among severe COVID-19 patients 65 years or younger hospitalized within the New York City area, without considering age, obesity, or other high-risk comorbidities (Lovinsky-Desir et al., 2020). Other studies carried out in Italy confirm that the proportion of asthmatic patients in hospitalized COVID-19 positive patients is very small and suggest that asthma itself cannot be considered an independent risk factor for COVID 19 (Caminati et al., 2021) and does not appear to be one of the most relevant risk factors for ICU admission (Grasselli et al., 2020). There are several possible explanations for these findings. One explanation is that asthmatic patients are usually treated with systemic or inhaled corticosteroids, which have been demonstrated to ameliorate the disease course in severe COVID-19 (Peters et al., 2020; Rogliani et al., 2020; Sterne et al., 2020). Moreover, many severe asthmatic patients are under treatment with biologicals that inhibit type 2 immune responses via various mechanisms. The cited position paper does not dissuade to avoid such biological therapies but emphasizes that the potential effects of biologicals on the immune response in COVID-19 are currently unknown.

The first reports show that the disease course is not worse in COVID-19 patients with eosinophilic diseases under biological therapy, compared to those infected patients with eosinophilic disorder not treated with biologicals (Heffler et al., 2020). The pharmacological blockade of type 2 inflammation by therapeutic antibodies against IgE, IL-5, or IL-5/IL-4/-13 receptors, so far has not been suspected to increase the risk of viral infections, also in the respective approving clinical trials (Klimek et al., 2020). For example, in the Italian Severe Asthma Network (SANI) cohort, 26 patients received a confirmed (11) or a suspected diagnosis of COVID-19 (15). 21 patients with COVID-19 used biologicals: 15 (71%) anti-IL-5 or anti-IL5R drugs (mepolizumab n = 13; benralizumab n = 2) and 6 (29%) anti-IgE drug (omalizumab). In this small population, all patients were treated with inhaled corticosteroids/long-acting β2-agonists, and only two patients deceased. The SANI registry cohort includes 1,504 patients, 65% of them receive biological treatments (anti-IL5 or anti-IL5R drugs: 52.9%, anti-IgE: 47.1%). In their large cohort of severe asthmatics, few COVID-19 diagnoses were made. The mortality rate among severe asthmatic patients with a SARS-CoV-2 diagnosis was 7.7%, lower than that recorded in the Italian population (14.5%). Among the severe asthmatic group with COVID-19, the majority were treated with anti-IL5 drugs (71%), with a minority with anti-IgE (29%) (Heffler et al., 2020). These preliminary data suggest that severe asthma subjects are not at higher risk of SARS-CoV-2 infection or development of a severe COVID-19 form of the disease.

Similar results were derived from the Belgian registry of Severe Asthma. Fourteen severe asthmatic patients with a SARS-CoV-2-confirmed infection were collected from the registry cohort; five of them were hospitalized and none of them displayed asthma exacerbation, required systemic treatment with corticosteroids, invasive ventilation, or death. Only three patients received oxygen supplementation. Additionally, there was no difference in the incidence of SARS-CoV-2 infection between asthmatic patients who were on treatment with biologic therapy (four patients received anti-IgE and seven patients received anti-IL-5 or anti-

IL-5R) and asthmatic patients not on treatment with biologics (Hanon et al., 2020). On the other hand, nine patients from the Dutch Severe Asthma Registry under biological treatment received a diagnosis of COVID-19, seven of them were hospitalized, and five entered to ICU. Six patients were on anti-IL5 therapy and three of them were admitted to ICU (all three patients were obese). Finally, only one patient died, but he had obesity and diabetes, known comorbidities for fatal outcome (Eger et al., 2020). Other case reports do not indicate a worse clinical course in COVID-19 patients exposed to anti-IgE omalizumab (Lommatzsch et al., 2020) and, anti-IL-5R benralizumab (Renner et al., 2020a; Renner et al., 2020b). Although only a few cases were reported, it can be speculated that biological inhibitors of type 2 response can have a possible impact on aberrant immune response, and thus can protect infected subjects from severe complications of COVID-19.

Besides the potential protective effects of asthma medications, it must be pointed out that a possible explanation for a limited prevalence of asthma in COVID-19 patients might be because asthmatic patients are more aware of the greater risk of exacerbations of their condition, and thus have paid more attention to hygiene prescriptions and have been even more protected than the general population.

On the other hand, several reports challenge the hypothesis that severely asthmatic patients do not display a higher risk for severe COVID-19 (Choi H. G. et al., 2020; Williamson et al., 2020; Zhu et al., 2020); therefore, further studies focused on different asthma phenotypes are needed to better understand the association between asthma and COVID-19 severity.

DISCUSSION

To date, there are five monoclonal antibodies addressed against type 2 immune activation authorized as a specific target therapy for the treatment of severe eosinophilic asthma (Chaplin, 2020):

- omalizumab, which binds to IgE, blocking their interaction to the relative IgE receptor on basophils and mast cells; it down-regulates the expression of IgE receptor (MacGlashan et al., 1997);
- benralizumab, an IL-5 receptor antagonist, which is expressed on the surface of eosinophils and basophils, provoking their apoptosis (Kolbeck et al., 2010);
- mepolizumab, targeted to IL-5, blocking the binding with the respective receptor expressed on eosinophils;
- reslizumab, targeted to IL-5, blocking the binding with the respective receptor expressed on eosinophils;
- dupilumab, which slows down type 2 inflammation, blocking IL-4 and IL-13;

The hypothesis presented here is based on the observation that in severe COVID-19 patients, Th2 immune response is stimulated and eosinophils may play a central role in precipitating immune derangement and aggravating SARS-CoV-2-induced pneumonia. Because IL-5 is essential for the survival, maturation, and activation of eosinophils, it is suggested that IL-5 inhibitor drugs might block eosinophils activation in severe COVID-19 patients.

Immunophenotyping studies showed a moderate increase of eosinophils in severe COVID-19 patients, at least in a subset of them, with a mean blood count of around 100 cells/μL (Lucas et al., 2020; Mathew et al., 2020). Anti-IL-5 drugs (benralizumab, mepolizumab, reslizumab) are currently indicated as an add-on therapy for subjects with severe eosinophilic asthma, not responding to standard treatments, diagnosed with a peripheral blood eosinophils count of 150 cells/μL or higher at the beginning of treatment (GlaxoSmithKline, 2015; Teva, 2016; AstraZeneca, 2018). So, the question that arises is why such drugs should be beneficial in COVID-19 patients, especially those most severely affected.

In the EMA's Summary of Product Characteristic of mepolizumab, a combined analysis of the MEA112997 (DREAM) and MEA115588 (MENSA) approving trials is reported. Mepolizumab given at 75 mg IV/100 mg s.c. provided a significant reduction rate of clinically asthma exacerbations when given to patients with severe refractory eosinophilic asthma with a baseline blood eosinophil count as low as <150 cells/μL, with a reduction of exacerbation rate of 0.67. The effect size was larger, with an increasing count of eosinophils (GlaxoSmithKline, 2015). Mepolizumab reduces blood eosinophils and decreases the active migration of these cells to the lungs after stimulation with an allergen, but has a limited effect on respiratory resident eosinophils in asthma (Johansson et al., 2013; Kelly et al., 2017).

IL-5 receptor α (IL-5Rα) has been found in bronchial epithelial cells and allows epithelial barrier maintenance (Barretto et al., 2020). IL-5 beneficial effects on a mouse model of influenza do not depend only on eosinophils. Hence, IL-5Rα is expressed on migrated neutrophils in the lungs and neutrophils from other tissues (Gorski et al., 2019). IL-5Rα on activated neutrophils can promote signal transduction and, when activated by low concentrations of IL-5, causes a reduction of ROS production (Gorski et al., 2019). Children with asthma exacerbation exhibit both neutrophils and eosinophils recruitment and activation (Norzila et al., 2000). Interestingly, patients with mild asthma and rhinovirus infection that received mepolizumab treatment displayed a lower increase of neutrophils and neutrophil-derived myeloperoxidase in both BALF and sputum but also an increment of B lymphocytes and secretory IgA (Sabogal Piñeros et al., 2019b).

The role of neutrophils during SARS-CoV-2 infection is currently under investigation (Borges et al., 2020; Tomar et al., 2020; Wang et al., 2020). Specifically, the peripheral blood count of neutrophils is significantly higher in severe COVID-19 patients than those with moderate disease and can be considered a prognostic factor for a severe course (OR 1.5, 95% CI: 1.0–2.1) (Wang et al., 2020). Moreover, neutrophils count increases within 7–9 days since symptoms onset and correlate with radiologic findings (Wang et al., 2020). Activated neutrophils drive the production of neutrophil extracellular traps (NET) composed of DNA and toxic proteins that lead to cell death (named NETosis) and tissue damage (Cheng and Palaniyar, 2013). Transcriptome analysis conducted in COVID-19 patients showed up-regulation

of NET-associated genes. Thus, neutrophils and NETs can contribute to immunopathology in infected lungs (Wang et al., 2020). Anti-IL-5 therapy might block neutrophils' contribution to COVID-19 pneumonia.

IL-5Rα has been found in B-cell progenitors and activated B cells. On B cells, IL-5 stimulation participates in the plasma cell differentiation process (Takatsu, 2011). However, mepolizumab promotes the activation of the antiviral immune response, like NK cells potentiation, B lymphocytes' survival, and IgA secretion (Contoli and Papi, 2019). Patients receiving mepolizumab should experience higher viral replication, so it should not be given in the first phase of the infection, like corticosteroids (Contoli and Papi, 2019).

Considering that eosinophils may participate in COVID-19's immunopathology and that anti-IL-5 drugs could be effective even starting from relatively modest levels of eosinophil counts, it is tempting to speculate that treating COVID-19 patients with such biologicals might prove beneficial. Because eosinophils participate in antiviral immune response, anti-IL-5 drugs should not be administered in patients in the first stage, characterised by viral replication and limited inflammation. In such a setting, these monoclonal antibodies might be detrimental, similarly to corticosteroids (Siddiqi and Mehra, 2020). High-risk patients with hypoxia in the second stage of the disease [namely pulmonary phase IIB according to Siddiqi et al. proposal (Siddiqi and Mehra, 2020)] might be the best setting to try using anti-IL-5 biologics and prevent eosinophils recruitment. This assumption is based on the observations made by Lucas et al. (2020), who showed an increase of IL-5 levels within 6–10 days from symptoms onset, and a subsequent increase of eosinophils count on 11–15 days from symptoms onset (Lucas et al., 2020). The clinical context is similar to that of the RECOVERY trial, whereby dexamethasone efficacy was demonstrated: namely patients who require oxygen therapy (from supplemental oxygen to mechanical ventilation) (Sterne et al., 2020; Horby et al., 2020). Consistently, the efficacy of dexamethasone on COVID-19 mortality maybe also due to eosinophils apoptosis induction (Ilmarinen et al., 2014).

It is predicted that hospitalised COVID-19 patients at risk of fatal outcome should be treated with anti-IL-5 drugs as soon as possible before peripheral eosinophil count falls. Clinical risk scores aiming to predict intensive care admission or death are still under investigation (Galloway et al., 2020), and the timing of changes in leucocyte counts (including that of eosinophils) is yet to be precisely determined in COVID-19.

An idea of the effect of IL-5 antagonism in severe COVID-19 may be inferred from the results of a small clinical trial that explored granulocyte-macrophage colony-stimulating factor (GM-CSF) antagonism (De Luca et al., 2020). GM-CSF is a growth factor produced by macrophages, T-cells, epithelial cells, endothelial cells, and fibroblasts: it promotes the survival of monocytes, the differentiation of macrophages, and the activation of T cells subpopulations (Bonaventura et al., 2020). GM-CSF facilitates the migration of eosinophils in the lung and promotes their survival, especially in a setting of allergic inflammation (Nobs et al., 2019). Mavrilimumab, a monoclonal antibody that blocks GM-CSF, has been shown to improve clinical outcomes compared to standard care in hospitalized patients (De Luca et al., 2020).

CONCLUSION

A reasoned timing and appropriate patient selection in a randomized controlled clinical trial is the only way to establish whether IL-5 antagonism in COVID-19 is beneficial or harmful. The second and early third stages of the disease, with high-risk moderate and severe patients, respectively, should be the appropriate setting to try using IL-5 drugs.

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MINI REVIEW
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Repurposing Anti-Malaria Phytomedicine Artemisinin as a COVID-19 Drug

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Artemisinin is an anti-inflammatory phytomedicine with broad-spectrum antiviral activity. Artemisinin and its antimalarial properties were discovered by the Chinese scientist Tu Youyu, who became one of the laureates of the 2015 Nobel Prize in Physiology or Medicine for this breakthrough in tropical medicine. It is a commonly used anti-malaria drug. Artemisinin has recently been repurposed as a potential COVID-19 drug. Its documented anti-SARS-CoV-2 activity has been attributed to its ability to inhibit spike-protein mediated and TGF-β-dependent early steps in the infection process as well as its ability to disrupt the post-entry intracellular events of the SARS-CoV-2 infection cycle required for viral replication. In addition, Artemisinin has anti-inflammatory activity and reduces the systemic levels of inflammatory cytokines that contribute to cytokine storm and inflammatory organ injury in high-risk COVID-19 patients. We postulate that Artemisinin may prevent the worsening of the health condition of patients with mild-moderate COVID-19 when administered early in the course of their disease.

Keywords: COVID-19, ARDS, TGF — Transforming growth factor, malaria, anti-inflammatory

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INTRODUCTION

New effective drugs are needed to prevent the potentially deadly complications of COVID-19 (Woolf et al., 2020; Faust et al., 2021; Woolf et al., 2021) and thereby reduce its fatality rate (Uckun, 2020a; Uckun, 2020b; Uckun et al., 2020a; Zheng et al., 2020; Zhou et al., 2020). The goal of this mini-review is to discuss the emerging evidence regarding the clinical potential of Artemisinin for the treatment of COVID-19.

Due to their favorable safety profiles, natural products and phytomedicines are being explored as potential therapeutic or prophylactic agents with different mechanisms of action against COVID-19 (Huang et al., 2020). Some natural products have the potential to impair the attachment of SARS-CoV-2 spike glycoprotein to its receptors on human cells, including the Heat Shock Protein A5 (HSPA5) substrate-binding domain β (SBDβ) and angiotensin-converting enzyme 2 (ACE2) receptor (Elfiky, 2020; Kumar et al., 2020). Others have been proposed as inhibitors of viral replication, such as the recently reported compounds derived from Alpinia officinarum and ginger that may affect SARS-CoV-2 replication by blocking the SARS-CoV-2 papain-like protease (PLpro) (Goswami et al., 2020), compounds derived from African plants that may inhibit the 3-chymotrypsin-like protease (3CL ^{pro}): (Gyebi et al., 2020), or natural polyphenols such as quercetin that may inhibit the RNA-dependent RNA polymerase (RdRp) (El-Aziz Abd et al., 2020). In addition, several natural products have immunomodulatory activities that may have

clinically beneficial anti-inflammatory effects, including Chinese herb prescriptions Huang et al., 2020; Xu and Zhang, 2020).

Artemisia species contain bioactive substances with pleiotropic biological effects (Li et al., 2018). For example, *Artemisia annua* contains anti-inflammatory sesquiterpenoids, including Artemisinin (viz.: artesunate). Artemisinin and its antimalarial properties were discovered by the Chinese scientist Tu Youyu, who became one of the laureates of the 2015 Nobel Prize in Physiology or Medicine for this discovery (Li et al., 2018).

Artemisinin and some of its derivatives exhibit *in vitro* antiviral activity against a number of pathogenic human viruses, such as human cytomegalovirus (HCMV), Epstein Barr virus (EBV), human herpes simplex virus-6 (HHV-6) (Efferth et al., 2008; D'alessandro et al., 2020). Case reports of clinical response have been reported in a child with HHV-6 myocarditis and a patient with ganciclovir-resistant, foscarnet-resistant HCMV (Efferth et al., 2008; D'alessandro et al., 2020). *In vivo* antiviral activity was observed in the rat CMV model and a murine model of herpes simplex encephalitis (HSE) as well (Efferth et al., 2008). Studies by Cao et al. (2020) and Gilmore et al. (2020) confirmed the antiviral activity of Artemisinin and its derivatives against SARS-2-CoV-2 at micromolar concentrations. Recent docking studies indicated that Artemisinin and its derivative Artesunate could bind the SARS-CoV-2 spike protein in a way that would interfere with its docking onto the human ACE2 receptor protein, which is the required first step in the host infection process of the coronavirus disease 2019 (COVID-19) (Sehailia and Chemat, 2020; Yan et al., 2020). Importantly, recent research by Cao et al. revealed that Artemisinin-related compounds Arteannuin B and Lumefantrine disrupted the post-entry intracellular events of the SARS-CoV-2 infection cycle required for viral replication (Cao et al., 2020). Therefore, when these artemisinins were added at clinically achievable micromolar concentrations throughout the infection process or post-entry (but not when added before or during virus entry), SARS-CoV-2 replication was effectively inhibited, as measured by quantitative RT-PCR or viral RNA and protein assays (Cao et al., 2020).

Clinical Safety Profile and Pharmacokinetics of Artemisinin

Orally administered Artemisinin and Artemisinin derivatives are generally well-tolerated, especially when used for a short treatment course (Duc et al., 1994; De Vries et al., 1997; Ashton et al., 1998; Gordi et al., 2002; Hien et al., 2011; Li et al., 2018; Wang et al., 2020; Li et al., 2021). Except for the rare occurrence of hepatotoxicity and mild-moderate headache, nausea, vomiting, fatigue, and anorexia, Artemisinin was found to be clinically safe in healthy volunteers as well as malaria patients (Duc et al., 1994; De Vries et al., 1997; Ashton et al., 1998; Gordi et al., 2002; Hien et al., 2011; Li et al., 2018; Wang et al., 2020; Li et al., 2021). Severe hemolytic anemia requiring transfusion is a well-documented complication encountered within 28 days of therapy initiation by 20–25% of malaria patients treated with parenterally administered Artusenate and it necessitates close clinical monitoring for risk mitigation (Jauréguiberry et al., 2014; Savargaonkar et al., 2020). Likewise, severe hemolytic anemia requiring blood transfusions after oral artemisinin therapy has been observed as a rare complication in malaria patients with high parasite loads (Conlon et al., 2020). Based on its overall favorable safety profile, the World Health Organization (WHO) recommends parenteral artesunate for the treatment of severe malaria (WHO) (WHO, 2010).

Clinical Activity of Artemisinin and Chemical Derivatives of Artemisinin in COVID-19 Patients

Some clinical trials also suggested that Artemisinin may contribute to a faster recovery of COVID-19. Li et al. reported the results from an open-label non-randomized study in which 41 COVID-19 patients received either standard of care (SOC) therapy (control) or SOC combined with Artemisinin plus piperaquine (AP) (Li et al., 2021). The average time to reach undetectable viral RNA was significantly shorter for the AP group (Li et al., 2021). Patients in the AP group showed a faster clearance of SARS-CoV-2 than control patients. Liver enzyme elevations, as well as QTc interval prolongations on ECGs were observed in the AP arm, consistent with hepatotoxicity and cardiac toxicity.

ArtemiC is a medical spray containing Artemisinin, curcumin, Frankincense resin from the Boswellia sacra tree and Vitamin C. In the controlled Phase II trial NCT04382040, patients with COVID-19 received ArtemiC spray in addition to standard care. Study data have not been published in a peer-review article, but a press release of the preliminary data suggested that ArtemiC may be more active than placebo in contributing to the improvement of the patients' condition (Health Care, 2020). Likewise, the efficacy signal for the Artemisinin derivative Artesunate during a recently completed prospective, controlled clinical COVID-19 study was promising. In Artesunate treatment group, time to significant improvement of the symptoms, time to conversion to negativity of SARS-CoV-2 tests, and length of hospital stay was shorter than in the control group (Lin et al., 2020).

There are several Phase II/III studies currently underway in which pharmaceutical compositions or supplements containing Artemisinin and/or its derivatives are being evaluated as adjuncts to the standard of care in COVID-19 patients, including but not limited to Artesunate plus Artemisinin (NCT04387240), Artesunate plus amodiaquine (NCT04502342); Artesunate plus pyronaridine (NCT04475107), Artusunate as well as Artemisia annua (NCT04374019). In the CTRI/2020/09/028044 randomized Phase 4 trial, the efficacy of ARTIVeda (Artemisinin) is being studied in COVID-19 patients with mild-moderate disease. The product, ArtiVeda™ (License # UK.AY-401/2018, Ministry of AYUSH, India), is a novel gelatin capsule formulation of the Artemisia extract Ayurveda for oral delivery of the active ingredient Artemisinin for treatment of COVID-19. Pending the comparative evaluation of the pending data, it would be helpful to evaluate the clinical

potential of specific artemisinin compounds in well-designed randomized proof of concept studies. Ultimately, adaptive clinical trials will be required for the identification of the most promising treatment regimens (Uckun, 2020b).

DISCUSSION

The pharmacokinetics of Artemisinin after a single oral dose was examined in multiple small clinical studies employing Artemisinin most often at the clinically active 500 mg dose level alone or in combination with other antimalarial drugs, such as piperaquine, and showed a rapid elimination within 2–3 h (Duc et al., 1994; De Vries et al., 1997; Ashton et al., 1998; Gordi et al., 2002; Hien et al., 2011; Wang et al., 2020; Li et al., 2021). Due to its time-dependent enzymatic metabolism in the liver by the liver microsomal enzymes CYP2B6 and CYP3A4, the daily systemic exposure level rapidly declines in 5–7 days treatment cycles. This time-dependent pharmacokinetics of Artemisinin and its derivatives have been implicated in the observed high recrudescence rates in malaria patients within 2–3 weeks after monotherapy (Gordi et al., 2002). Therefore, treatment schedules need to be rationally designed for optimal efficacy by taking into consideration both the pathophysiology of target disease, concomitant medications and the pharmacokinetics characteristics of Artemisinin.

High-risk COVID-19 patients have a higher probability of developing a potentially life-threatening multi-system inflammation caused by a cytokine release syndrome (CRS) (Uckun, 2020a; Uckun 2020b; Uckun et al., 2020a; Wu et al., 2020; Zheng et al., 2020; Zhou et al., 2020). Several pro-inflammatory cytokines, including interleukin-6 (IL6), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β), contribute to the inflammatory injury of lungs in COVID-19 patients during the CRS (Uckun, 2020b; Uckun et al., 2020b). Notably, infection with SARS-CoV increases the expression of TGF-β and potentiates the TGF-β-regulated MAPK-mediated inflammatory signals (He et al., 2006; Zhao et al., 2008; Li et al., 2016; Wang et al., 2017). These cytokines also contribute to the potentially fatal severe systemic inflammation and multi-organ dysfunction during the viral sepsis of high-risk COVID-19 patients (Uckun, 2020a; Uckun 2020b; Uckun et al., 2020a; Wu et al., 2020; Zheng et al., 2020; Zhou et al., 2020). The reported anti-inflammatory and immunomodulatory effects of Artemisinin and its derivatives have been attributed to their ability to inhibit the pro-inflammatory nuclear factor kappa B (NF-κB) signaling pathway leading to reduced TNF-α and IL-6 levels as well as the Smad2/3-dependent TGF-β signaling pathway (Aldieri et al., 2003; Xu et al., 2007; Wu et al., 2010; He et al., 2011; Mo et al., 2012; Li et al., 2013; Jiang et al., 2016; Zhang et al., 2020). Artemisinin is hoped to mitigate the cytokine-mediated inflammatory injury associated with the cytokine storm and viral sepsis in critically ill COVID-19 patients (Aldieri et al., 2003; Xu et al., 2007; Wu et al., 2010; He et al., 2011; Mo et al., 2012; Li et al., 2013; Jiang et al., 2016; Alhelfawi, 2020; Zhang et al., 2020), in part owing to its ability to block the TGF-β surge which contributes to the development of lung injury and ARDS (Pittet et al., 2001; Budinger et al., 2005; Bossman and Ward, 2014; Frank and Matthay, 2014; Hu and Huang, 2019; Chen, 2020; Zuo et al., 2020). Due to the pivotal role of TGF-β in the pathophysiology of lung fibrosis that develops after an inflammatory injury to the lungs (Xu et al., 2007; Wu et al., 2010; Mo et al., 2012; Wang, 2019; Zhang et al., 2020), the TGF-β pathway inhibitory effect of Artemisinin has the clinical potential to prevent pulmonary fibrosis in COVID-19 patients. It may also help prevent the development of TGF-β triggered serious coagulopathy (Lev et al., 2007; Fox et al., 2020; Stafford et al., 2020). In this regard, data from an ongoing randomized Phase 2 clinical trial of the intravenously administered RNA therapeutic OT101 targeting the TGF-β mRNA that is being conducted in Peru (REPEC (Regsitro Peruano de Ensayos Clinicos):EC INS # PER-067-20) and Argentina (ReNIS (Registro Nacional de Investigaciones en Salud): IS003024) (Uckun et al., 2020b, Uckun and Trieu, 2020). Uzun et al. recently reported that artemisinins might also help reduce the risk of neurologic complications that are encountered in COVID-19 patients (Uzun et al., 2020)

CONCLUSION

Artemisinin has a clinical impact potential in the treatment of COVID-19 because it can prevent the progression of the disease and accelerate the recovery of patients before they develop potentially life-threatening complications (Uzun and Toptas, 2020; Krishna et al., 2021). This dual-function COVID-19 drug candidate is hoped to mitigate the cytokine-mediated inflammatory injury associated with the cytokine storm and viral sepsis in critically ill COVID-19 patients.

AUTHOR CONTRIBUTIONS

Each author (VT, SS., HW., FU) has made significant and substantive contributions to the study, reviewed and revised the manuscript, provided final approval for submission of the final version. No medical writer was involved. VT and FU conceived the study, designed the evaluations reported in this paper, directed the data compilation and analysis, analyzed the data, and prepared the initial draft of the manuscript. Each author had access to the source data used in the analyses.

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REVIEW
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Repurposing Chloroquine Against Multiple Diseases With Special Attention to SARS-CoV-2 and Associated Toxicity

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Chloroquine and its derivatives have been used since ages to treat malaria and have also been approved by the FDA to treat autoimmune diseases. The drug employs pH-dependent inhibition of functioning and signalling of the endosome, lysosome and trans-Golgi network, immunomodulatory actions, inhibition of autophagy and interference with receptor binding to treat cancer and many viral diseases. The ongoing pandemic of COVID-19 has brought the whole world on the knees, seeking an urgent hunt for an anti-SARS-CoV-2 drug. Chloroquine has shown to inhibit receptor binding of the viral particles, interferes with their replication and inhibits "cytokine storm". Though multiple modes of actions have been employed by chloroquine against multiple diseases, viral diseases can provide an added advantage to establish the anti–SARS-CoV-2 mechanism, the *in vitro* and *in vivo* trials against SARS-CoV-2 have yielded mixed results. The toxicological effects and dosage optimization of chloroquine have been studied for many diseases, though it needs a proper evaluation again as chloroquine is also associated with several toxicities. Moreover, the drug is inexpensive and is readily available in many countries. Though much of the hope has been created by chloroquine and its derivatives against multiple diseases, repurposing it against SARS-CoV-2 requires large scale, collaborative, randomized and unbiased clinical trials to avoid false promises. This review summarizes the use and the mechanism of chloroquine against multiple diseases, its side-effects, mechanisms and the different clinical trials ongoing against "COVID-19".

Keywords: SARS-CoV-2, pH-dependent, autophagy, immunomodulatory, antiviral mechanism, toxicity

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INTRODUCTION

Chloroquine, commonly known for the anti-malarial applications has evolved gradually as a magic medicine, effective against many diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple types of cancer and viruses. It has also been a molecule of choice among research community for studying the mechanism of autophagy, nanoparticles internalization, endocytosis and interlinked role of multiple signalling pathways in various diseases including cancer and autophagy (Pelt et al., 2018; Varisli et al., 2019).

Recent onset of the Coronavirus Disease-2019, a pandemic which has put the world on its knees, has again brought this "age-old drug" chloroquine and its derivatives into bright limelight. The disease has already spread worldwide and has killed more than 9,534,437 of the world population (Coronavirus

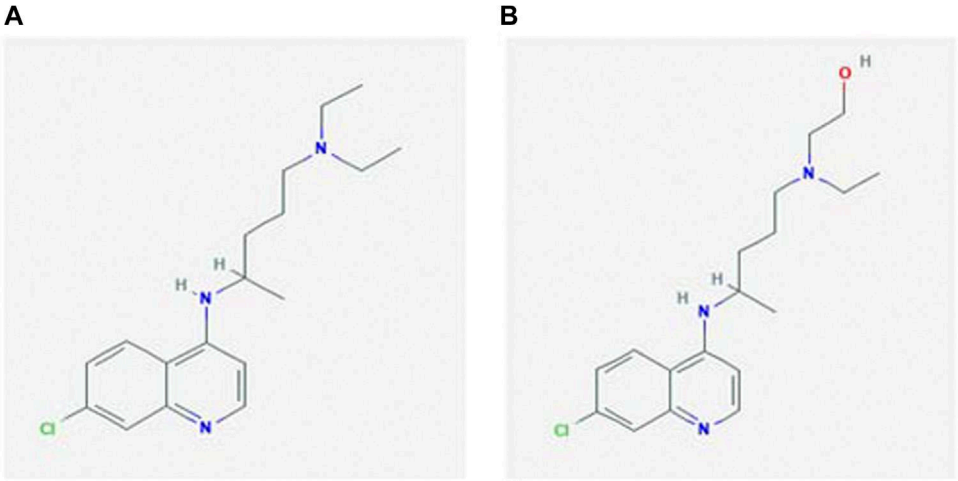

FIGURE 1 | Chemical structure of **(A)** chloroquine and **(B)** hydroxychloroquine (National Center for Biotechnology Information, 2004).

Death Toll and Trends-Worldmeter, n.d.) and is still affecting millions. Multiple drugs are being tested, and the research community leaves no stone unturned to come up with an effective vaccine or drug to treat this wide-spread disease. Chloroquine and its derivatives have also emerged as a potential drug for effective treatment of this novel coronavirus (Smith et al., 2020; Touret and Lamballerie, 2020). Other potential drugs being tested for COVID-19 are remdesivir (GS-5734), lopinavir;ritonavir, Interferon alfacon-1 in conjunction with corticosteroids and Ribavirin in conjunction with corticosteroids (Smith et al., 2020; Wang M. et al., 2020). However, none of the drugs being researched has been approved by the World Health Organization (WHO) for the treatment of COVID-19 till now, keeping the room open for further research on chloroquine and the derivatives.

4-N-(7-chloroquinolin-4-yl)-1-N, 1-N-diethylpentane-1, 4-diamine, commonly known as chloroquine is a 4-aminoquinoline approved by FDA for treatment of malaria and inflammation–related diseases. It is a colorless and odourless crystal with a molecular mass of 319.9g/mol and available as a generic medicine (PubChem ID: 2719). Chloroquine is an inexpensive, water-soluble, weakly basic tertiary amine, which at physiological pH (7.2–7.4) is highly membrane permeable. However, inside the acidic organelles, it gets protonated and accumulates, raising the pH of the respective organelle. It can interfere with all the pH-dependent signalling and functioning of the endosome, lysosome, Golgi network, phagosome, and autophagosomes (Weyerhäuser et al., 2018). However, due to some side-effects of chloroquine, several derivatives, including hydroxychloroquine have been synthesized with similar efficacy but reduced toxicity.

Chloroquine and its derivatives (**Figure 1**), emerging as one of the most probable drugs alone or in combination against the battle of COVID-19, needs a detailed compilation and review so that the mechanisms elucidated by them against multiple diseases can be understood and co-related or used for the further vaccine and drug development for COVID-19. This review summarises chloroquine's journey, from being an anti-malarial drug to a magic bullet against multiple diseases, its good and evil, results of clinical trials obtained so far and the future aspects, it holds along with its drawbacks as prophylaxis or drug to fight COVID-19.

CHLOROQUINE AS AN ANTI-MALARIA DRUG

Mechanism of Haemoglobin Degradation Inside the Human Body by the Malaria Parasite

To understand, how chloroquine inhibits malarial parasite, it is important to know the mechanism employed by *Plasmodium* sp. to hijack erythrocytes and use haemoglobin for their energy requirements. The *Plasmodium* sp. has a specialized acidic organelle known as digestive vacuole (DV) for degrading haemoglobin for its energy requirements following a cascade of protease activities (Pandey and Chauhan, 1998). The by-product of haemoglobin digestion is heme. Heme, when bound in haemoglobin is in the non-toxic ferrous form (Fe^{2+}), but when free, it converts into very toxic ferric form (Fe^{3+}) (Francis et al., 1994). To avoid toxicity, the parasite must evolve machinery to get rid of toxic heme, which is achieved by crystallization of heme called "hemozoin" or "malarial pigment" (Coronado et al., 2014). The formation of hemozoin takes place at considerably low pH where two heme units are linked together by iron carboxylate bonds. This unusual linkage is important for the synthesis and growth of an ordered insoluble crystal (Bohle et al., 1997; Coronado et al., 2014). Histidine rich protein (HRP) plays a vital role in the biocrystallization of hemozoin (Coronado et al., 2014). The hemozoin formed does not only detoxify the heme pigment for parasite but also adversely affects the human immune system, especially macrophages (Schwarzer et al., 2003).

How Chloroquine Works Against the Malaria Parasite

There were several theories proposed regarding the mode of action of chloroquine to kill malaria pathogen as DNA binding agent (Parker and Irvin, 1952) protein synthesis inhibitor (Surolia and Padmanaban, 1991) polyamine metabolism inhibitor (Slater, 1993) and inhibitor of hemozoin crystallization (Orjih, 1997; Gorka et al., 2013). Most of the studies have shown chloroquine as a potent inhibitor of hemozoin crystallization. Sullivan. et al. (1996) postulated that chloroquine inhibits hemozoin formation by inhibiting HRP II. Again, Sullivan. et al. (1998) in their study concluded that chloroquine blocked the polymerization of free heme released during haemoglobin proteolysis in intraerythrocytic *P. falciparum*. Later in a review, Sullivan (2017) summarized that quinolones block every step of toxic heme crystal growth. DVs are acidic organelles with pH 5.0, where chloroquine can diffuse inside easily. However, the acidic pH yields diprotonation of the drug, inhibiting its movement out of the DV. The trapped diprotonic chloroquine inhibits the crystal growth of hemozoin, toxifying the malaria pathogen (Goldberg, 1993). Pandey and Tekwani et al. (1997) in their study established that chloroquine initiates a reverse reaction of conversion of hemozoin to monomeric heme (ferriprotoporphyrin IX) after interaction with malarial hemozoin, also termed as termed "hemozoin depolymerization".

Developing Resistance by *Plasmodium* sp. Against Chloroquine and Alternative Strategies

Developing resistance by *Plasmodium* sp. against chloroquine attributes to a point mutation in the genes coding for the chloroquine resistance transporter (PfCRT) present in DV (Martin et al., 2009; Chinappi et al., 2010). This protein avoids the accumulation of chloroquine by facilitating the efflux of the diprotonic chloroquine. However, the action of protein as a channel or a carrier is still debatable. Chinappi et al. (2010) in their study proposed that the protein acts as a carrier to exclude out both mono and diprotonic chloroquine. Reiling et al. (2018) proposed that pharmacological responses of sensitive and resistant malaria parasite towards chloroquine are also different.

Different strategies including alternative drugs, derivatives of chloroquine and combinational drug therapies have been used to combat the chloroquine-resistant malarial parasite. Clindamycin in combination with quinine was successfully used for the treatment of uncomplicated multidrug-resistant *P. falciparum* malaria in Thai patients (Pukrittayakamee et al., 2000). Artesunate-atovaquone-proguanil combination has proven successful for the treatment of the similar case of malaria (van Vugt et al., 2002). Primaquine, mefloquine, artesunate and artemisinins are some of the drugs used in the treatment of resistant malaria in India (Kalra et al., 2002). Treatment of chloroquine-resistant malaria using a combination of pyrimethamine, berberine, tetracycline or cotrimoxazole has been used successfully to treat chloroquine-resistant malaria in Africa (Sheng et al., 1997).

CHLOROQUINE AS AN ANTI-RHEUMATOID ARTHRITIS AND LUPUS ERYTHEMATOSUS DRUG

RA and LE are autoimmune diseases, where healthy tissues are attacked by the hyper-immune system causing inflammatory responses. RA is mainly characterized by pain, inflammation and stiffness around the joints, whereas LE is characterized in the early phase with arthritis, skin lesions, inflammation around the lungs and kidneys. Rhupus, is a syndrome which presents symptoms associated with both RA and LE (Macfarlane and Manzel, 1998; Thome et al., 2013).

Both chloroquine (CQ) and 4-hydroxychloroquine (HCQ) are extensively used as immune-modulators to treat RA and LE. There are evidence of both, pH-dependent and pH-independent role of chloroquine and its derivatives to inhibit the generation of autoantibodies and reducing the secretion of inflammatory cytokines (Macfarlane and Manzel, 1998; Thome et al., 2013).

CQ and HCQ both can enter acidic endosome and lysosome, remain there as CQ^{+} and CQ^{++}, elevate their pH from 4.5 to 6.0, and interfere with their functions (Mindell, 2012). By interfering with endosome functions, it inhibits TLR7 and nine signalling and thus inhibits dendritic cell maturation. By changing the acidity of lysosome of antigen-presenting cells, CQ and HCQ, inhibits the presentation of the major histocompatibility (MHC) complex peptides to T cells, thus inhibiting the production of T helper cells and cytokines (Thome et al., 2013; Ponticelli and Moroni, 2017). It also inhibits calcium-dependent signalling, toll-like receptor signalling pathways, and iron metabolism in macrophages, thus suppressing production of IL-6, IL-1 and tumor necrosis factor-alpha (TNF-α) (Ponticelli and Moroni, 2017; Schrezenmeier and Dörner, 2020). Rand et al. (2008) in their experiment using atomic force microscopy (AFM) observed that hydroxychloroquine interferes with binding of antiphospholipid antibody–β2-glycoprotein I complexes to phospholipid bilayers, thus lowering down inflammation. Oh et al. (2016) concluded in their study that chloroquine reduces inflammation through p21-mediated suppression of T cell proliferation and Th1 cell differentiation.

Though the development of resistance against disease-modifying anti-rheumatic drugs (DMARDs) including chloroquine, has not been studied much, the role of ATP binding cassette (ABC) proteins responsible for drug efflux cannot be neglected (Jansen et al., 2003). A better understanding is needed in this field to establish alternative strategies and drug combination therapy for RA and LE.

CHLOROQUINE AS AN ANTI-CANCER DRUG

How Chloroquine Works Against Cancer

Inhibition of cancer cell growth by chloroquine is a complex process. **Table 1** summarizes the multi-ranged effects of chloroquine on multiple types of cancer cells. The primary mechanism employed by chloroquine and its derivative is

TABLE 1 | Examples of chloroquine used in treatment of cancer.

S. No	Name of drug	Type of Cancer Cell	Concentration of chloroquine	Mechanism	Reference
1	Chloroquine with C2 ceramide	Lung Cancer H460 and H1299 Cells	10 μM	Inhibition of autophagosome maturation and degradation during autophagy progression	Chou et al. (2019)
2	Chloroquine with Luteolin	Squamous Cell Carcinoma Cells	50 μM	Blocked autophagy	Verschooten et al. (2012)
3	Chloroquine as an adjuvant	Glioma cells	5–20 μM	Blocked autophagy and modulated several metabolic pathways, deficient DNA repair	Weyerhäuser et al. (2018)
4	Chloroquine	Bladder cancer cells	20 μM	Inhibition of cholesterol metabolism	King et al. (2016)
5	Chloroquine and GX15-070	Pancreatic cancer cells	20 μM	Blocked autophagy	Wang et al. (2014)
6	Chloroquine	Rat sarcoma	1–100 μM	Sensetized cells by inhibition of DNA repair and loss of mitochondrial potential	Eng et al. (2016)
7	Chloroquine with temozolomide	Glioma cells	5–20 μM	Sensetizing glioma cells by autophagy inhibition	Yan et al. (2016)
8	Hydroxycholoroquine with phytosterol	Lung cancer cell	20–120 μM	Autophagy inhibition	Elshazly et al. (2020)
9	Chloroquine with Tenovin-6	Gastric cancer	25–50 μM	Autophagy inhibition	Ke et al. (2020)
10	Hydroxychloroquine	HeLa cells	60 μg/ml	Loss of lysosome and mitochondrial membrane potential	Boya et al. (2003)
11	Chloroquine and NVP-BEZ235	Neuroblastoma cells	0–120 μM	Lysosome -mitochondria cross talk	Seitz et al. (2013)
12	Chloroquine	Pancreatic cancer	0.5–100 μg/ml	Inhibition of neutrophil extracellular traps	Boone et al. (2018)
13	Chloroquine	Prostate cancer	10–20 μM	Induces Par-4 response	Rangnekar (2019)
14	Chloroquine	Bladder cancer	10 μM	Enhances the radiosensitivity by inhibiting autophagy	Wang et al. (2018)
15	Chloroquine and oxaliplatin	Pancreatic cancer	—	modulating activity of cytosolic HMGB1	Lee et al. (2018)

inhibition of autophagy during cancer cell death. The pH-dependent accumulation of chloroquine inside lysosome leads to impairment of autophagosome degradation and thus inhibition of autophagy (Mauthe et al., 2018). It is also known to generate endoplasmic stress, lysosome and mitochondrial membrane depolarization in a reactive oxygen species (ROS) dependent manner, thus increasing apoptosis (Ganguli et al., 2014; Alam et al., 2016). Though chloroquine alone is not sufficient to depolarise membrane potential; it is generally used to sensitize chemo or radiotherapy, in an autophagy-dependent or independent manner (Maycotte et al., 2012; Makowska et al., 2016; Zhu et al., 2019). However, there are some severe kidney and organ injuries have also been reported after the use of chloroquine as the sensitizer to chemo and radiotherapy (Kimura et al., 2013).

Recent studies have revealed that chloroquine is also able to interfere with different metabolic pathways, including cholesterol, glucose, amino acids, and mitochondria metabolism (Weyerhäuser et al., 2018).

Chloroquine is also used to treat multidrug-resistant cancer by blocking drug extrusion by interfering with the ATP-binding cassette (ABC) transporter family and other transmembrane protein related to drug resistance (Szakács et al., 2006). A summary of mechanisms employed by chloroquine has been illustrated in **Figure 2**.

CHLOROQUINE AGAINST BACTERIAL AND FUNGAL DISEASES

Generally, in response to intracellular bacterial or fungal pathogens, the first-line antimicrobial defence is initiated by the phagocytes. After being internalised by the phagocyte, a phagosome forms which further fuses with lysosomes. Through oxygen dependent and independent mechanisms, the bacteria are killed. This acidifies the phagolysosome to pH 4.5 and activates lysosomal enzymes. Several intracellular pathogenic bacteria and fungi evade this line of defence through different mechanisms such as, they lack the lysosomal pathway (ex. *Bartonella* sp.), escape before the fusion of phagosome and lysosome and survive in the cytosolic region (ex. *Shigella* sp, *Rickettsia* sp.), block lysosomal fusion and multiply in the phagosome (ex. *Chlamydia* sp, *Salmonella* sp, *Mycobacterium* sp, *Yersinia* sp), resistance to survival in phagolysosome (*Coxiella burnetii*, *Tropheryma whipplei*). Chloroquine treatment inhibits the growth of these intracellular pathogens by pH dependent iron deprivation and neutralising the phagolysosomal pH (Rolain et al., 2017). Lagier et al. (2014) reported the bactericidal combination treatment of doxycycline and hydroxychloroquine against the classic *Tropheryma whipplei* caused Whipple's disease. The authors confirmed the effectivity of the combination treatment through *in vitro* studies and clinical trials (Lagier et al., 2014). Q fever, caused by *Coxiella burnetii* infection manifests into a severe complication of endocarditis. A combination of doxycycline and chloroquine derivates has been reported to reduce the mortality rate and is a prominent therapeutic intervention for Q fever. The mechanism of action is under investigation; however, it can be presumed that chloroquine increases the lysosomal pH and enhances the antibacterial activity of doxycycline (Alegre et al., 2012; Lagier et al., 2014).

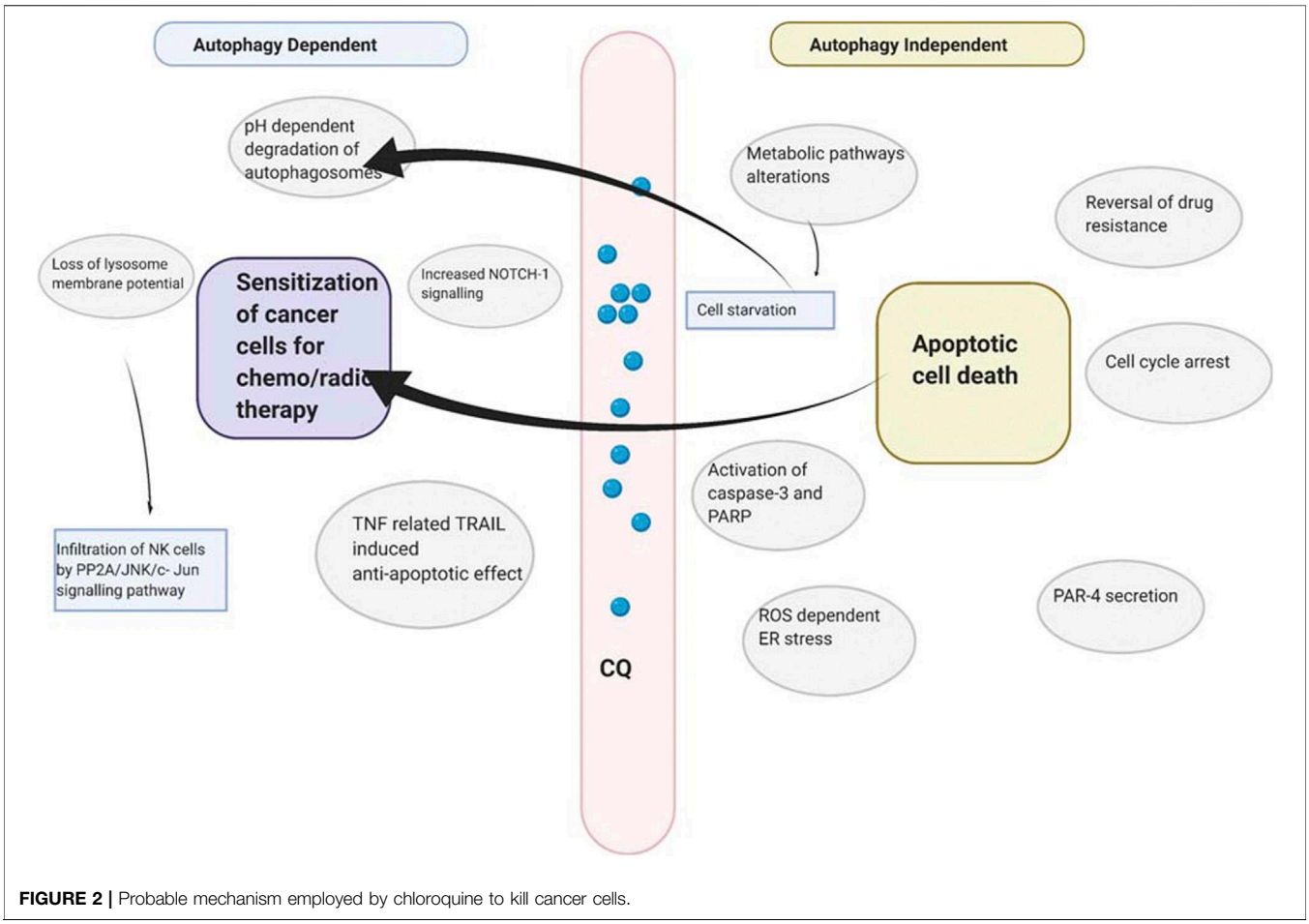

FIGURE 2 | Probable mechanism employed by chloroquine to kill cancer cells.

CHLOROQUINE AGAINST VIRAL DISEASES WITH SPECIAL ATTENTION TO SARS-COV-2

Viral Pathogenesis in Human With Special Attention to SARS-CoV-2

The catastrophic impact of viral diseases on human has been observed since ages. From Spanish flu to COVID-19, humankind has always struggled to make a way out of socio-economic burden slapped by viral pathogens. The COVID-19 pandemic crisis has worsened the economic and health condition worldwide to such a level that had not been observed in the last 70years (https://www.un.org/development/desa/dspd/2020/04/social-impact-of-covid-19/). The COVID-19 outbreak is detrimental to old age, immuno-suppressive people, and a significant economic burden on indigenous and poor people.

Each virus has a different virulence factor, and the pathological consequences also differ from virus to virus. The knowledge of viral pathogenesis is neither accurate nor complete for most viral infections, especially for SARS-CoV-2. Novel SARS-CoV-2 is an enveloped single-stranded RNA virus belonging to the family Coronaviridae (Zheng, 2020) responsible for ongoing pandemic COVID-19. The main symptoms of this disease include fever, cough and fatigue, and it can lead to severe complications, having a mortality rate of 5.7% (Lechien et al., 2020) 50% of the COVID-19 positive patients are asymptomatic. The main symptoms in the early stages are headache (70%) loss of smell, and nasal obstruction. Cough, fever and dyspnoea are a sign of late infection (8–10days) (Lechien et al., 2020; Sajna and Kamat, 2020).

Discussing complete progress details about the viral pathogenesis will be beyond this review, however, in general, pathogenic virus and in particular, SARS-CoV-2 follows the following events to cause an infection.

a. Entry inside the cells in an endocytosis-dependent or independent manner.

Most of the human viruses follow an endocytosis-dependent entry inside the cells. The envelope spike glycoprotein of SARS-CoV and SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor on target cells to facilitate entry (Li et al., 2020; Zhang et al., 2020). The spike "S" protein is responsible for the ACE2 receptor binding, whereas the cellular serine protease TMPRSS2 is required to prime the "S" protein (Hoffmann et al., 2020).

Xu et al. (2020) in their study found that the 3D structure of the receptor-binding domain in both the viruses is identical. SARS-CoV followed direct membrane fusion between the virus

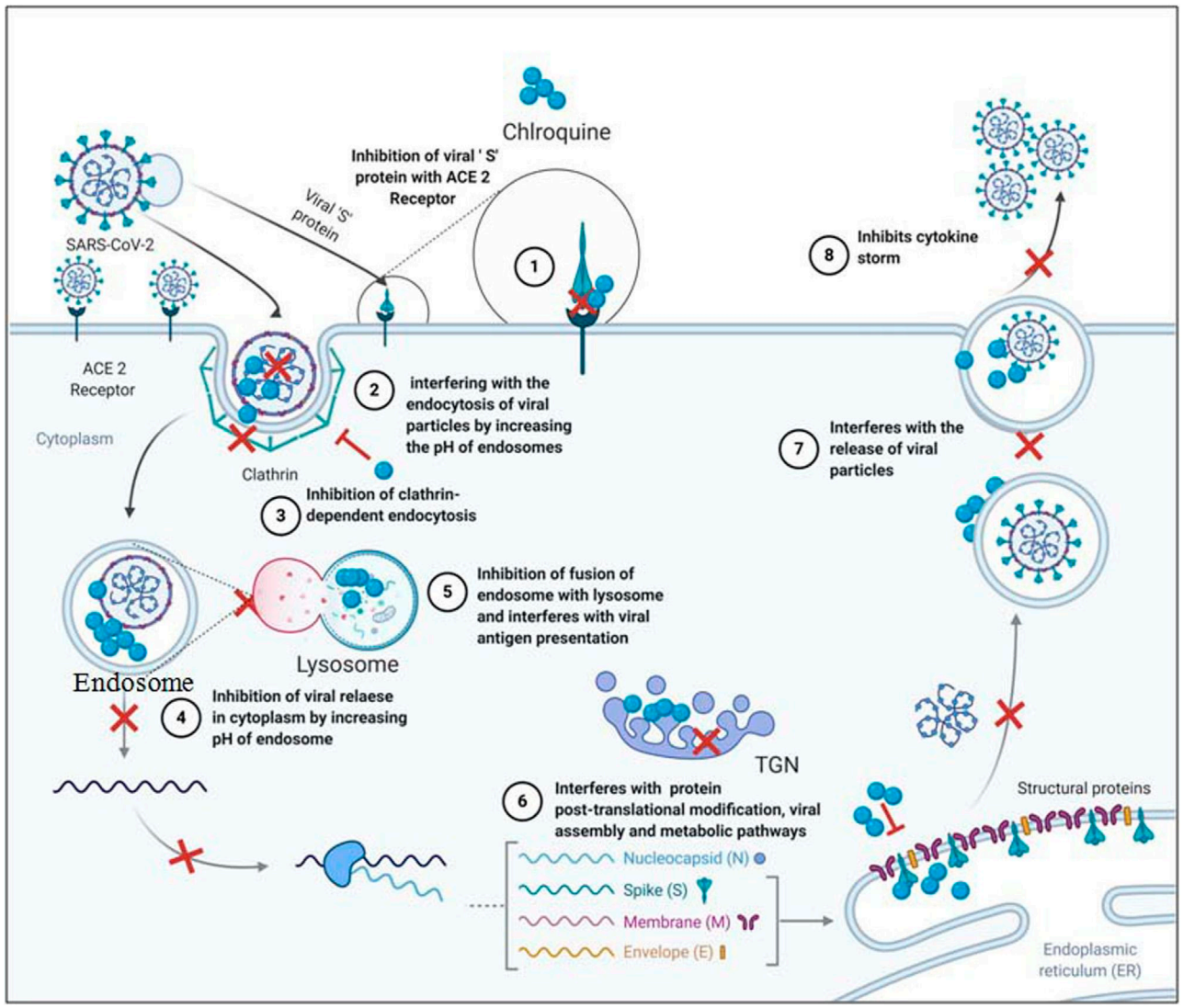

FIGURE 3 | Probable mechanism employed by chloroquine to inhibit SARS-CoV-2.

and plasma membrane as well as clathrin-dependent and -independent endocytosis mediated entry inside target cells (Wang et al., 2008; Kuba et al., 2010).

b. Viral replication inside target cells.

The replication mechanism of SARS-CoV and SARS-CoV2 is also found to be similar (Caly et al., 2020). After entry inside the target cells, the virus's RNA genome is released in the cytoplasm, translated, and posttranslational modifications occur in endoplasmic-reticulum or Golgi apparatus. After the assembly of RNA and nucleocapsid proteins, the replicated virus particles are released by membrane fusion (Li et al., 2020).

c. Escaping immune surveillance

Most of the viral diseases survive inside human by escaping immune surveillance. Viruses of the family Coronaviridae are no exception. During the initial infection, the SARS-CoV-2 delays type 1 IFN production and avoids the recognition by pattern recognition receptors (PRRs), allowing uncontrolled viral replication, activating pro-inflammatory cytokines triggering "cytokine storm" (Huang et al., 2005; Li et al., 2020; Rothan and Byrareddy, 2020). Further, activation of specific Th1/Th17 enhances the inflammatory responses.

SARS-CoV-2 escapes activation of adaptive immunity by interfering differentiation and function of dendritic cells and defensins and a severe decrease in $CD4^+$ and $CD8^+$ T cells (Li X. et al., 2020; Li G. et al., 2020).

How Chloroquine Works Against Viral Diseases

Chloroquine acts as a potent anti-viral agent by implying several mechanisms which have been listed in **Table 2**. The anti-viral

TABLE 2 | Examples of chloroquine used against viral diseases.

S. no	Drug	Viral Disease	Concentration	Mechanism	Reference
1	Chloroquine	Human Coronavirus OC43	15 mg of chloroquine per kg of body weight	Not established	Keyaerts et al. (2009)
2	Chloroquine	SARS-CoV	10–50 μM	Elevations of endosomal pH, terminal glycosylation of the cellular receptor, angiotensinconverting enzyme 2	Vincent et al. (2005)
3	Hydroxyferroquine Derivatives	SARS-CoV	IC_{50}- 0.3–1 μg/ml	Not established	Biot et al. (2006)
4	Chloroquine, 7-8-dihydroneopterin	SARS-CoV, MERS-CoV	EC_{50} 3–8mol/L, 4 mg/kg per day	Endosomal acidification	Al-Bari (2017)
5	Chloroquine	MERS-CoV	EC_{50} of 3 μM	Inhibited replication	Liang et al. (2018)
6	Chloroquine	Zika virus	20 mg/kg of body weight	Protection against ZIKV-induced inflammatory changes	Li et al. (2017)
7	Chloroquine	Ebola virus	10 μM	Lysosome acidification. Was able to inhibit *in vitro* but failed *in vivo*	Dowall et al. (2015)
8	Chloroquine	Zika virus	5–40 μM	obstructs fusion of the flaviviral envelope protein with the endosomal membrane	Shiryaev et al. (2017)
9	Chloroquine	Herpes simplex virus	15 μM	Interacts with endocytic viral entry	Dai et al. (2018)
10	Chloroquine	Influenza A virus	60 μM	Blocking autophagy	Calderon et al. (2019)
11	Chloroquine	Zika virus	0–300 μm/l	Blocking autophagy	Zhang et al. (2019)
14	Hydroxy-chloroquine	Dengue virus	0–100 μM	Activating ROS and a MAVS mediated host IFN anti-viral pathway	Wang et al. (2015)
15	Hydroxy-chloroquine	Influenza A virus	3–30 μM	Blocking autophagy	Yan et al. (2013)
16	Chloroquine	Influenza A virus	500 mg/day for 1week	Disrupts pH-dependent structural changes in viral-synthesized proteins	Paton et al. (2011)
17	Chloroquine	HIV	100 μM	Interferes with innate immunity-induced immune hyperactivation	Martinson et al. (2010)
18	Hydroxy-chloroquine	HIV	20 μM	Apoptosis in the memory T-cell compartment by inhibiting autophagy	van Loosdregt et al. (2013)
19	Hydroxy-chloroquine	HIV	—	Induction of a defect in the maturation of the viral envelope glycoprotein gp120	Tsai et al. (1990)
20	Chloroquine	Chikungunya	250 mg/day	Not established	Chopra et al. (2014)
21	Chloroquine	Prion (scrapie-infected neuroblastoma (ScN2a))	100 μM	Acidification of lysosome	Supattapone et al. (1999)
22	SGI-1027 (Derivative of Chloroquine)	Creutzfeldt-Jakob disease	0–1μm/L	reduce PrP^{Sc} formation via direct coupling with PrPC in prion-infected cells	Kim et al. (2019)
23	Chloroquine	Influenza B virus	0–10 μM	lysosomotropic alkalinizing agents (LAAs) and calcium modulators (CMs)	Marois et al. (2014)
24	Chloroquine	Human Papilloma Virus (HPV)	10 μM	Autophagy inhibition, inhibited the up-regulation of PD-L2	Baruah et al. (2019)
25	Chloroquine	Grass carp reovirus (GCRV)	50–400 μM	Inhibition of Lysosomal acidification	Wang et al. (2016)
26	Chloroquine and hydroxyl-chloroquine	Human Papilloma Virus (HPV) (Cutaneous warts)	400 mg/day	Inhibition of Lysosomal acidification	Bhushan et al. (2014)
27	Hydroxy-chloroquine	SARS-CoV-2	EC_{50} = 1.13μM	Interfering with the glycosylation of cellular receptors and endosome alkylatiation	Wang et al. (2020)
28	Hydroxy-chloroquine	SARS-CoV-2	400 mg given twice daily for 1day, followed by 200mg twice daily for 4 more	Not established	Yao et al. (2020)
29	Hydroxy-chloroquine	SARS-CoV-2	CC_{50} 249.50 μM	Inhibition of endocytosis	Liu et al. (2020)
30	Hydroxy-chloroquine and azithromycin	SARS-CoV-2	600 mg of hydroxyl-chloroquine daily	Not established	Gautret et al. (2020)
31	Chloroquine and hydroxyl-chloroquine	SARS-CoV-2	In silico study	Inhibition of viral S protein to bind with gangliosides	Fantini et al. (2020)
32	Hydroxy-chloroquine	SARS-CoV-2	400 mg given twice daily for 1 day, followed by 200 mg twice daily for 4 more days	Not established	Clementi et al. (2020)
33	Chloroquine and hydroxyl-chloroquine	SARS-CoV-2	IC_{50} 46 and 11μM	Not established	Weston et al. (2020)
34	Hydroxy-chloroquine and azithromycin	SARS-CoV-2	1, 2 and 5 μM for 78 hydroxy-chloroquine and 2, 5 and 10 μM for azithromycin	Not established	Andreani et al. (2020)
35	Chloroquine	SARS-CoV-2	EC_{50} of 1.13 μM	Not established	Gao et al. (2020)

mechanisms of chloroquine can further be exploited to develop it as a therapeutic agent against SARS-CoV-2.

Probable Mechanisms of Chloroquine Against SARS-CoV-2

Though studies are still ongoing on chloroquine as an inhibitor of SARS-CoV-2, the plausible mechanisms known from its use against various diseases can provide a substantial ground for further research and development of chloroquine as a potential drug against COVID-19. Multiple modes of actions of chloroquine against SARS-CoV-2 are as follows:

1. Inhibition of viral entry inside the target cells

Chloroquine can inhibit the binding of viral spike glycoprotein with ACE2 receptor on target cells to inhibit their entry. Chloroquine has shown potent inhibition of sugar modifying enzymes or glycosyltransferases and quinone reductase which have been involved in sialic acid biosynthesis of ACE2 receptor (Kwiek et al., 2004; Devaux et al., 2020). Wu et al. (2020) in their docking studies showed that chloroquine can potentially target Nsp3b or E-channel with the docking mfScores of–130.355 and–107.889, respectively, though experimental results are yet to be verified.

SARS-CoV-2 particles significantly resemble the nanoparticles with a size of 60–140nm and are spherical. Nanoparticles are known to exhibit their desired results by cell internalization (Kumari et al., 2017) which can effectively be inhibited by chloroquine. Chloroquine inhibits nanoparticles internalization by suppression of phosphatidylinositol binding clathrin assembly protein (PICALM), thus inhibiting clathrin-dependent endocytosis (Hu et al., 2020). The same principle can be applied for stopping the internalization of SARS-CoV-2 particles inside the target cells. Chloroquine can also play a vital role in interfering with the endocytosis of viral particles by increasing the pH of endosomes which has been explained earlier (Touret and Lamballerie, 2020). Interaction of TMPRSS2 with the ACE2 receptor is essential for facilitating SARS-CoV-2 entry (Matsuyama et al., 2020). Application of chloroquine with a known serine protease inhibitor can weaken the viral entry inside the cells (Markus et al., 2020). Serine protease inhibitor camostat mesylate has been observed to blocks TMPRSS2 activity in SARS-CoV-2 (Hoffmann et al., 2020).

2. Inhibition of viral replication and posttranslational modifications (PTM)

Chloroquine inhibits acidification of endosome and lysosome, stalling the virus inside endosomes and inhibiting the release of the viral RNA genome in the cytosol. Inhibition of lysosome acidification further hampers the fusion of endosome with the lysosome and upstream trafficking essential for viral replication (Devaux et al., 2020; Hu et al., 2020; Wang et al., 2020).

Inhibition of acidification further continues to work in favour of chloroquine against SARS-CoV-2 as it inhibits posttranslational modification in trans Golgi network (TGN). Lack of low pH in TGN interferes with functional proteases and glycosyl-transferases resulting in impaired PTM or non-infectious viral particles (Devaux et al., 2020; Touret and Lamballerie, 2020).

3. Inhibition of autophagy

Many of the human viruses employ autophagy for their replication inside the target cells (**Table 2**) (Yan et al., 2013; Calderon et al., 2019; Zhang et al., 2019). Though the role of autophagy in the proper functioning of SARS-CoV-2 is still under investigation, several results claim that autophagy is crucial for SARS-CoVs replication (Brest et al., 2020; Yang and Shen, 2020). Prentice et al. (2004) demonstrated the critical role of endogenous LC-3, a protein marker for autophagosomes in the replication of SARS-CoV. Chloroquine, being a well-established autophagy inhibitor can be a potential candidate for suppression of COVID-19.

4. Immuno-modulator and inhibition of "cytokine storm"

Chloroquine is widely used for the treatment of RA and SE based upon its immune-modulatory properties. As discussed in earlier sections, chloroquine inhibits pH-dependent toll-like signalling pathway in the endosome and inhibits the inflammatory response "cytokine storm". The inhibition of toll-like signalling pathway prevents the recognition of viral antigen by dendritic cells (Devaux et al., 2020). It also enhances cytotoxic $CD8^+$ T cell responses against viral antigens and exports soluble antibodies into the cytosol of the dendritic cell to fight the viral antigen.

Chloroquine interferes with viral antigen presentation via the lysosomal pathway and thus inhibits MHC II recognition of antigen, modulating the elevation of inflammatory responses (Kearney, 2020). Inhibition of TNFα, TNF α receptors and TNF α signalling by chloroquine plays a vital role in the suppression of "cytokine storm" (Touret and Lamballerie, 2020).

5. Interference with the metabolic pathways

As it is already known from the use of chloroquine against glioblastoma, this can regulate metabolic pathways especially lipid metabolisms in cells. Lipid metabolic pathways play an important role in viral entry and replication inside the target cells. SARS-CoV-2 infection interfered with the regulation of lipid metabolism with the higher concentration of free fatty acids, lysophosphatidylcholine, lysophosphatidylethanolamine, and phosphatidylglyceroland significant lower concentration of total cholesterol (TC), HDL-cholesterol and LDL-cholesterol levels in serum (Hu et al., 2020; Zheng et al., 2020). As observed during treatment of glioblastoma and SLE, chloroquine can also regulate metabolic pathways during SARS-CoV-2 infection as its therapeutic mode of action.

CHLOROQUINE AGAINST OTHER DISEASES

Apart from being an FDA approved anti-malaria, anti-RA, and anti-LE drug, chloroquine has been investigated against several other

TABLE 3 | Examples of chloroquine used in treatment of multiple diseases.

S. no	Name of Disease	Name of the drug	Mode of Action	Reference
1	Graft-versus-host disease (GVHD)	Chloroquine	Alterations in T-cell cytokine production	Schultz et al. (2002)
2	Graft-versus-host disease (GVHD)	Hydroxychloroquine	Immunomodulator	Gilman et al. (2012)
3	Porphyria cutanea tarda (PCT)	Chloroquine	Release of bound hepatic porphyrin and its rapid elimination	Scholnick et al. (1973)
4	Porphyria cutanea tarda (PCT)	Hydroxychloroquine	Interaction with large amounts of porphyrins	Singal et al. (2012)
5	Porphyria cutanea tarda (PCT)	Hydroxychloroquine	Interaction with large amounts of porphyrins	Singal et al. (2019)
6	Sarcoidosis	Chloroquine and hydroxychloroquine	Suppression of the granulomtous inflammation	Beegle et al. (2013)
7	Sarcoidosis	Chloroquine and hydroxychloroquine	Not established	Sharma (1998)
8	Granuloma annulare	Chloroquine and hydroxychloroquine	Not established	Masmoudi et al. (2006)
9	Granuloma annulare	Chloroquine and hydroxychloroquine	Anti-inflammatory responses	Rodriguez-Caruncho and Marsol (2014)
10	Lichen planus	Chloroquine	Not established	De Argila, et al. (1997)
11	Lichen planus	Hydroxychloroquine	Lowering the expression of regulatory T cells	Zhu et al. (2014)
12	Urticaria vasculitis	Chloroquine	Not established	Loricera et al. (2014)
13	Osteoporosis	Chloroquine	Decreases the intracellular pH in mature osteoclasts and stimulates cholesterol uptake	Both et al. (2018)
14	Osteoporosis	Chloroquine	Not established	Stapley (2001)
15	Avascular Necrosis	Chloroquine	Immunomodulator	Roberts et al. (2018)
16	Diabetes Type II	Chloroquine	Alterations in insulin metabolism and signaling through cellular receptors	Hage et al. (2014)
17	Diabetes Type II	Chloroquine	ATM activation	McGill et al. (2019)
18	Diabetes Type II	Chloroquine	Reduction in lysosomal degradation of the internal insulin-insulin receptor	Wondafrash et al. (2020)
19	Cardiovascular Diseases	Chloroquine and hydroxychloroquine	Decreased levels of total cholesterol, triglycerides, and low-density lipoprotein-cholesterol (LDL-c)	Liu et al. (2018)
20	Thrombosis	Chloroquine	Inhibition of neutrophil extracellular traps	Boya et al. (2003)
21	Thrombosis	Chloroquine	Disaggregation of ADP-stimulated platelets and inhibition of thrombin-and A23187-induced aggregation	Jancinová et al. (1994)
22	Glanders and melioidosis	Chloroquine	pH Alkalinization of type 6 Secretion System 1 and Multinucleated Giant Cells	Chua et al. (2016)
23	Q fever	Chloroquine	Restore intracellular pH allowing antibiotic efficacy for *Coxiella burnetii*	Calza et al. (2002)
24	Whipple's disease	Chloroquine	The downregulation of tumour necrosis factor-a expression	Lagier et al. (2014)
25	Whipple's disease	Hydroxychloroquine	Not established	Alegre et al. (2012)
26	Giardiasis	Hydroxychloroquine	Not established	Escobedo et al. (2014)
27	Antiphospholipid syndrome	Hydroxychloroquine	Reduces antiphospholipid antibodies levels	Nuri et al. (2017)
28	Antiphospholipid syndrome	Hydroxychloroquine	Reduces antiphospholipid antibodies levels	Wang and Lim (2016)
29	Antifungal activity against *H. capsulatum* and *C. neoforman*	Chloroquine and hydroxychloroquine	Inhibition of phagolysosomal fusion and by expression of a unique endogenous H+-ATPase	Rolain et al. (2017)
30	Antifungal activity against *Aspergillus niger*	Hydroxychloroquine	pH-dependent iron deprivation	Keshavarzi et al. (2016)

prevalent medical conditions. **Table 3** summarises the use of chloroquine in the treatment of other diseases and its mode of action.

CHLOROQUINE TOXICITY

Chloroquine and the derivatives while using as an anti-malaria drugs in Mâncio Lima, Acre, Brazil, caused itching, stinging sensation, epigastric pain, and diarrhoea (Braga et al., 2015). It was explained by enhanced production of IgE, degranulation of mast cells and basophils creating allergy like reaction. However, severe side-effects including mental confusion, seizures, coma, and cardiovascular symptom, was not reported. Adedapo et al. (2009) observed that higher dosage of chlorpheniramine plus chloroquine (10 mg/kg daily for 3 days) in children below 5 years caused drowsiness and lower respiratory rates, though no additional benefits were obtained. Chloroquine is known to induce concentration-dependent cytotoxicity, which should always be optimized before finalizing the dosage.

Though optimized dosage and short-term treatment of RA with CQ and HCQ was considered safe, long-term use of CQ in a 64-year-old woman resulted in both restrictive and hypertrophic myocardiopathy auricular-ventricular blocks due to long term pH alteration in the lysosome (Cervera et al., 2001). Kelly et al. (1990) also focussed on the narrow margin between therapeutic uses of chloroquine against RA and the chloroquine poisoning. They reported the death of a 12-month-old infant after receiving 300 mg of chloroquine. They also highlighted the different dose optimization of chloroquine for adults and infants. Scherbel et al., (1965) in

their clinical trials found that out of 741 patients treated with chloroquine derivative for SLE, 31-68% developed retinopathy and marked destruction of rod and con cells. However, no clear relationship between chloroquine dosage and retinal toxicity could be established. Lane et al., (2020) performed a multinational retrospective study to evaluate the risk of HCQ alone and in combination with azithromycin in 956,374 RA patients (18 years and above). It was observed that a 30-days dose of HCQ demonstrated no risk of adverse events. However, long term use of HCQ alone increased cardiovascular mortality. A combination of HCQ and azithromycin elevated the risk of heart failure even in the short term. Therefore, the authors suggest a careful consideration of benefit:risk ratio when starting HCQ treatment (Lane et al., 2020).

It is difficult to estimate the frequency of adverse events because many cases have been reported in more than one publication and lack a criterion for diagnosis (Scherbel et al., 1965). Hence, it is recommended to evaluate cardiac health with ECG and ophthalmological examinations for 6 months before prescribing a long-term treatment with chloroquine (Scherbel et al., 1965; Kelly et al., 1990; Cervera et al., 2001).

Chloroquine, as a chemotherapeutic agent against cancer, can act as a double-edged sword. It not only sensitizes the cancer cells but also the normal cells by blocking autophagy and impairing lysosome and endosomes' function (Kimura et al., 2013; Choi et al., 2018). Kidney is the most critically affected organ during chemotherapy with chloroquine with significant nephrotoxicity (Klionsky et al., 2016; Wang B. et al., 2020). Evangelisti et al. (2015) also reviewed the substantial side effects of chloroquine while treating acute leukaemia. Repurposing chloroquine against cancer was generally considered safe for short term treatment with optimized dosage. However, patients suffering from glucose-6-dehydrogenase deficiency, impaired hepatic and kidney diseases should always be cautious while practising chloroquine and derivatives as a chemotherapeutic agent (Verbaanderd et al., 2017). A clinical trial (ClinicalTrials.gov ID NCT04201457) by "Pediatric Brain Tumor Consortium" is ongoing to assess the safety and benefit of adding hydroxychloroquine to dabrafenib and/or trametinib in children with recurrent or progressive low grade or high-grade brain tumor with specific genetic mutations whose results are waited in February 2025 (https://clinicaltrials.gov/ct2/show/results/NCT04201457).

The standard and optimized dose of chloroquine as prophylaxis and during treatment of diseases do not bear significant toxicity, however, long term use of higher concentration of chloroquine can result in severe toxicity (**Table 4**). Use of less toxic derivatives such as hydroxychloroquine, optimized dosage, nanoencapsulation of the drug and combinational therapies have been used for reducing chloroquine toxicity and increasing efficacy (Amolegbe et al., 2018; Lima et al., 2018).

Hypokalemia toxicity is commonly observed in chloroquine and hydroxychloroquine overdose due to the intracellular shift of potassium. Clemessy et al. (1995), performed a retrospective study of 191 cases of chloroquine toxicity in which the initial clinical features included, gastro-intestinal disturbances, neurological impairment, and respiratory symptoms and eventual blockage of potassium channels contributed to hypokalaemia (Clemessy et al., 1995).

Neuropsychiatric manifestations including depression, psychosis, insomnia, agitation have also been reported due to acute or chronic use of chloroquine and hydroxychloroquine (Juurlink, 2020).

Hematologic toxicities are attributed to its long half-life in plasma which leads to accumulation in the blood cells. Lymphopenia, eosinophilia is typically observed immunologically mediated idiosyncratic drug reactions (Juurlink, 2020).

Prolongation of the QT interval due to both chloroquine and hydroxychloroquine has also been observed since the drugs interfere with vascular repolarization. It was observed that after a dose of 600 mg QTc increases 6.1 and 28 ms after a dose of 1,200 mg. However, this effect varied in younger age groups. In response to this treatment in COVID-19 patients, ventricular tachycardia and ventricular fibrillation and mortality were observed potentially due to the overdosage. Hence, in a COVID-19 setting FDA cautions the use of HCQ or CQ, but not in cases of malaria, lupus and RA (https://www.fda.gov/drugs/drug-safety-and-availability/fda-cautions-against-use-hydroxychloroquine-or-chloroquine-covid-19-outside-hospital-setting-or). In severe COVID-19 cases where azithromycin was co-prescribed in combination with either chloroquine and hydroxychloroquine, Molina et al. (2020) reported no evidence of rapid anti-viral clearance or any associated clinical benefit in only 11 patients, possibly because they had significant comorbidities such as obesity, cancer, HIV infection (Molina et al., 2020). However, in a retrospective study 3,737 COVID-19 patients treated with hydroxychloroquine/azithromycin and other regimens in Lagier et al. (2020) observed otherwise. Along with 3 days early treatment of HCQ-azithromycin resulting in faster viral load reduction, no cases of *torsade de pointe* or sudden death were observed. This could be because the patients belonged to mean age of 45 years, the treatment was initiated very early with a dosage of 200 mg of oral HCQ, three times daily for ten days and 500 mg of oral azithromycin on day 1 followed by 250 mg daily for the next four days, respectively.

Sacher et al., (2020) propose a pragmatic approach to mitigate the cardiac risk in the COVID-19 setting. The authors propose a cardiac algorithm for critically reviewing patient's clinal history (use of other drugs that may extend QT interval, levels of serum K^+, creatinine, and a recent 30 s ECG). In cases of QT intervals >500 ms, the authors recommend that a QT-prolonging drug should not be prescribed (Sacher et al., 2020).

Some rare immunologically mediated adverse reactions including Stevens-Johnson syndrome, toxic epidermal necrolysis, DRESS (drug reaction with eosinophilia and systemic symptoms), have been implicated in chloroquine and hydroxychloroquine treatment against viral diseases. Although rare, these conditions turn into serious entities when accompanied by liver or kidney injury (Juurlink, 2020).

RESULTS OF CLINICAL TRIALS DONE SO FAR WITH CHLOROQUINE AGAINST COVID-19

Currently, there are multiple clinical trials underway to investigate the potential use of hydroxychloroquine and chloroquine alone or

TABLE 4 | Toxic effects of chloroquine.

S. no	Drug	Toxicity[a]	Concentration/Duration/Dosage	Reference
1	Chloroquine	Ocular toxicity	250 mg of chloroquine per day for 6 monthe-14 years	Puavilai et al. (1999)
2	Hydroxy-chloroquine	Retinopathy	Inadequately Weight Adjusted Dosage	Arendt and Gerding (2017)
3	Hydroxy-chloroquine	Retinopathy	≥5 mg per kilogram per day	Zaidi et al. (2019)
4	Chloroquine	NeuromytoToxicity	200–500 mg per day for 7m-16 years	Estes et al. (1987)
5	Chloroquine	Neurotoxicity	Variable concentration in culture media	Bruinink et al. (1991)
6	Chloroquine	Renal toxicity	50 mg^{-1}kg for 4weeks	Wang et al. (2020)
7	Chloroquine	Renal toxicity	—	Wiwanitkit (2015)
8	Chloroquine and hyrdoxy-chloroquine	Cutaneous toxicity	200–500 mg/day for 7 years	Martin-Garcia et al. (2003)
9	Hyrdoxy-chloroquine	Stevens-Johnson syndrome	40 mg/day for 2 weeks	Leckie and Rees (2002)
10	Amodiaquine	Hematological toxicity	—	Parhizgar and Tahghighi (2017)
11	Chloroquine	Leukemia	For several months	Nagaratnam et al. (1978)
12	Chloroquine	Hepatotoxicity	Combination of proguanil 200 mg and chloroquine 100 mg	Wielgo-Polanin et al. (2005)
13	Hydroxy-chloroquine	Ototoxicity	Hydroxychloroquine 5 mg/kg/day (400 mg/day)	Fernandes et al. (2018)
14	Chloroquine	Cardiotoxicity	250 mg/day for 9 years	Teixeira et al. (2002)
15	chloroquine	Alveolitis	For two weeks	Mitja et al. (2000)
16	Chloroquine and hydroxyl-chloroquine	Myopathy	3.5 mg/kg/day for chloroquine and 6.5 mg/kg/day for hydroxychloroquine for 40.4 months	Casado et al. (2006)
17	Chloroquine	Pruritus	—	Aghahowa et al. (2010)

[a]*The frequency of chloroquine induced toxicity and adverse effects is difficult to estimate due to lack of common methods of diagnosis and metrics of evaluation.*

in combination against SARS-CoV-2 (**Table 5**). Some of the *in vitro* and *in vivo* results obtained with chloroquine and hydroxychloroquine supported their anti-viral role against SARS-CoV-2, (Andreani et al., 2020; Clementi et al., 2020; Fantini et al., 2020; Gao et al., 2020; Gautret et al., 2020; Liu et al., 2020; Weston et al., 2020; Yao et al., 2020), however, results of Gautret et al. (2020) faced severe criticism because of small sample size, overruling type I errors, inconsistency between study protocols and lack of blinding and a control arm even though the treatment resulted in viral load reduction. It is also very important to reproduce the *in vitro* results obtained with chloroquine in the *in vivo* studies and clinical trials to establish it as a safe and effective anti-SARS-CoV-2 drug.

The studies by Patel and coworkers (https://www.sciencemag.org/news/2020/06/mysterious-company-s-coronavirus-papers-top-medical-journals-may-be-unraveling; Mehra et al., 2020a; Mehra et al., 2020b), claimed to have performed a multinational registry analysis using a cloud-based health-care data analytics platform, Surgisphere Corporation, Chicago, IL, United States, on the usage of hydroxychloroquine or chloroquine with or without a macrolide for the treatment of COVID-19. They reported an increased risk of in-hospital mortality and de-novo ventricular arrhythmia in response to the treatment which led to the inference that hydroxychloroquine or chloroquine, when used alone or with a macrolide does not offer any benefit to the COVID-19 patients, which contributed to the halt in worldwide clinical trials by the WHO on May 25, 2020. The second study (Mehra et al., 2020b) claimed to negate the association of ACE inhibitors and angiotensin-receptor blockers (ARBs) with in-hospital COVID-19 deaths. Their analyses brought forth better survival rates due to the use of either ACE inhibitors or statins. However, the authors mentioned that since the study was not based on randomized trials, there could be a possibility of confounding and hence, concluded that an underlying cardiovascular disease is independently associated with an increased risk of in-hospital COVID-19 death.

Substantive red flags were raised by the rattled global scientific community because the doses in the reported cases were higher than those set by the United States FDA and discrepancies in the official COVID-19 mortality statistics, and sample size (https://www.sciencemag.org/news/2020/06/mysterious-company-s-coronavirus-papers-top-medical-journals-may-be-unraveling). Eventually, both the studies were retracted from the journals, The Lancet and The New England Journal of Medicine. Currently, clinal trials in various parts of the world have resumed to investigate the potential use of hydroxychloroquine in COVID-19 patients in response to WHO's green signal (https://www.sciencemag.org/news/2020/06/mysterious-company-s-coronavirus-papers-top-medical-journals-may-be-unraveling). In another report by Geleris et al. (2020) reported no positive or negative observational effect of hydroxychloroquine on death or incubation risk on COVID-19 patients, however, this study did support the further randomized clinical trials of hydroxychloroquine testing its efficacy.

The United Kingdom's mega RECOVERY trial (RECOVERY Collaborative Group, 2020) reported the ineffectiveness of hydroxychloroquine. The patients who received the treatment demonstrated a longer hospitalization duration, higher risk of mechanical ventilation or mortality than those who received the usual care. However, the study received sceptical reviews due to the high dosage issues: 800 mg at 0 and 6 h, followed by a 400 mg dose at 12 h and every 12 h thereafter for 9 days; which may have contributed to cardiovascular, neurological, and other toxicities. The authors chose this dosage based on extensive pharmacokinetic studies.

On December 2nd, 2020, the WHO Solidarity Trial Consortium published the findings of their trials on

TABLE 5 | Ongoing clinical trials to evaluate the potential of chloroquine and hydroxychloroquine against SARS-CoV-2.

S. no	Clinical trial no	Location	Details	Dosage	Current status	Results	Reference
1	NCT04328493 (April 7, 2020)	Vietnam	Randomized trial, 250 participants	250 mg chloroquine tablet Adult ≥53 kg: 4 tabs Adult 45–52 kg: 3.5 tabs Adult <38 kg: 2.5 tabs	Phase 2	Expected by April 1, 2022	https://clinicaltrials.gov/ct2/show/NCT04328493
2	NCT04358068 (May 1, 2020)	United States	Randomized, 2,000 participants	efficacy of hydroxychloroquine (HQ) and azithromycin (Azi) Day 0: HQ 400 mg (200 + 200) + Azi 500 mg (250 + 250) orally Day 1–6: HQ 200 mg (twice/day) + Azi 250 mg (4 days)	Phase 2	Expected by March 5, 2021	https://clinicaltrials.gov/ct2/show/NCT04358068
3	NCT04333654 April 12, 2020	United States, Belgium, France, Netherlands	Randomized, 210 participants	HQ loading dose on day 1, maintenance dose till day 9	Phase 1	Expected by August 2020	https://clinicaltrials.gov/ct2/show/NCT04333654
4	NCT04358081May 1, 2020	United States	Randomized, 444 participants	HQ monotherapy (600 mg) and in combination With Azi (200 mg) HQ (600 mg) with or without Azi (500 mg)	Phase 3	Expected by July 24, 2020	https://clinicaltrials.gov/ct2/show/NCT04358081
5	NCT04381936 (March 19, 2020)	United Kingdom	Randomized, 12,000 participants	Oral dose Initial: 800 mg, 6h: 800 mg, 12h:400 mg, 24h: 400 mg, every 12h thereafter for 9 days: 400 mg	Stopped	No clinical benefit. Out of 1,542 patients administered with hydroxychloroquine, no significant difference in primary endpoint of 28-days mortality. (25.7% HQ as compared with 23.5% usual care alone)	https://clinicaltrials.gov/ct2/show/record/NCT04381936; https://www.recoverytrial.net/files/hcq-recovery-statement-050620-final-002.pdf
6	NCT04308668 (March 17, 2020)	United States, Canada	Randomized, 3,000 participants	Oral dose 200 mg tab Initial: 800 mg orally once 4 days: 600 mg (every 6–8 h)	Phase 3	After high or moderate risk exposure to COVID-19, HQ did not prevent illness when used as postexposure prophylaxis within 4 days after exposure	https://clinicaltrials.gov/ct2/show/NCT04308668
7	NCT04304053 (March 18, 2020)	Spain	Randomized, 2,250 participants	Testing, treatment and prophylaxis of SARS-CoV-2 Oral dose 200 mg tabs Day 1: 800 mg Day 2–7: 400 mg Contacts Day 1: 800 mg Day 2–4: 400 mg	Phase 3	No significant results	https://clinicaltrials.gov/ct2/show/NCT04304053; https://www.sciencemag.org/news/2020/06/three-big-studies-dim-hopes-hydroxychloroquine-can-treat-or-prevent-covid-19
8	NCT04303507 (April 29, 2020)	Thailand, United Kingdom	Randomized, 40,000 participants	Prophylaxis Study Loading dose: 4 tabs of 155 mg/60 kg body weight 90 days: 155 mg daily	Not mentioned	Expected by April 2021	https://clinicaltrials.gov/ct2/show/NCT04303507

repurposed anti-viral drugs for COVID-19 (NCT04315948). The drugs included hydroxychloroquine, remdesivir, lopinavir, and interferon beta-1a in hospitalized COVID-19 patients. The randomized trials were evaluated for death rate according to age and requirement of mechanical ventilation. Like the RECOVERY trials, this one too reported negligible effect on mortality, ventilation, and hospitalization duration of COVID-19 patients (WHO Solidarity Trial Consortium, 2020).

POINTS TO BE CONSIDERED IN THE CURRENT PANDEMIC TIME WITH CHLOROQUINE AS A THERAPEUTIC

Several ongoing clinical trials against COVID-19 with chloroquine/hydroxychloroquine alone or in a combination of drugs are the outcome of promising *in vitro* results and the hype created worldwide over the drug (Cortegiani et al., 2020). Giving

too much of attention by the scientific community generates false promises and hampers the path of other potential drugs against COVID-19 in this pandemic era. Simultaneously, no negative feedback against chloroquine should be postulated without confirming the clinical trial results. Chloroquine being an age-old drug, has already been used against multiple diseases. If found effective, its inexpensive nature and already documented toxicity profile and dosage optimization can save time and a million lives. Though mixed results of chloroquine against COVID-19 have been obtained so far, there is an urgent need to test their effects and toxicity as a prophylactic drug, in mildly ill patients and severely ill patients of COVID-19. Moreover, one should never forget the thin line between chloroquine as a therapeutic agent and chloroquine poisoning (Kelly et al., 1990). An in-depth toxicity analysis of chloroquine and derivatives is required before confirming any comment for/against its use in time of COVID-19. The poor methods of clinal trials and its reporting has thus far been inadequate in proving the effective nature of hydroxychloroquine. Ferner and Aronson (2020) claim that the overuse of hydroxychloroquine will result in rare buy harmful cutaneous adverse reactions, hepatic problems and ventricular arrhythmias when prescribed in combination with azithromycin. In a recent study, Haque et al. (2021) reported the changes in purchasing patterns and pricing of hydroxychloroquine since March 2020 in India states. While no price and utilization changes were observed, hydroxychloroquine shortages were encountered due to the misinformation and management of COVID-19.

Among the rapidly changing guidelines in this pandemic era of COVID-19, WHO has revoked the ban on clinical trials with chloroquine against COVID-19 (https://www.sciencemag.org/news/2020/06/mysterious-company-s-coronavirus-papers-top-medical-journals-may-be-unraveling), however, FDA has cautioned its use outside of the hospital setting or a clinical trial due to risk of heart rhythm problems (https://www.fda.gov/drugs/drug-safety-and-availability/fda-cautions-against-use-hydroxychloroquine-or-chloroquine-covid-19-outside-hospital-setting-or). Though the preventive or prophylactic potential of chloroquine and the derivatives are yet to be confirmed against COVID-19, extensive, collaborative, unbiased and random clinical trials are required instead of small and individual trials to conclude. Results of unprejudiced, statistically significant and ethical outcomes of clinical trials are eagerly awaited before sealing the fate of this age-old drug against COVID-19.

CONCLUSION AND FUTURE ASPECTS

The "age-old" drug used to treat multiple diseases has generated mixed therapeutic responses against "COVID-19." Chloroquine has been recognized as a miracle medicine to treat malaria, autoimmune diseases, cancer, viral, dermatological, and fungal infections. Different *in vitro* and *in vivo* studies have suggested the positive, neutral, and negative role of chloroquine and derivatives against SARS-CoV-2. Though some studies are still ongoing, different probable mechanisms have been reported in literature employed by chloroquine to inhibit SARS-CoV-2 infection or cause more harm than good. In this difficult situation where an effective anti-viral drug is urgently needed, a biased decision against or in favour of chloroquine can either generate a false sense of security or can add more anxiety in an already worse situation. However, the previous research done on chloroquine against multiple diseases can help establish its anti-SARS-CoV-2 mechanism, precautions to be taken to avoid chloroquine's toxicity, and dosage–optimization to reach any conclusion.

AUTHOR CONTRIBUTIONS

MK conceptualized the idea. The manuscript was written, literature was collected and finalized by SK and MK.

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ORIGINAL RESEARCH
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No Efficacy of the Combination of Lopinavir/Ritonavir Plus Hydroxychloroquine Versus Standard of Care in Patients Hospitalized With COVID-19: A Non-Randomized Comparison

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Objectives: No specific treatment has been approved for COVID-19. Lopinavir/ritonavir (LPV/r) and hydroxychloroquine (HCQ) have been used with poor results, and a trial showed advantages of combined antiviral therapy vs. single antivirals. The aim of the study was to assess the effectiveness of the combination of antivirals (LPV/r and HCQ) or their single use in COVID-19 hospitalized patients vs. standard of care (SoC).

Methods: Patients ≥18 years with SARS-CoV-2 infection, defined as positive RT-PCR from nasal/oropharyngeal (NP/OP) swab or positive serology, admitted at L. Spallanzani Institute (Italy) were included.

Primary endpoint: time to invasive ventilation/death. Secondary endpoint: time to two consecutive negative SARS-CoV-2 PCRs in NP/OP swabs. In order to control for measured confounders, a marginal Cox regression model with inverse probability weights was used.

Results: A total of 590 patients were included in the analysis: 36.3% female, 64 years (IQR 51–76), and 91% with pneumonia. Cumulative probability of invasive ventilation/death at 14 days was 21.2% (95% CI 17.6, 24.7), without difference between SOC, LPV/r, hydroxychloroquine, HCQ + LPV/r, and SoC. The risk of invasive ventilation/death in the groups appeared to vary by baseline ratio of arterial oxygen partial pressure to fractional inspired oxygen (PaO2/FiO2). Overall cumulative probability of confirmed negative nasopharyngeal swabs at 14 days was 44.4% (95% CI 38.9, 49.9), without difference between groups.

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Conclusion: In this retrospective analysis, we found no difference in the rate of invasive ventilation/death or viral shedding by different strategies, as in randomized trials performed to date. Moreover, even the combination HCQ + LPV/r did not show advantages vs. SoC.

Keywords: SARS-CoV-2, antivirals, drug repurposing, viral shedding, invasive ventilation

INTRODUCTION

In December 2019, an outbreak of viral pneumonia cases of unknown cause was identified in Wuhan, China. A novel coronavirus was quickly identified in some of these patients and it has been designated as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (European Centre for Disease Prevention and Control, 2020). Currently, there are no approved therapeutic agents available for SARS-CoV-2, and great efforts have been unfolded for the discovery of possible treatment strategies. Many repurposed drugs have shown some preclinical activity against SARS-CoV-2 and have been experimented *in vivo* (Tobaiqy et al., 2020). Prompt identification and implementation of life support therapies are pivotal steps in order to prevent the spreading of the infection and improve patient's clinical outcome. Some data about treatment from observational studies, compassionate use programs, and few RCT results are available up-to-date.

Among the antiviral strategies, the antiretroviral drug lopinavir/ritonavir (LPV/r) had already demonstrated activity against SARS-CoV-2 and Middle East respiratory syndrome (MERS)-CoV (Ford et al., 2020). Two initial RCTs about lopinavir/ritonavir for treatment of SARS-Cov-2 showed inconclusive results. A small randomized, controlled, open-label trial conducted in China did not observe any benefit of lopinavir/ritonavir treatment vs. standard care in reducing the time to clinical improvement in hospitalized adult patients with severe COVID-19. However, the study appeared to be underpowered and post hoc analyses showed accelerated clinical recovery (16.0 vs. 17.0 days) and reduced mortality (19.0 vs. 27.1%) in the subgroup of patients treated within 12 days after the onset of symptoms (Cao et al., 2020). Another very small RCT comparing lopinavir/ritonavir vs. arbidol for treating patients with mild/moderate COVID-19 showed no differences in term of viral clearance, symptoms resolution, and radiological improvement between the arms (Li et al., 2020).

Follow-up retrospective studies did not show evidence of effectiveness of lopinavir/ritonavir and of other antiretrovirals, as recently systematically reviewed (Ford et al., 2020).

A possible antiviral activity against SARS-CoV-2 of hydroxychloroquine (HCQ) has been supposed. This drug inhibits the glycosylation of ACE II, the receptor used by SARS-CoV-2 to enter the cells, and could result in a reduced ligand recognition and internalization of the virus (Vincent et al., 2005). This activity, together with the best known immunomodulatory and anti-inflammatory effects, yielded HCQ an interesting drug in this contest, but the most recent results showed lack of efficacy.

A review of seven clinical trials has shown contrasting results, but the analyzed studies posed significant risk for bias in the randomization process, in measurement of outcomes, or in deviations from planned interventions (Chowdhury et al., 2020). Recent data from the RECOVERY trial, a large randomized study, showed no evidence of benefit for mortality or other outcomes (duration of hospitalization and need for invasive ventilation) of HCQ treatment in hospitalized patients with COVID-19. Indeed, day-28 mortality was reported as 25.7% with HCQ and 23.5% with comparator (hazard ratio 1.11, 95% CI 0.98–1.26, p = 0.10), so the investigators announced closure of the HCQ arm due to lack of effectiveness (Recovery Randomised Evaluation of COVID-19 Therapy, 2020a). Similarly, also the LPV/r arm in RECOVERY was halted for the same reasons (World Health Organization, 2020).

Even the ORCHID study, a clinical trial evaluating the safety and effectiveness of hydroxychloroquine for the treatment of hospitalized adults with COVID-19, has been halted by NIH (NIH, 2020b). Solidarity trial showed no effect of hydroxychloroquine or lopinavir/ritonavir on hospitalized patients with COVID-19, as indicated by overall mortality, initiation of ventilation, and duration of hospital stay (WHO Solidarity Trial Consortium et al., 2020). Even in terms of antivirals' effectiveness on ending SARS-COV-2 shedding, conflicting results about the clinical role of HCQ have been published. In fact, one report observed a positive impact on viral shedding (Huang et al., 2020), but a large RCT showed no difference in probability of negative conversion (Tang et al., 2020). Currently, NIH guidelines recommend against the use of HCQ or LPV/r for treatment of COVID-19 because of lack of effectiveness (NIH, 2020a).

However, triple combination of interferon beta-1b, lopinavir/ritonavir, and ribavirin resulted to be superior to lopinavir/ritonavir alone in alleviating symptoms and shortening the duration of viral shedding and hospital stay in patients with mild-to-moderate COVID-19 (Hung et al., 2020). Thus, these data provided a proof of concept for the possible synergic effect of using a combination of two or more antivirals to improve effectiveness like that seen for other infections such as HIV. More recently, an in silico approach proposed a possible synergistic effect of 16 compounds with independent mechanism of action in SARS-CoV-2 (Bobrowski et al., 2021).

The purpose of this study was to explore the difference in effectiveness of single antivirals (lopinavir/ritonavir and HCQ) and their combination when compared to current standard of care (SoC) in COVID-19 hospitalized patients, by emulating a RCT using the observational retrospective data of the INMI COVID-19 database.

MATERIALS AND METHODS

We conducted a retrospective cohort study on the INMI COVID-19 database of L. Spallanzani Institute in Rome (Italy) that contains data from consecutive hospitalized patients (≥18 years of age) who had a positive test result for the SARS-CoV-2 virus at any time during their hospitalization from January 29 to June 13, 2020. Participants' follow-up of those not yet discharged was administratively censored on July 1st, 2020. INMI COVID-19 database was approved by the local INMI, Rome Ethical Committee and patients provided written informed consent. The study was performed in accordance with the Declaration of Helsinki. INMI COVID-19 database retrieves epidemiological, demographic, clinical, and laboratory data of patients, as well as therapy prescribed (antiviral, immunomodulatory drugs, oxygen therapy, and need for ventilation) for COVID-19 patients.

Patients were included in this study if the following inclusion criteria were satisfied: ≥18 years of age, a diagnosis of SARS-CoV-2 infection, defined as positive RT-PCR from nasal/oropharyngeal (NP/OP) swab or positive serology, and admitted at INMI L. Spallanzani Institute.

This is a retrospective study which was conducted in exceptional conditions during the first wave of COVID-19 pandemic in Italy, so sample size was not preplanned.

In all included patients, diagnosis of SARS-CoV-2 infection was confirmed by the detection of SARS-CoV-2 RNA through real-time polymerase chain reaction (RT-PCR) targeting the E and RdRp viral genes on NP/OP swab. Subsequently, during the hospitalization, all patients underwent follow-up NP/OP swab to assess the clearance of viral RNA. The timing of follow-up NP/OP swab was variable, according to treating physician's judgment.

We compared four treatment strategies initiated after hospital admission: 1) starting hydroxychloroquine; 2) starting lopinavir/ritonavir; 3) starting hydroxychloroquine plus lopinavir/ritonavir; and 4) a control group receiving none of the previous drugs (standard of care). Standard of care included any supportive therapy: fluids, antibiotics, oxygen supplementation, and any concomitant therapy except for HCQ and LPV/r. Concomitant use of therapy with immunomodulants (e.g., anti-IL6 and anti-JAK), corticosteroids, heparin, and antibiotics (including azithromycin) was controlled for in the analysis. The decision of whether to treat patients with off-label hydroxychloroquine or lopinavir/ritonavir or other drugs was based on local medical consensus, guidelines, and the clinicians' own opinion.

The most commonly prescribed dosage of HCQ was 400 mg orally bid in the first day, followed by 200 mg bid for a total of 10 days and of lopinavir/ritonavir was 400/100 mg orally bid for 14 days.

The start of follow-up (baseline) for each patient was the first start of any therapy. All patients were followed up from baseline until death, discharge, last available visit, or the administrative censored date of July 1st, 2020, whichever occurred first.

The ratio of arterial oxygen partial pressure (PaO2) to fractional inspired oxygen (FiO2) between ≤300 mmHg was used as marker of severe respiratory disease, according to NIH guidelines (NIH, 2020a), for the stratified analysis.

The primary endpoint of this study was the evaluation of time from starting of therapy to invasive ventilation or death (whichever occurred first).

The secondary endpoints were 1) the evaluation of time from treatment initiation to two consecutive negative SARS-CoV-2 PCRs in nasal/oropharyngeal swabs, without an in-hospital relapse; 2) the evaluation of time from starting of therapy to noninvasive or invasive ventilation or death (whichever occurred first). Noninvasive ventilation includes CPAP or NIV.

For the secondary endpoint of evaluation of viral shedding, patients with diagnosis of COVID-19 infection made by SARS-CoV-2 serology were excluded from the analysis.

For the statistical analysis, chi-square or nonparametric Kruskal–Wallis tests were used to compare categorical or continuous variables in descriptive analysis, respectively. Besides age and PaO2/FiO2, which showed approximately symmetric distribution, all other continuous variables showed skewed distributions and are therefore expressed as median values with interquartile ranges (IQR). Comparison of age and PaO2/FiO2 by the parametric ANOVA test led to identical conclusions (data not shown).

Unweighted Kaplan–Meier curves were used to compare cumulative probabilities of invasive ventilation/death and of confirmed negative NP/OP swabs by treatment group. Stratification for baseline PaO2/FiO2 (0–300 mmHg vs. > 300 mmHg) and interaction test were used to test whether response to treatment groups might differ in subsets of participants.

A marginal Cox regression model with inverse probability of treatment weighting approach (IPW) was used to balance the differences in baseline and time-varying variables between treatment groups. Propensity scores to construct the weights were based on a vector of potential confounders identified a priori on the basis of axiomatic knowledge and previously published results. These included time-fixed variables measured at entry (i.e., gender, age, extent of comorbidity, and duration of symptoms), as well as time-varying confounders affected by initial treatment choice such as intensification by use of azithromycin, anticoagulants, steroids, and immunomodulatory drugs. A double-robust estimator was used, controlling also for potential informative censoring. The assumption of no positivity was checked by inspecting the distribution of the standardized combined weights. In a subset of participants with available data, we further controlled for baseline levels of inflammation and coagulation (CRP, ferritin, and d-dimer). In an additional sensitivity analysis, severity of disease at baseline was controlled using a diagnosis of pneumonia at entry in the study instead of the PaO2/FiO2 level.

An intention-to-treat approach was used. For endpoints including individual components of the composite endpoint (e.g., separately only invasive ventilation or death), the first event occurred was counted.

All statistical analyses were performed with SAS statistical package version 9.4 (Carey NC, United States).

TABLE 1 | Demographics, comorbidities, sign, and symptoms of the overall population and of the 4 groups.

Characteristic	Intervention					
	LPV/r	HCQ	LPV/r + HCQ	SoC	p-value[a]	Total
	N = 124	*N* = 109	*N* = 244	*N* = 113		*N* = 590
Age, years, median (IQR)	64 (53, 75)	69 (55, 79)	61 (50, 74)	69 (49, 82)	0.076	64 (51, 76)
Female gender, *n* (%)	41 (33.1%)	51 (46.8%)	73 (29.9%)	49 (43.4%)	0.006	214 (36.3%)
Baseline Po2/FiO2, median (IQR)	252 (170, 326)	348 (277, 429)	333 (256, 386)	381 (300, 467)	<0.001	324 (244, 398)
Baseline Po2/FiO2 < 300, *n* (%)	53 (66.3%)	28 (35.4%)	64 (37.2%)	17 (26.2%)	<0.001	162 (40.9%)
Pneumonia, *n* (%)	118 (95.2%)	104 (95.4%)	238 (97.5%)	77 (68.1%)	<0.001	537 (91.0%)
Follow-up, days	10 (5, 31)	12 (5, 26)	12 (6, 23)	10 (4, 21)	0.560	11 (5, 23)
≥2 comorbidities[b]	26 (21.0%)	20 (18.3%)	35 (14.3%)	24 (21.2%)	0.287	105 (17.8%)
Comorbidities, *n* (%)						
Diabetes	19 (15.3%)	33 (30.3%)	34 (13.9%)	19 (16.8%)	0.091	105 (17.8%)
Hypertension	48 (38.7%)	68 (62.4%)	109 (44.7%)	39 (34.5%)	0.195	264 (44.7%)
Cardiovascular disease	34 (27.4%)	39 (35.8%)	65 (26.6%)	39 (34.5%)	0.008	177 (30.0%)
Chronic renal insufficiency	8 (6.5%)	8 (7.3%)	8 (3.3%)	9 (8.0%)	0.215	33 (5.6%)
Cancer	10 (14.7%)	43 (53.8%)	37 (23.7%)	26 (31.0%)	0.251	116 (29.9%)
HIV	2 (1.6%)	4 (3.7%)	0 (0.0%)	4 (3.5%)	0.213	10 (1.7%)
Days from symptoms onset to hospitalization, median (IQR)	8 (5, 11)	8 (3, 11)	9 (7, 12)	10 (3, 22)	0.020	9 (5, 12)
Sign and symptoms, *n* (%)						
Fever	101 (82%)	64 (62%)	211 (86%)	65 (62%)	<0.001	441 (77%)
Cough	88 (71.5%)	46 (44.7%)	153 (62.7%)	50 (50.0%)	<0.001	337 (59.1%)
Myalgia	10 (8.1%)	9 (8.7%)	28 (11.5%)	16 (16.0%)	0.338	63 (11.1%)
Conjunctivitis	3 (2.4%)	4 (3.9%)	6 (2.5%)	11 (11.0%)	0.479	24 (4.2%)
Headache	13 (10.6%)	10 (9.7%)	23 (9.4%)	17 (16.7%)	0.535	63 (11.0%)
Dyspnea	53 (43.1%)	36 (35.0%)	72 (29.5%)	44 (42.7%)	0.080	205 (35.8%)
Diarrhea	12 (9.8%)	12 (11.7%)	26 (10.7%)	17 (16.8%)	0.482	67 (11.7%)

[a]*Chi-square or Kruskal–Wallis test as appropriate.*
[b]*Asthma, cardiovascular disease, diabetes, hepatic disease, HIV, renal disease, hypertension, cancer, and TB.*

RESULTS

Population Characteristics

A total of 590 patients with diagnosis of COVID-19 were included in this analysis (demographic characteristics, signs, and symptoms are shown in **Table 1** and biomarkers in **Table 2**): 36.3% female, median age of 64 years (IQR 51–76), 91% with a diagnosis of pneumonia, median baseline PaO2/FiO2 of 324 (IQR 244–398) mmHg, days from onset of symptoms to hospitalization were 9 (IQR 5–12), and 17.8% with 2 or more comorbidities. The

TABLE 2 | Biomarkers and other baseline characteristics.

	Intervention					
	LPV/r	HCQ	LPV/r + HCQ	SoC	p-value[a]	Total
	N = 124	*N* = 109	*N* = 243	*N* = 112		*N* = 588
Neutrophils, N	4.2 (2.9, 6.4)	4.0 (2.8, 5.8)	3.7 (2.7, 5.6)	4.8 (3.2, 6.9)	0.031	4.1 (2.8, 5.9)
Neutrophils, %	72.0 (62.3, 82.3)	67.9 (57.9, 77.5)	70.1 (60.3, 79.7)	69.4 (56.0, 78.4)	0.095	69.9 (59.6, 79.7)
Total lymphocytes, N	1.0 (0.7, 1.4)	1.2 (0.9, 1.8)	1.1 (0.8, 1.4)	1.4 (1.0, 1.9)	<0.001	1.1 (0.8, 1.6)
Total lymphocytes, %	17.5 (11.1, 26.9)	22.6 (14.4, 30.5)	21.0 (12.8, 28.3)	20.5 (13.1, 31.2)	0.079	20.5 (12.7, 28.4)
Aspartate amino-transferase (AST), U/L	32.0 (23.5, 42.5)	25.0 (19.0, 41.0)	28.0 (22.0, 42.0)	24.0 (18.0, 38.0)	0.002	27.0 (21.0, 41.0)
Alanine amino-transferase (ALT), U/L	26.0 (18.5, 43.5)	22.0 (14.0, 41.0)	26.5 (16.0, 40.0)	22.0 (14.0, 35.0)	0.072	24.0 (16.0, 40.0)
Bilirubin, mg/L	0.6 (0.5, 0.9)	0.6 (0.5, 0.8)	0.7 (0.5, 1.0)	0.7 (0.5, 1.0)	0.003	0.7 (0.5, 0.9)
Hemoglobin, mg/L	13.8 (12.7, 14.9)	12.9 (11.6, 14.0)	13.7 (12.7, 15.0)	13.2 (11.6, 14.6)	<0.001	13.6 (12.3, 14.7)
Creatinine, mg/L	0.9 (0.8, 1.1)	0.9 (0.7, 1.0)	0.9 (0.8, 1.1)	0.9 (0.7, 1.1)	0.083	0.9 (0.8, 1.1)
D-dimer, mg/L	790.0 (423.0, 1254)	698.0 (436.0, 1245)	660.0 (441.0, 1266)	796.5 (435.0, 1501)	0.923	711.0 (437.0, 1299)
Lactate dehydrogenase, U/L	267.0 (217.0, 368.0)	224.0 (177.0, 282.0)	251.0 (202.0, 326.0)	203.0 (166.0, 264.0)	<0.001	245.0 (192.0, 311.0)
C-reactive protein, mg/L	3.4 (1.5, 9.8)	2.4 (1.2, 7.0)	3.7 (1.6, 8.8)	1.8 (0.2, 4.7)	<0.001	3.0 (1.2, 8.0)
Platelets, 10^9/L	198.0 (158.5, 274.0)	231.0 (181.0, 309.0)	207.0 (161.0, 276.0)	234.5 (173.5, 289.5)	0.068	217.0 (167.0, 284.5)
Potassium, mmol/L	3.7 (3.4, 3.9)	3.7 (3.3, 3.9)	3.6 (3.4, 3.8)	3.7 (3.4, 4.0)	0.388	3.6 (3.4, 3.9)
Ferritin, mg/L	374.0 (176.0, 839.0)	297.0 (104.0, 637.0)	536.5 (266.5, 1045)	277.5 (128.5, 602.5)	<0.001	427.5 (186.0, 841.0)

All values are expressed as median (IQR).
[a]*Kruskal–Wallis test.*

TABLE 3 | Endpoint events and other drugs disposition.

	Intervention					
	LPV/r	HCQ	LPV/r + HCQ	SoC	p-value[a]	Total
	N = 124	*N* = 109	*N* = 244	*N* = 113		*N* = 590
Events, *n* (%)						
Invasive ventilation	23 (18.5%)	8 (7.3%)	37 (15.2%)	11 (9.7%)	0.04	79 (13.4%)
Death	21 (16.9%)	12 (11.0%)	27 (11.1%)	15 (13.3%)	0.41	75 (12.7%)
Invasive ventilation/death	36 (29.0%)	19 (17.4%)	51 (20.9%)	23 (20.4%)	0.15	129 (21.9%)
Noninvasive ventilation/invasive ventilation/death	38 (30.7%)	23 (21.1%)	61 (25.0%)	26 (23.0%)	0.36	148 (25.0%)
Stop of shedding	50 (47.6%)	41 (47.7%)	117 (56.0%)	42 (59.2%)	0.26	250 (53.1%)
Other drugs, *n* (%)						
Anticoagulants	46 (37.1%)	69 (63.3%)	107 (43.9%)	55 (48.7%)	0.0005	227 (47.0%)
Steroids	64 (51.6%)	26 (23.9%)	81 (33.2%)	25 (22.1%)	<0.0001	196 (33.2%)
Azithromycin	45 (36.3%)	27 (24.8%)	35 (14.3%)	25 (22.1%)	<0.0001	132 (22.4%)
Immunomodulatory drugs	20 (16.1%)	18 (16.5%)	51 (20.9%)	3 (2.7%)	0.0002	92 (15.6%)

[a]*Chi-square test.*

most represented comorbidities were hypertension (44.7%), followed by cardiovascular disease (30%) and cancer (29.9%). The median time from hospital admission to baseline was 0 days (IQR 0–1).

Among the 590 patients included in the analysis, 109 received hydroxychloroquine, 124 received lopinavir/ritonavir, 244 received hydroxychloroquine plus lopinavir/ritonavir, and 113 did not receive any of them (standard of care). The latter group consisted of 44 people who did not start any drug, 55 who started anticoagulants, 25 steroids, 25 azithromycin, and 3 immunomodulatory drugs. The different treatment groups were not homogenous for sex, timing of hospitalization, pneumonia, baseline PaO2/FiO2, and some inflammatory biomarkers. In particular, patients in the SoC group had a higher PaO2/FiO2 at baseline (median 381, IQR 300–467 mmHg) and were less frequently diagnosed with pneumonia (68.1% of them); had lower LDH, AST, CRP, and ferritin; and higher neutrophils and lymphocytes count than the other three groups. Overall, 132 (22%) were treated also with azithromycin, 196 (33%) with corticosteroids at various dosage, 92 (16%) with immunomodulatory drugs, and 277 (47%) received heparin at various dosage. Concomitant use of azithromycin was most prevalent in the lopinavir/r group (n = 45, 36 vs. 22% in SoC), while immunomodulatory drugs were most frequently used in the dual antiviral combination (n = 51, 21% vs. 3% in SoC) (**Table 3**).

Primary Endpoint: Invasive Ventilation/Death

Overall, 79 patients over 590 (13.4%) underwent invasive ventilation and 75/590 (12.7%) did not survive (**Table 3**).

By Kaplan–Meier analysis, the estimated probabilities of invasive ventilation or death were 17.3% (95% CI 14.1, 20.4) at 7 days and 21.2% (95% CI 17.6, 24.7) at 14 days in the overall population (**Figure 1**). The estimated probabilities of invasive ventilation or death at 14 days were 16.2% (95% CI 8.8, 23.5) with SoC, 26.9% (95% CI 18.7, 35.2) with LPV/r, 16.2% (95% CI 8.9, 23.6) with HCQ, and 20.5% (95% CI 15.1, 26.0) with LPV/r + HCQ, without any evidence of a difference between the groups (log rank p = 0.20) (**Figure 2A**).

Even considering only the strata of moderate patients (PaO2/FiO_2 > 300 mmHg at baseline), no difference among the groups was detected (log rank p = 0.43, **Figure 2B**).

Unadjusted and adjusted marginal relative hazards of invasive ventilation/death from fitting a marginal Cox regression model are shown in **Table 4**. This model was adjusted for age, gender, presence of comorbidities, duration of symptoms, time-varying use of immunomodulatory drugs, heparin and azithromycin, and censoring using IPW. There was no evidence of a difference in risk of invasive ventilation/death in the three treatment groups when compared to standard of care. The aHR was 1.09 (95% CI 0.60, 1.98) with LPV/r + HCQ, 0.81 (95% CI 0.38, 1.72) with HCQ, and 1.55 (95% CI 0.82, 2.93) with LPV vs. SoC.

The risk of invasive ventilation/death in the three groups appeared to vary by PaO2/FiO2, driven by the use of LPV/r or HCQ alone. In fact, for HCQ vs. SoC, the aHR resulted 1.48 (95% CI 0.35, 6.18) in the strata of patients with PaO2/FiO2 < 300 mmHg at baseline and 0.83 (95% CI 0.22, 3.18) in the strata of patients with PaO2/FiO2 > 300 mmHg at baseline, suggesting a more beneficial effect of HCQ in people with less severe disease. Results were similar for LPV/r vs. SoC, with an aHR of 2.48 (95% CI 0.65, 9.43) in the strata of patients with PaO2/FiO2 < 300 mmHg at baseline and 0.73 (95% CI 0.14, 3.95) in the strata of patients with PaO2/FiO2 > 300 mmHg at baseline (p-value for interaction <0.001) (**Table 4**).

Results were similar in two additional sensitivity analyses. First, after we further controlled for baseline level of inflammation and coagulation in a subset of participants with available values of these markers (ferritin, CRP, and d-dimer), the aHR was 0.84 (95% CI 0.41, 1.70) with LPV/r + HCQ, 0.63 (95% CI 0.26, 1.54) with HCQ, and 1.15 (95% CI 0.54, 2.47) with LPV vs. SoC (**Supplementary Table S1**). The second sensitivity analysis was done after controlling for pneumonia instead of baseline levels of PaO2/FiO2, to try to remove bias due to imbalance in the severity of disease at entry. The HR in this

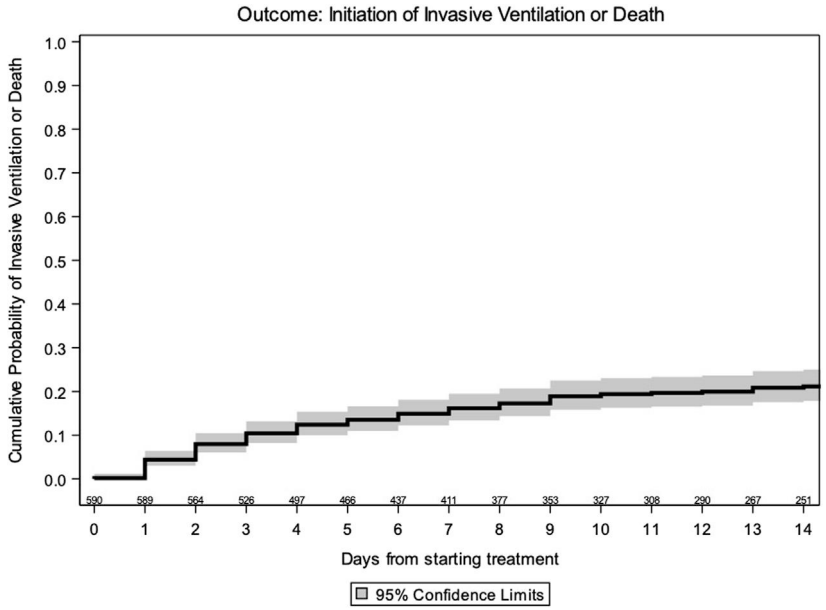

FIGURE 1 | Kaplan–Meier estimate of time to invasive ventilation/death–overall.

case were 0.93 (95% CI 0.53, 1.62) with LPV/r + HCQ, 0.60 (95% CI 0.32, 1.12) with HCQ, and 0.65 (95% CI 0.36, 1.20) with LPV vs. SoC.

Secondary Endpoint i): Viral Shedding

Overall, confirmed negative SARS-CoV-2 PCR in nasal/oropharyngeal swabs, without a relapse, was obtained in 215 patients over 441 during hospitalization (**Table 3**).

By Kaplan–Meier analysis, the estimated probabilities of confirmed negative SARS-CoV-2 PCR in nasal/oropharyngeal swabs were 22.7% (95% CI 18.5, 26.9) at 7 days and 44.4% (95% CI 38.9, 49.9) at 14 days in the overall population (**Figure 3**). The estimated probabilities in the different groups at 14 days were 49.7% (95% CI 35.5, 63.8) with SoC, 32.2% (95% CI 21.6, 42.8) with LPV/r, 37.1% (95% CI 23.7, 50.5) with HCQ, and 44.7% (95% CI 36.7, 52.7) with LPV/r, without evidence of a difference between the groups (log rank $p = 0.15$).

Unadjusted and adjusted marginal relative hazards of confirmed negative SARS-CoV-2 PCR in nasal/oropharyngeal swabs from fitting a marginal Cox regression model are shown in **Table 5**. This model was adjusted for age, gender, presence of comorbidities, duration of symptoms, time-varying use of immunomodulatory drugs, heparin and azithromycin, and censoring using IPW. Again, these data were compatible with the null hypothesis of no difference between the groups. The aHR was 1.09 (95% CI 0.66, 1.79) with LPV/r + HCQ, 0.72 (95% CI 0.41, 1.26) with HCQ, and 0.77 (95% CI 0.44, 1.32) with LPV/r vs. SoC. In the subset of patients with PaO2/FiO2 > 300 mmHg at baseline, the aHR was 0.60 (95% CI 0.32, 1.12) with LPV/r + HCQ, 0.40 (95% CI 0.19, 0.84) with HCQ, and 0.46 (95% CI 0.19, 1.12) with LPV/r vs. SoC.

Results were similar when we further adjusted for baseline PaO2/FiO2 levels. The aHR was 1.12 (95% CI 0.68, 1.85) with LPV/r + HCQ, 0.79 (95% CI 0.45, 1.36) with HCQ, and 0.78 (95% CI 0.45, 1.36) with LPV/r vs. SoC, and these risks did not vary by stratification for duration of symptoms (more or less than 9 days from symptoms' onset) (**Supplementary Table S2**).

Secondary Endpoint ii): Noninvasive or Invasive Ventilation or Death

Overall, 101 patients over 590 (17.1%) underwent noninvasive or invasive ventilation (**Table 3**).

By Kaplan–Meier analysis, the estimated probabilities of noninvasive/invasive ventilation or death was 20.8% (95% CI 17.4, 24.2) at 7 days and 25.7% (95% CI 21.8, 29.6) at 14 days in the overall population (**Figure 4**). The estimated probabilities of noninvasive or invasive ventilation or death at 14 days were 18.0% (95% CI 10.4, 25.7) with SoC, 24.7% (95% CI 18.9, 30.5) with LPV/r + HCQ, 20.3% (95% CI 12.2, 28.3) with HCQ, and 29.3% (95% CI 20.8–, 7.8) with LPV/r, without evidence of a difference between SOC, LPV/r, HCQ, and LPV/r + HCQ (log rank p = 0.42).

DISCUSSION

Herein, we report a retrospective study with real-world data collected from routine care to assess the clinical and virological efficacy of hydroxychloroquine, lopinavir/ritonavir, or the combination of hydroxychloroquine plus lopinavir/ritonavir vs. SoC in a population of 590 patients admitted to our hospital for COVID-19 infection. We found that none of the antivirals investigated or their combination were associated with a reduction of invasive ventilation or death 14 days after starting of therapy compared with standard of care alone. Additionally, a

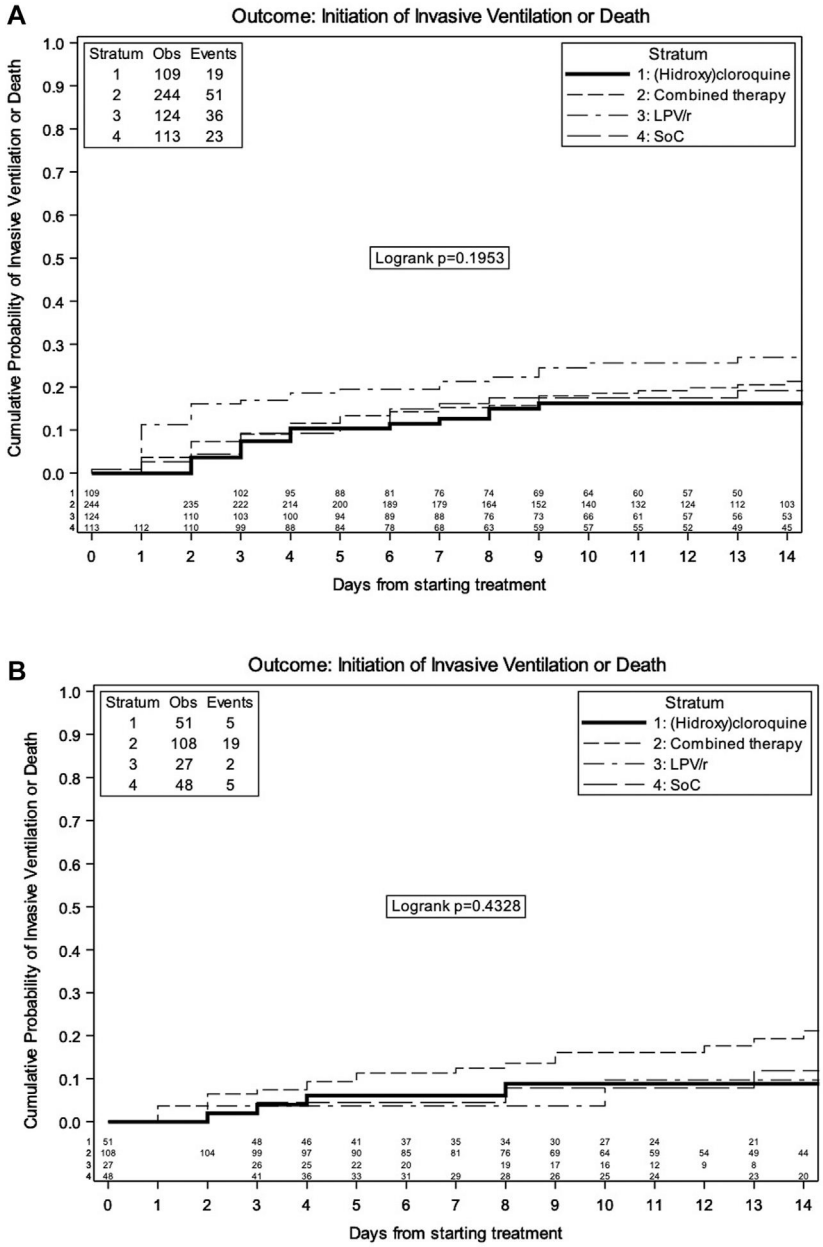

FIGURE 2 | Kaplan–Meier estimate of time to invasive ventilation/death by treatment group in overall population **(A)** and in the strata of moderate patients **(B)**.

reduction in estimated probability of any ventilation (noninvasive or invasive ventilation) or death was not demonstrated. Even in terms of ending of the viral shedding, in the subgroup of patients with positivity to SARS-CoV-2 in nasal/oropharyngeal swabs, we found no benefit with hydroxychloroquine, lopinavir/ritonavir, or the combination of hydroxychloroquine plus lopinavir/ritonavir in comparison to the standard of care.

Our population is well-characterized and clinical features of our patients were consistent with many other reports, with a predominance of men, mean aged in the seventh decade of life. In contrast with other reports, we found a lower prevalence of comorbidities in our population (Docherty et al., 2020; Richardson et al., 2020), but higher number of symptomatic patients with fever (Richardson et al., 2020).

As expected, results of the comparison between HCQ or LPV/r monotherapy and SoC were similar to those of randomized trials, where no advantage in the use of HCQ or LPV/r was observed (Cavalcanti et al., 2020; NIH, 2020b; Recovery Randomised Evaluation of COVID-19 Therapy 2020a; Recovery Randomised Evaluation of COVID-19 Therapy, 2020b; World Health Organization, 2020). All our analyses took into account heparin use and corticosteroids, and this is particularly relevant in light of the possible association between anticoagulant treatment and decreased mortality in severe COVID-19 and above all in

TABLE 4 | HR of invasive ventilation/death from fitting a marginal Cox regression model.

	Unadjusted and adjusted marginal relative hazards of invasive ventilation/death			
	Unadjusted HR (95% CI)	***p*-value**	**Adjusted[a] HR (95% CI)**	***p*-value**
All patients				
SoC	1.00		1.00	
LPV/r + HCQ	1.01 (0.61, 1.66)	0.972	1.09 (0.60, 1.98)	0.772
HCQ	0.78 (0.42, 1.44)	0.423	0.81 (0.38, 1.72)	0.584
LPV/r	1.42 (0.83, 2.45)	0.201	1.55 (0.82, 2.93)	0.173
Baseline PaO2/FiO2 0–300				
SoC	1.00		1.00	
LPV/r + HCQ	0.51 (0.24, 1.08)		1.41 (0.37, 5.33)	
HCQ	0.53 (0.22, 1.30)		1.48 (0.35, 6.18)	
LPV/r	0.89 (0.41, 1.94)		2.48 (0.65, 9.43)	
				p-value for interaction <0.001
Baseline PaO2/FiO2 > 300				
SoC	1.00		1.00	
LPV/r + HCQ	1.57 (0.59, 4.17)		1.63 (0.56, 4.78)	
HCQ	0.87 (0.25, 3.00)		0.83 (0.22, 3.18)	
LPV/r	0.67 (0.13, 3.42)		0.73 (0.14, 3.95)	

[a]*Adjusted for age, gender, presence of comorbidities, duration of symptoms and time-varying use of immunomodulatory drugs, azithromycin, steroids, anticoagulants, and censoring using IPW.*

NB. The stratified analysis is based on the subset of 396/590 (67%) participants with available PaO2/FiO2 values at baseline.

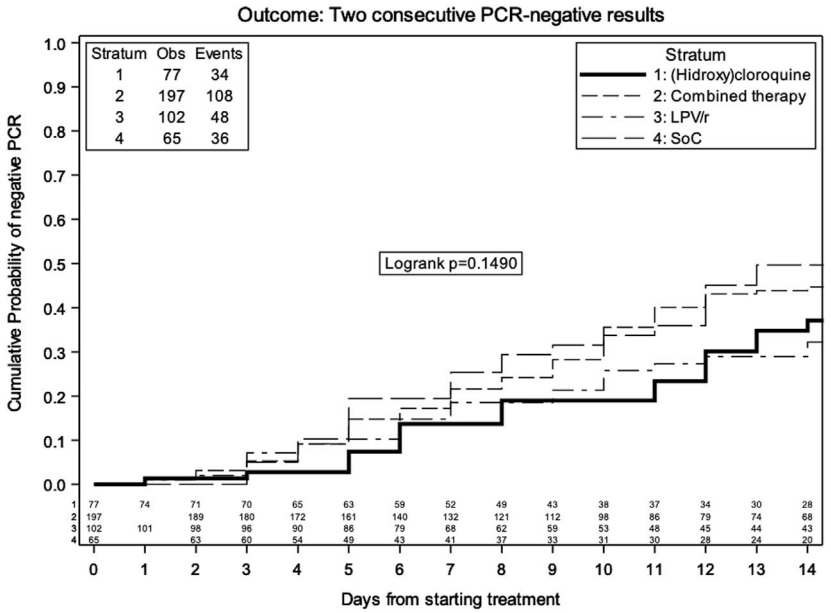

FIGURE 3 | Kaplan–Meier estimate of time to confirmed negative NP/OP swabs by treatment group.

light of recently published data from randomized and observational studies about benefits of corticosteroids in terms of clinical evolution (Group TRC, 2020; Salton et al., 2020) and mortality (Fadel et al., 2020; Fernández Cruz et al., 2020; Group TRC, 2020; Salton et al., 2020; Wu et al., 2020).

The risk of invasive ventilation/death in the groups appeared to vary by PaO2/FiO2 at baseline; its putative mechanism was not clear and could deserve further investigation. Antivirals were administered approximately 9 days after symptoms' initiation, similarly to other studies (Cao et al., 2020; Goldman et al., 2020). It is possible that therapies that may limit viral replication may be more effective earlier in the course of the disease, so this could explain our signal for a greater potential benefit of HCQ and LPV/r vs. SoC in the subset of people with less severe disease. According to Hung et al. (Hung et al., 2020), antivirals could also potentially have a role in reducing viral shedding, but we

TABLE 5 | HR of reversing to PCR negative from fitting a marginal Cox regression model.

	Unadjusted and adjusted marginal relative hazards of reverting from PCR + to PCR negative			
	Unadjusted HR (95% CI)	***p*-value**	**Adjusted[a] HR (95% CI)**	***p*-value**
All patients				
SoC	1.00		1.00	
LPV/r + HCQ	1.11 (0.72, 1.71)	0.625	1.09 (0.66, 1.79)	0.732
HCQ	0.79 (0.48, 1.29)	0.339	0.72 (0.41, 1.26)	0.244
LPV/r	0.82 (0.50, 1.32)	0.409	0.77 (0.44, 1.32)	0.337
Baseline PaO2/FiO2 > 300				
SoC	1.00		1.00	
LPV/r + HCQ	0.62 (0.37, 1.03)		0.60 (0.32, 1.12)	
HCQ	0.42 (0.22, 0.78)		0.40 (0.19, 0.84)	
LPV/r	0.50 (0.21, 1.16)		0.46 (0.19, 1.12)	

[a]*Adjusted for age, gender, presence of comorbidities, duration of symptoms and time-varying use of immunomodulatory drugs, azithromycin, steroids, anticoagulants, and censoring using IPW.*

NB. The stratified analysis is based on the subset of 317/471 (67%) participants with available PaO2/FiO2 values at baseline included in the analysis for this endpoint. Estimates in the PaO2/FiO2 0–300 mmHg stratum could not be calculated due to the small sample size and positivity in the distribution of the weights.

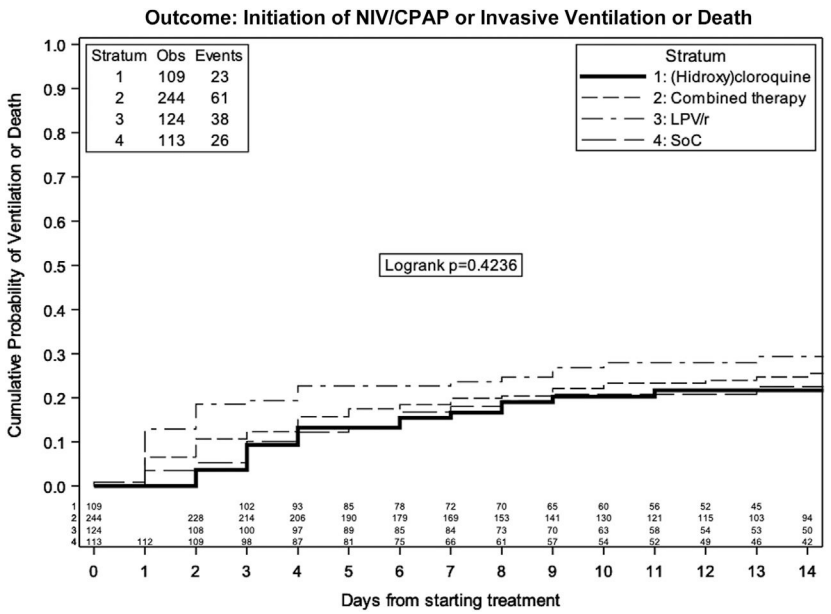

FIGURE 4 | Kaplan–Meier estimate of time to ventilation/death by treatment group.

found no such evidence in our cohort. The analysis has been prompted by a recent RCT showing the beneficial effect of combining antivirals for the treatment of COVID-19 patients (Hung et al., 2020). Indeed, these strategies have been seldom compared in randomized studies and more research is needed. Of note, the effectiveness of the triple combination in that study was mainly ascribed to the use of interferon beta, which has potential to prevent SARS-CoV-2 from shutting down the host innate immunity in the first few days from infection (Richardson et al., 2020). Interferon beta was not used in this case, so this might explain why we could not replicate these earlier results. In general, COVID-19 is a complex disease from the standpoint of pathogenesis with different stages, so broad comparisons of drug A vs. B might not be as useful as trials designed to compare interventions tailored to patients following specific pathogenic pathways (e.g., cytokines storm as opposed to microcirculatory platelet aggregation, etc.). Indeed, the case-mix of our study population was quite heterogeneous. The ending of viral shedding seemed to be possibly favored by HCQ, but only in patients with moderate COVID-19 and not confirmed in a further analysis adjusted for PaO2/FiO2 at baseline and stratified for duration of symptoms. Anyway, this is a subset analysis, so p-values should be considered with caution. Even previously published data showed conflicting

results, but considering all the evidence cumulated to date on the lack of efficacy of HCQ, its beneficial role on SARS-COV-2 shedding should be considered unlikely (NIH, 2020a; Tang et al., 2020; Huang et al., 2020).

Our study presents some limitations. First of all because it is retrospective and observational, we cannot rule out unmeasured confounding. The analysis also relies on specific assumptions regarding the underlying causal structure of the data (time-fixed and time-varying confounding factors) and the linear predictor of the model, with or without interaction terms, to be correctly specified. Thus, it is possible that a key variable was missing in our propensity scores; however, after further controlling for markers of inflammation/coagulation, results were similar. Also, although there was a standard treatment protocol in place, untreated patients in the SoC group might have been a selected population in which treatment was withheld because of predicted poor prognosis or conversely because of initial better evaluation. In fact, the SoC group showed healthier profiles at baseline, in terms of inflammation biomarkers and of leukocyte count and a rate of admission to the ICU which was comparable with the overall mortality (10.3% vs. 9.7%), suggesting that all participants had been equally considered for critical care. Further, other sub-analyses have been performed, taking into account a diagnosis of pneumonia and the baseline difference in biomarkers among groups and similar results were obtained. Indeed, in our population, patients allocated to SoC showed a much less COVID-19 disease severity and our propensity score adjustment should have minimized this imbalance. Also, because time zero of the survival analyses was the date of starting treatment, immortal bias also cannot be completely ruled out. However, the average time from hospital admission to therapy initiation was <1 day for 75% of the study population. Finally, safety data (i.e., risk of arrhythmia in people receiving HCQ) have not been analyzed in this work.

On the other hand, key strengths of this work were the detailed characterization of the study population, including the coadministered drugs, the possibility of comparing combination treatment strategies seldom investigated in randomized studies, and the use of a sophisticated counterfactual prediction framework to appropriately control for time-fixed and time-vary confounding factors.

CONCLUSION

In our retrospective analysis of real-life data of hospitalized patients with mild-to-severe COVID-19, we did not find a significant difference in clinical and virological outcome among lopinavir/ritonavir, hydroxychloroquine, and lopinavir/ritonavir plus hydroxychloroquine or standard of care. Our results are consistent with those of randomized trials comparing mono-antiviral treatment arms vs. placebo which led to the recommendation against the use of these antivirals for treatment of COVID-19 patients in national and international guidelines. Indeed, some of the early RCTs were of poor quality and risk of bias was high, but larger more recent and reliable studies confirmed these results. Additional RCTs specifically addressing the timing of initiation of these and other interventions according to patients' disease course and specific pathogenic pathways as well as the use a combination of approaches are further needed.

COLLABORATIVE AUTHORS

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Database was approved by the local INMI, Rome Ethical Committee. The study was performed in accordance with the declaration of Helsinki. INMI COVID-19 Database retrieves demographic, clinical and laboratory data of patients, as well therapy prescribed (antiviral, immuno-modulatory drugs, oxygen therapy, need for ventilation) for COVID-19 patients. Patients provided written informed consent.

AUTHOR CONTRIBUTIONS

RG was a major contributor in writing the manuscript; AL analyzed data and contributed in writing the manuscript; AM, FT, AV, AA, FG, VM, AAM, GO, FP, LM, PP, CA, EN, MC, NP, GI, FV, and EG have participated in the research and in the acquisition of data; and AAN made substantial contributions to the conception of the work and to interpretation of data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.621676/full#supplementary-material.

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High Dose Intravenous Vitamin C for Preventing The Disease Aggravation of Moderate COVID-19 Pneumonia. A Retrospective Propensity Matched Before-After Study

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Background: Coronavirus disease 2019 (COVID-19) pandemic is continuing to impact multiple countries worldwide and effective treatment options are still being developed. In this study, we investigate the potential of high-dose intravenous vitamin C (HDIVC) in the prevention of moderate COVID-19 disease aggravation.

Methods: In this retrospective before-after case-matched clinical study, we compare the outcome and clinical courses of patients with moderate COVID-19 patients who were treated with an HDIVC protocol (intravenous injection of vitamin C, 100 mg/kg/day, 1 g/h, for 7 days from admission) during a one-month period (between March 18 and april 18, 2020, HDIVC group) with a control group treated without the HDIVC protocol during the preceding two months (January 18 to March 18, 2020). Patients in the two groups were matched in a 1:1 ratio according to age and gender.

Results: The HDIVC and control groups each comprised 55 patients. For the primary outcomes, there was a significant difference in the number of patients that evolved from moderate to severe type between the two groups (HDIVC: 4/55 vs. control: 12/55, relative risk [RR] = 0.28 [0.08, 0.93], $P = 0.03$). Compared to the control group, there was a shorter duration of systemic inflammatory response syndrome (SIRS) ($P = 0.0004$) during the first week and lower SIRS occurrence (2/21 vs 10/22, $P = 0.0086$) on Day 7 (6–7 days after

Abbreviations: HDIVC, high dose intravenous vitamin C; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease 2019; SIRS, systemic inflammatory response syndrome; CRP, C-reactive protein; APTT, activated partial thromboplastin time; ROS, reactive oxygenase species; ESR, erythrocyte sedimentation rate; LDH, lactate dehydrogenase (LDH); TB, total bilirubin; ALT, alanine transaminase; CK, creatine kinase; cTNI, cardiac troponin I; IQR, interquartile range; RR, relative risk; CI, confidential interval; Ethical Approval and Consent to participate.

admission). In addition, HDIVC group had lower C-reactive protein levels ($P = 0.005$) and higher number of $CD4^+$ T cells from Day 0 (on admission) to Day 7 ($P = 0.04$)." The levels of coagulation indicators, including activated partial thromboplastin time and D-dimer were also improved in the HDIVC compared to the control group on Day 7.

Conclusion: HDIVC may be beneficial in limiting disease aggravation in the early stage of COVID-19 pneumonia, which may be related to its improvements on the inflammatory response, immune function and coagulation function. Further randomized controlled trials are required to augment these findings.

Keywords: COVID-19, vitamin C, therapy, inflammatory response, disease aggravation

INTRODUCTION

The potentially fatal disease, coronavirus disease 2019 (COVID-19), has caused a worldwide pandemic since December 2019 (Mahase, 2020; Spinelli and Pellino, 2020). By September 10, 2020, SARS-CoV-2 had affected more than 200 countries, resulting in more than 28 million confirmed cases, and over 900,000 confirmed deaths. Besides Corticosteroids for severe and critical COVID-19, few agents have been shown to be definitively effective according to the latest guideline of World Health Organization (Anonymous, 2020). By severity, COVID-19 is classified into mild, moderate, severe, and critical type according to the guidelines of the National Health and Family Planning Commission of the People's Republic of China (National Health and Family Planning Commission of the People's Republic of China, 2020).

The severe type is mainly characterized by deteriorating respiratory function and rapid progression of radiological lesions, while the critical type further requires mechanical ventilation and is accompanied by shock or multiple organ failure. These two types are reported to be associated with a mortality rate as high as 66% (Wu et al., 2020). One of the keys to improving the prognosis of COVID-19 is to prevent disease aggravation, especially when the disease severity ranges from moderate, through severe, to critical type.

High dose intravenous vitamin C (HDIVC) has been suggested to exert beneficial effects on various critical illnesses in animal and clinical studies (Oudemans-van Straaten et al., 2014). HDIVC was shown to reduce 28-days all-cause mortality (29.8 vs 46.3%, $P = 0.01$) by sepsis in the CITRIS-ALI study (Fowler et al., 2019), and this result was recently reanalyzed by Hemilä and Chalker (2020) who revealed stronger evidence when the analysis is restricted to the four days during which vitamin C was administered (mortality, 4.8 vs 22.9%, $P = 0.0007$). Conversely, another recent trial, ACT, found that a combination of vitamin C, corticosteroid and thiamine exerted no beneficial effect on organ function (Moskowitz et al., 2020). The rationale for HDIVC administration in the treatment of COVID-19 patients, as we speculated, relies on its ability to effectively eliminate the surge of reactive oxygen species and the ensuing uncontrolled inflammatory response and organ dysfunction. Additionally, vitamin C has been demonstrated to have potential immune-enhancing properties, which may help to improve lymphopenia, the main characteristic of COVID-19 that is associated with severity (Wang et al., 2020). The administration of HDIVC in COVID-19 has already received much attention (Carr and Rowe, 2020; Cerullo et al., 2020). In this retrospective before-after case-matched study, we investigate whether HDIVC could prevent disease aggravation from the moderate to the severe type and its effect on the inflammatory response, immune function, and organ function.

METHODS

Study Design and Participants

This study was an electronic health record-based retrospective before-after case-matched clinical study. It was conducted in accordance with the amended Declaration of Helsinki (as revised in 2013) and approved by the Institutional Ethics Board of the Ruijin Hospital, Shanghai Jiao Tong University school of medicine, and has been retrospectively registered in the Chinese Clinical Trail Registry (ChiCTR2000033050). This study was conducted at the Shanghai Public Health Clinical Center. From March 18, 2020, we began to use the HDIVC protocol in the treatment of COVID-19 patients. To investigate the effect of HDIVC in the prevention of disease aggravation, we screened the patients admitted between March 18, 2020 and april 18, 2020 who accepted HDIVC treatment. The inclusion criteria for the HDIVC group were:

1) COVID-19 patients with a diagnosis of moderate type on admission; 2) age >18 years; 3) patients who were not pregnant and had no malignant tumors. The diagnosis and severity classification followed the guidelines of the National Health and Family Planning Commission of the People's Republic of China (National Health and Family Planning Commission of the People's Republic of China, 2020). For the control group, we retrospectively screened the patients who had been admitted during the two previous months (between January 17, 2020 and March 17, 2020) according to the same criteria as those in the HDIVC group. These patients had not received the HDIVC protocol Propensity score matching was conducted to minimize the impact of potential confounders and selection bias between 2 groups of patients. A propensity score for each patient was calculated through logistic regression modeling and covariates of age and gender were matched. A 1:1 matching was used to

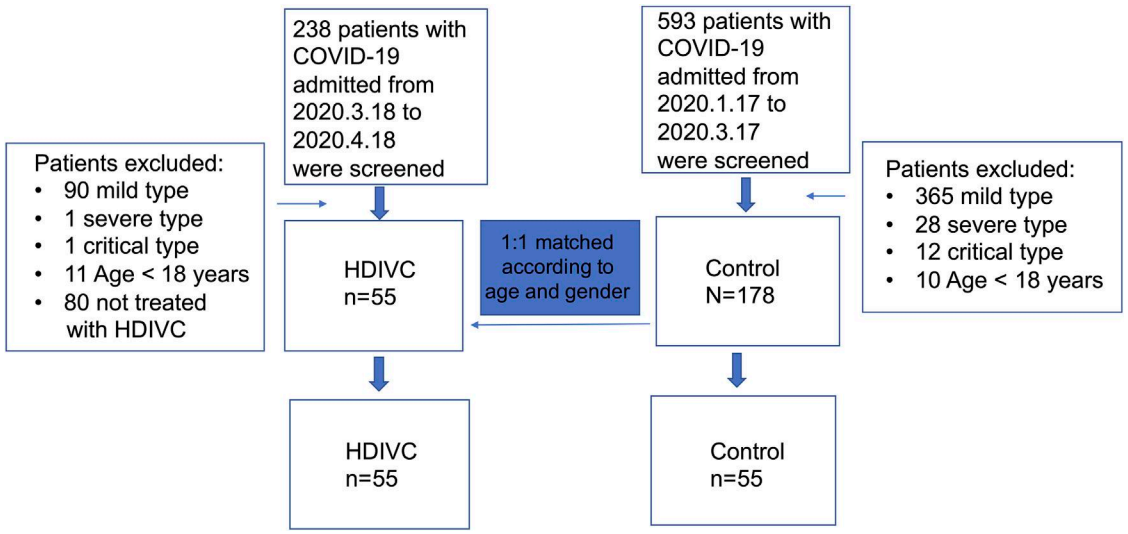

FIGURE 1 | Study flowchart. HDIVC, high dose intravenous vitamin C; COVID-19, coronavirus disease 2019.

select patients in the 2 groups, with the caliper width set as 0.1 for the standard deviation (**Figure 1**).

Data Collection

Data were collected from an electronic medical records and reviewed by two trained physicians. The observation period was the first week after admission. The information or data were collected mainly on admission ("Day 0"), 3–4 days ("Day 3"), and 6–7 days ("Day 7") after admission. Information regarding age, gender, body weight, co-existing diseases, and epidemiology was obtained. The definition of systemic inflammatory response syndrome (SIRS) has been described previously (Kaukonen et al., 2015). Data regarding the serum levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and the occurrence and duration of SIRS were also collected. Additionally, data regarding immune indicators, including counts of $CD4^+$ T cells, $CD8^+$ T cells, and lymphocytes were collected. Indicators of organ function, including lactate dehydrogenase (LDH), total bilirubin (TB), alanine transaminase (ALT), activated partial thromboplastin time (APTT), creatine kinase (CK), cardiac troponin I (cTNI), and pre-albumin levels were also recorded.

The primary outcome was disease aggravation, defined as a progression of the disease severity from moderate type on admission to severe type within one week after admission. The clinical symptoms of the mild type are non-severe, with no pneumonia on imaging examination. The moderate type is characterized by symptoms and pneumonia-related imaging findings. The severe type is diagnosed if any of the following criteria was met: 1) respiratory rate ≥30 cycles/minute; 2) in the resting state, arterial oxygen saturation (SaO2) ≤93%; arterial partial pressure of oxygen/fraction of inspired oxygen ≤300 mmHg; 3) pulmonary imaging shows lesions that have progressed by more than 50% within 24–48 h. The critical type is diagnosed if any of the followings criteria was met: 1) patient require mechanical ventilation; 2) shock occurs; 3) combination with other organ failure that requires ICU monitoring and treatment. The secondary outcomes included indicators for inflammatory response, immune function, organ function and time to viral load negative (**Supplementary Table S1**).

Treatment Protocol

All patients received treatment based on the guidelines of the National Health and Family Planning Commission of the People's Republic of China (National Health and Family Planning Commission of the People's Republic of China, 2020) and the Shanghai expert consensus on comprehensive treatment of COVID-19 (Shanghai Expert Group on Clinical Treatment of New Coronavirus Diseases, 2020). The HDIVC protocol for moderate COVID-19 consisted of an intravenous injection of vitamin C (ascorbic acid) at a dosage of 100 mg/kg/day and a rate of 1 g/h for 7 days, starting from the time of admission. Other associated therapies included antiviral therapy, nutrition support, the low-molecular-weight heparin (if D-dimer was above the normal value), antibiotics in cases of suspected bacterial infections, nasal tube oxygen support if necessary, and/or physical cooling and medical treatment (non-steroidal anti-inflammatory drugs or glucocorticoid) if the body temperature was above 38°C.

Statistical Analysis

Continuous variables were presented as medians and interquartile range (IQR, shown in square brackets) and compared using the Mann-Whitney U test, or reported as the mean with standard deviation and compared using the *t*-test as per distribution type. Categorical variables were compared using Fisher's exact test. The generalized estimating equations (GEE) were performed to investigate the difference in inflammatory markers, immune function, and organ function between the

TABLE 1 | Characteristics of COVID-19 patients.

	HDIVC (*n* = 55)	Control (*n* = 55)	*P* Value
Age, median (IQR), y	36 (31–47)	36 (31–46)	0.96
Sex (male, n)	33	35	0.69
Weight, median (IQR), kg	70 (58–80)	65 (55–76)	0.26
Interval from first symptom to admission, median (IQR), days	4 (2–6)	3 (2–7)	0.65
Symptoms on Day 0			
Fever (n)	34	43	0.06
Dry cough (n)	27	32	0.33
Diarrhea (n)	6	4	0.51
Olfactory dysfunction (n)	3	0	0.07
Gustatory dysfunction (n)	2	0	0.15
Co-existing disease			
Hypertension (n)	1	6	0.05
Diabetes (n)	3	4	0.69
Contemporary treatments, n			
Antiviral (n)	52	54	0.31
Antibiotic (n)	12	20	0.06
Low molecular heparin (n)	16	10	0.44
Glucocorticoid (n)	2	5	0.24

HDIVC, high dose intravenous vitamin C; IQR, interquartile range; Day 0, the day on admission. P, HDIVC vs control group.

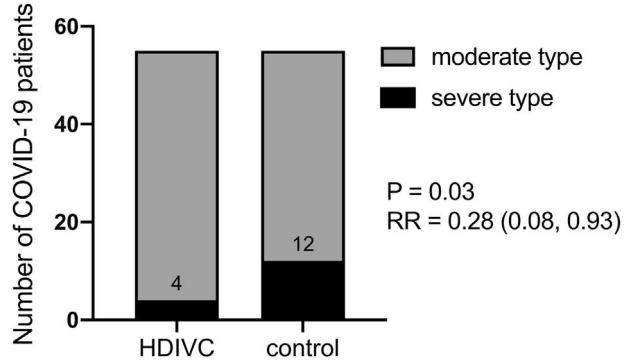

FIGURE 2 | Effect of HDIVC on preventing the disease aggravation. The number of patients who experienced disease aggravation in HDIVC and the control group were compared (4/55 vs 12/55, RR = 0.28 [0.08, 093], *P* = 0.03). HDIVC, high dose intravenous vitamin C; COVID-19, coronavirus disease 2019; RR, relative risk; CI, confidential interval.

HDIVC and control groups. All statistical analyses were performed using SAS v. 9.2 (SAS Institute Inc., United States) and GraphPad prism 8.0 (version 8.2.0). Two-sided P values of less than 0.05 were considered statistically significant.

RESULTS

Characteristics of the Patients

As **Figure 1** shows, 238 patients, admitted between March 18, 2020 and april 18, 2020, were retrospectively screened, and 55 patients met the inclusion criteria for the HDIVC group. Between January 17, 2020 and March 17, 2020, 593 patients admitted to the Shanghai Clinic Public Health Center were screened for the purpose of matching. One hundred and seventy-eight patients diagnosed with moderate COVID-19 on admission were selected to match patients in the HDIVC group in a 1:1 ratio according to age and gender. Fifty-five patients were included in the control group. Patient characteristics were similar between the HDIVC and control groups (**Table 1**). The main associated therapies within the first weeks after admission included antiviral therapy, antibiotics, low-molecular-weight heparin, and glucocorticoids. No significant difference in therapies was found between the two groups.

Effect of High Dose Intravenous Vitamin C on Primary Outcome

The primary outcome is to investigate if HDIVC could prevent disease aggravation. All enrolled patients were diagnosed with moderate COVID-19 on admission (Day 0). As **Figure 2** showed, at the end of the observational period (Day 7), 4 patients in the HDIVC group and 12 in the control group suffered the disease aggravation with a final diagnosis of severe or critical COVID-19 (relative risk [RR] 95% confidential interval [CI] = 0.28 [0.08, 0.93], *P* = 0.03). IQR is shown in square brackets.

Effect of High Dose Intravenous Vitamin C on Secondary Outcomes

As **Table 2** shows, SIRS occurrence at Day 0 was similar between the two groups (HDIVC: 21/55vs. control: 22/55; RR = 0.93 [0.43–1.93], *P* = 0.86). On Day 7, there were fewer patients with SIRS in the HDIVC group (*N* = 2/21) than the control group (*N* = 10/22, RR = 0.13 [0.02–0.68], *P* = 0.0086). Among the patients with SIRS on admission, the duration of SIRS was further analyzed, and we found that patients who accepted the HDIVC protocol experienced a significantly shorter lasting time of SIRS (2 [1, 3], days) than the ones who did not (6 [1, 7], days, *P* = 0.0004). There was no significant difference in the serum levels of CRP between the HDIVC group and the control

TABLE 2 | Effect of HDIVC on inflammatory response.

Variables	Time points	n	HDIVC	N	Control	RR (95%CI)	P Value
Patients with SIRS, n/total	Day 0	55	21/55	55	22/55	0.93 (0.43–1.93)	0.85
Patients with SIRS, n/total	Day 7	21	2/21	22	10/22	0.13 (0.02–0.68)	**0.008**
Duration of SIRS, days, median (IQR)	Day 0 to day 7	55	2 (1, 3)	55	6 (1, 7)	—	**0.0006**
Serum level of CRP	Day 0	55	1.2 (0.5, 7.6)	55	0.5 (0.5, 7.3)	—	0.19
mg/L, median (IQR)	Day 3	55	0.5 (0.5, 8.5)	55	0.5 (0.5, 10.2)	—	0.18
	Day 7	55	0.5 (0.5, 0.6)	54	0.5 (0.5, 7.7)	—	**0.02**
Serum level of ESR	Day 0	55	33 (10, 76)	50	40.5 (21, 74.3)	—	0.23
ml/h, median (IQR)	Day 3	45	44 (21, 75)	49	39 (23.5, 72)	—	0.39
	Day 7	48	30 (11, 49.8)	47	38 (21, 73)	—	0.09

HDIVC, high dose intravenous vitamin C; RR, relative risk; CI, confidential interval. SIRS, systemic inflammatory response syndrome; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IQR, interquartile range; Day 0, the day on admission; Day 3, 3–4 days after admission; Day 7, 6–7 days after admission. P, HDIVC vs control group.

TABLE 3 | Effect of HDIVC on the recovery of immune function deficiency.

Variables (median [IQR])	Time points	n	HDIVC	n	Control	RR (CI)	P Value
Patients with $CD4^+$ T cell (<410/μL) deficiency on day 0, n/total	Day 0	12	12/55	18	18/55	0.6 (0.2–1.3)	0.19
Counts of $CD4^+$ T cell, n/μl	Day 0	12	289.5 (262.3, 339.3)	18	340 (203, 375)	—	0.29
Counts of $CD4^+$ T cell, n/μl	Day 7	12	638 (452.3, 746.5)	9	493 (281.5, 641.5)	—	0.17
Increase of $CD4^+$ T cell, n/μl	Day 0 to day 7	12	334 (191.9, 409.3)	9	151 (43.5, 240)	—	**0.04**
Patients with $CD8^+$ T cell deficiency (<190/μL) on day 0, n/total	Day 0	4	4/55	9	9/55	0.4 (0.1–1.4)	0.14
Counts of $CD8^+$ cell, n/μl	Day 0	4	143 (95.5, 163.5)	9	125 (108, 166)	—	>0.9
Counts of $CD8^+$ cell, n/μl	Day 7	4	240 (215.5, 346.3)	6	287 (147, 339.5)	—	>0.9
Increase of $CD8^+$ T cell, n/μl	Day 0 to Day 7	4	123 (65, 211.8)	6	153 (51.5, 242.9)	—	0.76
Patients with lymphocyte deficiency (<1.1 *109/L) on Day 0, n/total, %	Day 0	13	13/55, 23.6	19	19/55, 34.5	0.6 (0.3–1.4)	0.21
Counts of lymphocyte, n*109/L	Day 0	13	0.9 (0.7, 1.1)	19	0.8 (0.7, 1)	—	0.22
Counts of lymphocyte, n*109/L	Day 7	13	1.4 (1.2, 1.9)	18	1.2 (0.7, 1.6)	—	0.11
Increase of lymphocyte, n*109/L	Day 0 to Day 7	13	0.5 (0.4, 1.1)	18	0.35 (-0.02, 0.76)	—	0.09

The COVID-19 patients with a deficiency of $CD4^+$ T cells, $CD8^+$ T cells and lymphocytes on Day 0 were selected. The increases in these immune cells from Day 0 to Day 7 were compared between the HDIVC and control group. IQR, interquartile range; RR, relative risk; CI, confidential interval; HDIVC, high dose intravenous vitamin C; Day 0, the day on admission; Day 3, 3–4 days after admission; Day 7, 6–7 days after admission. P, HDIVC vs control group.

group on Day 0 and Day 3. However, on Day 7, CRP levels were significantly lower in the HDIVC group than in the control group (0.5 [0.5, 0.6] vs 0.5 [0.5, 7.7], mg/L, P = 0.005). Another inflammatory indicator, ESR, showed no significant difference between the two groups.

As **Table 3** shows, for the patients with $CD4^+$ T lymphocyte deficiency (<410/μL) on admission, HDIVC exerted a significant improving effect (334 [191.9, 409.3] vs 151 [43.5, 240] P = 0.04), but not for the patients with deficiencies in $CD8^+$ (190/μL) and lymphocytes on admission. There was no obvious effect of HDIVC on the $CD4^+$ T cell counts, $CD8^+$ T cell counts, and lymphocytes counts on Day 3 and Day 7 for the entire study population (**Supplementary Table S2**).

As **Table 4** shows, D-dimer levels in the HDIVC group (0.3 [0.2, 0.4], μg/ml) were lower than those in the control group (0.4 [0.2, 0.7], μg/ml, P = 0.05). APTT in the HDIVC group (seconds) was significantly shorter than that in the control group on Day 3 (37.7 [35.2, 39.3] vs 40.1 [36.8, 44.2], seconds, P = 0.02) and Day 7 (36.9 (34.9, 38.9) vs 40.8 (36.5, 43.5), seconds, P = 0.02). Other organ function indicators including LDH, TB, ALT, D-Dimer, APTT, cTNI, and CK-MB were within the normal ranges on Day 0 and showed no obvious changes in either of the two groups on Day 3 and Day 7.

No significant difference in the time to achieve negative viral load of nasopharyngeal swab (**Figures 3A,B**) and stool (**Figures 3C,D**) was observed between the HDIVC group and the control group.

DISCUSSION

In this retrospective study, we found that after application of the HIDVC protocol since March 23, 2020, fewer (4/55 vs 12/55, RR = 0.28 [0.08, 0.93], P = 0.03) patients with moderate COVID-19 on admission evolved to the severe type during the week after admission. These patients also demonstrated a shorter SIRS duration and a lower CRP level. The patients with $CD4^+$ T cell deficiency on admission who accepted HDIVC showed a better recovery ability of the $CD4^+$ T cell count than those who had not received HDIVC. Coagulation function indicators, including APTT and D-dimer, were improved in the HDIVC group compared to the control group.

TABLE 4 | Effect of HDIVC on organ functions.

Variables	Time points	HDIVC		Control		*P* Value
		n	Median (IQR)	n	Median (IQR)	
DD (μg/ml)	Day 0	55	0.3 (0.2, 0.5)	55	0.3 (0.2, 0.4)	0.84
(0–0.5)	Day 3	45	0.4 (0.3, 0.5)	50	0.3 (0.2, 0.4)	0.80
	Day 7	51	0.3 (0.2, 0.4)	52	0.4 (0.2, 0.7)	**0.05**
APTT (seconds)	Day 0	55	36.9 (35.4, 39.8)	55	38.6 (36.3, 42.9)	0.20
(31.5–43.5)	Day 3	45	37.7 (35.2, 39.3)	50	40.1 (36.8, 44.2)	**0.02**
	Day 7	58	36.9 (34.9, 38.9)	52	40.8 (36.5, 43.5)	**0.02**
LDH (U/L)	Day 0	55	203 (189, 240)	55	203 (178, 234)	0.43
(120–250)	Day 3	45	210 (176.5, 236.5)	53	199 (172, 226)	0.95
	Day 7	52	207 (179.3, 237.3)	52	200 (172.5, 246.8)	0.19
TB (μmol/L)	Day 0	55	11.3 (9.4, 14.8)	55	7.3 (6, 10.5)	<0.001
(3.4–20.5)	Day 3	45	8.8 (7.3, 11.7)	53	9.5 (7.2, 11.6)	0.64
	Day 7	53	9.3 (7.4, 11.7)	52	8.1 (7.2, 11.3)	0.67
ALT(U/L)	Day 0	55	29 (16, 45)	55	22 (13, 33)	0.30
(8–38)	Day 3	45	25 (15.5, 39)	53	19 (11, 28)	0.36
	Day 7	53	30 (18.5, 51.5)	52	20 (11.5, 34.5)	0.08
CK (U/L)	Day 0	55	89 (54, 126)	55	86 (56, 135)	0.42
(**30–200U/L**)	Day 3	39	60 (39, 85)	46	61.5 (41.5, 99.3)	0.42
	Day 7	39	60 (40, 80)	51	56 (40, 87)	0.19
cTNI (ng/ml)	Day 0	55	0.02 (0.02, 0.02)	55	0.02 (0.01, 0.04)	0.28
(<0.04)	Day 3	35	0.02 (0.02, 0.02)	50	0.02 (0.01, 0.03)	0.26
	Day 7	39	0.02 (0.02, 0.02)	49	0.02 (0.01, 0.02)	0.48
Pre-albumin (mg/L)	Day 0	55	195.2 (156.9, 256.1)	55	206 (137.7, 248.6)	0.35
(180–400)	Day 3	45	194.9 (152.7, 241.3)	52	199.2 (132.4, 261.7)	0.99
	Day 7	51	257.5 (215.5, 295.7)	52	236.9 (157.8, 284.4)	0.09

HDIVC, high dose intravenous vitamin C; DD, D-Dimer; APTT, activated partial thromboplastin time LDH, lactate dehydrogenase (LDH); TB, total bilirubin; ALT, alanine transaminase (ALT); CK, creatine kinase (CK); cTNI, cardiac troponin I; RR, relative risk; CI, confidential interval; IQR, interquartile range. P, HDIVC vs control group.

According to the recent report (Chiscano-Camón et al., 2020), the level of vitamin C is almost undetectable in the COVID-19 patients with severe or critical condition. Another recent study also reported low vitamin C plasma levels in COVID-19 patients, and non-survivors had half the plasma level of survivors (Arvinte et al., 2020). Therefore, early application of HIDVC may assist the quick recovery of its level and gain the benefits as we observed. We found obvious differences in the primary outcome, the disease aggravation, between the two groups. This finding implies the effect of HDIVC in the prevention of disease aggravation. This was partially consistent with the mortality reducing effect of HDIVC on sepsis with acute respiratory distress syndrome reported by Folwer (CITRIS-ALI study) (Fowler et al., 2019) and Hemilä, et al. (reanalysis of CITRIS-ALI study) (Hemilä and Chalker, 2020).

Recently, Zhang et al. (2021) reported that HDIVC (12 g every 12 h, 7 days) failed to improve invasive mechanical ventilation-free days in 28 days (the primary outcome). Compared to our study, the patients enrolled in their study were with higher severity of disease and the duration from onset of symptom to administration of HDIVC (median [IQR], 17 [11–25], days) of their study was longer than ours (control group: 3 [2–7], HDIVC group: 4 [2–6], days). Therefore, it is speculated that the early application of HDIVC routinely in COVID-19, especially when there is a potential risk of disease aggravation, may gain benefits. It should be noted that our study design was a comparison between two groups of patients before and after HDIVC protocol initiation. We matched the two groups strictly and the other therapy showed no significant difference, but as the understanding and management of COVID-19 improves, the outcomes may be better during the time of HDIVC administration than in the previous two months. Therefore, high quality randomized controlled trials are warranted for the prevention of disease aggravation using HDIVC.

SIRS, characterized by the release of huge amounts of pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin-1β, interleukin-6, and interferon-γ named as "cytokine storm", has been reported to be correlated with higher mortality in severe sepsis (Kaukonen et al., 2015). The Cytokine storm is regarded as an important characteristic in the early stages of COVID-19 (Fink-Neuboeck et al., 2016). The relevance of the cytokine storm to COVID-19 is still in debate and several clinical trials are underway (NCT04306705, NCT04322773) to investigate its potential role as a therapeutic target (Sinha et al., 2020). Although we did not directly show the effect of HDIVC on cytokines, we have demonstrated the shorter duration of SIRS and less SIRS prevalence in the HDIVC compared to the control group during the first week after admission. Serum levels of CRP are usually used to track and monitor the inflammatory response caused by infection due its short half-life of 19 h (Williams et al., 2019). CRP levels were shown to be reduced rapidly by HDIVC (200 mg/kg/day) in a previous before-after study in a cohort of sepsis patients (Fowler et al., 2014). In this study, we found that CRP levels in the HDIVC group were significantly lower than the ones in the control group. Therefore, we concluded that HDIVC might be

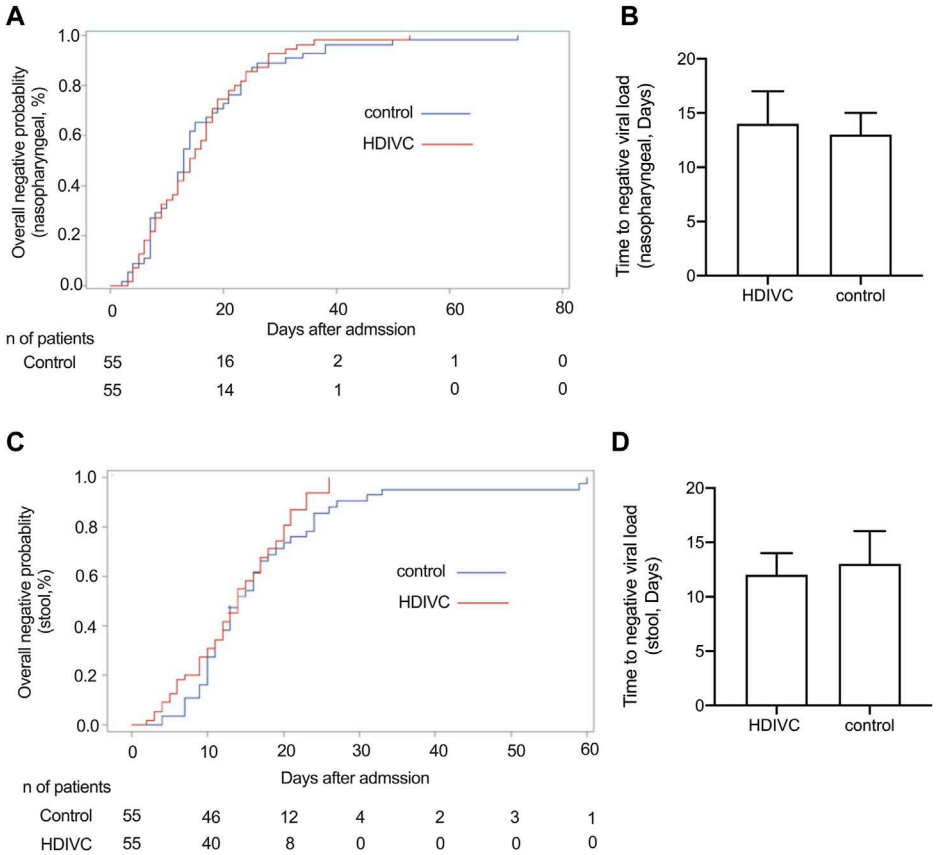

FIGURE 3 | Effect of HDIVC on the time to negative nucleic acid load. The overall negative probability of nasopharyngeal swab **(A)** and stool **(C)** at admission between the HDIVC and control groups were compared and no significant difference was found. The time to negative nucleic acid was compared between HDIVC and control groups for nasopharyngeal swab **(B)**, median [IQR], days, 14 [8, 21] vs 13 [7, 21], $P = 0.79$) and for stool **(D)**, median [IQR], days, 12 [7, 17] vs 13 [10, 20], $P = 0.12$). HDIVC, high dose intravenous vitamin C; COVID-19, coronavirus disease 2019. IQR, interquartile range.

beneficial for the inhibition of the inflammatory response in COVID-19 patients.

A reduction of lymphocytes, especially in the $CD4^+$ T cell subgroup, has been reported to correlate with COVID-19 severity (Xu et al., 2020). SARS-CoV-2 infects and kills T lymphocyte cells. This might be due to growth inhibition and apoptosis of hematopoietic cells by the production of autoimmune antibodies (Yang et al., 2004) or certain cytokines (Channappanavar et al., 2014). In our study, 12 out of 55 patients in the HDIVC group and 18 out of 55 patients in the control group had $CD4^+$ T cell deficiency on admission. Lymphocytes, especially T lymphocytes, have been extensively studied in the context of vitamin C biology (van Gorkom et al., 2018). Both *in vitro* and *in vivo* studies have shown that vitamin C is essential for the development, maturation, and proliferation of functional T lymphocytes, and epigenetic regulation of gene expression is one of the underlying mechanisms (Manning et al., 2013). We showed among the patients with $CD4^+$ T cell deficiency on admission, the increase in $CD4^+$ was more obvious in the HDIVC group than in the control group. This finding might imply the immune-enhancing property of HDIVC in the treatment of COVID-19.

Coagulopathy is a common feature of SARS-CoV-2 infection, and an increase in D-dimer level is the most common finding (Iba et al., 2020a), occurring in 43% of non-severe case (Guan et al., 2020). Higher D-dimer and fibrin degradation product levels, longer prothrombin time, and longer APTT have been reported to correlate with disease severity (Cheng, 2020). In our study, the APTT and D-dimer values were also in the normal range on admission, and we found that the APTT was shorter in the HDIVC than in the control group on Day 3 as well as Day 7, and the level of D-Dimer was lower in the HDIVC group than in the control group on Day 7. This confirmed the beneficial effect of HDIVC on coagulation disorders. This finding might be explained by the fact that vitamin C exerts an improving effect on endothelial damage (Barabutis et al., 2017), which promotes microvascular clot formation and angiopathy in COVID-19 pneumonia (Iba et al., 2020b).

CONCLUSION

In this retrospective before-after study, we found that fewer COVID-19 pneumonia patients suffered disease aggravation

after HDIVC application. Significant differences in the duration of SIRS, CRP level, $CD4^+$ T cell recovery, and coagulation function indicators were found between the HDIVC and control groups. These results imply that HDIVC may have a role in prevention of the disease aggravation, possibly due to its improvement of the inflammatory response, immune function and coagulation function. Anyway, these observations require evaluation in prospective clinical trials.

This study was conducted in accordance with the amended Declaration of Helsinki (as revised in 2013) and approved by the institutional ethics board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. Oral consent was obtained from each participated patient.; Consent for publication; All the authors approved the publication.; Availability of supporting data; All data are fully available without restriction.; Competing interests; The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ruijin Hospital, Shanghai Jiaotong University school of medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BZ conceived the hypothesis and wrote the manuscript. ML and PL contributed to data collection. YP, JH, ML, YW, LX, and XQ provided supporting data and contributed intellectual input. YL and JL contribute statistical analysis. WZ and EM conceived hypothesis, provided supporting data, contributed intellectual input and reviewed the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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SYSTEMATIC REVIEW
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Comparative Effectiveness of Pharmacological Interventions for Covid-19: A Systematic Review and Network Meta-Analysis

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Background: Several pharmacological interventions are now under investigation for the treatment of Covid-19, and the evidence is evolving rapidly. Our aim is to assess the comparative efficacy and safety of these drugs.

Methods and Findings: We performed a systematic review and network meta-analysis searching Medline, Pubmed, Embase, Cochrane Covid-19 register, international trial registers, medRxiv, bioRxiv, and arXiv up to December 10, 2020. We included all randomised controlled trials (RCTs) comparing any pharmacological intervention for Covid-19 against any drugs, placebo or standard care (SC). Data extracted from published reports were assessed for risk of bias in accordance with the Cochrane tool, and using the GRADE framework. Primary outcomes were all-cause mortality, adverse events (AEs) and serious adverse events (SAEs). We estimated summary risk ratio (RR) using pairwise and network meta-analysis with random effects (Prospero, number CRD42020176914). We performed a systematic review and network meta-analysis searching Medline, Pubmed, Embase, Cochrane Covid-19 register, international trial registers, medRxiv, bioRxiv, and arXiv up to December 10, 2020. We included all randomised controlled trials (RCTs) comparing any pharmacological intervention for Covid-19 against any drugs, placebo or standard care (SC). Data extracted from published reports were assessed for risk of bias in accordance with the Cochrane tool, and using the GRADE framework. Primary outcomes were all-cause mortality, adverse events (AEs) and serious adverse events (SAEs). We estimated summary risk ratio (RR) using pairwise and network meta-analysis with random effects (Prospero, number CRD42020176914). We included 96 RCTs, comprising of 34,501 patients. The network meta-analysis showed in terms of all-cause mortality, when compared to SC or placebo, only corticosteroids significantly reduced the mortality rate (RR 0.90, 95%CI 0.83, 0.97; moderate certainty of evidence). Corticosteroids significantly reduced the mortality rate also when compared to hydroxychloroquine (RR 0.83, 95%CI 0.74, 0.94; moderate certainty of evidence). Remdesivir proved to be better in

terms of SAEs when compared to SC or placebo (RR 0.75, 95%CI 0.63, 0.89; high certainty of evidence) and plasma (RR 0.57, 95%CI 0.34, 0.94; high certainty of evidence). The combination of lopinavir and ritonavir proved to reduce SAEs when compared to plasma (RR 0.49, 95%CI 0.25, 0.95; high certainty of evidence). Most of the RCTs were at unclear risk of bias (42 of 96), one third were at high risk of bias (34 of 96) and 20 were at low risk of bias. Certainty of evidence ranged from high to very low.

Conclusion: At present, corticosteroids reduced all-cause mortality in patients with Covid-19, with a moderate certainty of evidence. Remdesivir appeared to be a safer option than SC or placebo, while plasma was associated with safety concerns. These preliminary evidence-based observations should guide clinical practice until more data are made public.

Keywords: COVID-19, systematic (literature) review, network meta analysis, adults (MeSH), pharmacologic (drug) therapy

INTRODUCTION

The emergence of the novel coronavirus SARS-CoV-2 in December 2019 has posed both the scientific community and wider society challenges of an unprecedented scale and nature. It is highly transmissible resulting in a rapid outbreak globally and was declared a pandemic by the world health organisation (WHO) on March 11th.

Coronavirus disease (Covid-19) can be asymptomatic or can manifest with a wide range of symptoms ranging from mild respiratory ailments to a fatal acute respiratory syndrome and multi-organ failure. The mortality rate is associated with age, gender and comorbidity (Horby and Lim, 2020). Until recently there has been no compelling evidence that any pharmacological treatment of Covid-19 improves outcomes, meaning that supportive care has been the mainstay of management. Dexamethasone has been shown in a large multi-arm trial to be superior to standard care for all-cause mortality (Karagiannidis et al., 2020).

Various other pharmacological agents have been touted as potential treatments for Covid-19, with a preponderance for established antiviral drugs licensed in the treatment of other infections (Sanders et al., 2020). None of these has yet come to the forefront or obtained a strong evidence base as an effective and safe treatment for Covid-19. Since the outbreak of the SARS-CoV-2 epidemic anecdotal evidence, non-peer reviewed articles and strong claims from small clinical trials have exposed clinicians and patients to the risks associated with the use of off-label medicines with very low level evidence (Fauci et al., 2020; Kalil, 2020).

This study comes at a pivotal time whereby a substantial amount of research has been simultaneously carried out in a coordinated global effort and over a short timescale. Prospectively designed network meta-analyses based on existing and future randomised trials can generate high quality comparative evidence, which can be used to assess drugs used against Covid-19 (Cipriani et al., 2020; Naci et al., 2020). Therefore, in this study, we aimed to do a systematic review and network meta-analysis of randomised controlled trials to inform clinical practice and regulatory agencies by comparing different pharmacological interventions versus standard care, placebo or any other intervention for the treatment of Covid-19.

MATERIALS AND METHODS

This study is part of a living review of pharmacological agents for the treatment of Covid-19 conducted by the Department of Epidemiology of the Regional Health Service Lazio, Italy, to inform national regulatory agencies and clinicians, available at https://www.deplazio.net/farmacicovid. This living review is also part of the rolling collaborative reviews published on a monthly basis with the European Network of Health Technology Assessment (EUnetHTA) and available at https://eunethta.eu/covid-19-treatment/.

This living review was conducted following a pre-established protocol registered on PROSPERO (CRD42020176914). The amended protocol with a full search strategy is detailed in **Supplementary Appendix S1** and the review is hereby reported according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines, detailed in **Supplementary Appendix S2** (Hutton et al., 2015). In order to have a full evaluation of the safety, the evaluation of adverse events and serious adverse events were included as primary outcomes in the amended version of the protocol.

Search Strategy and Selection Criteria

We searched Medline, PubMed, and embase from December 2019 to December, 10 2020. We searched medRxiv.org (https://www.medrxiv.org/), bioRxiv.org (https://www.bioRxiv.org/), and arXiv.org (https://www.arXiv.org/) for preprints of preliminary reports of randomised trials. We also searched the Cochrane Covid-19 Study Register (https://covid-19.cochrane.org/), ClinicalTrials.gov (www.clinicaltrials.gov) and World Health Organization (WHO) International Clinical Trials Registry Platform (ICTRP) (www.who.int/ictrp/en/). Additional sources included journal alerts, contact with researchers, websites such as Imperial College, London School of Hygiene and Tropical

Medicine, and Eurosurveillance. We applied no restriction on language of publication.

We included parallel randomised controlled trials (RCTs) comparing any pharmacological intervention against another pharmacological intervention or placebo or standard care (SC), for the treatment of individuals with Covid-19. We included individuals >18 years of age affected by Covid-19 as defined by the authors of the studies. There were no limits in terms of gender or ethnicity or severity of disease. We included pharmacological interventions without restrictions on dosage, regimen, dosing interval, route of administration, or intervention duration. We included standard care as defined by study authors. All studies had standard care underlying the control arms, and we grouped together standard care and placebo as a common comparator. We did not include quasi-randomized controlled trials, cross-over trials, or pilot studies with a single arm.

We excluded studies comparing two dosages of the same pharmacological agent. We did not exclude studies on individuals with a comorbid disorder.

Data Extraction

Four authors (FC, GLD, SV, ZM) independently screened the references retrieved by the search, selected the studies, and extracted the data, using a predefined data-extraction sheet, including the following data:

Methods: first author or acronym, year of publication, study design.
Participants: diagnosis, sample size, mean age, gender distribution, severity of illness, setting.
Interventions: number of patients allocated to each arm, drug name, dose, duration of the interventions and follow-up.
Outcomes: all-cause mortality, adverse events and serious adverse events.
Additional outcomes: Patients with SARS-CoV-2 nasal or pharyngeal swab RT-PCR clearance, time to nasal or pharyngeal swab RT-PCR clearance, number of patients with improvement of pulmonary disease (CT imaging), number of patients experiencing disease progression, number of patients discharged from the hospital, and length of hospital stay.
Notes: Country, funding source.

The same reviewers discussed any uncertainty regarding study eligibility and data extraction until consensus was reached; conflicts of opinion were resolved with other members of the review team (FDC, LA, RS). Two authors (FC, RS) independently assessed the risk of bias of the included studies with the Cochrane tool (Higgins and Green, 2011). Three authors (FC, FDC, GLD) used the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach (Salanti et al., 2014), through the Confidence in Network Meta-Analysis Software (University of Bern Institute of Social and Preventive Medicine, 2017), to evaluate the strength of evidence for results at the end of treatment from the network meta-analysis. We did rate the double blinded studies using placebo as having lower risk of bias, which is reflected on the GRADE evaluation (see **Supplementary Appendix S1**). We considered an OR of 0.80 for mortality and an OR of 1.25 for adverse events and serious adverse events as clinically meaningful, following Cipriani et al. (2018). Using the GRADE approach, we assessed each network estimate according to the following criteria: study limitation, indirectness, inconsistency, imprecision, publication bias. We derived the overall judgment of the certainty of evidence considering the domains altogether and downgraded the evidence by one if a domain was rated as "some concerns" and by two if a domain was rated as "major concerns". Finally, we assigned to each comparison an overall qualitative judgment based on four levels of certainty of evidence: high, moderate, low, very low.

Outcomes

We considered as primary outcomes all-cause mortality at the longest follow up and safety (number of patients experiencing any adverse event and serious adverse event) at the end of treatment. Secondary outcomes were measured at study endpoint and included number of patients with SARS-CoV-2 nasal or pharyngeal swab RT-PCR clearance, time to nasal or pharyngeal swab RT-PCR clearance, number of patients with improvement of pulmonary disease (CT imaging), number of patients experiencing disease progression, number of patients discharged from the hospital, and length of hospital stay.

Dealing With Missing Data

When dichotomous outcome data were missing, they were managed according to the intention-to-treat (ITT) principle, and we assumed that patients who dropped out after randomisation had a negative outcome. Missing continuous outcome data were analysed using the last observation carried forward to the final assessment (LOCF). Where LOCF data were not reported by the trial authors, continuous outcomes data were analysed on an endpoint basis, including only participants with a final assessment. When p values, t-values, CIs or standard errors were reported in articles, we calculated SDs from their values as in Higgins et al. (2011).

Data Analysis

First, we performed pairwise meta-analyses using a random-effects model to estimate pooled risk ratios (RRs) for dichotomous outcomes. We narratively reported hazard ratios (HRs) when RRs were not available. We reported standardised mean differences (SMDs) for continuous outcomes with their 95% confidence intervals (CIs) using The Cochrane Collaboration, 2014. We assessed statistical heterogeneity in each pairwise comparison with τ^2, I^2 statistic, and p value (Higgins and Green, 2011).

We incorporated indirect comparisons with direct comparisons for primary outcomes using random-effects network meta-analyses within a frequentist framework using STATA 16 (network package), and results are presented with the network graphs package (Chaimani et al., 2013). We report the results of network meta-analyses in league tables with effect sizes (RR) and their 95% CIs. While in the pairwise meta-analyses we included all the treatments, we included in our network meta-analysis only those treatments with >100 individuals randomised

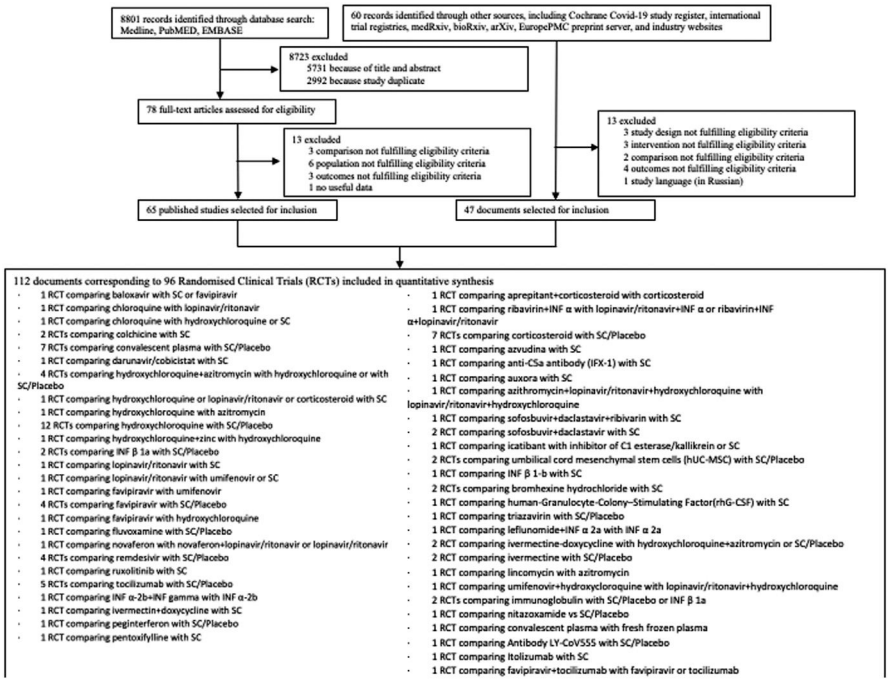

FIGURE 1 | Flow chart.

as some treatment nodes with few total participants resulted in implausible and imprecise effect estimates, as described in Siemieniuk et al. (2020).

We assessed inconsistency between direct and indirect sources of evidence using local and global approaches. Consistency is an important assumption to check in network meta-analyses because it is the manifestation of transitivity in the data from a network of interventions: consistency exists when treatment effects from direct and indirect evidence are in agreement (subject to the usual variation due to heterogeneity in the direct evidence) (Cipriani et al., 2013). A network-meta-analysis can be misleading if the network is substantially inconsistent. Inconsistency can be present if the trials in the network have very different protocols and their inclusion/exclusion criteria are not comparable or may result as an uneven distribution of the effect modifiers across groups of trials that compare different treatments. We first checked for any erroneous data abstraction. Then, to evaluate the presence of inconsistency locally, we used the loop-specific approach (which identified inconsistent loops of evidence) (Chaimani et al., 2014). This method evaluates the consistency assumption in each closed loop of the network separately as the difference between direct and indirect estimates for a specific comparison in the loop (inconsistency factor). The magnitude of the inconsistency factors and their 95% CIs were used to infer about the presence of inconsistency in each loop. We assumed a common heterogeneity estimate within each loop. Global inconsistency was measured with the between-studies standard deviation (SD) (heterogeneity parameter) by using both a consistency and inconsistency model and by measuring the chi-squared inconsistency, with its p value.

We estimated the presence of publication bias and small effect studies by plotting comparison-adjusted funnel plots for the network meta-analyses with a linear regression line (Salanti et al., 2011).

We also estimated the ranking probabilities for all treatments, i.e., their probability of being at each possible rank for each intervention. We report the treatment hierarchy as the surface under the cumulative ranking curve (SUCRA), the probability of being the best and as the mean rank (Salanti et al., 2011).

To determine whether the results were affected by study characteristics, we performed subgroup network meta-analyses for all-cause mortality according to the severity of disease as defined in Jin et al., 2020.

RESULTS

Study Characteristics

We identified 8,861 citations from the search and included 112 articles, comprising 96 trials, which randomised 34,501 patients to 59 pharmacological treatments or combination of treatments or SC or placebo (**Figure 1**). A total of 47 articles were included in the form of preprints or unpublished reports. **Table 1** summarizes the characteristics of included studies, and a full list of references for the included studies is available in **Supplementary Appendix S3**. Further characteristics of the included studies are included in **Supplementary Appendix S4**.

The mean study sample size was 343 participants (SD 1312). In total, 21,846 participants were randomly assigned to an active drug (see **Supplementary Appendix S7** in the supplementary

TABLE 1 | Characteristics of included randomized controlled trials.

Study, year	Country	Study design	Setting	Study duration (days)	Longest follow-up (days)	Intervention	N randomised	Mean (SD)/Median (IQR) age (in years)*	% male*	Disease severity (N)
Abbaspour kasgari, 2020	Iran	OL	Hospital (single-centre)	NR	14	Sofosbuvir plus daclastavir plus ribivarin	24	Median: 45	46	Moderate (all)
						Standard care	24	Median: 60	29	
Abd-elsalam, 2020a	Egypt	NR	Tertiary care units (multicentre)	15	28	Hydroxychloroquine	97	40.4 (18.7)	57.7	Severe (all)
						Standad care	97	41.1 (20.1)	59.8	
Abd-elsalam, 2020b	Egypt	NR	Hospital (NR)	6	28	Hydroxychloroquine plus zinc	96	43.48 (14.62)	54.2	Mild (9), moderate (58), severe (18), critical (11)
						Hydroxychloroquine	95	43.64 (13.17)	67.4	Mild (12), moderate (55), severe (20), critical (8)
Agawal, 2020	India	OL	Hospitals (multicentre)	2	28	Convalescent plasma	235	Median: 52 (42–60)	75	Moderate (all)
						Standard care	229	Median 52 (41–60)	77	
AlQathani, 2020	Bahrain	OL	Hospitals (multicemtre)	2	NR	Convalescent plasma	20	52.6 (14.9)	85	Moderate (all)
						Standard care	20	50.7 (12.5)	75	
Ansarin, 2020	Iran	OL	Univerity hospital (single-centre)	14	28	Bromhexine hydrochloride	39	58.4 (13.7)	48.7	NR
						Standard care	39	61.1 (6.1)	61.5	
Avendaño-solà, 2020	Spagna	OL	Hospitals (multicentre)	1	29	Convalescent plasma	38	61.3 (16.3)	52.6	Moderate (all)
						Standard care	43	60.3 (15)	55.8	
Bajpal 2020	India	OL	Hospital (single-centre)	2	28	Convalescent plasma	15	48.1 (9.1)	78.6	Severe (all)
						Frozen fresh plasma	16	48.3 (10.8)	73.3	
Beigel, 2020	United States, Denmark, United Kingdom, Greece, Germany, korea, Mexico, Spain, Japan, Singapore	DB	Hospitals (multicentre)	10	29	Remdesivir	541	58.6 (14.6)	65.1	Severe (476); mild/ moderate (62)
						Placebo	521	59.2 (15.4)	63.7	Severe (464); mild/ moderate (57)
Brown, 2020	United States	OL	Hospitals (multicentre)	5	28	Hydroxychloroquine	42	Median: 51 (42–60)	56	NR
						Azithromycin	43	Median: 58 (43–68)	67	
Cao B, 2020	China	OL	Hospital (single-centre)	14	28	Lopinavir/ritonavir	99	58 (50–68)	61.6	Severe (all)
						Standard care	100	58 (48–68)	59.0	
Cao Y, 2020	China	SB	Hospital (multicentre)		28	Ruxolitinib	20	63 (51–65	60	Severe (all)
						Standard care	21	64 (59–71)	57.1	
Cavalcanti AB, 2020	Brazil	OL	Hospital (multicentre)	7	15	Hydroxychloroquine plus azithromycin[a]	217	49.6 (14.2)	56.7	Mild (NR), moderate (NR)
						Hydroxychloroquine[a]	221	51.3 (14.5)	64.3	
						Standard care[a]	229	49.9 (15.1)	54.2	
Chen C, 2020	China	OL	Hospital (multicentre)	7–10[b]	10	Favipiravir	116	NR	50.9	Severe (18); moderate (98)
						Umifenovir	120		42.5	Severe (9); moderate (111)
Chen CP, 2020	Taiwan	OL	Hospital (single-centre)	7	14	Hydroxychloroquine	21	33 (12)	52.4	Mild (29), moderate (4)
						Standard care	12	32.8 (8.3)	66.7	
Chen J, 2020a	China	OL	Hospital (single-centre)	5	7	Hydroxychloroquine	15	50.5 (3.8)	60.0	Moderate (all)
						Standard care	15	46.7 (3.6)	80.0	
Chen J, 2020b	China	OL	Hospital (single-centre)	5	14	Darunavir/Cobicistat	15	51.5 (12.2)	60.0	Moderate (all)
						Standard care	15	42.9 (17.7)	60.0	

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TABLE 1 | (*Continued*) Characteristics of included randomized controlled trials.

Study, year	Country	Study design	Setting	Study duration (days)	Longest follow-up (days)	Intervention	N randomised	Mean (SD)/Median (IQR) age (in years)*	% male*	Disease severity (N)
Chen L, 2020	China	OL		10	28	Chloroquine	25	45.22 (13.66)	38.89	Moderate (all)
						Hydroxychloroquine	28	45.67 (14.37)	44.4	
						Standard care	14	51.33 (15.36)	58.30	
Chen P, 2020	United States	DB	Outpatients (single-centre)	11	1 hour	Neutralized antibody LY-CoV555	317	Median: 45 (18–86)	44.7	Mild (all)
						Placebo	150	Median: 46 (18–77)	45.5	
Chen Z, 2020	China	OL	Hospital (single-centre)	5	6	Hydroxychloroquine	31	44.10 (16.1)	45.2	Moderate (all)
						Standard care	31	45.20 (14.7)	48.3	
Cheng L, 2020	China	OL	Hospitals (multicentre)	2	21, 28, 60	Human-granulocyte-colony–Stimulating Factor (rhG-CSF)	100	Median: 45 (40–55)	58	Moderate to severe (NR)
						Standard care	100	Median 46 (38–54)	54	
Chowdhury, 2020	Bangladesh	NR	Outpatients (single-centre)	10	35	Ivermectin plus doxycycline	63	35.72 (15.1)	71.7	Mild (all)
						Hydroxychloroquine plus azithromycin	62	31.9 (12.72)	83.9	
Corral-gudino, 2020	Spain	OL[f]	Hospitals (multicentre)	6	28	Corticosteroid (metylprednisolone)	34	73 (11)	68	Severe (all)
						Standard care	29	66 (12)	55	
Dabbous, 2020	Egypt	OL	Hospitals (multicentre)	10	30	Favipiravir	50	36.3 (12.5)	50	Mild (NR), moderate (NR)
						Standard care*	50	36.4 (11.5)	50	
Davoudi-monfared, 2020	Iran	OL	Hospital (single-centre)	14	28	Interferon β-1a	46	56.50 (16)	52.4	Moderate (NR) to critical (NR)
				7–10 days		Standard care	46	59.53 (14)	56.4	
Deftereos, 2020	Greece	OL	Hospital (multicentre)	21	21	Colchicine	56	63 (55–70)	56	Severe (NR), moderate (NR)
						Standard care	50	65 (54–80)	60	
Dequin, 2020	France	DB	Hospitals (multicentre)	14	28	Corticosteroid (hydrocortison)	76	63.1	71.1	Severe (28), critical (121)
						Placebo	73	66.3	68.5	
Duarte, 2020	Argentina	OL	Hospitals (multicentre)	14	30	Telmisartan	41	60 (17.8)	67.5	NR
						Standard care	41	63.8 (18.7)	55.3	
Dubèe, 2020	France	DB	Hospitals (multicentre)	9	28	Hydroxychloroquine	125	Median: 76 (60–85)	52	Mild (99), moderate (151)
						Standard care	125	Median: 78 (57–87)	44.8	
Edalatifard, 2020	Iran	SB	Hospitals (multicentre)	3	60	Corticostroid (methylprednisolone)	34	55.8 (16.3)	70.6	Severe (all)
						Standard care	34	61.7 (16.6)	53.6	
Entrenas castillo, 2020	Spain	OL	University hospital (single-centre)	Until discharge	28	Calcifediol	50	53.1 (10.8)	54	Moderate to severe (NR)
						Standard care	26	53.8 (9.3)	69	
Esquivel-moynelo, 2020	Cuba	OL	Hospital (single-center)	14	14	Interferon α 2b plus interferon γ	41	Median 42 (19–82)	46.7	Mild (NR) moderate (NR)
						Interferon α 2b	38	Median 31 (19–57)	60.6	
Furtado, 2020	Brazil	OL	Hospitals (multicentre)	10	29	Hydroxychloroquine plus azithromycin	237	Median: 59.4 (49.3–70)	65	Moderate to critical (NR)
						Hydroxychloroquine	210	Median 46 (38–54)	67	
Gharbharan A, 2020	Netherlands	OL	Hospitals (multicentre)	NR	60	Convalescent plasma	43	63 (55–77)	77	Moderate (NR), critical (NR)
						Standard care	43	61 (56–70)	67	
Gharebaghi, 2020	Iran	DB	Hospital (single-centre)	3	NR	Immunoglobulin	30	55.5 (45.6)	70	Severe (all)
						Placebo	29	56 (47.7)	68.9	
Guvenmez, 2020	Turkey	OL	Hospital (single-centre)	5	6	Lincomycin	12	58.4 (15.4)	66.7	Moderate (all)
						Azithromycin	12	59.1 (16.6)	58.3	

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TABLE 1 | (*Continued*) Characteristics of included randomized controlled trials.

Study, year	Country	Study design	Setting	Study duration (days)	Longest follow-up (days)	Intervention	N randomised	Mean (SD)/Median (IQR) age (in years)*	% male*	Disease severity (N)
Hashim, 2020	Iraq	NR	Hospital (critical and severe ill)/Outpatients (mild/moderate)	10	NR	Ivermectin + Doxycycline	70	50.1 (9.3)	53	Mild/moderate (48), severe (11), critical (11)
						Standard care	70	47.2 (7.8)	51	Mild/moderate (48), severe (11)
Hermine 2020	France	OL	Hospitals (multicentre)	1	90	Tocilizumab	64	Median: 64 (57.1–74.3)	70	Moderate (NR), severe (NR)
						Standard care	67	Median: 63.3 (57.1–72.3)	66	
Huang, 2020	China	OL	Hospital (single-centre)	10	14	Chloroquine	10	41.5 (33.8–50)	30.0	Severe (3); moderate (7)
						Lopinavir/ritonavir	12	53.0 (41.8–63.5)	50.0	Severe (5); moderate (7)
Huang Y-Q, 2020	China	OL	Hospital (single-centre)	14	28	Ribavirin	33	40.3 (12.5)	55	Moderate (all)
						Lopinavir/ritonavir plus interferon α	36	43.3 (10.4)	53	
						Ribavirin plus lopinavir/ritonavir plus interferon α	32	43.8 (11.7)	28	
Hung, 2020	China	OL	Hospitals (multicentre)	14	14	Lopinavir/ritonavir + ribavirine + interferon β-1b	86	51 (31–61.3)	52.0	Mild (NR); moderate (NR)
						Lopinavir/ritonavir	41	52 (33.5–62.5)	56.0	
Ivashchenko, 2020	Russia	OL	Hospitals (multicentre)	14	29	Favipiravir (1,600/600 mg)	20	51 (15.6)	40	Moderate (all)
						Favipiravir (1800/800 mg)	20	52.6 (15)	65	
						Standard care	20	48.6 (16.1)	45	
Jagannathan, 2020	United States	SB	Outpatients	1	28	Peginterferon Lambda-1a	60	Median: 37 (18–66)	60	Mild/ moderate (all)
						Placebo	60	Median: 34 (20–71)	54	
Jeronimo, 2020	Brazil	DB	Hospital (single-centre)	5	28	Corticosteroid	209	54 (14.9)	65.9	Moderate to critical (NR)
						Placebo	207	56 (15.5)	64.7	
Kamran, 2020	Pakistan	OL	Hospital (single-centre)	5	14	Hydroxychloroquine	349	34 (11.8)	93.2	Mild (all)
						Standard care	151	34 (9.8)		
Khamis, 2020	Oman	OL	Hospital (single-centre)	10 + 5 8	14	Favipiravir plus interferon β 1b	44	54 (15)		Moderate to severe (NR)
						Hydroxychloroquine	45	56 (16)		
Krolewiecki, 2020	Argentina	OL	Hospitals (multicentre)	5	30	Ivermectin	30	42.3 (12.8)	50	Mild/ moderate (all)
						Standard care	15	38.1 (11.7)	67	
Kumar, 2020	India	OL	Hospitals (multicentre)	NR	30	Itolizumab	22	49.55 (12.49)	95	Severe (all)
						Standard care	10	48.3 (14.62)	70	
Lenze, 2020	United States	DB	Outpatients	15	15	Fluvoxamine	80	Median: 46 (35–58)	30	NR
						Placebo	72	Median: 45 (36–54)	26	
Li L, 2020	China	OL	Hospital (multicentre)	2–3 (hours)	28	Convalescent plasma	52	70 (62–80)	59.9	Severe (45), critical (58)
						Standard care	51	69 (63–76)	64.7	
Li T, 2020	China	OL	Hospital (single-centre)	14	28	Bromhexine hydrochloride	12	Median: 53	83.3	Mild/ moderate (NR)
						Standard care	6	Median: 47	66.7	
Lopes, 2020	Brazil	DB	Hospital (NR)	10	28	Colchicine	19	Median: 48 (41.5–64)	52.9	Moderate to severe (NR)
						Placebo	19	Median: 53 (35.5–65.5)	27.8	

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TABLE 1 | (*Continued*) Characteristics of included randomized controlled trials.

Study, year	Country	Study design	Setting	Study duration (days)	Longest follow-up (days)	Intervention	N randomised	Mean (SD)/Median (IQR) age (in years)*	% male*	Disease severity (N)
Lou Y, 2020	China	OL	Hospital (single-centre)	7	14	Baloxavir	10	53.5 (12.5)	70.0	Moderate (NR);
						Favipiravir	10	58 (8.1)	77.0	severe (NR);
						Existing antiviral treatment	10	46.6 (14.1)	70.0	critical (NR)
Maldonado, 2020	Mexico	NR	Hospital (single-centre)	Until discharged	Until discharged	Pentoxyfilline	36	55.3 (9.2)	53.8	NR
						Standard care	18	62.3 (15.3)	58.3	
Mansour, 2020	Brazil	OL	Hospital (single-centre)	4	28	Icatibant	10	51.6 (9.1)	70	Severe (all)
						Inhibitor of C1 esterase/kallikrein	10	54.4 (14.8)	40	
						Standard care	10	48.9 (10.5)	50	
Mehboob, 2020	Pakistan	OL	Hospital (single-centre)	3–5	5	Aprepitant plus corticosteroid	8	47.63 (12.1)	37.5	Moderate (5),
						Corticosteroid	10	60.9 (9.8)	80	severe (6), critical (7)
Miller, 2020	United States	OL	Hospitals (multi-centre)	3	28	Auxora[e]	20	59 (12); 64 (14)e	41, 33	Severe (all)
						Standad care[e]	10	61 (13), 36e	56, 100	
Mitijà O, 2020	Spain	OL	Outpatients	7	14	Hydroxychloroquine	136	41.6 (12.4)	72.1	Mild (all)
						Standard care	157	41.7 (12.6)	65.6	
Monk, 2020	United Kingdom	DB	Hospitals (multi-centre)	14	28	Interferon β 1a	50	57.8 (14.6)	56	Mild/moderate (11), severe (37)
						Placebo	51	56.5 (11)	62	Mild/moderate (21), severe (29)
Morteza, 2020	Iran	OL/DB	Hospital (NR)	5	NR	Ivermectin (200 mg/kg)	30	Median:61 (42–69)	40	Mild/moderate (29), severe (1)
						Ivermectin (200,200,200 mg/kg)	30	Median: 53 (47–60)	63.3	Mild/moderate (22), severe (26)
						Ivermectin (400 mg/kg)	30	Median: 54 (46–65)	53.3	Mild/moderate (25), severe (5)
						Ivermectin (400,200,200 mg/kg)	30	Median: 54 (46–65)	43.3	Mild/moderate (25), severe (5)
						Standard care	30	Median: 55 (45–70)	53.3	Mild/moderate (27), severe (3)
						Placebo	30	Median: 58 (45–68)	46.7	Mild/moderate (28), severe (2)
Nojomi, 2020	Iran	OL	Hospitals (multicentre)	7–14	30	Umifenovir plus hydroxychloroquine	50	56.6 (17.8)	66	Mild (9), moderate (29), severe (12)
						Lopinavir/ritonavir plus hydroxychloroquine	50	52.6 (14.8)	54	Mild (10), moderate (29), severe (11)
Omrani, 2020	Qatar	DB	Outpatients	7	21	Hydroxychloroquine + Azitromycin	152	Median: 42 (38–48)	98.7	Mild (all)
						Hydroxychloroquine	152	Median: 40 (31–47)	98	
						Placebo	152	Median: 41 (31–47)	98.7	

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TABLE 1 | (*Continued*) Characteristics of included randomized controlled trials.

Study, year	Country	Study design	Setting	Study duration (days)	Longest follow-up (days)	Intervention	N randomised	Mean (SD)/Median (IQR) age (in years)*	% male*	Disease severity (N)
Pan (SOLIDARITY trial), 2020	Albania, Argentina, Austria, Belgium, Brazil, Canada, Colombia ecypt, Honduras, India, Indonesia, Iran, Ireand, Italy, kwait, Lebanon, Luxembourg, Lithuania, Malaysia, north Macedonia, Pakistan, Norway, Peru, Philippines, Saudi Arabia, soputh africa, Spain, Switzerland	OL	Hospitals (multicentre)	10, 14, 6	28	Remdesivir	2750	NR	62.2	Mild/moderate (4964), severe (487)
						Standard care	2725		63.7	
						Hydroxychloroquine	954		60.6	Mild/moderate (1,686), severe (167)
						Standard care	909		59	
						Lopinavir-ritonavir	1,411		60.8	Mild/moderate (2545), severe (226)
						Standard care	1,380		58.5	
						Interferon beta 1a	2050		63.6	Mild/moderate (3831), severe (269)
						Standard care	2064		62.3	
Rahamani, 2020	Iran	OL	Hospitals (multicentre)	14	28	Interferon β 1b	40	Median: 60	60.6	NR
						Standard care	40	Median: 61	57.6	
Ray, 2020	India	OL	Hospital (NR)	1	30	Convalescent plasma	40	Total: 61.43 (11.33)	75	Severe (all)
						Standard care	40		67.5	
Recovery trial, 2020	United Kingdom	OL	Hospital (multicentre)	10	28	Hydroxychloroquine	1,561	65.2	62	Moderate (NR) to critical (NR)
						Standard care	3155	65.4	63	
						Dexamethasone	2104	66.9	64	
						Standard care	4321	65.8	64	
						Lopinavir-ritonavir	1,596	NR	NR	
						Standard care	3376	NR	NR	
REMAP-CAP trial, 2020	United Kingdom, Europe, Australia	OL	ICU (multicentre)	7	21	Corticosteroid (Hydrocortisone)_fixed dose	143	60.1 (15.8)	59.6	Severe (all)
						Corticosteroid (Hydrocortisone) _shock-dependent	152	62.7 (13.1)	65.6	
						Standard care	108	60.1 (15.8)	59.6	
Ren, 2020	China	OL	Hospital (single-centre)	5	NR	Azvudina	10	Median: 52 (17–61)	60	Mild (3), moderate (17)
						Standard care	10	Median: 50.5 (29–76)	60	
Rocco, 2020	Brazil	DB	Outpatient	5	6	Nitazoxanide	238	18–77	52	Mild/ moderate (all)
						Placebo	237	18–77	42	
Rosas, 2020	Canada, Denmark, France, Germany, Netherlands, Spain, United States	DB	Hospitals (multicentre)	7	28, 60	Tocilizumab	301	60.9 (14.6)	69.7	Severe (all)
						Placebo	151	60.6 (13.7)	70.1	
Ruzhentsova, 2020	Russia	OL	Outpatients/hospitals (multicentre)	10	28	Favipiravir	112	41.7 (10.6)	43.8	Mild/ moderate (all)
						Standard care	56	42 (10.4)	53.6	
Sadeghi, 2020	Iran	OL	Hospitals (multicentre)	14	30	Sofosbuvir plus daclastavir	35	Median: 58	61	Moderate (NR), severe (NR)
						Standard care	35	Median: 62	42	
Sakoulas, 2020	United States	OL	Hospitals (multicentre)	3	30	Intravenous immunoglobulin	17	56.6 (17.8)	66	Moderate (NR), severe (NR)
						Standard care	17	52.6 (14.8)	54	
Salama, 2020	United States, Mexico, Kenya, South Africa, peri, Brazil	DB	Hospitals (multicentre)	1	28, 60	Tocilizumab	259	56 (14.03)	60.2	Severe (all)
						Placebo	129	55.6 (14.9)	57	
Salvarani C, 2020	Italy	OL	Hospitals (multicentre)	8–12 (hours)	30	Tocilizumab	60	Median: 61.5 (51.5–73.5)	66.7	Severe (all)
						Standard care	66	Median: 60 (54–69)	56.1	
Sekhavati, 2020	Iran	OL	Hospital (single-centre)	5	30	Azytromicin plus lopinavir/ritonavir plus hydroxychloroquine	56	54.4 (15.9)	50	NR
						Lopinavir/ritonavir plus hydroxychloroquine	55	59.9 (15.5)	41.8	

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TABLE 1 | (*Continued*) Characteristics of included randomized controlled trials.

Study, year	Country	Study design	Setting	Study duration (days)	Longest follow-up (days)	Intervention	N randomised	Mean (SD)/Median (IQR) age (in years)*	% male*	Disease severity (N)
Self, 2020	United States	DB	Hospitals (multicentre)	5	28	Hydroxychloroquine	242	Median: 58 (45–69)	55.8	Severe (all)
						Placebo	237	Median: 57 (43–68)	55.7	
Shi, 2020	China	DB	Hospital (single-centre)	6	28	Umbilical cord_ mesenchymal stem cells (hUC-MSC)	66	60.7 (9.1)	56.9	Severe (all)
						Placebo	35	59.9 (7.8)	54.3	
Shu, 2020	China	OL	Hospital (single-centre)	5	30	Umbilical cord_ mesenchymal stem cells (hUC-MSC)	12	61 (17.9)	66.7	Mild (3), moderate (28), severe (10)
						Standard care	29	57.9 (15.8)	51.2	
Simonovic, 2020	Argentina	DB	Hospitals (multicentre)	1	30	Convalescent plasma	228	Median: 62.5 (53–72.5)	70.6	Severe (all)
						Placebo	106	Median: 62 (49–71)	61	
Spinner, 2020	United States, Italy, Spain, Germany, Hong Kong, Singapore, South Korea, and Taiwan	OL	Hospitals (multicentre)	5–10	11	Remdesivir 5 days	197	Median: 56	61	Moderate (all)
						Remdesivir 10 days	199	Median: 58	60	
						Standard care	200	Median: 57	63	
Stone, 2020	United States	DB	Hospitals (multicentre)	1	28	Tocilizumab	161	Median: 61.6 (46.4–69.7)	60	Moderate (NR), severe (NR)
						Placebo	82	Median: 56.5 (44.7–67.8)	55	
Tabarsi, 2020	Iran	OL	Hospital (single-centre)	14	NR	Immunoglobulin	52	54.29 (12.85)	76.9	Severe (all)
						Standard care	32	52.47 (14.49)	78.1	
Tang, 2020	China	OL	Hospitals (multicenter)	14–21[b]	28	Hydroxychloroquine	75	48 (14.1)	56.0	Severe (1); moderate (59); mild (15
						Standard care	75	44.1 (15)	53.0	Severe (1); moderate (67); mild (7)
Tomazini, 2020	Brazil	OL	ICU (multicentre)	10	28	Corticosteroid (dexamethasone)	151	60.1 (15.8)	59.6	Critical (all)
						Standard care	148	62.7 (13.1)	65.6	
Udwadia, 2020	India	OL	Hospitals (multicentre)	14	28	Favipiravir	75	43.6 (12.2)	70.8	Mild (47), moderate (28)
						Standard care	75	43 (11.7)	76	Mild (45), moderate (30)
Ulrich, 2020	United States	DB	Hospitals (multicenter)	6	14, 30	Hydroxychloroquine	67	65.5 (16.4)	67.2	Mild (NR),
						Standard care	61	65.8 (16)	50.8	moderate (NR), severe (NR)
Vlaar, 2020	Netherlands	OL	Hospital (single-centre)	22	28	Anti-c5a antibody (IFX-1)	15	58 (9)	73	Moderate (4),
						Standard care	15	63 (8)	73	severe (8), critical (18)
Wang, 2020	China	DB	Hospital (multicentre)	10	28	Remdesivir	158	Median 66	56.0	Severe (all)
						Placebo	79	Median 64	65.0	
Wang D, 2020	China	OL	Hospitals (multicentre)	1	14	Tocilizumab	33	Median: 65.3 (58–71)	69.7	Moderate (37), severe (28)
						Standard care	32	Median: 63 (54–69	70.1	
Wang M, 2020	China	OL	University hospital (single-centre)	10	60	Leflunomide + Interferon α 2a	26	Median: 56 (43–67.3)	54.2	NRg
						Interferon α 2a	24	55.5 (47.8–66.5)	37.5	

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TABLE 1 | (*Continued*) Characteristics of included randomized controlled trials.

Study, year	Country	Study design	Setting	Study duration (days)	Longest follow-up (days)	Intervention	N randomised	Mean (SD)/Median (IQR) age (in years)*	% male*	Disease severity (N)
Wu, 2020	China	DB	Emerrgency dept., isolation wards, ICU (multicentre)	7	28	Triazavirin	26	Median: 53 (46–62)	53.9	Mild to severe
						Placebo	26	Median 59 (51–69)	46.1	
Yakoot, 2020	Egypt	OL	Hospital (NR)	10	21	Sofosbuvir + daclastavir	44	Median: 48 (34–59)	41	Mild (6), moderate (30), severe (8)
						Standard care	45	Median: 50 (31–60)	45	Mild (6), moderate (31), severe (8)
Yueping, 2020	China	OL	Hospital (single-centre)	14	21	Lopinavir/ritonavir	34	50.7 (15.4)	50	Mild (11); moderate (NR)
						Umifenovir	35	50.5 (14.6)	45.7	
						Standard care	17	44.3 (13.1)	41.2	
Zhao, 2020	China	OL	Hospitals (multicentre)	7	60	Favipiravir + tocilizumab	14	Median: 75 (34–81)	42.9	Moderate to critical (NR)
						Favipiravir	7	Median: 70 (45–89)	71.4	
						Tocilizumab	5	Median: 71 (48–77)	60	
Zheng, 2020	China	OL	Hospitals (multicentre)	7–10[a]	9	Novaferon	30	50.1	56.7	Severe (2); moderate (28)
						Novaferon plus lopinavir/ritonavir	30	48.8	43.3	Severe (2); moderate (28)
						Lopinavir/ritonavir	29	41.1	41.4	Severe (1); moderate (28)

*Note: DB= double blind, NR=not reported, OL= open label, SB=single blind *: in some studies the information was reported only for the analysed participants (e.g. ITT population), a:172 in Hydroxychloroquine plus Azithromycin arm, 159 Hydroxychloroquine arm and 173 Standard care confirmed with COVID-19 by RT-PCR test b: the course of treatment in both groups was 7-10 days. c: the course of treatment in moderate patients was 14 day and in severe patients was 21 days; d: in Standard care arm the course of treatment was 7-10 days; e: 26 patients received low flow supplemental oxygen (17 assigned to Auxora, 9 assigned to SC) and 4 patients received high flow supplemental oxygen (3 assigned to Auxora, 1 assigned to SC); f:partially randomized controlled trial. g: prolonged PCR positivity. *: quote: "50 patients who received oseltamivir 75 mg 12 hourly for 10 days and hydroxychloroquine 400 mg 12 hourly on day-one followed by 200 mg 12 hourly daily on day-2 to10 days conforming to the national.*

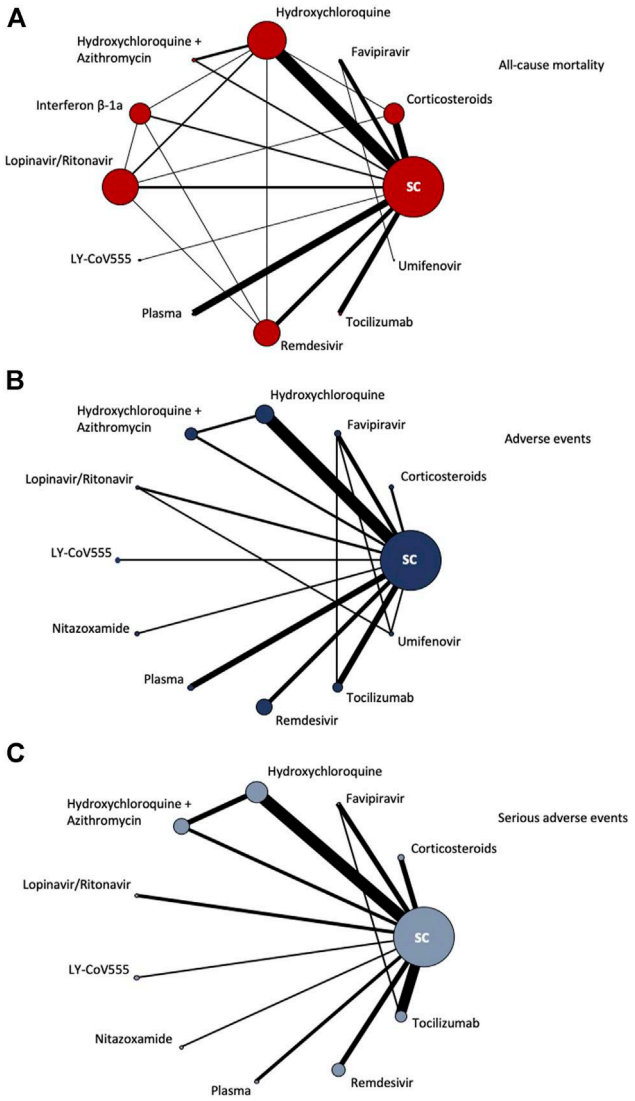

FIGURE 2 | Network of eligible comparisons for all-cause mortality, adverse events and serious adverse events. The figure plots the network of eligible direct comparisons for all-cause mortality (42 studies) **(A)**, adverse events (30 studies) **(B)** and serious adverse events (30 studies) **(C)**. The width of the lines is proportional to the number of trials comparing every pair of treatments, and the size of every node is proportional to the number of randomized participants.

material) and 12,655 were randomly assigned to placebo or SC. The mean age was 51.7 years (SD 8.4), while two third (40.8%) of the sample population were women. The average duration of the treatment in the studies was 7.9 days (SD 4.8), while the average duration of follow up was 26.1 days (SD 12.9). The evaluation of transitivity assessment was evaluated in all trials included in the network irrespectively of the outcome being reported for the following effect modifiers: age, gender, disease severity (mild to moderate, severe, critical) and is reported in **Supplementary Appendix S5**.

Seventy-two studies compared active drugs only with SC or placebo, eighteen studies compared active drugs only with other active drugs and six three-arm studies compared active drugs with other active drugs and with SC or placebo. Most of the studies were conducted in China (25 of 96), thirteen studies were conducted in Europe (i.e. France, Greece, Italy, Netherlands, Spain, United Kingdom). Eleven studies were conducted in United States, eleven in Iran, seven studies in Brazil, five in India, four in Egypt and three in Argentina. Two studies were conducted in Russia and two in Pakistan, while six studies were intercontinental. Other nine countries contributed to the pool of the evidence with one study each (see **Table 1** for more details). In terms of risk of bias, 35% of the RCTs were at high risk of bias (34 of 96), 44% were at unclear risk of bias (42 of 96) and 21% at low risk of bias (20 of 96) (See **Supplementary Appendix S6** for the full risk of bias assessment).

Figure 2 shows the network of eligible comparisons for all-cause mortality, adverse events and serious adverse events. An analysis of the geometry of the network showed a well-connected polygon for all-cause mortality, with some single-connected nodes which included LY-CoV555, plasma, tocilizumab and umifenovir. The single-connected nodes are poorly connected to the rest of the network and will provide more imprecise estimates. For the safety outcomes (i.e. AEs and SAEs), we can see from **Figure 2** more single-connected nodes and overall poorer connected networks which therefore depended extensively on indirect comparisons.

Pairwise Meta-Analysis

The pairwise meta-analysis and data on heterogeneity are presented in the Supplementary Material (**Supplementary Appendix S7**). The pairwise meta-analysis for the primary outcomes showed a reduction of all-cause mortality for Human-Granulocyte-Colony–Stimulating Factor (rhG-CSF) (RR 0.25, 95%CI 0.07 to 0.86, 1 RCT, n = 200) compared to SC. Regarding safety, a number of pharmacological interventions were worse than SC in terms of adverse events, including colchicine (RR 2.17, 95%CI 1.29–3.65), hydroxychloroquine (RR 1.99, 95%CI 1.13–3.51), the combination of hydroxychloroquine and azithromycin (RR 1.39, 95%CI 1.06–1.82), rhG-CSF (RR 2.02, 95%CI 1.62–2.50). In terms of serious adverse events remdesivir was safer than SC (RR 0.75, 95% CI 0.63–0.89).

Regarding secondary outcomes, the pairwise meta-analysis showed that azvudine, nitazoxamide and convalescent plasma were better than SC in terms of SARS-CoV-2 clearance rate (RR ranging from 1.6 to 2.33). Telmisartan and tocilizumab compared to SC reduced length of hospital stay (HR 2.02 and 1.24, respectively). Hydroxychloroquine and ruxolitinib compared to SC showed in one small trial each to improve pulmonary disease in CT imaging (RR 3.80 and 1.45, respectively). In one RCT, rhG-CSF had a reduction in the progression of COVID-19 disease when compared to SC (RR 0.13, 95%CI 0.03–0.57). Remdesivir and telmisartan were superior compared to SC for number of patients discharged from hospital (RR 1.13 and 1.61, respectively).

Network Meta-Analysis

The results of the network meta-analysis are presented in **Figure 3** for the primary outcomes. In terms of all-cause mortality, we evaluated 42 studies. When compared to SC or placebo, only corticosteroids significantly reduced the mortality rate (RR 0.90,

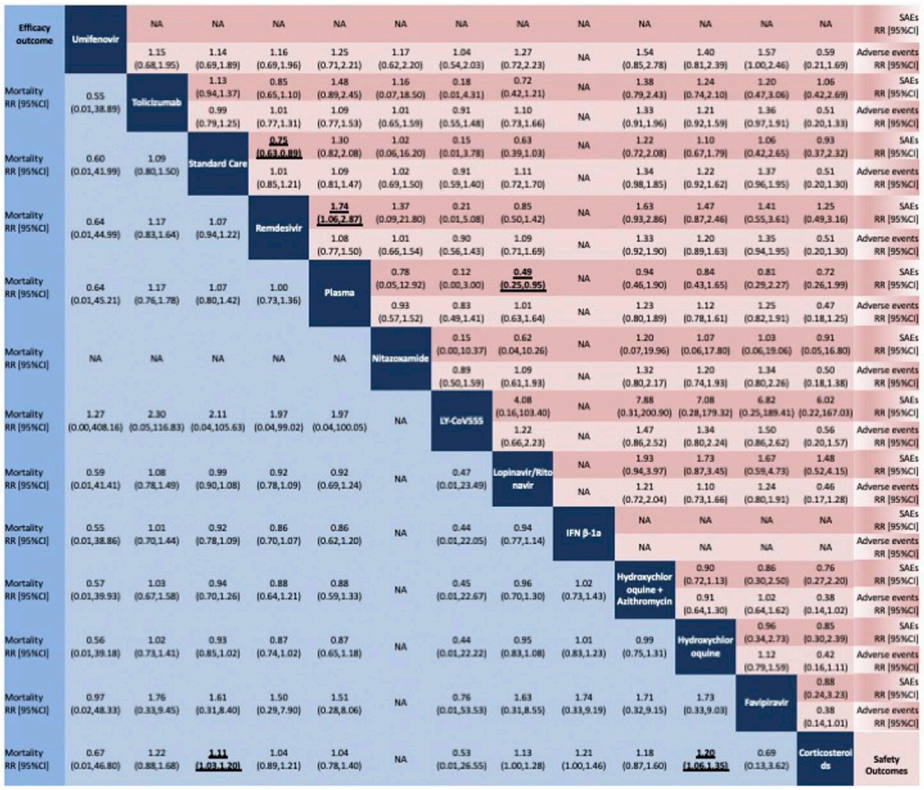

FIGURE 3 | Network meta-analysis of all-cause mortality (blue), adverse events (light red) serious adverse events (red). Pharmacological treatments are reported in alphabetical order. Comparisons should be read from left to right. All-cause mortality and safety estimates are located at the intersection between the column-defining and the row-defining treatment. For all-cause mortality, RRs above 1 favor the column-defining treatment. For safety, RRs above 1 favor the row-defining treatment. We incorporated the GRADE judgments in the figure. Estimates in gray have a very low or low certainty of evidence.

95%CI 0.83 to 0.97, moderate certainty of evidence). Corticosteroids significantly reduced the mortality rate also when compared to hydroxychloroquine (RR 0.83, 95%CI 0.74 to 0.94, moderate certainty of evidence).

In terms of AEs, we evaluated 30 studies. No significant differences were found between the included compounds. Remdesivir proved to be better in terms of SAEs (30 studies included in the whole network) when compared to SC or placebo (RR 0.75, 95%CI 0.63 to 0.89, high certainty of evidence) and plasma (RR 0.57, 95%CI 0.34 to 0.94, high certainty of evidence). The combination of lopinavir and ritonavir proved to reduce SAEs when compared to plasma (RR 0.49, 95%CI 0.25 to 0.95, low certainty of evidence). The global inconsistency was not significant for all the outcomes considered (See **Supplementary Appendix S9**). Tests of local inconsistency did not show any inconsistent loops (See **Supplementary Appendix S9**). The comparison-adjusted funnel plots of the network meta-analysis were suggestive for some publication bias for all-cause mortality (42 studies evaluated) (see **Supplementary Appendix S10**). Few studies reported similar comparisons for AEs and SAEs (30 studies evaluated for both AEs and SAEs), which makes difficult the interpretation of the funnel plots for safety outcomes. **Supplementary Appendix S11** in Supplementary Material presents the ranking of treatments based on cumulative probability plots and SUCRAs.

The certainty of evidence for the relative treatment effects of all-cause mortality and safety outcomes varied from high to very low (See **Supplementary Appendix S12**).

Subgroup Analysis

The subgroup network meta-analysis for all-cause mortality according to the severity of disease showed a positive effect for corticosteroids compared to SC or placebo for individuals with a severe (RR 1.13, 95% CI 1.01–1.27) or critical condition (RR 1.28, 95%CI 1.05–1.58). Remdesivir showed to be effective compared to SC or placebo only for individuals with a severe condition (RR 1.18, 95%CI 1.01–1.38). No pharmacological treatments proved to be useful for individuals with a mild to moderate disease (see **Figure 4**).

DISCUSSION

This study includes 96 trials randomising a total of 34,501 patients to receive one of 59 therapeutic options and comparing these to either SC or placebo. This is part of a living systematic review and network meta-analysis previously registered on Prospero (number CRD42020176914) investigating pharmacological

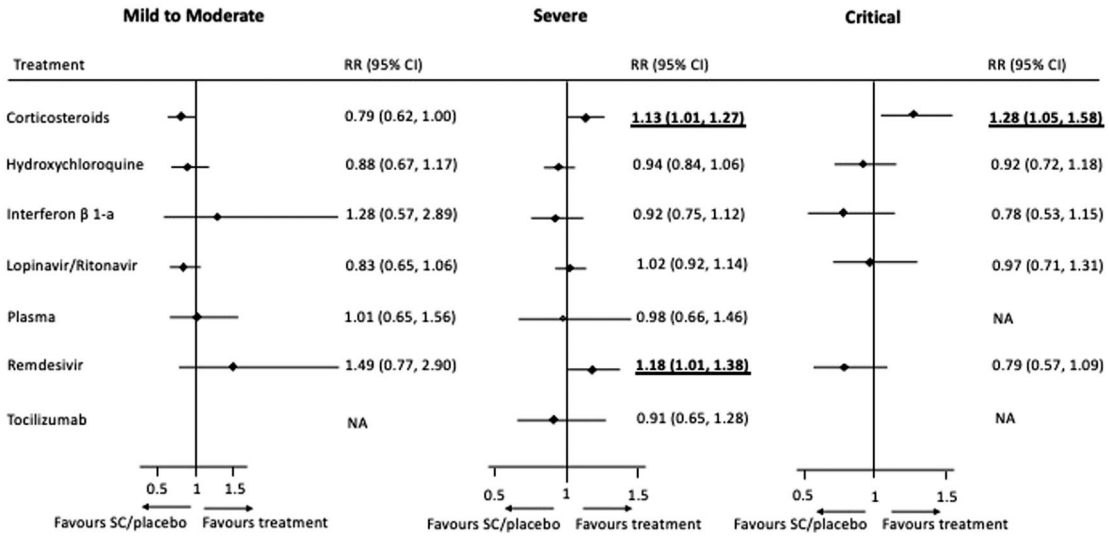

FIGURE 4 | Forest plots of network meta-analysis by subgroup for disease severity: Mild to Moderate (17 trials), Severe (21 trials), and Critical (9 trials). Treatments are compared for each treatment to standard care or placebo, and performed for treatments with >100 individuals randomised.

interventions against Covid-19 and encompasses all of the comparative RCTs until this point (December 10, 2020). The 59 options comprise both single agents and combination therapies. Our work is registered as a prospective network meta-analysis, which confers several advantages over the more common practice of meta-analyses done on retrospective collection of RCTs. It is a living study and so will extend synchronously with the evidence as new data is published. Our results come at an opportune time because clinical practice can be informed based on the evidence already available.

We found that corticosteroids reduced all-cause mortality in patients with Covid-19. Remdesivir was safer than SC in terms of SAEs, while no treatment proved superiority over others in terms of AEs. High value and clinically important objective outcomes were chosen in the form of mortality, adverse events and serious adverse events in order to give this study credence and help us to make clearer recommendations.

In general, according to our analysis, we can recommend corticosteroids as they reduced mortality significantly with a moderate certainty of evidence. However, based on our subgroup analysis we would recommend corticosteroids only for individuals with a severe or critical disease as they did not prove to be superior to SC or placebo for individuals with a mild to moderate disease.

There are a plethora of secondary outcomes of lesser importance than mortality. Other agents have appeared superior in these outcomes however paint an unconvincing picture with a low certainty of evidence.

Recently, several systematic reviews on the effectiveness of pharmacological compounds for Covid-19 have been published. This report has several originalities: it focused on all pharmacological treatments now under investigation, compared versus placebo, standard care or active control; it was the result of one of the first protocols on this subject registered on the Prospero database (CRD42020176914); it produced continuous analyses which were integrated into a platform ready to be used by decision-makers in the context of this pandemic (https://eunethta.eu/covid-19-treatment/). Our data are consistent with a recent systematic review that summarised evidence about the benefits and harms of hydroxychloroquine or chloroquine for the treatment or prophylaxis of Covid-19 either from observational and randomised clinical trials (Hernandex et al., 2020). The authors concluded that evidence on the benefits and harms of using hydroxychloroquine or chloroquine to treat COVID-19 was very weak and conflicting. Our study differs with another network meta-analysis that was recently published (Siemieniuk et al., 2020). One of the differences it that our network analysis was performed under a frequentist framework and the other was a Bayesian. A second difference is that our search strategy is more recent, extending to include studies for one month later. Moreover we included important unpublished data that were not included by Siemieniuk et al. (Siemieniuk et al., 2020), such as the SIMPLE trial (Spinner et al., 2020) and the hydroxychloroquine and lopinavir/ritonavir arms of the RECOVERY trial (Horby et al., 2020; RECOVERY Collaborative Group, 2020). We are aware of an initiative that has been taken by some Cochrane groups which performs comprehensive and living systematic reviews and network meta-analyses of preventative treatment, rehabilitation, pharmacological and non-pharmacological treatments for Covid-19 (Boutron et al., 2020 - available at: https://covid-nma.com/). The aim of this systematic review is more targeted to pharmacological treatments.

Our study has some limitations.

Firstly, outcomes are not being consistently reported by different trials and although we included a total of 96 RCTs, only 42 studies were used in the network meta-analysis for all-

cause mortality, 30 in the network meta-analysis for adverse events and 30 in the network meta-analysis for serious adverse events.

A second limitation of our work is the small number of compounds currently included in our network meta-analysis, due to the low number of patients randomised to many treatments. Despite this several significant results were able to be achieved. Although we currently face a limitation in that many eligible studies are not numerous, this living study will become more substantial and comprehensive as time progresses. This will allow the evidence base to be drawn from an ever-greater number of studies. Studies are being released at a rapid rate reducing bias from differing times of data collection. In this context, we will be able to produce comparative evidence earlier and more efficiently as new evidence is published.

A third limitation to consider is that a number of the trials we have used are unpublished. On a positive note it is helpful to extract unpublished data because it gives us more information, however this might potentially produce less reliable analysis as results have not been through the process of peer review (Zhao et al., 2021). We plan to conduct in future a meta-regression to evaluate the impact of unpublished data on the effect estimates.

Fourth, 'standard care' is heterogeneously defined and can consist of supportive care with intravenous resuscitation fluid, antibiotics, analgesics and anti-pyretics but also antiviral agents and glucocorticoids. This means that some drugs that are used as experimental in some trials are used as SC. One reason for this is that many clinicians have resorted to using off-label medications with a lack of other viable options. This could create a confounder for the trial analysis and can dampen the internal validity of the trial.

Fifth, only few of the trials were double-blinded, while most were open-label. This resulted in a high rating for risk of bias and low certainty of evidence according to GRADE. However given our objective outcome measures this will be less relevant than if our outcome measures had been subjective.

We believe that the results of our research can be informative for patients, clinicians and policy-makers. Corticosteroids reduced all-cause mortality with a moderate certainty of evidence compared to SC in individuals with a severe or critical disease. The safety profile of remdesivir was better than SC and we have also a moderate certainty of evidence that hydroxychloroquine and lopinavir/ritonavir do not affect all-cause mortality compared to SC. Corticosteroids were better than hydroxychloroquine for all-cause mortality with moderate certainty of evidence. Data emerging from observational studies culminated in regulatory decisions by World health Organization and national authorities that limited the use of hydroxychloroquine outside clinical trials (Ledford, 2020). Our analysis supports this decision overcoming several potential biases associated with analysis based on observational studies. However, debate as to which patients should receive hydroxychloroquine is continuing. Results from rhG-CSF are encouraging but we must wait for further research before commenting on whether they affect mortality as it was studied in one small RCT. Clearly, drugs repurposed for the treatment of Covid-19 showed limited effectiveness (Kotecha et al., 2020).

We registered this as a prospective study in order to capitalise on the benefits that this provides. Consistently agreed outcome measures between researchers is one of these, and as this living study proceeds, we hope to attain that. The differentiation of patients by mild, moderate and severe disease would also be helpful. Future research should be prospectively planned in this way, and refocused in a coordinated effort to improve critical patient outcomes. This was a network meta-analysis of aggregate data which comprises the highest certainty evidence available at the present time. However we would like to stress the importance to researchers of sharing their data which increases transparency. Meta-analysis of individual patient data from RCTs would be the next logical step allowing tailored treatments dependent on patient characteristics.

DATA AVAILABILITY STATEMENT

Publicly available data were analyzed in this study. Our living review can be found at https://www.deplazio.net/farmacicovid/index.html.

AUTHOR CONTRIBUTIONS

FD, GD'A, LA, and SV conceived and designed the study. FC, SV, RS, and ZM, selected the articles and extracted the data. FD and FC analysed data. FD and LM wrote the first draft of the report. LA, VD, AA, MD, and SV interpreted data and contributed to the final version of this report. All authors agreed with the results and conclusions reported.

FUNDING

The funder of the study had no role in study design, data collection, data analysis, data interpretation or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. FD is supported by a National Institute for Health Research (NIHR) Research Professorship to Professor Andrea Cipriani (grant RP-2017-08-ST2-006) and an NIHR Oxford Health Biomedical Research Centre Grant (grant BRC-1215-20005). Views expressed are those of the authors and not necessarily those of the United Kingdom National Health Service, the NIHR, or the United Kingdom Department of Health.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.649472/full#supplementary-material

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A Survey Among Italian Physicians During COVID-19 Outbreak. Could Bacillus Calmette–Guérin Vaccine Be Effective Against SARS-CoV2?

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Background: Epidemiological studies show that BCG-vaccinated population seems to be more likely protected from COVID-19 infection, but WHO gave a stark warning on use of BCG vaccine without confirmed COVID-19 trials. The aim of the study is to evaluate whether TB vaccination, performed several years earlier, could confer protection against COVID-19.

Methods: After the Ethical Committee authorization, professional orders were used to contact physicians with an online survey. Specialty, COVID-19 infection and previous BCG vaccination were recorded. Statistical data analysis was performed.

Results: 1906 physicians answered the questionnaire, (M = 1068; F = 838; mean age 50.7 ± 13.3 years; range 24–87), more than half (1062; 55.7%) experienced BCG vaccination. Professional activity was recorded, and only 49 subjects (2.6%) of them were infected by SARS-CoV2. Among the group of infected people, asymptomatic form occurred in 12 subjects (24.5%); a pauci-symptomatic form in 24 subjects (49.0%); and a severe form (pneumonia and/or respiratory distress) in 13 (26.5%). Considering only the clinically relevant form of COVID-19, period prevalence was 2.2% (23/1062) in the vaccinated group and 1.7% (14/844) in the unvaccinated group (OR: 1.31, 95% C.I.: 0.68–2.63, $p = 0.427$).

Conclusion: Our experience does not confirm the possible protective role of BCG vaccination, performed years earlier, against COVID-19. Although recent epidemiological studies point out in BCG-vaccinated population a lower prevalence of SARS-CoV2 infection, in our cohort of physicians no significant difference was found in terms of prevalence of COVID-19 infection. Our data underline the necessity to follow the WHO warning about the indiscriminate use of BCG vaccine, until clear evidence of protection by BCG vaccination against COVID-19 is fully demonstrated.

Keywords: SARS-CoV2, COVID-19, trained immunity, natural killer cells, innate immunity, epidemiology, Bacille Calmette-Guerin vaccine

INTRODUCTION

At the end of December 2019, the Chinese authorities informed the World Health Organization (WHO) of a series of pneumonia-like cases in the city of Wuhan, likely originating from a fish and animal market in the city (Li et al., 2020). Only in January 2020, the first news about the viral outbreak detected in the city of Wuhan was confirmed by the Chinese government, about a new virus belonging to the coronavirus family (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020). Subsequently, the infection spread rapidly throughout the world with a significant intensity of infection in Europe, especially in Italy (Grasselli et al., 2020). On March 11, 2020, the General Director of the WHO Tedros Adhanom Ghebreyesus, given the dramatic increase in the cases and countries involved, declared COVID-19 a pandemic (WHO Director-General's opening remarks at the media briefing on COVID-19", 2020).

An old vaccine, *Bacillus* Calmette–Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, was originally developed by Albert Calmette and Camille Guérin at the beginning of the 20th century, with the aim to prevent tuberculosis (TB) (Calmette et al., 1927). It is well known that BCG vaccine protects against serious tuberculosis disease for up to 15 years after vaccination, while in some cases is more effective than it was thought, offering protection for at least 20 years (Mangtani et al., 2018).

In recent weeks, some studies hypothesize whether BCG vaccine can protect against SARS-CoV2 infection. There are a lot of expert opinions, but available data related to epidemiological studies are limited and controversial (Faust et al., 2020; Hamiel et al., 2020; Ozdemir et al., 2020). Several trials are currently investigating the hypothesis that heterologous immune response induced by BCG could protect against severe COVID-19.[1,2]

In addition to inducing a specific immune response against Mycobacterium tuberculosis, anti-TB vaccination with BCG also seems to promote a nonspecific protection against other viral and bacterial agents (Biering-Sørensen et al., 2012; Hirve et al., 2012), acting as an immune enhancer, in particular on the innate component of the immune system. This phenomenon, called "trained immunity," has been studied extensively from an immunological point of view. The induced "non–antigen-specific immunological memory" in innate immunity cells such as macrophages, monocytes, and natural killer cells can be the effector of this phenomenon (Ponticiello et al., 2014; Netea et al., 2016). After stimulation with BCG these cells undergo an epigenetic reprogramming of some transcription factors; promoters of cytokine genes are de-phased or *de novo* created. This epigenetic upgrade is maintained even after the disappearance of the primary stimulus ("non–antigen-specific innate immune memory"), giving to these cells the ability to respond more powerfully to a secondary stimulus, even if not correlated with the primary stimulus (Uthayakumar et al., 2018; Netea et al., 2020). All these mechanisms could justify a protective potential of "trained immunity" against SARS-CoV2.

In this study, we investigated with a questionnaire the relationship between BCG vaccine and SARS-CoV2 infection among a group of Italian physicians, who still currently are highly exposed to SARS-CoV2 (Patella et al., 2020c). In Italy, anti-TB vaccination is not mandatory, but it was up to few years ago, only for students of Medicine and Dentistry who were starting their degree course; for this reason most physicians have been vaccinated with BCG.

The aim of the study is to evaluate whether TB vaccination, performed several years earlier, could confer protection against COVID-19. The results could confirm recent epidemiological observations according to which in countries where the vaccine is performed in childhood (therefore many years before), there is a lower spread of COVID-19 (Gursel and Gursel, 2020; Ozdemir et al., 2020).

MATERIALS AND METHODS

Ethics

The studies for this survey involving human participants were reviewed and approved by an Independent Ethics Committee: "*Comitato Etico Interaziendale Campania SUD*" (Note: n. 19_53, 2020).

Questionnaire

The anonymous questionnaire was administered to all the medical doctors belonging to the Campania region (Southern Italy) Professional Orders (44,203 subjects, at time of survey), reached through their mailing list, by a computer-based platform with a survey technique. Participation was voluntary and uncompensated; after giving informed consent, the participants could access the questionnaire. To avoid incomplete questionnaires, the registration of the questionnaire was possible only if all questions were answered.

A web-based data collection tool was used to collect non-identified data over the period between April 10 and May 7, 2020. Data collected included each individual subject's gender, age, type of care activity carried out, results of nasopharyngeal swab, and serological examination, any contagion with SARS-CoV2 (considered in case of positive swab or serology), the level of the clinical signs and symptoms in case of infection, the previous BCG vaccine, and the year of vaccination (**Table 1**).

Statistical Analysis

Statistical platform R (vers. 3.5.1) was used for all the statistical analyses. Descriptive statistics were used to characterize the sample; mean ± standard deviation or median [25th; 75th percentile] with range in case of numerical variables and absolute frequencies with percentages in case of categorical factors. Association between vaccination and infection was

[1]Reducing healthcare workers absenteeism in the COVID-19 pandemic through BCG vaccine (BCG-CORONA). Radboud University, the Netherlands. ClinicalTrials.gov Identifier: NCT04328441.

[2]BCG vaccination to protect healthcare workers against COVID-19 (BRACE). Murdoch Children's Research Institute, Australia. ClinicalTrials.gov Identifier: NCT04327206.

TABLE 1 | Questionnaire.

1. Basic epidemiological data
Age, years
Sex
Type of care activity
A. Intensive care or first aid doctor
B. Hospital internal medicine physicians
C. Dentistry
D. Doctor working with discharged patients during the pandemic
E. General practitioner and pediatrician
F. Outpatient specialist
G. Other
2. Did you receive the swab for COVID-19?
3. If yes, indicate the result among these:
Positive
Negative
4. Did you receive the serological examination for COVID-19?
5. If yes, indicate the result among these:
Negative
Positive only for IgM
Positive only for IgG
Positive for both IgM and IgG
6. Have you been infected by the new SARS-CoV2?
(answer yes only in case of positive swab and/or serology)
7. If yes, indicate the clinical feature among the following:
Asymptomatic form
Pauci-symptomatic form (fever> 37.5°, cough, and cooling symptoms)
Pneumonia and/or respiratory distress
8. Did you receive BCG vaccine?
9. If yes, indicate the year of execution of the vaccine
(if you do not remember the year, indicate the year of enrolment in the degree course)

TABLE 2 | Population Characteristic.

Gender	
Male	838 (44%)
Female	1068 (56%)
Age, years	50.7 ±13.3 (24–87)
Age class	
≤40	528 (27.7%)
41–60	783 (41.1%)
>60	595 (31.2%)
Care activity	
Intensive care or first aid doctor	136 (7.1%)
Hospital internal medicine physicians	251 (13.2%)
Dentistry	283 (14.9%)
Doctor working with discharged patients during the pandemic	64 (3.4%)
General practitioner and pediatrician	332 (17.4%)
Outpatient specialist	192 (10.1%)
Other	648 (34%)
Year of vaccination	
Overall	1062 (55.7%)
After 2000	145 (7.6%)
After 2005	54 (2.8%)
Age at vaccination	19 years [18; 20]
Time since vaccination	34 years [24; 43]
Number of swabs	385 (20.2%)
Number of serological tests	453 (23.8%)

assessed using the chi-square test and further quantified using odds ratio (OR) with the corresponding 95% confidence intervals (95% CIs). Logistic regression models were used to adjust the association between vaccination and infection for potential confounding factors (age, gender, and care activity). Statistical significance was set at $p < 0.05$.

RESULTS

Epidemiological Data and Bacille Calmette–Guerin Vaccination

The questionnaire collection phase lasted one month, from April 10 to May 7, 2020. During this period, a total of 1906 physicians joined the study, corresponding to a participation rate in the survey of 4.3%: 1068 men (56.0%) and 838 (44.0%) women. The mean age (±standard deviation) was 50.7 ± 13.3 years, with a minimum age of 24 years and a maximum age of 87 years. In the whole sample, more than half (55.7%) had undergone BCG vaccine. Median age at vaccination was 19 years [18; 20] (**Table 2**), which is in line with the age at which medical students enroll in the degree course. Vaccination coverage, stratified by gender and age groups, is reported in **Table 3**. The highest coverage was observed in the middle-age class (41–60 years) and a slightly higher prevalence of BCG vaccination was observed in the female sample.

In Italy, the mandatory TB vaccination became voluntary for medical students in 2001. Students who enroll in medicine are usually about 19 years old, and BCG vaccination was performed at that exact moment. Considering the end of the obligation in 2001, all the students enrolled at that time and who were no longer required to take the vaccine would now be around 40 years old. Therefore, analyzing the sample by age, we noticed that, in the group of subjects <40 years, the number of vaccinated people is significantly lower (194/491—39.5%) than the group ≥40 (866/1415—61.2%).

Infected Population and Period Prevalence with Respect to Care Activity

Forty-nine physicians were infected with SARS-CoV2 out of the sample analyzed of 1906 subjects, with a period prevalence of 2.6% (**Table 4**), a much higher value than in the general population of the Campania region (0.08%) calculated on the basis of the cumulative data of the Civil Protection Department on May 07, 2020.

TABLE 3 | Vaccination coverage in the sample stratified by gender and age groups.

Age class	Overall (%)	Male (%)	Female (%)
≤30	22 (14)	8 (12.9)	14 (14.7)
31–40	196 (52.8)	63 (47.4)	133 (55.9)
41–50	264 (71.7)	120 (71.4)	144 (72)
51–60	253 (61)	155 (58.7)	98 (64.9)
60–70	302 (55.6)	213 (54.2)	89 (59.3)
>70	25 (48.1)	23 (47.9)	2 (50)
Overall	1062 (55.7)	582 (54.5)	480 (57.3)

TABLE 4 | Infection rates related to gender, age, and care activity.

	Infection rate	
	All forms of infection	**Clinically relevant infections**
Overall	49 (2.6)	37 (1.9)
Gender		
Male	25 (2.3)	20 (1.9)
Female	24 (2.9)	17 (2)
Age class		
≤40	19 (3.6)	13 (2.5)
41–60	21 (2.7)	17 (2.2)
>60	9 (1.5)	7 (1.2)
Care activity		
Intensive care or first aid doctor	7 (5.1)	6 (4.4)
Hospital internal medicine physicians	15 (6)	12 (4.8)
Dentistry	4 (1.4)	4 (1.4)
Doctor working with discharged patients during the pandemic	3 (4.7)	2 (3.1)
General practitioner and pediatrician	4 (1.2)	2 (0.6)
Outpatient specialist	3 (1.6)	2 (1)
Other	13 (2)	9 (1.4)

The clinical features registered among infected physicians were as follows: an asymptomatic condition in 12 subjects (24.5%); a pauci-symptomatic condition characterized mainly by fever, cough, and nonspecific symptoms, which occurred in 24 subjects (49.0%); and a severe condition characterized by pneumonia and respiratory distress syndrome, which occurred in 13 subjects (26.5%) (**Figure 1**).

With respect to all forms of infection (**Table 4**), average age of infected physicians was 47.1 ± 11.4 years with the highest period prevalence (3.6%) observed in the youngest age group (≤40). Depending on the type of care activity, a different percentage of contagion with SARS-CoV2 was observed. The most at-risk categories were the physicians of the critical area, the hospital internal medicine physicians, and those working with discharged patients during the emergency, with a period prevalence of 5.1; 6.0; 4.7%, respectively. A similar pattern was observed when only clinically relevant infections were considered (**Table 4**).

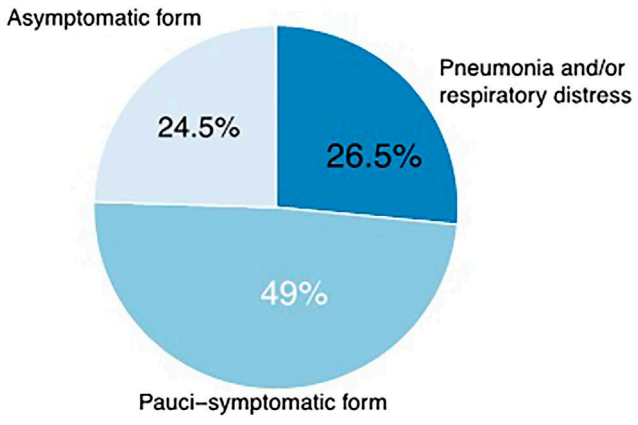

FIGURE 1 | Clinical features among infected physicians. An asymptomatic state occurred in 12 subjects (24.5%); a pauci-symptomatic state in 24 subjects (49.0%); and a severe state in 13 subjects (26.5%).

Relationship Between Bacille Calmette–Guerin Vaccine and Clinically Relevant COVID-19

In these comparisons between *vaccinated* and *unvaccinated*, we took into consideration only the cases that had any clinical relevance. In fact, subjects positive for the swab and therefore infected, but without clinical features, can be considered, in terms of hypothetical vaccine protection, in the same way as noninfected subjects.

In the "*unvaccinated*" group, 14 out of 844 subjects (1.7%) were infected by a clinical form of COVID-19 (pauci-symptomatic or respiratory distress); the rate raised to 2.2% (23 out of 1062) in the "*vaccinated*" group (unadjusted OR: 1.31, 95% C.I. 0.68–2.63; $p = 0.427$). When adjusting the analysis by age, gender, and care activity the difference remains not significant (OR: 1.35, 95% C.I.: 0.68–2.71, $p = 0.393$; **Table 5**).

When considering in the "*vaccinated* group" only those physicians who received vaccine within the last 20 years (vaccination year after 2000), a similar pattern was observed (in this model three questionnaires were excluded because they did not report the year). In the group of "*unvaccinated*" (not vaccinated or vaccinated before 2000), 31 out of 1758 individuals (1.8%) showed a clinical form of COVID-19, and in the group of "*vaccinated,*" 6 out of 145 individuals (4.1%) showed a clinical form of COVID-19 (unadjusted OR: 2.40, 95% C.I.: 0.89–5.47; $p = 0.054$). In the full logistic model, the odds of infection were two-fold higher in the vaccinated group than in the unvaccinated group without reaching statistical significance (OR: 2.02, 95% C.I.: 0.72–5.68; $p = 0.184$; **Table 5**).

Finally, in considering in the "*vaccinated* group," only those physicians who received vaccine within the last 15 years

TABLE 5 | Impact of COVID-19 related to timing BCG vaccination[a].

	Model 1		Model 2		Model 3	
	OR (95%C.I.)	*p*	OR (95%C.I.)	*p*	OR (95%C.I.)	*p*
Vaccination	1.35 (0.68–2.71)	0.393	2.02 (0.72–5.68)	0.184	0.74 (0.1–5.78)	0.778
Age, years						
≤40	Ref	—	Ref	—		
41–60	0.83 (0.39–1.8)	0.643	1.11 (0.48–2.57)	0.808	0.88 (0.41–1.88)	0.743
>60	0.55 (0.2–1.45)	0.225	0.71 (0.25–2.03)	0.524	0.56 (0.21–1.5)	0.247
Male gender	1.19 (0.6–2.39)	0.619	1.17 (0.58–2.33)	0.661	1.18 (0.59–2.35)	0.648
Care activity						
Intensive care or first aid doctor	Ref	—	Ref	—		
Hospital internal medicine physicians	1.15 (0.42–3.17)	0.781	1.18 (0.43–3.26)	0.746	1.11 (0.4–3.03)	0.844
Dentistry	0.31 (0.08–1.12)	0.074	0.32 (0.09–1.16)	0.082	0.3 (0.08–1.11)	0.072
Doctor working with discharged patients during the pandemic	0.72 (0.14–3.67)	0.689	0.76 (0.15–3.89)	0.740	0.74 (0.14–3.77)	0.715
General practitioner and pediatrician	0.16 (0.03–0.79)	0.025	0.16 (0.03–0.83)	0.029	0.15 (0.03–0.76)	0.022
Outpatient specialist	0.24 (0.05–1.21)	0.083	0.24 (0.05–1.22)	0.085	0.23 (0.05–1.18)	0.079
Other	0.33 (0.12–0.97)	0.043	0.35 (0.12–1.02)	0.054	0.33 (0.11–0.94)	0.038

[a]*Model 1 considers in the vaccinated group all physicians who received BCG vaccination regardless year of vaccination.*
Model 2 considers as vaccinated only those physicians who received BCG vaccination after 2000.
Model 3 considers as vaccinated only those physicians who received BCG vaccination after 2005.
OR with the corresponding 95% C.I. was estimated using logistic regression models and was adjusted for all the predictors reported in the table.

(vaccination year after 2005), in the group of "*unvaccinated,*" 36 subjects out of 1849 (1.9%) showed a clinical form of COVID-19, while in the "*vaccinated*" *group*, only 1 subject out of 54 physicians (1.9%) was infected with SARS-CoV2 presenting a pauci-symptomatic form (unadjusted OR: 0.95, 95% C.I.: 0.05–4.53, $p = 0.96$). Also, in this model, three questionnaires were excluded because they did not report the year. When adjusting the analysis by age, gender, and care activity, the association remains not significant (OR: 0.74, 95% C.I.: 0.1–5.78, $p = 0.778$; **Table 5**; **Figure 2**).

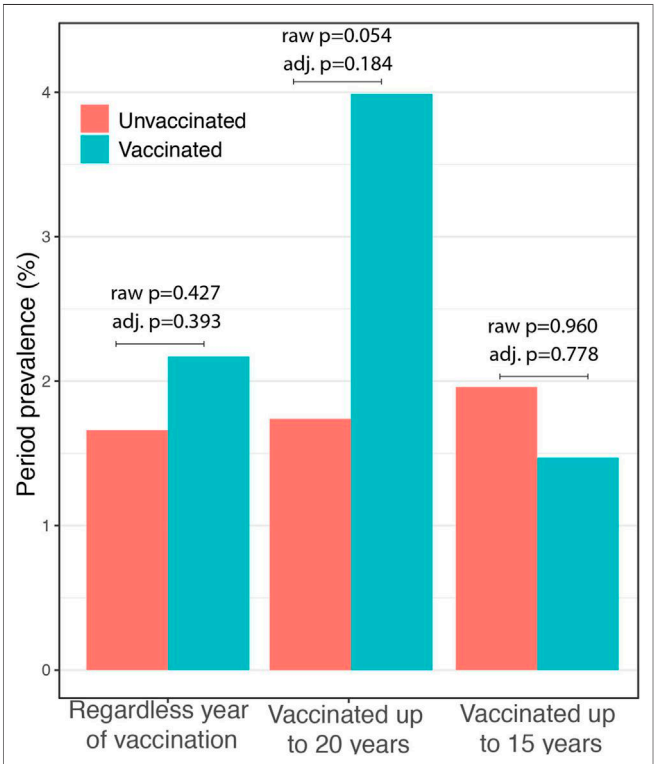

FIGURE 2 | Impact of COVID-19 in relation to timing of BCG vaccination. In all three analyses, no significant difference was observed between the vaccinated and the unvaccinated group neither in unadjusted (raw *p*) or in adjusted analysis (adj. *p*).

Relationship Between BCG Vaccine and Clinically Relevant COVID-19 Among the Subjects Who Had Subjected to the Swab

We also evaluated the association between BCG and COVID-19 among the subjects who had underwent the swab testing (385 subjects—20.2% of the sample): the "*vaccinated*" were 229 (and the *unvaccinated* 156). The subjects infected with SARS-CoV2 were 45 (four subjects are missing compared to the total of 49 infected because these four were judged affected only on the basis of serology), of these 34 presented a clinical form of COVID-19 (pneumonia, respiratory distress, and pauci-symptomatic form) and 11 were asymptomatic. Among "*vaccinated,*" 22 individuals (9.6%) were infected by a clinical form of COVID-19, while among "*unvaccinated,*" 12 individuals (7.7%) were infected by a clinical form of COVID-19 (OR: 1.28, 95% C.I.: 0.62–2.74; $p = 0.516$); the association remained not significant after adjusting the analysis by age, gender, and care activity (OR: 1.20, 95% C.I. 0.56–2.66; $p = 0.6418$). Taking into account year of vaccination (after 2000 or 2005) the association remained not significant both in the unadjusted and in the adjusted analysis (data not shown).

DISCUSSION

The results allow a rapid epidemiological review of the relationship between BCG vaccination and COVID-19 in a population exposed to SARS-CoV2. We verified that there was

no difference between the vaccinated and unvaccinated population about the prevalence of the disease during the observation, which was the period of greatest exposure to such disease. In a recent study concerning general population, these epidemiological data seem to be confirmed (Hamiel et al., 2020).

In our sample, the majority of medical cohort was BCG vaccinated, and the prevalence of COVID-19 was much higher than the prevalence in regional population in the same period, confirming the high exposure of medical personnel during the COVID-19 pandemic. According to the various physicians' categories, the most exposed to the contagion were those working in critical care (intensive care and emergency department) and internal medicine hospital. These data confirm the absolute need for protective tools in the most exposed categories (Houghton et al., 2020; Patella et al., 2020b) and the need for weighted choices in diagnostics and preventive medicine to control the infection spread among health care workers.

It should be noted that the incidence of COVID-19 differs between the various age groups regardless of the anti-TB vaccination (**Table 4**—"age class" section), resulting double in subjects ≤40 years of age compared to subjects >60 years. This finding could be explained by the higher exposure to the virus of younger doctors. In fact, among the subjects ≤40 years of age, 24.2% were intensive care or doctors in internist branches compared to subjects >60 where only 13.1% were at high exposure (intensive care doctor and/or internist). Moreover, among the 13 young subjects with clinically relevant forms of COVID-19, only two presented a more severe form with pneumonia and/or respiratory distress (15.4%), while among the older subjects, 4 out of 7 infected individuals (57, 1%) presented a more severe form with pneumonia and/or respiratory distress. On the other hand, the association between age class and incidence of infection is not statistically significant both in the case in which all forms of infections are considered ($p = 0.085$) and in the case of clinically relevant infections ($p = 0.247$). Moreover, considering the individual age groups, the difference in the incidence of COVID-19 between the vaccinated and unvaccinated remains insignificant.

We conducted a reassessment of the data according to three approaches related to the time elapsed since BCG vaccination. The first analysis was considered regardless of the year of vaccination; in the second analysis, we considered protection from BCG-induced "trained immunity" only who had undergone the vaccination in the last 20 years, and in the third analysis, we considered only the vaccination in the last 15 years. In all the approaches, we adjusted the analysis by age, gender, and care activity. None of the approaches showed a significant difference between the two groups.

Clearly, the study refers to vaccinations performed several years before exposure to SARS-CoV2 (on average 34 years before and in the two models, up to 20 years and up to 15 years). The immunological processes underlying "trained immunity" have a duration that is still unclear today; work by Kleinnijenhuis and Netea show that the "trained immunity status" is maintained for at least 1 year (Kleinnijenhuis et al., 2014) (the maximum time point measured), even if the duration of BCG induced "trained immunity" in terms of longevity of induced innate memory is not known. Therefore, these data can only conclude on the absence of long-term effects of BCG on COVID-19. Only trials currently underway will be able to answer the short-term effects of BCG on COVID-19.

Our study arises in response to several epidemiological observations according to which the BCG vaccine performed in childhood (therefore many years before) could protect against COVID-19, in particular it is reported that in the countries where the vaccine is regularly performed, there is a lower spread of COVID-19 (Gursel and Gursel, 2020; Ozdemir et al., 2020). Several reasons may explain the different global prevalence of the disease: for example, the difference in infections between the northern and southern hemisphere could be due, at least in part, to their different temperature, since the outbreak of COVID-19 occurred during winter in the most affected countries (Caminati et al., 2020; Wu et al., 2020). Furthermore, the main problem in studies comparing mortality rates between different countries is the different ability of national systems to report epidemiological data. In those countries with more precarious welfare systems and greater population density, where COVID-19 epidemic presented a multilevel emergency (health, social, and political), not all deaths were adequately assessed for the cause, resulting in an underestimation of COVID-19 mortality rate. This phenomenon is likely to be more frequent in poorer countries, where tuberculosis is still widespread and therefore with active BCG vaccination programs (Patella et al., 2020a).

In contrast, our study verified that a BCG vaccination performed years earlier cannot confer protection. Our data appear to be in line with the epidemiological study by Hamiel et al. conducted on a cohort of Israeli adults aged 35–41 years, who had received BCG vaccination in childhood, where the authors found a similar rate of positive test results for SARS-CoV2 compared with no vaccination (Hamiel et al., 2020). Our work suffers from the limitations of the retrospective nature of the study, and being the participation on a voluntary basis, the sample studied is not standardized. Moreover, the questionnaire-based survey, which relies only on the memory of the voluntary participant, may have introduced recall bias. Our results suffer from relatively low number of examined subjects; in particular among who had subjected to the swab, furthermore they present the limitation of not considering participants' co-morbidities.

The possibility that BCG vaccination may protect against SARS-CoV2 infection remains an open discussion with conflicting data and opinions in the international scene (Curtis et al., 2020). Protection of tuberculosis vaccination could derive from trained immunity, a phenomenon relating to innate immunity, which, once stimulated by BCG, would drive a reprogramming of nonspecific immunological response towards even other infections, as SARS-CoV2. Epigenetic reprogramming of innate immune cells by a primary stimulus such as BCG vaccine may allow activation of transcription factors in myeloid cells (Freyne et al., 2018). In BCG vaccinated subjects, monocytes showed increased expression of activation surface markers and produced more IL-1β, IL-6, IFN-γ, and TNF-α in response to Staphylococcus aureus or *Candida* albicans

(Kleinnijenhuis et al., 2012; Kleinnijenhuis et al., 2014). Kleinnijenhuis J et al. reported that NK cells isolated from volunteers 3 months after BCG vaccination, produced more pro-inflammatory cytokines on stimulation, in particular IL1β, but also IL-6 and TNFα. In another study, Kleinnijenhuis and Smith et al. reported that BCG vaccinated children showed a significant increase in surface expression of the CD69 activation marker on NK cells in response to stimulation of Pam3Cys (Smith et al., 2017).

Taken together, these data could justify the protective potential of trained immunity against SARS-CoV2. In particular, it would seem that one of the fundamental elements in the immune response against SARS-CoV2 could be just the rapidity of the pathogen elimination: indeed the escape of the immune system and the viral permanence could entail an uncontrolled response with vicarious production of inflammatory cytokines, and the clinical progression towards the most severe forms of pneumonia as has been demonstrated for SARS-CoV (Channappanavar et al., 2016). Therefore, the enhancement of the innate immune responses that represents the earliest defense line could be a fundamental step to block the progression of immune activation toward more massive and uncontrolled responses that are ultimately harmful to the host organism itself.

Probably only clinical trials with active administration of BCG vaccine will give an evident answer about the protective role of BCG: Giamarellos–Bourboulis *et al.* reported preliminary results about an ongoing clinical trial (ACTIVATE), which evaluates the role of active vaccination with BCG versus placebo on the time to first infection in the elderly. The first data show that the ratio of new infections during the 12-month period of follow-up after BCG vaccination was significantly decreased. The difference in the incidence according to the type of infection showed most of the benefit on the prevention of respiratory infections of probable viral origin. Unfortunately, the outcomes of the ACTIVATE trial did not include the specific assessment for SARS-CoV2 infection (Giamarellos-Bourboulis et al., 2020).

All over the world, several clinical trial protocols (see footnotes 1 and 2) have been developed with active administration of BCG vaccine in selected populations, to evaluate the efficacy during COVID-19. The results of these trials will clarify whether or not there is a real protection by BCG vaccine against SARS-CoV2 infection.

However, in the light of current knowledge and data from our study, we consider appropriate to follow the WHO warning about indiscriminate use of BCG vaccine, until clear evidence of protection by BCG vaccination against COVID-19 is fully demonstrated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

VP and GD contributed equally to ideation, to write and analysis of data in this paper, DB contributed to statistical analysis, the other authors GiF, GaF, RB, and AS contributed equally to ideation and analysis of paper.

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Profiling Ribonucleotide and Deoxyribonucleotide Pools Perturbed by Remdesivir in Human Bronchial Epithelial Cells

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Remdesivir (RDV) has generated much anticipation for its moderate effect in treating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. However, the unsatisfactory survival rates of hospitalized patients limit its application to the treatment of coronavirus disease 2019 (COVID-19). Therefore, improvement of antiviral efficacy of RDV is urgently needed. As a typical nucleotide analog, the activation of RDV to bioactive triphosphate will affect the biosynthesis of endogenous ribonucleotides (RNs) and deoxyribonucleotides (dRNs), which are essential to RNA and DNA replication in host cells. The imbalance of RN pools will inhibit virus replication as well. In order to investigate the effects of RDV on cellular nucleotide pools and on RNA transcription and DNA replication, cellular RNs and dRNs concentrations were measured by the liquid chromatography-mass spectrometry method, and the synthesis of RNA and DNA was monitored using click chemistry. The results showed that the IC_{50} values for BEAS-2B cells at exposure durations of 48 and 72 h were 25.3 ± 2.6 and 9.6 ± 0.7 μM, respectively. Ten (10) μM RDV caused BEAS-2B arrest at S-phase and significant suppression of RNA and DNA synthesis after treatment for 24 h. In addition, a general increase in the abundance of nucleotides and an increase of specific nucleotides more than 2 folds were observed. However, the variation of pyrimidine ribonucleotides was relatively slight or even absent, resulting in an obvious imbalance between purine and pyrimidine ribonucleotides.

Abbreviations: RDV, Remdesivir; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; COVID-19, Coronavirus disease 2019; RdRps, RNA-dependent RNA polymerases; RR, Ribonucleotide reductase; RRM1, Ribonucleotide reductase subunit M1; RRM2, Ribonucleotide reductase subunit M2; p53R2, p53-controlled ribonucleotide reductase; RDV-MP, Remdesivir nucleoside monophosphate; RNs, Ribonucleotides; dRNs, Deoxyribonucleotides; NTPs, Ribonucleoside triphosphates; dNTPs, Deoxyribonucleoside triphosphates; AMP, Adenosine monophosphate; GMP, Guanosine monophosphate; IMP, Inosine monophosphate; UMP, Uridine monophosphate; CMP, Cytidine monophosphate; ADP, Adenosine diphosphate; GDP, Guanosine diphosphate; UDP, Uridine diphosphate; CDP, Cytidine diphosphate; ATP, Adenosine triphosphate; GTP, Guanosine triphosphate; CTP, Cytidine triphosphate; dAMP, Deoxyadenosine monophosphate; dGMP, Deoxyguanosine monophosphate; dUMP, Deoxyuridine monophosphate; TMP, Thymidine monophosphate; dCMP, Deoxycytidine monophosphate; dADP, Deoxyadenosine diphosphate; dGDP, Deoxyguanosine diphosphate; dUDP, Deoxyuridine diphosphate; TDP, Thymidine diphosphate; dCDP, Deoxycytidine diphosphate; dATP, Deoxyadenosine triphosphate; dGTP, Deoxyguanosine triphosphate; dUTP, Deoxyuridine triphosphate; TTP, Thymidine triphosphate; dCTP, Deoxycytidine triphosphate; HPLC-MS/MS, High-performance liquid chromatography– tandem mass spectrometry.

Interestingly, the very marked disequilibrium between cytidine triphosphate (CTP) and cytidine monophosphate might result from the inhibition of CTP synthase. Due to nucleotides which are also precursors for the synthesis of viral nucleic acids, the perturbation of nucleotide pools would block viral RNA replication. Considering the metabolic vulnerability of endogenous nucleotides, exacerbating the imbalance of nucleotide pools imparts great promise to enhance the efficacy of RDV, which possibly has special implications for treatment of COVID-19.

Keywords: remdesivir, perturbation of nucleotide pools, inhibition of RNA and DNA synthesis, inhibition of CTP synthase, cell cycle arrest, Covid-19 therapy

INTRODUCTION

Remdesivir (RDV), an adenine nucleotide analog when inserted into viral RNA chains results in their premature termination (Warren et al., 2016), has shown a broad spectrum of antiviral activity against severe acute respiratory syndrome coronavirus (SARS–CoV) (Sheahan et al., 2017), Nipah virus (Lo et al., 2019), Middle East respiratory syndrome coronavirus (MERS–CoV) (de Wit et al., 2020; Sheahan et al., 2020), Ebola virus (Jacobs et al., 2016; Dornemann et al., 2017; Mulangu et al., 2019), and SARS-CoV-2 (Beigel et al., 2020; Holshue et al., 2020; Wang et al., 2020a). Because of the advantage of RDV in shortening the time for recovery in adults infected with SARS-CoV-2, the US Food and Drug Administration issued an Emergency Use Authorization for the use of remdesivir for the treatment of hospitalized patients with coronavirus disease 2019 (COVID-19) (US Food and Drug Administration, 2020). Based on the previous studies, RDV exerts its antivirus activity by specifically inhibiting the activity of viral RNA-dependent RNA polymerases (RdRps), which are crucial to virus survival not only through replication but also as engines of genome variability and evolution, without interference with human RNA polymerase (Tchesnokov et al., 2019; Ju et al., 2020).

Successful applications of metabolic reprogramming to treat cancer (Luengo et al., 2017) and inflammation (Ip et al., 2017) have prompted us to explore the potentials of RDV treatment. Like other nucleotide analogues, RDV is subjected to phosphorylation to form bioactive triphosphate, which is substrate-competitive with ATP for incorporation by viral RdRp and inhibition of viral RNA synthesis (Ray et al., 2016; Lee et al., 2017). The Phosphoramidate (ProTide) approach is used to establish phosphate prodrug of RDV to either bypass the rate-limiting step during translation of the parent nucleoside into its monophosphate, or overcome the low bioavailability due to the inefficient cellular uptake and poor *in vivo* stability (Cho et al., 2012; Elsharif and Dakhil, 2017; Siegel et al., 2017). *In vivo*, two enzymatic activation steps remove the masks to release the nucleoside monophosphate (RDV-MP), in which the ubiquitous esterases (cathepsin A/carboxylesterase1) and phosphoramidases (HINT1-3) are involved (Williamson et al., 2020; Yan and Muller, 2020). Meanwhile, some RDV is hydrolyzed to its parent nucleoside (GS-441524) (Yan and Muller, 2020). Subsequently, both the monophosphorylated RDV and the parent nucleoside are converted to diphosphate and triphosphate by natural kinases. Due to metabolic competition with natural nucleotides such as AMP, ADP, and ATP, RDV inevitably results in perturbation of endogenous RNs, which could restrict the synthesis of viral RNA in turn (Boccardo and Accotto, 1988; Fahima et al., 1993). However, to date, it remains uncertain how RDV exposure affects cellular nucleotides.

Besides alteration of adenine nucleotides, RDV might change the levels of other nucleotides by affecting enzymes in nucleotide synthesis and metabolism. Previous studies have shown that guanine analogues, ribavirin, and 5-ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide (EICAR), depleted the GTP pool through inhibition of inosinate dehydrogenase (Stridh, 1983; Balzarini et al., 1993). Similarly, RDV as an adenine analog was hypothesized to inhibit S-adenosylhomocysteine (SAH) hydrolase and adenylate kinase, consequently interfering with the biosynthesis of adenine derivatives (Bistulfi et al., 2009; De Clercq, 2019). Moreover, Kim et al. have reported previously that SARS coronavirus may require more ATP to promote stable helicase translocation necessary for delicate RNA replication (Jang et al., 2020). Endogenous RNs and dRNs pools also affect the response of RDV against viral infection because the disturbance of adenine derivatives will affect the function of RdRps (Vander Heiden and DeBerardinis, 2017). Furthermore, unbalanced changes in dRNs caused by RDV could induce potential side effects because of failure to maintain the dNTPs level causing genetic abnormalities or cell death (Mathews, 2014). This has already been proven that adaptive metabolic reprogramming of RNs and dRNs pools could promote chemotherapy at the early stage of treatment (Brown et al., 2017). Thus elucidation of the disturbances of RDV treatment on RNs and dRNs pool sizes will not only permit us to understand the exact mechanism of action of RDV, but also enhance the antivirus activity based on the targeted-regulation of RNs and dRNs.

So far, there has been no report on the effects of RDV on RNs and dRNs pool sizes due mainly to the difficulty of quantifying these pool sizes, particularly for the monophosphate and diphosphate nucleotides. Recently, we described a simpler, selective and highly sensitive HPLC-MS/MS method for quantification of RNs and dRNs pools in cells after trimethylsilyl diazomethane (TMSD) derivatization (Li et al., 2019). In the present study, the effects of RDV incubation over different timeperiods on RNs and dRNs pool sizes of human bronchial airway epithelial cells (BEAS-2B cells) were

investigated using our well-established HPLC-MS/MS methodology. Furthermore, the influence of RDV on cell cycle, RNA and DNA synthesis and protein expression were studied. The results obtained from this study should facilitate understanding the action mechanisms of RDV and assessment of its efficacy and toxicity for developing individualized therapy.

MATERIALS AND METHODS

Reagents and Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), paraformaldehyde, propidium iodide (PI), and 0.05% RNase A were provided by Sigma-Aldrich Inc. (St. Louis, MO, United States). RDV was purchased from Manhey Chemical Limited (Hong Kong, China). For our experiments, the stock solution of RDV was prepared in DMSO, stored at −20°C, and serially diluted in Dulbecco's Modified Eagle's Medium (DMEM) when needed. The final DMSO concentration did not exceed 0.1% throughout this study. 5-ethynyl uridine (EU) and 5-ethynyl-2′-deoxyuridine (EdU) were supplied by Tokyo Chemical Industry Co., Ltd. (Shanghai, China). 4′, 6-Diamidino-2-phenylindole (DAPI) and Alexa Fluor™ 594 were purchased from Invitrogen Co. (Carlsbad, CA, United States). Glycine, Tris, $CuSO_4$, ascorbic acid, EDTA, Triton™ X-100, and TWEEN® 20 were also obtained from Sigma-Aldrich Inc. RIPA buffer (Cell Signaling Technologies Inc. Beverly, MA, United States), Bradford reagent (Bio-Rad Laboratory, Hercules, CA, United States), nitrocellulose membrane (Merck Millipore, United States), and the enhanced chemiluminescence reagents (Invitrogen, Paisley, Scotland, United Kingdom) were also used in this study. For cell culture, DMEM, fetal bovine serum (FBS), penicillin-streptomycin solution, phosphate buffer saline (PBS), and 0.25% trypsin-EDTA solution were obtained from GIBCO (Grand Island, NY, United States).

The stable isotope labeled adenosine-$^{13}C_{10}$,$^{15}N_5$-triphosphate (ATP-$^{13}C_{10}$,$^{15}N_5$) and adenosine-$^{13}C_{10}$,$^{15}N_5$-monophosphate (AMP-$^{13}C_{10}$,$^{15}N_5$), other nucleotide standards and ammonium acetate (NH_4OAc) were purchased from Sigma-Aldrich Inc (St. Louis, MO, United States). TMSD and tetrafluoroboric acid (HBF_4) were obtained from Alfa Aesar Co. (Ward Hill, MA, United States). The methanol (LC-MS grade) and acetonitrile used for the HPLC-MS/MS analysis were bought from Anaqua Chemical Supply (Houston, TX, United States). Formic acid was bought from Fisher Scientific Co. (Fair Lawn, NJ, United States) and diethyl ether was obtained from Tedia Co. (Fairfield, OH, United States), while acetic acid (AcOH) and 30% ammonium hydroxide aqueous solution (NH_4OH) were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ, United States). The solid phase extraction (SPE) cartridges (WAX, 3 cm^3; 30 mg, 60 μm) was bought from Waters Co. (Milford, MA, United States), and the chromatographic column Sepax GP-C_{18} (2.1 × 150 mm, 1.8 μm) from Sepax Technologies (Newark, DE, United States) was also used. Ultrapure water was obtained on the basis of a Milli-Q Gradient water system (Millipore, Bedford, MA, United States).

Cell Culture and Colorimetric MTT Assay

Normal human bronchial epithelial cells BEAS-2B were purchased from the ATCC (Manassas, VA, United States). They were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin-streptomycin in a humidified incubator at 37°C, and 5% CO_2. Cell viability was determined by a modified colorimetric MTT assay (van Meerloo et al., 2011). Briefly, BEAS-2B cells in the exponential phase were seeded in a 96-well plate for 24 h at 37°C, then treated with RDV at different concentrations (0–100 μM) for 24, 48, and 72 h, respectively. After the appropriate incubation time, 10 μL MTT solution (5 mg/ml) was added for another 4 h incubation and 100 μL DMSO was dispensed to dissolve formazan crystals before spectrophotometric measurement at 570 nm using a microplate ultraviolet-visible spectrophotometer. Cell viability was calculated as follows: cell viability (%) = (absorbance of the test group/absorbance of the control group) × 100. The IC_{50} value was taken as the concentration that caused 50% inhibition of cell viability and was calculated using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, United States).

EU and EdU Detection Using Click Chemistry

In order to label and visualize specifically newly synthesized DNA and RNA, the click chemistry method was used. The experiments were conducted based on previous publications with a slight modification (Jao and Salic, 2008; Akbalik et al., 2017). In short, BEAS-2B cells were grown in 6-well plates at 2.0×10^5 cells/well for 24 h and then incubated for 14 h with 1.0 mM EU or 10 μM EdU in the presence or absence of 10 μM RDV. After the labeling, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. The fixed cells were neutralized with 2 mg/ml glycine, rinsed with PBS, and stained for 30 min at room temperature with a click reaction buffer including 100 mM Tris, 1 mM CuSO4, 10 μM Alexa594-azide, and 100 mM ascorbic acid. After staining, cells were washed several times by using PBS with 0.5 mM EDTA, 1% TWEEN® 20, and 0.1% Triton™ X-100, and then stained with 0.5 μg/ml DAPI for 30 min. Finally, the cells were imaged by IncuCyte ZOOM Live-Cell Analysis Platform.

Cell Cycle Analysis

BEAS-2B cells were seeded in 6-well plates at 2.0×10^5 cells/well, cultured for 24 h, and then treated with/without RDV for 12, 24, and 48 h, respectively. Cells were then harvested, resuspended in ice-cold PBS, and fixed with 70% ethanol at −20°C overnight. The fixed cells were washed again using ice-cold PBS and incubated with 500 μL PI containing 0.05% RNase A for 30 min at room temperature in the dark. Finally, a cell cycle distribution profile was accessed by flow cytometry after the staining treatment. The percentages of cells in G0/G1, S, and G2/M phases were analyzed using ModFit LT software (Verity Sofware House, Topsham, ME, United States).

Western Blot Assay

BEAS-2B cells were treated with RDV at the indicated concentration for 12, 24, and 48 h, respectively. Then the cells

were washed with cold PBS twice and lyzed with RIPA buffer on ice. The lysates were centrifuged at 12,000 g for 30 min at 4°C in order to acquire the protein samples. The concentration of cellular total protein was measured by using the Bradford reagent at 595 nm according to the manufacturer's instructions. 30 μg protein samples were loaded on 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk for 1.5 h, followed by the incubation of primary antibodies diluted in Tris-buffered saline with a Tween® 20 (TBST) buffer (1:1,000 for β-tubulin, ribonucleotide reductase subunit M1 (RRM1), ribonucleotide reductase subunit M2 (RRM2), and p53-controlled ribonucleotide reductase ((p53R2), Cell Signaling Technologies, Danvers, MA, United States) overnight at 4°C. After that, the membranes were washed with TBST and incubated with secondary horseradish peroxidase-conjugated antirabbit IgG antibody (Cell Signaling Technologies, Inc. Danvers, MA, United States) for 1 h at room temperature. The immunoreactive protein bands were finally detected with an Amersham Imager 600 Western blotting system. Densitometry analysis of protein band was performed by Quantity One software (Version 4.6.2, Bio-Rad, United States).

Sample Preparation and HPLC-MS/MS Analysis

BEAS-2B cells were plated in 10 cm Petri dishes and cultured with medium for 24 h before treatment with RDV. The seeded cell number for 12, 24, and 48 h RDV treatments were 2.5×10^6, 2.0×10^6 , and 1.5×10^6 cells/dish, respectively. After that, the cells were resuspended with ice-cold PBS. The number of cells was counted before centrifugation at 1,200 rpm for 5 min, and the cell pellet was washed with 1.0 ml ice-cold PBS again and centrifuged at 1,200 rpm for 5 min. Subsequently, cell pellets were treated with 150 μL 80% methanol containing 4 μM AMP-$^{13}C_{10}$,$^{15}N_5$ and 2 μM ATP-$^{13}C_{10}$,$^{15}N_5$ as an internal standards (IS). The following sample preparation and the determination of endogenous RNs and dRNs were performed based on the method previously described (Li et al., 2019). The concentrations of cellular nucleotides were finally calculated according to dividing the absolute amount of each RN and dRN in each sample by the corresponding cell number.

Statistics Analysis

Data analyses were performed using GraphPad Prism software and values were expressed as mean ± standard deviation (SD) from three independent replicate experiments. The statistical significance of the comparison between control and treated groups was determined by Kruskal–Wallis tests. Statistical significance is indicated as $^*p < 0.05$ and $^{**}p < 0.01$.

RESULTS

Remdesivir Decreased the Viability of BEAS-2B Cell Line

At the beginning of this study, we investigated the cytotoxicity of RDV on BEAS-2B cells using MTT assays. The cells were treated with RDV at various concentrations (0–100 μM) for 24, 48, and 72 h. **Figure 1A** shows that the cell number gradually decreased as the concentration of RDV increased in all the time points of incubation. The viability of cells presented a dose- and time-dependent reduction. The calculated IC_{50} values in 48 and 72 h were 25.3 ± 2.6 and 9.6 ± 0.7 μM, respectively. 10 μM was chosen for the subsequent experiments.

Remdesivir Induced S Phase Arrest in BEAS-2B Cells

Based on its significant inhibitory effect on cell viability and proliferation, we investigated the effect of RDV treatment on the distribution of cells in cell cycle at different time points. BEAS-2B cells were treated with or without 10 μM RDV for 12, 24, and 48 h and analyzed by flow cytometry. As shown in **Figure 1B**, an altered pattern of cell cycle was observed in BEAS-2B cells exposed to 10 μM RDV compared to control. With increase in incubation time, the proportion of cells in S phase significantly increased while the percentage of cells in G2/M phases obviously decreased in comparison to untreated cells. After incubation for 24 h, the percentage of cells in S phase was 35.3 ± 0.75% in control, which gradually increases to 48.69 ± 1.8% in the RDV group ($p < 0.01$). The number of cells in G2 phase decreased from control from 29.67 ± 1.59% to 20.81 ± 1.92% of RDV ($p < 0.05$). Similar results at 48 h were obtained. In summary, RDV could arrest the cells in S phase.

Remdesivir Inhibited RNA and DNA Synthesis

In order to detect the effects of RDV on RNA and DNA synthesis in proliferating cells, we performed EU and EdU staining based on click chemistry. EU and EdU are the structural analogues of uridine and deoxyuridine, respectively. Their triphosphate metabolites compete with UTP and TTP to incorporate into newly synthesized RNA and DNA and subsequently react with azide-modified fluorophores. The fluorescence intensity is proportional to the amount of the incorporated EU and EdU in nascent RNA and DNA. As shown in **Figures 2A–B**, after incubation with RDV for 14 h, the fluorescence intensity of Alexa594-azide decreased significantly compared to control group, indicating the reduction of RNA and DNA synthesis and the inhibition of proliferation of BEAS-2B cells. Interestingly, not all DAPI stained cells were labeled with EdU. The reason for this phenomenon is that the incorporation of EdU only occurs in S phase during DNA replicating, while DAPI is a nonspecific fluorescent dye with the strong binding ability to the existing or nascent DNA (Qu et al., 2011).

Perturbation of RNs and dRNs Pool Size by Remdesivir in BEAS-2B Cells

To examine metabolic reprogramming events that influence the cellular response to virus, we used targeted LC/MS-MS via selected reaction monitoring (SRM) to examine changes in the

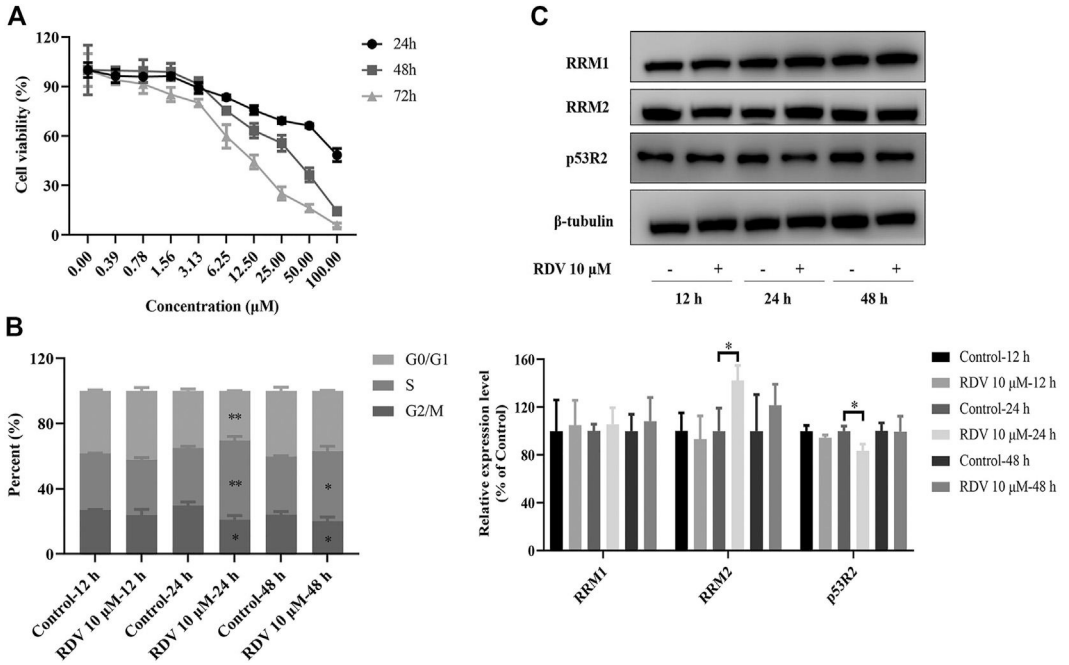

FIGURE 1 | Effects of RDV on **(A)** cell viability **(B)** cell cycle and **(C)** riboreductase expression in BEAS-2B cells treated with 10 μM RDV (RDV, remdesivir; RRM1, ribonucleotide reductase subunit M1; RRM2, ribonucleotide reductase subunit M2; p53R2, p53-controlled ribonucleotide reductase; ∗: $p < 0.05$; ∗∗: $p < 0.01$, compared with control group).

steady-state metabolomic profile of BEAS-2B cells after exposure to 10 μM RDV for 12, 24, and 48 h. The specific nucleotide levels are shown in **Supplementary Tables S1, S2**. The fold changes of the nucleotides were evaluated by comparison of their concentrations in cells treated with RDV and in the parallel controlled RDV-free cells at the same time points. Significant differences in the metabolite profiles of cells with or without RDV were observed. In general, RDV increased the abundance of the majority of RN and dRN species after 24 h incubation, including a greater than 2-fold increase in AMP, GTP, dAMP, dGDP, dGTP, dCTP, and TMP levels, and then decreased to the normal levels at 48 h (**Figures 2C–F**). A rational interpretation is that RDV significantly inhibited the synthesis of nascent RNA and DNA, and arrested the cell cycle in S phase, inevitably resulting in the accumulation of (deoxy)nucleoside triphosphates and subsequently the increase of their respective di- and monophosphates (Du et al., 2019). However, it was observed that most of the pyrimidine ribonucleotides remained unchanged or even reduced, among which the significant decrease of CTP was in stark contrast to the 3-fold increase of CMP after incubation for 24 h (**Figure 2D**).

The possible mechanism of remdesivir-induced CTP depletion and the imbalance of other nucleotides in *de novo* and salvage pathways is shown in **Figure 3**. CTP is synthesized from UTP by CTP synthase, which is the rate-limiting step of *de novo* CTP biosynthesis and probably a practical target just as in the treatment of leukemia (Verschuur et al., 1998) and parasitic infestations (Hofer et al., 2001; Fijolek et al., 2007; Tamborini et al., 2012). In this study, the ratio of CTP/UTP was calculated showing a significant decrease after 24 h incubation (**Figure 4D**), implying the inhibition effect of RDV on CTP synthase. Besides the *de novo* pathway, the salvage pathway plays an important role in metabolism of cellular nucleotides too. The relative low level of CTP might allosterically activate the recycle of free bases and nucleosides to promote the production of CMP, resulting in the abnormal elevation of CMP (**Figure 3**).

The alterations in nucleotide pools were also evaluated by comparing the percent of each NTP in the whole nucleotide pools. It showed that RDV exposure (10 μM) stimulates an increase in GTP and a decrease in CTP (**Figure 4A**). Consequently, a significant increment of GTP/CTP was observed (**Figure 4C**), indicating the huge disequilibrium in RN pools. Although there were no statistically significant differences, the ATP level reduced and UTP level increased slightly (**Figure 4A**), resulting in the elevated ratio of ATP/UTP (**Figure 4C**). From the aspect of drug disposition, RDV was hydrolyzed to RDV-MP in cell, and furtherly metabolized to RDV-TP. Due to the structural similarity of RDV-MP to AMP, the further phosphorylation of RDV-MP was achieved through the competitive inhibition of adenylate kinase, which inevitably resulted in the accumulation of AMP and the decrease in ADP and ATP. Meanwhile, the accumulation of AMP might inhibit the activity of adenylosuccinate synthase and the whole purine biosynthesis pathway in a negative feedback mode, which would simultaneously decrease the production of GMP, and ultimately GDP and GTP (Nelson et al., 2008). This speculation was proven by the relatively high AMP/GMP ratio and the reduced ATP/GTP and ADP/GDP ratios at 24 and 48 h

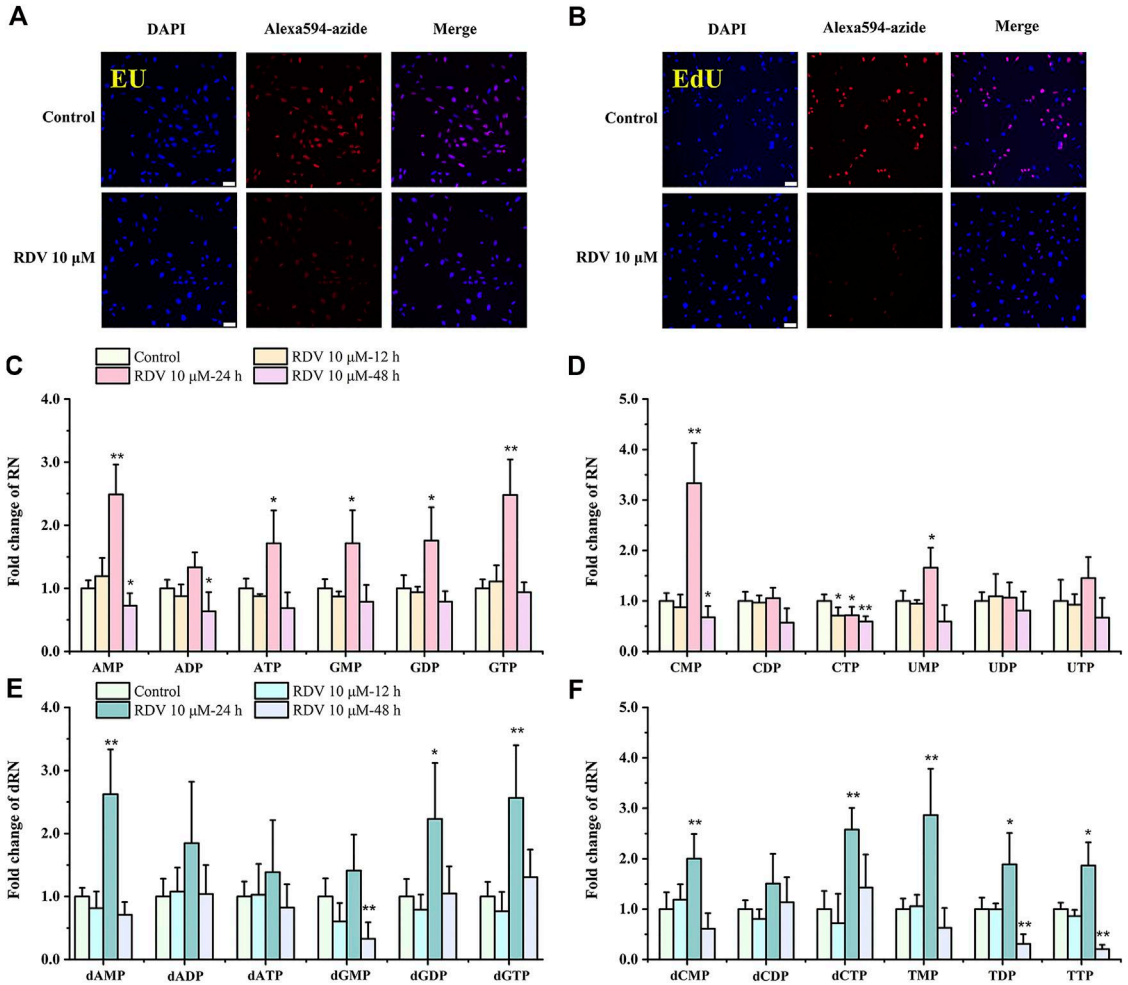

FIGURE 2 | Effects of RDV on RNA and DNA synthesis **(A–B)**, and on nucleotide pools **(C–F)**. Fluorescence microscope images (scale bars = 100 μm) of EU or EdU-mediated click chemistry indicated that RDV treatment for 14 h inhibited the synthesis of **(A)** RNA and **(B)** DNA. Fold changes in nucleotide abundances, as measured by LC/MS-MS, in 10 μM RDV-treated or vehicle-treated BEAS-2B cells for 12, 24 and 48 h **(C–F)** (RDV, remdesivir; EU, 5-ethynyl uridine; EdU, 5-ethynyl-2′-deoxyuridine; RN, ribonucleotides; dRN, deoxyribonucleotides; ∗: $p < 0.05$, ∗∗: $p < 0.01$, compared with control group).

(**Figures 4C–D**). The relative percent of dNTPs pools is shown in **Figure 4B**. The changes in dNTPs percent were contrary to that of NTPs, and there was obvious hysteresis, which was probably because of the allosteric regulation of NTPs to ribonucleotide reductase (RR). In summary, RDV exerted the antiviral activity partly via aggravating the imbalance of nucleotide pools, especially by reducing CTP.

Remdesivir Upregulated the Riboreductase R2 Expression

The remarkably elevated dNTP pools in cells are probably related to the dNTP synthesis enzymes, especially RR that catalyzes the formation of dRNs from RNs (Mathews, 2006; Liu and Grosshans, 2019). Mammalian RR comprises three subunits including RRM1, RRM2, and p53R2, which are expressed in a cell cycle-dependent manner (Yousefi et al., 2014). In cycling cells, the RRM1 protein is metabolically stable throughout the cell cycle, while the expression and degradation of RRM2 protein limit the S-phase–dependent activity of RR complex, leading to the high cellular dNTPs pools at S phase and low dNTPs pools outside S phase (Engström et al., 1985). In quiescent cells, p53R2 substitutes for protein RRM2 to supply precursor deoxyribonucleotides, which is fundamental to mitochondrial DNA replication and DNA repair. To further investigate whether the growth inhibitory activity of RDV had resulted from the induction of RR, we determined the expression of RRM1, RRM2, and p53R2 by using the western blot assay. **Figure 1** C shows that there was no obvious difference of RRM1 level after the BEAS-2B cells incubated with RDV for 12, 24, and 48 h. However, the expression of RRM2 was significantly increased after 24 h exposure to RDV at 10 μM ($p < 0.05$) caused by the S phase arrest. Simultaneously, the p53R2 level presented distinct down-regulated tendency ($p < 0.05$). In addition, there were also no changes in the levels of RRM2 and p53R2 after 48 h incubation

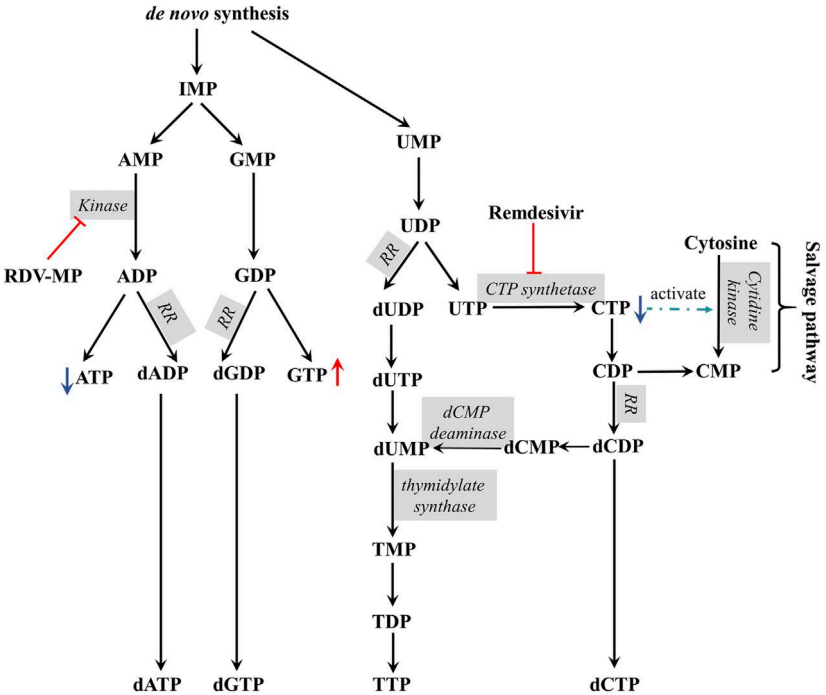

FIGURE 3 | The proposed mechanism of remdesivir induced CTP depletion and the imbalance of other nucleotides in *de novo* and salvage pathways (RR, ribonucleotide reductase).

with RDV. Taken together, it suggested that RDV inhibited the proliferation of BEAS-2B cells through the impact on RR expression.

DISCUSSION AND CONCLUSION

Although vaccination is widely considered as the most promising strategy to eliminate COVID-19, virus mutation may be a real threat to the effectiveness of vaccines. SARS-CoV-2 has infected and killed millions of people globally. Before the successful and complete implementation of vaccination for achieving herd immunity, it is urgent to cure infected patients by utilizing the currently available drugs. Among the candidate drugs, RDV was developed as a broad-spectrum antiviral drug, but cannot meet the clinical needs of COVID-19 treatment due to the unsatisfactory therapeutic outcome and high mortality (Beigel et al., 2020). Therefore, it is critical to develop new treatment modalities with high efficacy, among which the combined therapy is a practical strategy.

BEAS-2B was originally established as an immortalized but nontumorigenic epithelial cell line from human bronchial epithelium. The BEAS-2B cell line has been widely used as an *in vitro* cell model in a large variety of studies associated with respiratory diseases including SARS-CoV-2 infection (Wang et al., 2020b). In BEAS-2B, obvious inhibition of biosynthesis of nascent RNA and DNA and arrest of cell cycle in S phase were observed, indicating that remdesivir probably has some negative impact on cell proliferation.

RDV is an adenine nucleotide analog that has been targeted to the process of virus RNA synthesis. In general, nucleotide analogues exert the anticancer or antivirus activity via regulating the activity or expression of the related enzymes in nucleotide synthesis and metabolism pathways to deplete some specific nucleotides and to inhibit the progress of transcription and translation (Nikolaos et al., 2018). Thus, what are the effects of RDV on RNA and DNA synthesis in human cells? Do these effects have any relationship with its efficacy and toxicity? Is it feasible to enhance the antivirus activity of RDV through regulating the related enzymes and metabolites? To our knowledge, no relevant studies have been reported. To answer these questions, the EU staining assay was conducted to evaluate the extent of RDV influence on RNA transcription, and the EdU staining assay to detect the proliferating ability of host cells. As reported, RDV targets the viral RdRps and inhibits RNA chain extension through incorporating the active triphosphate form of RDV into RNA. However, the mechanism of action of RDV on DNA synthesis has not been studied previously.

RR plays a key role in the formation of deoxyribonucleoside diphosphates during DNA synthesis. Experimental results show that RDV inhibited DNA biosynthesis, thus it is rational to investigate the possible effect of RR on DNA synthesis inhibition after RDV treatment. Regulation of RR activity takes place at two levels: through allosteric control of the activity and specificity of RR by nucleoside triphosphate effectors (Nordlund and Reichard, 2006) and by regulation of transcription of the RR

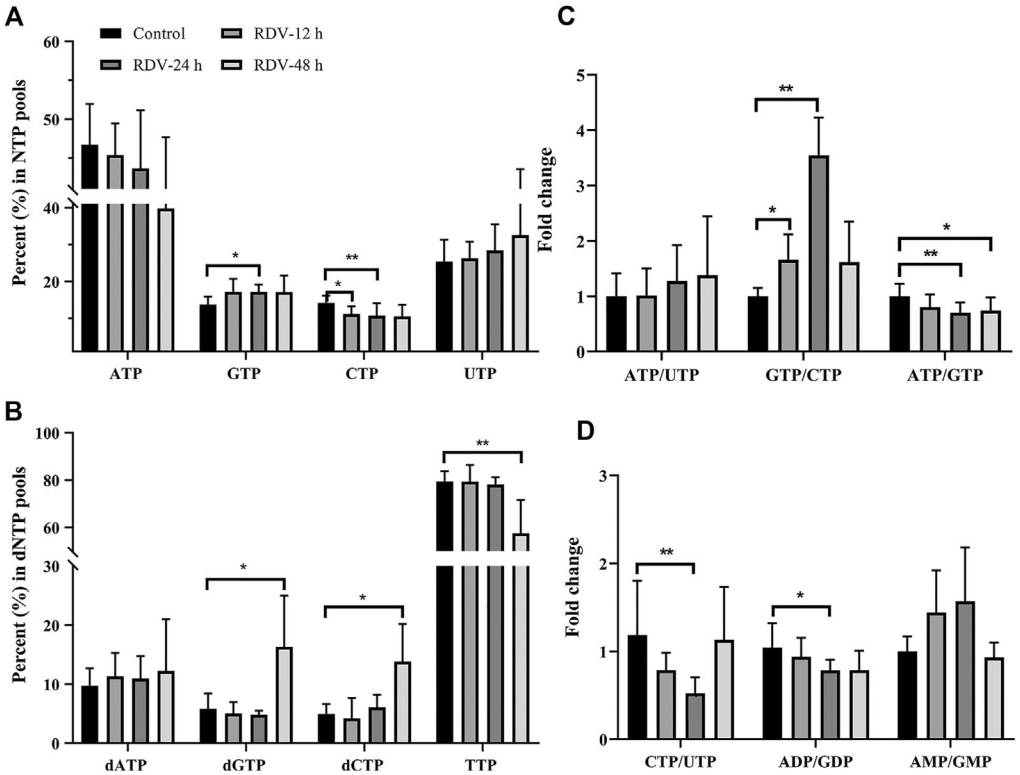

FIGURE 4 | RDV exposure (10 μM) perturbed the balance of **(A)** NTPs and **(B)** dNTPs, and altered the relative ratios of specific nucleotides **(C and D)** (NTP, ribonucleoside triphosphates; dNTP, deoxyribonucleoside triphosphates); ∗: $p < 0.05$; ∗∗: $p < 0.01$, compared with control group.

genes as a function of the cell cycle (Sun et al., 1992), in response to stresses to the replication machinery, or in response to oxygen content or oxidative stress. Given the structural similarity of RDV diphosphate with the natural ADP, the possible mechanism of RDV affecting RR activity was competitive inhibition by ribonucleoside diphosphate. However, after comparing the ratios of ribonucleoside diphosphate to the corresponding deoxyribonucleoside diphosphate in RDV and control groups (data was not shown), no significant change was observed, indicating that the inhibition of RDV on RR activity was insignificant. Meanwhile, RR expression was investigated. Commonly, RRM1 expression remains relatively constant in actively proliferating cells, while RRM2 expression is controlled by cell cycle. The synthesis of RRM2 starts when DNA replication forks are initiated and goes to a maximum in S phase (Gon and Beckwith, 2006). In this study, the expression of RRM2 increased rather than decreased after 24 h RDV exposure. The probable reason might be the delayed hydrolysis of RRM2 due to DNA replication arrest in S phase. p53R2 gene expression occurs mainly in nonproliferating cells. In postmitotic mammalian cells, protein p53R2 substitutes for protein RRM2, as a subunit of ribonucleotide reductase, and is a prerequisite for mitochondrial DNA replication and DNA repair (Pontarin et al., 2012). Due to the higher proportion of cells arrested in S phase, it is rational for the lower p53R2 level in RDV group at 24 h than in the corresponding control group.

According to the results of the changes of nucleotide, especially the abnormal elevation of CMP and the significant imbalance between CTP with GTP or UTP, we preliminarily speculated that RDV or its metabolites 1) upregulated the biosynthesis of CMP in the salvage pathway, and 2) inhibited the conversion of UTP into CTP by CTP synthase in the *de novo* pathway. In order to clarify these issues, the effect of RDV on purified uridine-cytidine kinase (UCK) and CTP synthase should be further researched. Additionally, as a potential target for RDV treatment, CTP synthase attracted our attention. In the clinical applications of antiviral nucleoside analogues, combination therapy is potent for overcoming drug resistance, such as lamivudine plus adefovir (Yatsuji, et al., 2008; Cai et al., 2016), lamivudine and zidovudine (Mandelbrot et al., 2001), and tenofovir DF plus stavudine (Gallant et al., 2004). Similarly, it was reported that zidovudine could increase the radiosensitizing effects of (E)-2′-Deoxy-(fluoromethylene)cytidine (FMdC) by regulating the alteration of dNTP pools in FMdC treatment. Here, we proposed a combined strategy targeting the metabolic vulnerability sites to enhance RDV's antiviral efficacy. Cyclopentenyl cytosine, a CTP synthase inhibitor (Kang et al., 1989), has broad-spectrum antiviral activity (De Clercq et al., 1991; Clercq, 1994). Leflunomide, as an inhibitor of *de novo* pyrimidine synthesis (Rückemann et al., 1998), possesses antiviral and immunosuppressive activities (Chong et al., 2006). Based on the action mechanism and the results of nucleotides induced by

RDV, a co-administration of RDV with cyclopentenyl cytosine or leflunomide might be a powerful approach and deserves further study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

WZ, YL, and HZ designed the study; YL performed the experiments; HZ and YL analyzed and plotted the data; WL and CW validated the data; LB investigated the study; HZ and YL drafted the manuscript; CWKL, WZ, and VKWW reviewed and edited the manuscript; WZ and ZJ supervised the project; WZ funded the experiments for the study. All authors edited and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.647280/full#supplementary-material

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frontiers in Pharmacology

MINI REVIEW
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The Rise and Fall of Chloroquine/ Hydroxychloroquine as Compassionate Therapy of COVID-19

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The emergence and rapid spread of novel coronavirus disease (COVID-19) has posed a serious challenge to global public health in 2020. The speed of this viral spread together with the high mortality rate has caused an unprecedented public health crisis. With no antivirals or vaccines available for the treatment of COVID-19, the medical community is presently exploring repositioning of clinically approved drugs for COVID-19. Chloroquine (CQ) and hydroxychloroquine (HCQ) have emerged as potential candidates for repositioning as anti–COVID-19 therapeutics and have received FDA authorization for compassionate use in COVID-19 patients. On March 28, 2020, the U.S. Food and Drug Administration (FDA) issued an Emergency Use Authorization (EUA) for HCQ in the treatment of COVID-19. However, it was later revoked by the FDA on June 15, 2020, after analyzing the emerging scientific data from ongoing clinical trials. Similarly, the World Health Organization (WHO) also conducted a Solidarity trial of chloroquine, hydroxychloroquine, remdesivir, lopinavir, and ritonavir. However, on May 23, 2020, the executive body of the "Solidarity trial" decided to put a temporary hold on the HCQ trial. On June 17, 2020, the WHO abruptly stopped the Solidarity trial of HCQ. The current review strives to examine the basis of compassionate use of CQ and HCQ for the treatment of COVID-19 in terms of literature evidence, establishing the antiviral efficacy of these drugs against corona and related viruses. Furthermore, the review presents a critical analysis of the clinical trial findings and also provides an insight into the dynamically changing decision on the authorization and withdrawal of HCQ as anti–COVID-19 therapy by the U.S. FDA and the WHO. Ultimately, our study necessitates an evidenced-based treatment protocol to confront the ongoing COVID-19 pandemic and not the mere observational study that mislead the public healthcare system, which paralyzes the entire world.

Keywords: chloroquine, hydroxychloroquine, coronavirus, COVID-19, SARS-CoV-2

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INTRODUCTION

An emergence of a mysterious viral disease causing a cluster of unexplained pneumonia and bronchiolitis cases was first registered in Wuhan, Hubei Province, China (Huang C. et al., 2020). This severe acute respiratory (SAR) disease was recognized in late December 2019 and reportedly given the name coronavirus disease, 2019 (COVID-19) [coronavirus disease (COVID-2019) technical

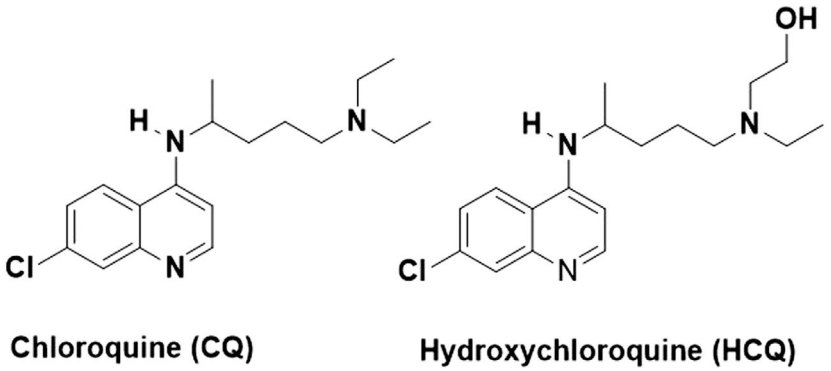

FIGURE 1 | Chemical structures of chloroquine (CQ) and hydroxychloroquine (HCQ).

guidance, WHO]. The causative agent for the disease was identified to be a novel coronavirus (SARS-CoV-2) (Wu et al., 2020). In a short period, COVID-19 spread rapidly and progressed to an epidemic proportion throughout the world with substantial morbidity and mortality. Therefore, COVID-19 has been declared a major public health emergency by the World Health Organization (WHO). On March 11, 2020, the WHO confirmed the COVID-19 outbreak as a global pandemic (Cucinotta and Vanelli, 2020). As of March 29, 2021, COVID-19 is responsible for more than 126,890,643 infections and 2,778,619 deaths worldwide (novel coronavirus 2019 status report, WHO). In India, there are 12,039,644 confirmed COVID-19 cases, and 161,843 deaths have been recorded as of March 29, according to the COVID-19 web portal, Ministry of Health and Family Welfare, Govt. of India.

Since the outbreak of the COVID-19 pandemic, tremendous efforts have been invested in the development of vaccines and antiviral therapeutics that target SARS-CoV-2 (Amanat and Krammer, 2020). At present, there is no Food and Drug Administration (FDA)-approved specific drug available for the treatment of COVID-19. Therefore, infection control measures like quarantine, isolation, social distancing, and travel ban are strictly imposed worldwide to contain the disease [Institute of Medicine (US) Forum on Microbial Threats, 2007]. COVID-19 patients are given supportive care such as fluid support, oxygen, and ventilatory support. The severe cases of COVID-19 are given mechanical ventilation or extracorporeal membrane oxygenation (ECMO) as life support (Nakamura et al., 2020). Besides this, several FDA-approved drugs have been repurposed based on preliminary clinical findings for the treatment of COVID-19 patients on a compassionate basis. The putative treatment based on the concept of drug repurposing includes chloroquine (CQ) (Shukla et al., 2020), hydroxychloroquine (HCQ) (Shukla et al., 2020), lopinavir (Chu et al., 2004), ritonavir (Cao et al., 2020), remdesivir (Wang et al., 2020), ribavirin (Khalili et al., 2020), griffithsin (Li and Clercq, 2020), tocilizumab (Marotto and Sarzi-Puttini 2020), sarilumab (Sallard et al., 2020), interferon (Long et al., 2020), immunoglobulins (Jiang et al., 2020; Long et al., 2020), and corticosteroids (Zha et al., 2020) used to reduce the viral load and prevent lung damage.

This mini-review aimed to provide a summary of the therapeutic potential and experimental use of CQ and HCQ in fighting COVID-19. Herein, we summarize the basis for compassionate use of CQ and HCQ, their *in vitro* and *in vivo* antiviral activities on coronaviruses, and clinical trials on COVID-19 patients. The review presents a critical analysis of the clinical trial findings and also provides an insight into the dynamically changing decision on the authorization and withdrawal of HCQ as anti–COVID-19 therapy by the U.S. FDA and the WHO.

COMPASSIONATE THERAPY OF COVID-19

Antiviral Activities of Chloroquine

CQ is chemically represented as N4-(7-chloroquinolin-4-yl)-N1,N1-diethylpentane-1,4-diamine, as shown in **Figure 1** (Tse et al., 2019). It is an inexpensive drug that has been used for more than 70 years for the treatment of malaria (Arrow et al., 2004). Although some malaria strains have developed resistance against CQ, it is one of the most widely prescribed drugs for malaria even today. Besides having clinically proven antimalarial activity, CQ demonstrates a wide range of pharmacological activities such as anti-inflammatory, immunomodulatory, and antiviral activities (Browning, 2014).

A large number of publications cite the *in vitro* studies on antiviral properties of CQ against a variety of viruses (Hashem et al., 2020; Huang M et al., 2020). The *in vitro* antiviral effect of CQ was identified for the first time in 1969 (Inglot, 1969), and this is followed by many published reports on antiviral properties of CQ in subsequent years (Shimizu et al., 1972) and in 1981 (Glushakova and Lukashevich, 1989). Further, the anti–SARS-CoV activity (growth inhibition of coronaviruses in cell culture) of both CQ and HCQ was reported in 2005 (Vincent et al., 2005). In addition to antiviral effects, CQ also caused a significant reduction in the expression of pro-inflammatory cytokines, interferons (IFN-β and IFN-g), tumor necrosis factor (TNF-α), and interleukins (IL-6 and IL-12) (Jang et al., 2006). Farias et al.

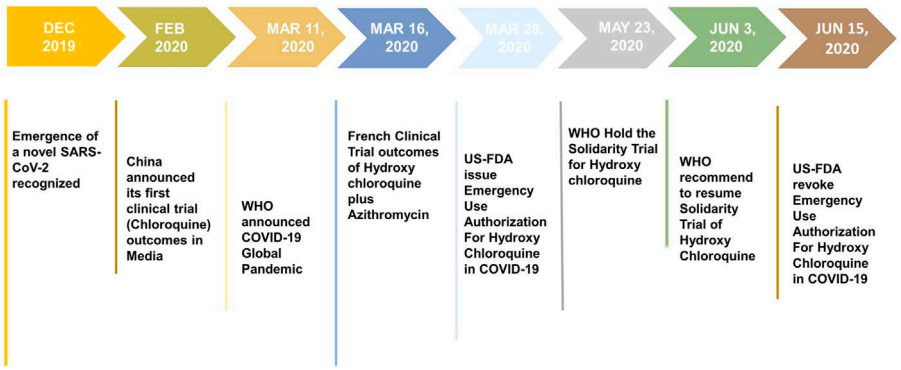

FIGURE 2 | Various stages of development of chloroquine (CQ) and hydroxychloroquine (HCQ) as compassionate therapy.

(2014) reported that CQ treatment (dose: 50 mg/ml) resulted in a significantly low virus production in dengue (DENV-2)-infected U937 cells. However, CQ was found to be nontoxic to the normal cells at the same dose (Farias et al., 2014).

Several articles reported the *in vivo* antiviral activity of CQ against human coronavirus OC43 (Keyaerts et al., 2009), enterovirus EV-A71 (Tan et al., 2018), Zika virus (Li et al., 2017), and influenza A H5N1 (Yan et al., 2013). CQ also showed promising *in vitro* antiviral effects on numerous viruses, but the *in vivo* antiviral efficacy of CQ in the primate model of CHIKV infection was not found satisfactory. CQ was found to worsen the disease in the primate model of CHIKV infection by exacerbating the acute fever and delaying the cellular immune response to an incomplete CHIKV viral clearance (Roques et al., 2018). Similarly, CQ was found active *ex vivo* but not *in vivo* in the case of Ebolavirus (Dowall et al., 2015), Nipah virus (Pallister et al., 2009), and influenza virus (Vigerust and McCullers, 2007). CQ has also been tested in chronic hepatitis C and HIV patients for viral clearance. CQ exhibited only a modest level of antiviral effect against chronic hepatitis C infection, and a transient viral load reduction was observed with CQ treatment in a small sample size pilot trial in nonresponder HCV patients (Peymani et al., 2016). However, this was found inadequate for inclusion of the drug in the standardized treatment protocols for hepatitis C–infected patients (Helal et al., 2016). The therapeutic use of CQ in HIV-infected patients has been considered indecisive, and the drug has not been recommended for further use in acquired immune deficiency syndrome (AIDS) treatment (Chauhan and Tikoo, 2015). Overall, CQ exhibited promising *in vitro* antiviral activities against a variety of viruses; however, this preexisting knowledge has not yet been translated into meaningful preclinical studies.

Farias et al. (2014) reported that CQ inhibits the replication of a human coronavirus (HCoV-OC43) in HRT-18 cells, with an effective concentration (EC_{50}) of 0.306 ± 0.0091 μm and a lethal concentration (LD_{50}) of 419 ± 192.5 μm. The selectivity index observed in HCoV-OC43 was SI. 1,369, which shows the wide safety margin of CQ (Farias et al., 2014). Further, the *in vivo* study of CQ on newborn C57BL/6 mice infected with a lethal HCoV-OC43 infection showed the highest survival rate (98.6%). Overall, the results show the favorable *in vitro* and *in vivo* antiviral effects of CQ against HCoV-OC43 (Farias et al., 2014). Similar *in vitro* antiviral activity studies of CQ on different types of viruses including SARS-CoV-2 were observed in the recent past (Wang et al., 2020). Therefore, it was hypothesized that CQ may be the potential clinical candidate against SARS-CoV-2. As a result of these findings, CQ has already been incorporated into the treatment protocols of certain COVID-19 patients.

Antiviral Activities of Hydroxychloroquine

HCQ is a hydroxylated derivative of CQ and an effective antimalarial agent (Liu et al., 2020). HCQ is also broadly used for the treatment of various autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (Silva et al., 2013). HCQ exhibits a better safety profile than CQ, hence is more tolerable than CQ in COVID-19 patients (Gevers et al., 2020). Furthermore, studies have demonstrated that the immunomodulatory activity of HCQ may play an important role in controlling the cytokine storm in severely infected SARS-CoV-2 patients (Silva et al., 2013).

Yao et al. (2020) studied the *in vitro* antiviral properties and rationalized the prophylactic activity of CQ and HCQ. The authors also built physiologically based pharmacokinetic models (PBPKs) for both of these drugs to predict drug concentrations under different dosing regimens. The *in vitro* antiviral activity evaluation of both these drugs was carried out in SARS-CoV-2–infected Vero cells. HCQ (EC_{50} = 0.72 μm) was found to be more active than its counterpart CQ (EC_{50} = 5.47 μm). The EC_{50} values estimated for CQ in Vero cells were 23.90 and 5.47 μm at 24 and 48 h, respectively (Yao et al., 2020). However, the EC_{50} values for HCQ were observed to be 6.14 and 0.72 μm at 24 and 48 h, respectively. It was reported that EC_{50} values for CQ in the drug pretreatment method were >100 and 18.01 μm at 24 and 48 h, respectively. In the same method, EC_{50} values for HCQ were found to be 6.25 and 5.85 μm at 24 and 48 h, respectively. The inhibition rate of CQ did not exceed 50% even at the maximum concentration tested. Both CQ and HCQ were found to decrease viral replication in a concentration-dependent manner. All these results suggested that HCQ

demonstrated superior *in vitro* SARS-CoV-2 inhibition in comparison to CQ (Yao et al., 2020).

It was evident from several *in vitro* and *in vivo* antiviral activity studies that HCQ has exhibited potent antiviral activity against coronaviruses (Yao et al., 2020). Furthermore, the drug also elicits tremendous immunomodulatory potential in addition to established clinical safety at appropriate doses (Chandler et al., 2020). All these findings support the inclusion of HCQ in the treatment of COVID-19. However, few reports suggest that even short-term treatment with HCQ can cause cardiac arrhythmias, dermatological reactions, hypoglycemia, and seizures, triggering serious concerns over the use of HCQ in this critical situation (Pereira, 2020). Despite these side effects, both these drugs have been used in the clinical practice of malaria and inflammatory disease for many years. HCQ being a more water soluble and less toxic than CQ is most suitable for repurposing. The promising *in vitro* antiviral activity results of HCQ against SARS-CoV-2 together with better safety profile positioned HCQ as a potential therapeutic option for the treatment of COVID-19.

CLINICAL TRIALS OF CHLOROQUINE AND HYDROXYCHLOROQUINE

The high mortality rate and tremendous pressure faced by public health systems to save lives during this devastating COVID-19 pandemic allowed the experimental use of CQ and HCQ in the treatment of severely infected COVID-19 patients (Becker, 2020). Researchers all over the world have initiated clinical trials of the repurposed drugs to find an effective cure for COVID-19. As a result, an enormously large number of clinical trials are underway to generate the robust data needed to establish the therapeutic efficacy and clinical safety of these drugs in COVID-19 cases (Lythgoe and Middleton, 2020). Until December 25, 2020, there were 354 clinical trials that have been registered in various national and international clinical trial databases for CQ and HCQ either alone or in combination with some other drugs in the treatment of COVID-19 (clinicaltrials.gov, WHO).

Last year, Gao et al. (2020) recorded the first clinical trial outcomes of CQ as reported in a news briefing by the Chinese government agency in February 2020. The news briefing revealed that the study was conducted with more than 100 COVID-19 patients. The clinical trial candidate CQ phosphate was found to be much superior to the control treatment in COVID-19. It was also stated that CQ successfully inhibited the exacerbation of pneumonia, improved lung imaging findings, promoted a virus-negative conversion, and shortened the disease course. No specific adverse events were observed in the trial (Gao et al., 2020). It appears that these findings were a result of a compilation of clinical data from several ongoing trials conducted in different Chinese hospitals from a variety of studies. So far, no such clinically validated data are available in the public domain supporting these findings.

A series of studies carried out in the outpatient (COVID-19) setting to test the triple therapy consists of zinc, low-dose hydroxychloroquine, and azithromycin (Derwand et al., 2020). In this retrospective study, a total of 141 COVID-19 patients received triple therapy for 5 days. The results of the study showed that among all treated patients, only 4 were hospitalized and only one patient in the treatment group died compared to 13 patients in the untreated group. Further, the study also reported that no cardiac side effects were observed using triple therapy.

Another pilot study on clinical benefits of HCQ in the treatment of COVID-19 was carried out in China. It was a randomized clinical trial involving 30 confirmed COVID-19 cases. The patients were randomized 1:1 to the HCQ group and the control group. They were given an HCQ plus conventional therapy or conventional treatment alone (Chen J. et al., 2020). On day 7, 13 patients administered with HCQ and 14 of those in the control group were found to be negative for COVID-19 nucleic acid in throat swabs. Other COVID-19 clinical measures like fever (the time required to achieve normal body temperature), progress in pneumonia, and overall clinical improvement were observed similarly in both groups. In contrast to previous studies, few adverse events were reported in the treatment group (Chen Y. et al., 2020). One patient who was given HCQ developed severe lung disease during the course of treatment. This study appears to be an open-label clinical trial with few participants. However, the trial gives no sufficient information on the therapeutic and prophylactic value of HCQ on COVID-19 patients.

Another clinical trial of HCQ was conducted at the University Hospital Institute Méditerranée Infection in Marseille, France. The study included a trial treatment of HCQ and a combination of HCQ with azithromycin in hospitalized COVID-19 patients (Gautret et al., 2020). The trial included a single-arm protocol carried out from early March to March 16th. The patients received 600 mg of HCQ on daily basis, and the viral load was tested every day in their nasopharyngeal swabs (Gautret et al., 2020). Further, azithromycin was added to the regular HCQ treatment depending on the clinical presentation of COVID-19 patients. On day 6 post-inclusion, either the presence or absence of SARS-CoV-2 virus in nasopharyngeal swabs was considered as the endpoint. Among all, twenty COVID-19 patients treated in this study presented a significant reduction of the viral load compared to control groups. It was also observed in the study that a combination of azithromycin and HCQ was more efficient in virus elimination. The overall results of the study showed that all patients treated with HCQ and azithromycin combination were 100% virologically cured as compared to patients (57.1% cured) treated with HCQ alone. A recovery rate of 12.5% was observed in the control group. The major outcome of this clinical trial was that all the patients who were treated with a combination of HCQ and azithromycin tested negative for COVID-19 on day 6 (Gautret et al., 2020). Therefore, the authors advocate the clinical effectiveness of HCQ and a synergistic effect in combination with azithromycin in the treatment of SARS-CoV-2 infection. However, the article received greater attention of scientific community with severe criticism and major concern for the clinical trial results presented in the study (International Society of Antimicrobial Chemotherapy, 2020).

USE OF HYDROXYCHLOROQUINE IN INDIA TO FIGHT COVID-19

In a large and densely populated country like India, the battle against COVID-19 is an enormous challenge. With the onset of the COVID pandemic, India has been facing several issues, such as shortage of diagnostic tools, medical equipment, and related medical supplies. It has directly challenged our public healthcare system and forced to quickly respond. As no drugs were available to fight against the ugly battle of COVID-19, the WHO and the FDA authorized the use of HCQ based on previously available clinical observational studies. Similarly, the Indian Council of Medical Research (ICMR), functioning under the Ministry of Health and Family Welfare, Govt. of India, recommended the use of HCQ in the treatment protocol for COVID-19 patients. India is the major producer and supplier of HCQ, and several countries were helped by additional supplies from India during the pandemic (Batumalai K., 2020; Brian et al., 2020; Channnel News Asia., 2020; NIH Clinical Trials.gov. 2020; Sibbal, 2020; Ying, 2020). The ICMR has also recommended a prophylaxis therapy with HCQ (400 mg twice on day 1, then 400 mg once a week thereafter) for asymptomatic healthcare workers in COVID-19 hospitals and household contacts of confirmed COVID-19 cases (Chatterjee et al., 2020; Rathi et al., 2020; Tilangi et al., 2020). The WHO Solidarity trial is the world's largest global randomized controlled clinical trial, and India contributed one-tenth of the participants in this trial. The authorization to use azithromycin in combination with hydroxychloroquine (HCQ) to treat patients with severe SARS-CoV-2 infections has been rolled back after the interim trial results that showed no potential benefit. During June, 2020, the Ministry of Health and Family Welfare, Govt. of India, issued an updated clinical management protocol for COVID-19 based on the clinical severity of the disease. The revised protocol allows the use of HCQ to COVID-19 patients in the early course of the disease and not on critically ill patients. Furthermore, it was advised to be administered only after "shared decision making with the patients" and also recommends an ECG before prescription. As part of India's COVID-19 therapeutic management, the Indian government has distributed 111.6 million pills of hydroxychloroquine or HCQ (Government Information in Parliament 2021).

WHAT WENT WRONG WITH THE HYDROXYCHLOROQUINE?

Some clinical observational studies have suggested therapeutic benefits of HCQ in COVID-19, whereas other studies have shown mixed results. Risch et al. reported that hydroxychloroquine has demonstrated significant major outpatient treatment efficacy by reviewing five observational studies, including two controlled clinical trials (Risch, 2020a). This study received strong scientific criticism and raised serious concern for openly promoting HCQ without strong clinical trial evidence (Fleury, 2020). The author has published a follow-up to that study that described seven additional studies in support of his earlier findings (Risch, 2020b). However, the data used in the study were either unpublished or without data. Further, none of the studies presented in the article was found to be large randomized controlled trials.

The WHO launched a huge international clinical trial called "Solidarity" to assess the effectiveness and safety of certain drugs that could be rapidly deployed in the battle against COVID-19. CQ and HCQ are enlisted in the Solidarity trial along with four other antiviral drugs. A research group studied the use of hydroxychloroquine or chloroquine with or without azithromycin for the treatment of COVID-19 by critically analyzing an enormously large COVID-19 clinical data (96,032 patients) obtained from 671 hospitals (Mehra et al., 2020a). The retrospective analysis of these data did not confirm any potential benefit for in-hospital patients, and further reported that the drugs decreased the survival of COVID-19 cases and resulted in an increased risk of ventricular arrhythmias. The same research group published a similar research that did not confirm the potential risk of angiotensin-converting enzyme (ACE) inhibitor or angiotensin II receptor blockers (ARBs) for in-hospital COVID-19 patients (Mehra et al., 2020b). These findings greatly influenced the WHO to take major decision over ongoing clinical trials of CQ and HCQ. On May 23, 2020, the executive body of the "Solidarity trial" decided to put a temporary hold on the HCQ trial, because of some safety concerns. Shortly after the retraction of the studies that rattled the scientific community (Mehra et al., 2020a; Mehra et al., 2020b), on June 3, 2020, the executive group received a recommendation based on the mortality data and endorsed the continuation of the HCQ Solidarity trial. The stagewise development of CQ and HCQ in COVID-19 is summarized in **Figure 2**. Till March 29, 360 clinical trials are registered to evaluate the therapeutic efficacy and prophylactic action of both CQ (88) and/or HCQ (272) in COVID-19 patients (Clinical trials report table 2021, WHO). However, only a few of the researchers published preliminary results, while other studies are under process. Although existing clinical trial data support some beneficial effects of CQ and HCQ in COVID-19, some of the ongoing trials were canceled or stopped due to possible adverse effects. Thus far, clinical trial results obtained for HCQ from different studies majorly suffer due to limitations of small sample sizes. Neither the French nor Chinese studies conducted for CQ and HCQ were randomized clinical trials. The clinical trial investigators of University Hospital Institute Méditerranée Infection acknowledged the "small sample size" and also the side effects of HCQ. The results of a RECOVERY trial (Randomized Evaluation of COVID-19 therapy) were carried out in the United Kingdom; a large clinical trial aimed to identify potential treatments for hospitalized COVID-19 cases that did not support the use of HCQ (The Recovery Collaboration group, 2020).

The global pandemic of SARS-CoV-2 infection has spread out of control in several countries and caused considerable morbidity and mortality (Elissa et al., 2020). Thus, there is an urgent need for an effective treatment to cure COVID-19 patients and also to prevent community transmission. Overall, the antiviral activities

of CQ and HCQ against several viral diseases, including novel coronaviruses, low costs, good safety profile, and preexisting supply chain, pave the path for entry of these drugs into the treatment guideline of COVID-19. CQ and HCQ have been currently authorized by many countries for treating COVID-19 on a compassionate basis with caution. On March 28, 2020, the U.S. Food and Drug Administration (FDA) has issued the EUA for the inclusion of HCQ in the treatment of COVID-19. Subsequently, on June 15, 2020, the U.S. FDA revoked the emergency use authorization (EUA) based on its ongoing analysis. The U.S. FDA further stated that both of these drugs show no benefit on mortality or in speeding recovery, and hence are unlikely to be effective in treating COVID-19 patients. Recently, on June 17, 2020, the WHO also announced to stop the Solidarity trial of HCQ in COVID-19. However, the WHO decided not to prohibit the use or evaluation of hydroxychloroquine in pre- or postexposure prophylaxis in COVID-19 patients.

CONCLUSION

The worldwide spread of SARS-CoV-2 infection made the global healthcare system to confront an entirely new and unprecedented situation. Clinicians worldwide employed a drug repurposing strategy to find drugs that can stem the progression of this highly contagious disease. A plethora of literature evidence on the antiviral potential of CQ and HCQ against several types of viruses including coronaviruses and preliminary clinical data on therapeutic benefits observed with CQ and HCQ treatment in COVID-19 patients led to the FDA authorization of both CQ and HCQ for compassionate use against COVID-19. Furthermore, clinical trial reports from China and France speculated the claims on the anti–SARS-COV-2 efficacy of HCQ either alone or in combination with other drugs like azithromycin. Although preliminary reports supported the use of the antimalarial agents such as CQ and HCQ to treat this rampant COVID-19, subsequent hospital observations and evidence from the large randomized clinical trials of HCQ did not demonstrate any clinical benefits. Ultimately, HCQ as COVID-19 therapy has come to an end on June 15, 2020, as the U.S. FDA revoked the EUA authorization. However, the global search for an effective drug or vaccine remains continues inspiring hope in the battle against COVID-19. Furthermore, our study emphasizes the need for evidence-based treatment approaches from large randomized clinical trials to confront the ongoing COVID-19 pandemic and not the mere observational study that mislead the public healthcare system, which paralyzes the entire world.

AUTHOR CONTRIBUTIONS

EM, CK, NM, and SC conceived the idea and conceptualize the manuscript. EM and NM wrote the first draft of the manuscript. EM made the figures. EM and CK finalized the manuscript. All authors reviewed and approved the manuscript.

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Adverse Effects Associated With the Use of Antimalarials During The COVID-19 Pandemic in a Tertiary Care Center in Mexico City

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Background: Antimalarial drugs were widely used as experimental therapies against COVID-19 in the initial stages of the pandemic. Despite multiple randomized controlled trials demonstrating unfavorable outcomes in both efficacy and adverse effects, antimalarial drugs are still prescribed in developing countries, especially in those experiencing recurrent COVID-19 crises (India and Brazil). Therefore, real-life experience and pharmacovigilance studies describing the use and side effects of antimalarials for COVID-19 in developing countries are still relevant.

Objective: To describe the adverse effects associated with the use of antimalarial drugs in hospitalized patients with COVID-19 pneumonia at a reference center in Mexico City.

Methods: We integrated a retrospective cohort with all adult patients hospitalized for COVID-19 pneumonia from March 13th, 2020, to May 17th, 2020. We compared the baseline characteristics (demographic and clinical) and the adverse effects between the groups of patients treated with and without antimalarial drugs. The mortality analysis was performed in 491 patients who received optimal care and were not transferred to other institutions (210 from the antimalarial group and 281 from the other group).

Results: We included 626 patients from whom 38% (n = 235) received an antimalarial drug. The mean age was 51.2 ± 13.6 years, and 64% were males. At baseline, compared with the group treated with antimalarials, the group that did not receive antimalarials had more dyspnea (82 vs. 73%, p = 0.017) and cyanosis (5.3 vs. 0.9%, p = 0.009), higher respiratory rate (median of 28 vs. 24 bpm, $p < 0.001$), and lower oxygen saturation (median of 83 vs. 87%, $p < 0.001$). In the group treated with antimalarials, 120 patients had two EKG evaluations, from whom 12% (n = 16) prolonged their QTc from baseline in more than

50 ms, and six developed a ventricular arrhythmia. Regarding the trajectories of the liver function tests over time, no significant differences were found for the change in the mean value per day between the two groups. Among patients who received optimal care, the mortality was 16% (33/210) in those treated with antimalarials and 15% (41/281) in those not receiving antimalarials (RR 1.08, 95% 0.75–1.64, and adjusted RR 1.12, 95% CI 0.69–1.82).

Conclusion: The adverse events in patients with COVID-19 treated with antimalarials were similar to those who did not receive antimalarials at institutions with rigorous pharmacological surveillance. However, they do not improve survival in patients who receive optimal medical care.

Keywords: hydroxychloroquine, chloroquine, COVID-19, arrythmia, adverse-effects, antimalarial

INTRODUCTION

Hydroxychloroquine (HCQ) and chloroquine (CLQ) are antimalarial drugs recently repurposed as a possible therapy against COVID-19 due to their immunomodulatory properties and the *in-vitro* antiviral effect against SARS-CoV-2 observed in experimental models (Chowdhury et al., 2020; Gautret et al., 2020). As a consequence, the Food and Drug Administration (FDA) provided an emergency authorization use against SARS-CoV-2 infection (US Food and Drug Administration, 2021a), which was later revoked (US Food and Drug Administration, 2021b) due to the negative results observed in randomized controlled trials (RCTs) (Boulware et al., 2020; Mehra et al., 2020a). The World Health Organization (WHO) withdrew hydroxychloroquine from its clinical trial *Solidarity* in July 2020 (RECOVERY Collaborative Group et al., 2020; Borba et al., 2021; Geleris et al., 2020; Self et al., 2020). In addition, several trials have demonstrated a high prevalence of significant adverse effects (primarily cardiovascular) in patients receiving this drug (RECOVERY Collaborative Group et al., 2020; WHO.int, 2021). Despite the compelling evidence, antimalarial drugs have resurged in developing countries experiencing recurrent outbreaks. On the past April 22, 2021, the AIIMS/ICMR—COVID 19 National Task Force/Joint Monitoring Group of the Ministry of Health and Family Welfare of India updated the clinical guidelines for managing adult patients with COVID-19 considering both ivermectin and HCQ in the category of "May Do" with low certainty of evidence (Icmrgovin, 2021). Similarly, the Brazilian government provides a "Covid-Kit" consisting of antimalarials and ivermectin (Kmietowicz, 2021).

Overall, antimalarial drugs are safe. The most frequent adverse effects are nausea, diarrhea, headache, diplopia, pruritus, urticaria, lichenoid rash, hair discoloration, seizures, and anxiety. The accumulation of high doses (>1 g/kg), usually due to a prolonged use, may develop ototoxicity, retinopathy, myopathy, heart toxicity, and peripheral neuropathy (Mercuro et al., 2020; Tang et al., 2020). Less frequently, acute and potentially fatal adverse effects such as QT prolongation, T wave abnormalities, and vasodilation may occur (Rosenberg et al., 2020; Tang et al., 2020). The current FDA-approved indications for these medications are predominantly in the ambulatory setting (both as an immunomodulatory medication for autoimmune disorders or as malaria prophylaxis) and rarely in the clinical context of hospitalized patients.

We aimed to describe the prevalence and severity of adverse effects in a cohort of patients with severe COVID-19 who received antimalarial drugs as therapy in a tertiary care center in Mexico City.

MATERIALS AND METHODS

Treatment and Study Design

We retrospectively collected data regarding treatment and adverse effects, as well as baseline characteristics, complications and mortality, from the institutional COVID-19 cohort (Ortiz-Brizuela et al., 2020), which included hospitalized patients from March 13th, 2020 to May 17th, 2020. Antimalarial drug administration was allowed by institutional protocol during the study period and was prescribed by the treating medical team in agreement with patients after a discussion regarding potential risks and benefits. Due to the shortage of HCQ, the most used antimalarial drug was CLQ. The initial dose was 400 mg bid the first day, then 200 mg bd for 5–14 days for HCQ and 300 mg bid on day 1, and then 150 mg bd for CLQ, or until an adverse effect appeared.

This study was approved by the Institutional Review Board (Comité de Investigación and Comité de Ética en Investigación, reference number 3333), who waived the informed consent requirement due to the minimal risk characteristics of an observational study. All the patients admitted to our institution during the pandemic agree with releasing their medical data (*via* standardized consent) for research purposes (and had the option to decline).

Patients and Follow up

During the hospital stay, patients were clinically evaluated twice a day, at least. Patients had blood testing on days 3 and 7 to assess clinical status and toxicity [complete blood count, blood glucose, serum ferritin, creatine phosphokinase, D-dimer, kidney and liver function tests (LFT), as well as prothrombin time and partial thromboplastin time]. The adverse effects regarding laboratorial

TABLE 1 | Demographic characteristics and comorbidities of patients with COVID-19 treated with and without antimalarial drugs in a tertiary care center in Mexico City.

Characteristics	*N*	Overall (*N* = 626)	HCQ/CLQ (*N* = 235)	No antimalarial (*N* = 391)	*p*–value
S	626	51.2 ± 13.6	50.0 ± 13.2	52.0 ± 13.8	0.062
Male gender, no.—(%)	626	402 (64)	152 (65)	250 (64)	0.92
BMI, mean (SD)—kg/m^2	591	30.6 ± 5.8	29.9 ± 5.6	31.0 ± 5.8	0.023
Diabetes—no. (%)	626	166 (26)	59 (25)	107 (27)	0.60
Hypertension—no. (%)	626	194 (31)	68 (28)	126 (32)	0.44
COPD—no. (%)	626	5 (0.8)	2 (0.9)	3 (0.8)	>0.99
CVD—no. (%)	625	26 (4.2)	10 (4.3)	16 (4.1)	>0.99
CKD—no. (%)	626	16 (2.6)	6 (2.6)	10 (2.6)	>0.99
Immunosuppression—no. (%)	626	29 (4.6)	12 (5.1)	17 (4.3)	0.81
Smoking—no. (%)	621	105 (16.9)	44 (18.9)	61 (15.7)	0.36

HCQ, hydroxychloroquine; CLQ, chloroquine; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; CKD, chronic kidney disease; HIV, human immunodeficiency virus.

testing were classified according to the FDA score: grade 1 (mild), grade 2 (moderate), grade 3 (severe) and grade 4 (life threatening) (US Food and Drug Administration, 2021c), and the specific definitions were: hypoglycemia G1 (55 mg/dl), G2 (<55–40 mg/dl), G3 (<40–30 mg/dl) and G4(<30 mg/dl); neutropenia G1 (2,000–1,500/ mm^3), G2 (1,500–1,000/ mm^3), G3 (1,000–500/ mm^3), G4 (<500/ mm^3); leukopenia G1 (4,000–3,000 mm^3), G2 (<3,000–2,000/ mm^3, G3(<2,000–1,000/ mm^3, G4 (<1,000/ mm^3); lymphopenia G1 (1,000–800/ mm^3, G2 (<800–500/ mm^3), G3 (<500–200/ mm^3), G4 (<200 mm^3); and thrombocytopenia G1 (150,000–75,000/ mm^3), G2 (<75,000–50,000/ mm^3), G3 (<50,000–25,000/ mm^3) and G4 (<25,000/ mm^3).

We performed active surveillance of pre-existing arrhythmias through an initial electrocardiogram (EKG) recorded before the first dose for most of the patients and after the second dose for those with borderline QTc in the baseline EKG. Patients were monitored *via* telemetry, abnormal tracing noted by the treating physician triggered additional EKG recordings for further analysis. The medication was stopped in case of ventricular polymorphism. We intentionally looked for ventricular arrhythmias and considered as positive the presence of ventricular premature activity and monomorphic ventricular tachycardia (Zipes et al., 2019). The information related to adverse effects was obtained retrospectively from the electronic records and from laboratory databases. The EKGs were analyzed by experienced cardiologists (JOM, CBM, JDG, ACD).

Statistical Analysis

Numerical variables are described in mean and standard deviation or in median and interquartile range (IQR) as appropriate, categorical variables are described in frequencies and percentages. The clinical and demographic characteristics on admission were compared between groups defined by the treatment received (antimalarial vs. no antimalarial) through Student's *t* test, Mann Whitney test or chi square test as appropriate. To identify potential hepatoxicity due to the use of antimalarial, we compare the dynamic profile in LFT between groups defined by the treatment received using generalized linear mixed models adjusted for age, sex, baseline laboratory values and allowing for interaction between time and therapy received (antimalarial vs. no antimalarial). This analysis was performed only on patients with at least two liver function test measurements and we assumed the missing data as missing at random. The mortality analysis was performed in patients who received optimal medical care and who either died or were discharged to home. That is, we excluded patients with ICU requirements who were not admitted to the ICU (due to not intubate/resuscitate order or to lack of ICU-bed) as well as those who were discharged against medical advice or transferred to another institution. The effect of the therapy received (antimalarial vs. no antimalarial) was estimated using Targeted Maximum Likelihood Estimation with a super learning algorithm for the treatment assignment model, adjusting for age, sex, diabetes, body mass index (BMI), period time of admission (before April 15th vs. April 15th or after) and NEWS score (this includes respiratory rate, oxygen saturation, need for supplemental oxygen, heart rate, systolic blood pressure, and responsiveness) and site of admission as confounders. A two-sided *p* value of less than 0.05 was considered statistically significant. All the analyses were performed using R software, version 3.6.3.

RESULTS

Demographic Characteristics

During the study period, 626 patients were hospitalized, of whom 235 (37.5%) received an antimalarial drug. **Table 1** shows the baseline characteristics, mean age was 51.2 ± 13.6 years and 64% were male. Overall, the most frequent comorbidities were obesity in 48%, overweight in 38%, hypertension in 31% and diabetes in 26%. When comparing baseline characteristics between both groups, only the BMI was significantly different, being lower in patients taking antimalarial drugs (mean of 30 vs. 31 kg/m^2, $p = 0.023$).

Clinical Manifestations and Laboratory Findings

The clinical and laboratorial findings at admission are summarized in **Tables 2**, **3**, respectively. Patients not receiving

TABLE 2 | Clinical manifestations, physical findings and value of the severity scales on admission on admission of patients with COVID-19 treated with and without antimalarial drugs in a tertiary care center in Mexico City.

Characteristic	*N*	Overall (*N* = 626)	HCQ/CLQ (*N* = 235)	No antimalarial (*N* = 391)	*p*-value
Symptoms—no. (%)					
Fever	625	548 (87.7)	212 (90.2)	336 (86.2)	0.17
Cough	623	569 (91.3)	220 (93.6)	349 (89)	0.15
Headache	620	471 (76)	177 (76)	294 (76)	>0.99
Dyspnea	624	492 (78)	173 (73)	319 (82)	**0.017**
Chest pain	611	206 (33)	75 (32)	131 (34.6)	0.63
Cyanosis	608	22 (3.6)	2 (0.9)	20 (5.3)	**0.009**
Physical findings					
Temperature—mean (SD)—°C	612	37.2 ± 0.8	37.2 ± 0.8	37.2 ± 0.8	0.36
Heart rate—mean—(SD)—bpm	624	102 ± 17.5	101.4 ± 17.5	102.6 ± 17.5	0.40
Respiratory rate, median (IQR)—bpm	623	27 (22–32)	24 (20–30)	28 (24–35)	<**0.001**
Mean arterial pressure, mean (SD)—mmHg	614	91 ± 11.7	90 ± 11.6	91 ± 11.8	0.68
Oxygen saturation, median (IQR)—%	602	85 (74.0–88)	87 (81–89)	83 (70–88)	<**0.001**
Time from symptoms to admission, median (IQR)—days	626	7 (5–10)	7 (5–9)	7 (6–10)	0.075
qSOFA	618				<**0.001**
0		110 (18%)	63 (27%)	47 (12%)	
1		455 (74%)	154 (66%)	301 (78%)	
2		50 (8.1%)	17 (7.3%)	33 (8.6%)	
3		3 (0.5%)	0 (0%)	3 (0.8%)	
Severity scales on admission					
NEWS	617	8.27 (2.28)	7.55 (2.48)	8.72 (2.03)	<**0.001**
NIH Severity	623				**0.006**
Moderate		24 (3.9%)	16 (6.8%)	8 (2.1%)	
Severe		573 (92%)	206 (88%)	367 (94%)	
Critical		26 (4.2%)	12 (5.1%)	14 (3.6%)	

SD, standard deviation; IQR, interquartile range HCQ, hydroxychloroquine; CLQ, chloroquine; qSOFA, quick sequential organ failure assessment; NEWS, National Early Warning Score. Bold values are the statistically significant variables.

antimalarials had more cyanosis (5.3 vs. 0.9%, $p = 0.009$), higher respiratory rate (median of 28 vs. 24 bpm, $p < 0.001$) lower oxygen saturation (median of 83 vs. 87%, $p = 0.001$) and higher NEWS score (mean of 8.7 vs. 7.6, $p < 0.001$). Regarding the laboratory findings, patients not receiving antimalarials had higher leukocyte counts ($p = 0.004$), absolute neutrophils count ($p < 0.001$), platelets count ($p = 0.001$), DHL ($p < 0.001$), C-reactive protein ($p < 0.001$), procalcitonin ($p = 0.003$), ferritin ($p = 0.004$), fibrinogen ($p < 0.001$), D-Dimer (D-D) ($p < 0.001$), troponin ($p < 0.001$), lactate serum concentration ($p < 0.001$) and lower PaO2/FiO2 index ($p < 0.001$). Patients in both groups received empiric antibiotics (including macrolides), corticosteroids, anticoagulant therapy, and were enrolled in clinical trials for COVID-19 therapies in the same proportion; however, more patients in the group of antimalarial drugs received oseltamivir (Supplementary Table S1).

Adverse Effects

During the hospitalization period, there were no significant differences between both groups in the presence of hypoglycemia; however, two patients receiving antimalarials had grade-4 hypoglycemia. Although there were not significant differences in cytopenias between both groups, severe cases of lymphopenia and neutropenia were more frequent in the group receiving antimalarial drugs and only one case of grade-3 thrombocytopenia was observed in both groups (**Table 4**). No neurological effects were reported.

EKG Alterations

A baseline EKG was obtained in 292 patients (177 from the antimalarial group and 115 from the group without antimalarials) from whom 132 had a follow-up EKG (120 in the group receiving antimalarials and 12 in the group without antimalarials). In the group receiving antimalarials, 13% (16/120) prolonged their QTc at least 50 ms and six developed a ventricular arrhythmia. From the 12 patients with a follow-up EKG in the group not receiving antimalarials, no one prolonged their QTc in more than 50 ms and two developed a ventricular arrhythmia. The characteristics of the eight patients in whom serious arrhythmias were documented are described in Supplementary Table S3.

Dynamic Profile of Liver Function Tests

During their follow-up, 308 patients had at least two LFT determinations, 132 (43%) from the HCQ/CLQ group and 176 (57%) from the group not receiving antimalarials. The dynamic profiles of the total bilirubin, alanine transferase (ALT), aspartate transferase (AST) and alkaline phosphatase (ALP) are displayed in **Figure 1**; the change in the mean value per day for each of these LFT was not significantly different between groups.

TABLE 3 | Laboratory findings on admission of patients with COVID-19 treated with and without antimalarial drugs in a tertiary care center in Mexico City.

Characteristic	*N*	Overall (*N* = 497)	HCQ/CLQ (*N* = 211)	No antimalarial (*N* = 286)	*p*-value
Hemoglobin, g/dl	617	15.3 ± 2.0	15.4 ± 1.9	15.3 ± 2.0	0.41
Leukocytes, × 10^3/μl	615	7.9 (5.8–11.0)	7.1 (5.3–9.9)	8.2 (6.1–12.4)	**0.001**
Absolute neutrophil count	613	6,438 (4,468–9,523)	5,833 (4,026–8,483)	6,757 (4,802–10,545)	**<0.001**
Absolute lymphocyte count	613	800 (558–1,057)	806 (560–1,078)	799 (558–1,041)	0.094
Platelets, K/μl	615	214 (174–275)	204 (165–256)	226 (179–292)	**0.001**
BUN, mg/dl	617	15.4 (11.1–22.8)	14.3, (10.6–20.1)	16.0 (11.6–23.9)	**0.006**
Creatinine, mg/dl	617	0.9 (0.8–1.2)	0.9 (0.8–1.2)	1.0 (0.8–1.2)	0.53
Total bilirubin, mg/dl	608	0.7 ± 0.5	0.7 ± 0.5	0.7 ± 0.5	0.31
Albumin, g/dl	607	3.7 ± 0.5	3.8 ± 0.5	3.6 ± 0.5	**0.004**
Globulin, g/dl	604	3.3 ± 0.5	3.2 ± 0.5	3.3 ± 0.5	**0.002**
ALT, u/l	608	36 (24–55)	34 (23–53)	38 (25–58)	0.11
AST, u/l	608	43 (30–64)	41 (27–60)	44 (31–66)	**0.026**
ALP, u/l	608	88 (70–114)	82 (67–106)	92 (74–118)	**<0.001**
LDH, u/l	573	382 (290–504)	348, (261–479)	395, (313–537)	**<0.001**
CRP, mg/dl	596	14 (7–22)	13 (5–20)	15 (8–23)	**<0.001**
CPK, u/l	538	116 (63–239)	112 (61–238)	116 (64–245)	0.79
Ferritin, ng/ml	588	629 (320–1,066)	539 (250–910)	704 (350–1,105)	**0.004**
Fibrinogen, mg/dl	508	688 (499–834)	618 (468–774)	715 (554–883)	**<0.001**
D-dimer, ng/ml	584	701 (437–1,138)	568 (390–1,020)	770 (595–1,208)	**<0.001**
Troponin I, pg/ml	532	5.5 (3.6–11.4)	5.0 (3.5–7.3)	6.1 (3.8–13.6)	**<0.001**
pO2, mmHg	599	62.9 (54.0–77.8)	63.8 (55.2–76.0)	62.5 (52.8–76.0)	0.077
Lactate, mmol/L	470	1.3, (1.0–1.9)	1.2, (0.9–1.6)	1.5, (1.1, 2.1)	**<0.001**
PaO2/FiO2 index	595	209 (124–266)	235 (160–281)	185 (109–250)	**<0.001**

HCQ, hydroxychloroquine; CLQ, chloroquine; BUN, blood urea nitrogen; ALT, alanine transaminase; AST aspartate transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CRP, C reactive protein; CPK, creatine phosphokinase; pO2, partial pressure of oxygen; FiO2, fraction of inspired oxygen. Bold values are the statistically significant variables.

TABLE 4 | Adverse effects of patients with COVID-19 treated with and without antimalarial drugs in a tertiary care center in Mexico City.

	N	Overall, *N* = 626	HCQ/CLQ, *N* = 235	No antimalarial, *N* = 391	*p*-value
Hypoglycemia, grade	494				0.57
0		473 (96%)	200 (95%)	273 (96%)	
1		10 (2.0)	5 (2.4%)	5 (1.8%)	
2		4 (0.8%)	1 (0.5%)	3 (1.1%)	
3		5 (1.0%)	2 (1.0%)	3 (1.1%)	
4		2 (0.4%)	2 (1.0%)	0 (0%)	
Leukopenia, grade	453				0.074
0		404 (89%)	171 (86%)	233 (92%)	
1		38 (8.4%)	23 (12%)	15 (5.9%)	
2		11 (2.4%)	6 (3.0%)	5 (2.0%)	
3		0	0	0	
4		0	0	0	
Neutropenia, grade	451				0.41
0		422 (94%)	185 (92%)	237 (94%)	
1		22 (4.9%)	10 (5.0%)	12 (4.8%)	
2		7 (1.6%)	5 (2.5%)	2 (0.8%)	
3		0	0	0	
4		0	0	0	
Thrombocytopenia, grade	453				0.90
0		409 (90%)	179 (90%)	230 (91%)	
1		41 (9.1%)	20 (10%)	21 (8.3%)	
2		1 (0.2%)	0 (0%)	1 (0.4%)	
3		2 (0.4%)	1 (0.5)	1 (0.4%)	
4		0 (0%)	0 (0%)	0 (0%)	

HCQ, hydroxychloroquine; CLQ, chloroquine.

Effect on Mortality

The mortality analysis was performed on 491 patients who received optimal care, 210 from the HCQ/CLQ group and 281 from the other group. The risk of in-hospital mortality was 16% (33/210) in patients who received HCQ/CLQ and 15% (41/281) in those who did not, with a risk ratio of 1.08, 95% CI 0.71—1.64, and an adjusted risk ratio of 1.12, 95% CI 0.69—1.82. See **Supplementary Table S4** for futher information.

DISCUSSION

This is the first real-life experience and pharmacovigilance study describing the use and side effects of antimalarials for COVID-19 in Mexico and Latin America during the early months of the pandemic. Despite patients not receiving antimalarials were more severely ill than those receiving antimalarials; the adverse effects regarding hypoglycemia, liver function tests and cytopenias were similar between both groups. Furthermore, when restricting to patients that received optimal care, both groups had similar mortality. This provides evidence against the use of antimalarials for COVID-19; they do not improve mortality over optimal care and their adverse effects are comparable with those experienced by more severely ill patients.

The disparity in disease severity at admission between both groups might be explained by the fact that the therapy was decided by the treating physician. It is possible that those patients that the treating physicians observed less severe were proposed treatment with antimalarials, while the more serious patients were avoided the risk of any potential arrhythmia with the use of the antimalarial.

Although antimalarials have shown an acceptable safety profile in the treatment of malaria, recent studies in COVID-19 have reported significant cardiac effects (Haeusler et al., 2018; Chowdhury et al., 2020). The most reported cardiac effect is prolongation of the QT interval, that increases the risk of torsade de pointes or sudden death (Patil et al., 2020), and it can be triggered by the concomitant use of macrolides as occurred in the early months of the pandemic. (Mason, 2017; Jeevaratnam, 2020). In a study performed in New York City, Mehra et al. reported that 23% of the patients who received antimalarials plus azithromycin prolonged their QTc interval in at least 60 ms (Mehra et al., 2020b). In the same way, in another study performed in New York City, Chorin et al. reported that, out of 84 patients who received antimalarials plus azithromycin, 30% prolonged their QTC more than 40 ms and 11% prolonged their QTc more than 50 ms (Chorin et al., 2020). In our study, 13% of patients receiving antimalarials prolonged their QTc in more than 50 ms; however, no additional electrocardiographic follow-up was performed. Furthermore, we detected eight ventricular arrhythmias, all in the context of hypoxemia and concomitant administration of macrolides, six occurred in the group receiving antimalarials from whom three died, and two in the group not receiving antimalarials from whom both died (Supplementary Table S2). Although it seems ventricular arrhythmias were more fatal in patients not receiving antimalarials, the follow-up EKG in these patients was recorded due to clinical deterioration and not by protocol as occurred in the group receiving antimalarials.

While severe cases of cytopenias were more frequent in the group that received antimalarial drugs, it cannot be solely attributed to these drugs. Regarding neuropsychiatric adverse effects, they have

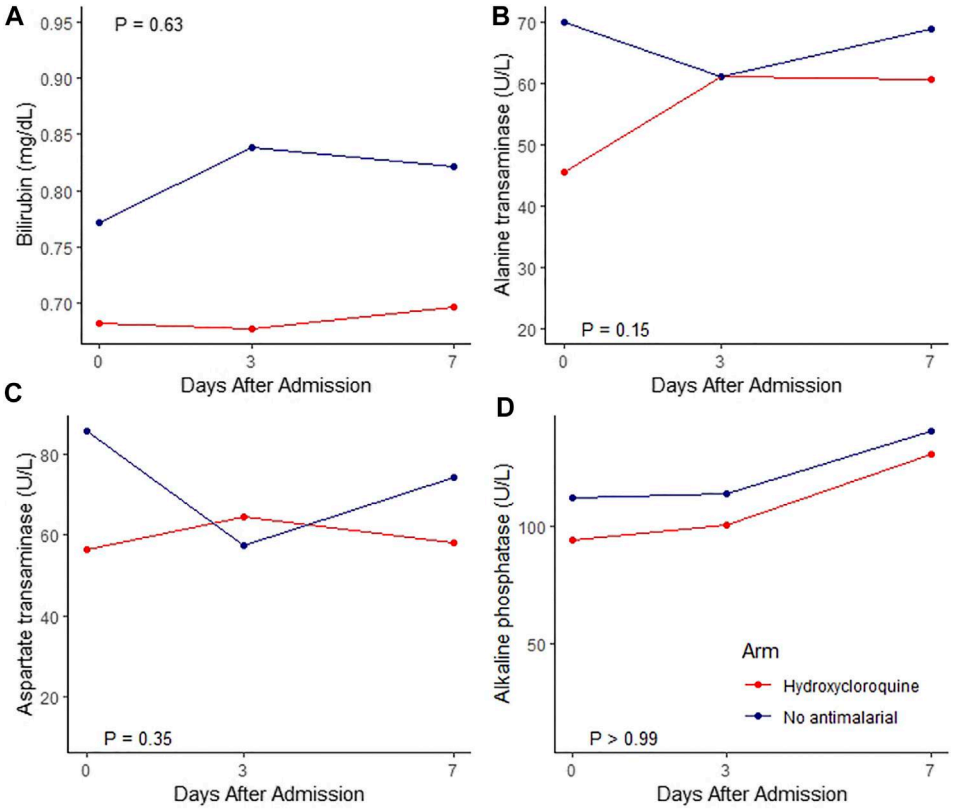

FIGURE 1 | Linear Mixed Effect Modelor for bilirubin, alanine transaminase, aspartate transaminse aminotransferase, alkaline phosphatase adjusted for age, sex and basal. Red line: hydroxycloroquine, Blue line: no antimalarial.

been observed in up to 12% of patients receiving antimalarials (Sato et al., 2020); however, we did not find any neuropsychiatric adverse effect in our study. This could be explained by underreported signs or symptoms in clinical records and by the limited interaction allowed with COVID-19 patients which compromised neurological examinations. In relation to hepatotoxicity in antimalarial users, few cases of liver failure have been described during the COVID-19 pandemic (Falcão et al., 2020). In our cohort, we did not find a difference in the change of the mean value per day for each liver function test between both groups. Finally, although the number of hypoglycemia cases were similar in boths groups, two cases of grade-4 hypoglycemia occurred among patients receiving antimalarials.

Previous studies have associated a higher in-hospital mortality in patients receiving antimalarials or macrolides for COVID-19. (Rosenberg et al., 2020). In this study almost all patients received macrolides and we found a similar mortality in both groups when restricting to patients who received optimal care (adjusted RR for mortality of 1.12, 95% CI 0.69—1.82. Although we performed an adjusted analysis that accounts for potential confounders, we acknowledge the existence of residual bias due to unmeasured confounders. However, our results are similar to those reported by Calvacanti; they did not find a difference in mortality, even when patients were also taking macrolides (Cavalcanti et al., 2020).

Nowadays, antimalarials are hardly recommended by any COVID-19 treatment guideline and it might seem obvious the evidence discouraging their use is compelling, nonetheless, real-life facts suggest the opposite. Recently Brazil have reported their use despite a lack of effectiveness (Kmietowicz, 2021). This study provides a real-life/pharmacovigilance experience with the use of these medications in the setting of a developing country.

LIMITATIONS

We acknowledge the limitations of our study. Although significant outcomes (mortality, discharge, ICU requirement) were completely collected, most of the adverse effects data is incomplete. There was limited identification of adverse effects *via* electronic medical records, which may underestimate the true incidence, and there was scarcity of follow-up EKGs, which may underestimate the incidence of QTc interval prolongation. The reduction in the number of EKG assessments was considered to minimize healthcare workers' exposure and because significant EKG abnormalities couldn't be solely accounted for the effect of antimalarial drugs. However, we consider it relevant to report these effects for future applications.

CONCLUSION

The use of HCQ/CLQ during the first months of the COVID-19 pandemic was widespread, especially among mild to moderate cases. Although the adverse events in patients with COVID-19 treated with antimalarials were similar to those who did not receive antimalarials at our institution that has a rigorous pharmacological surveillance, they do not improve survival in patients who receive optimal medical care.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the Institutional Review Board (Comité de Ética en Investigación, reference number 3333), who waived the informed consent due to the minimal risk characteristics of an observational study.

AUTHOR CONTRIBUTIONS

JS-O, DK-S, OL-C, and JC-F contributed to conception and design of the study. DA-L, SR-R, OL-C, CR-M, and JC-F organized the database. AO-M performed the statistical analysis OL-C, JC-F, and JJ wrote the first draft of the manuscript. JD-G, JO-M, AC-D, and CB-M contributed to the EKG interpretation All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.668678/full#supplementary-material

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BRIEF RESEARCH REPORT
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A Repurposed Drug Screen Identifies Compounds That Inhibit the Binding of the COVID-19 Spike Protein to ACE2

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Repurposed drugs that block the interaction between the SARS-CoV-2 spike protein and its receptor ACE2 could offer a rapid route to novel COVID-19 treatments or prophylactics. Here, we screened 2,701 compounds from a commercial library of drugs approved by international regulatory agencies for their ability to inhibit the binding of recombinant, trimeric SARS-CoV-2 spike protein to recombinant human ACE2. We identified 56 compounds that inhibited binding in a concentration-dependent manner, measured the IC_{50} of binding inhibition, and computationally modeled the docking of the best inhibitors to the Spike-ACE2 binding interface. The best candidates were Thiostrepton, Oxytocin, Nilotinib, and Hydroxycamptothecin with IC50's in the 4–9 μM range. These results highlight an effective screening approach to identify compounds capable of disrupting the Spike-ACE2 interaction, as well as identify several potential inhibitors of the Spike-ACE2 interaction.

Keywords: COVID-19, drug screen, IP-FCM, inhibition assay, repurposed

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INTRODUCTION

COVID-19 is currently a global pandemic, causing extensive mortality and economic impact. While the success of rapidly developed vaccines offers hope to control the virus (Polack et al., 2020), treatments that improve disease outcomes are also critically needed. Dexamethasone, an anti-inflammatory steroid, is FDA-approved to treat COVID-19, as is Remdesivir, a nucleoside analogue prodrug that inhibits viral RNA polymerase (Beigel et al., 2020), though its efficacy is disputed (WHO Solidarity Trial Consortium, 2021). Blocking the interaction between the Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) spike protein and its obligatory receptor Angiotensin-converting enzyme 2 (ACE2), has also shown promise as a therapy; recombinant soluble ACE2 is effective in a cell culture model (Monteil et al., 2020), and three different monoclonal antibody drugs are now FDA approved (Marovich et al., 2020). However, these biologic drugs are expensive and suffer production limitations. Repurposing already-approved small molecule drugs, particularly those that might block the interaction between ACE2 and spike, could allow for rapid deployment of low-cost and widely available therapeutics (Saul and Einav, 2020). Thus far, all repurposed drug candidates have failed to reduce mortality, initiation of ventilation or hospitalization duration in robust clinical trials (Cao et al., 2020; BJM, 2020; WHO Solidarity Trial Consortium, 2021); however none of these candidates act trough a mechanism that involves blocking the ACE2-spike interaction.

Here, we aimed to identify repurposed drugs that could block the interaction between the SARS-CoV-2 spike protein and ACE2. We screened 2,701 drugs approved by global regulatory agencies using a previously published assay (Gniffke et al., 2020) that measures inhibition of binding between the trimeric SARS-CoV-2 spike protein (Wrapp et al., 2020) and latex-bead-conjugated recombinant human ACE2. We identified 56 compounds that inhibited the spike-ACE2 interaction by < 90% at 1 mM and that produced dilution curves that yielded an IC_{50} value, and further characterized the 12 compounds with the lowest half-maximal inhibitory concentration (IC_{50}) using in silico modeling of the compounds' interaction with the binding interface.

METHODS

Drug Screening

The "FDA-approved drug screening library" (Cat #L1300) was purchased from Selleck Chemicals. Recombinant ACE2 and trimeric spike protein were produced in-house using previously published protocols (Gniffke et al., 2020). In a 96-well plate format, we briefly incubated recombinant, biotinylated, trimeric spike protein (Wrapp et al., 2020) with either 200 μM or 1 mM of each drug, in duplicate, then added 5-micron flow cytometry beads (Luminex) coated with recombinant ACE2 [for detailed methods, see (Gniffke et al., 2020)]. Three replicates per plate of positive (vehicle) controls and negative no-spike-protein controls were included, 31 plates in total. After washing and the addition of streptavidin-PE to bind spike attached to ACE2, plates were washed again on a magnetic plate washer and read on an Acea Novocyte flow cytometer. Data were expressed as the median PE fluorescence intensity (MFI), and converted to % inhibition using the formula 1-($MFI_{drug}/MFI_{positive\ control}$). For IC_{50} studies, two independent serial dilutions of drugs were performed and run as above. IC_{50} was calculated in Graphpad Prism using the Hill equation for a normalized response with variable slope (four parameter).

In Silico Modeling

Docking experiments were performed with the SARS-COV-2 spike protein (PDB ID: 6VSB) and ACE2 (PDB ID: 2AJF). The region selected for docking studies was the receptor binding domain (RBD) of the Spike protein and the corresponding region of ACE2, with docking grids generated around key binding residues at the Spike-ACE2 interface. The missing loops in the S1 subunit of the 6VSB structure, which contains the RBD, were reconstructed using the SWISS-MODEL server (Waterhouse et al., 2018). The FASTA sequence (residues 316-530) was retrieved from UniProtKB-P0DTC2 and used as a query sequence, PDB ID: 6VSB with 100% sequence identity was used as a template (Berman et al., 2000; Pundir et al., 2016). The structure quality of the modelled protein was validated on PROCHECK, which showed 89.2% residues in the core regions, the quality factor of 89.77% was obtained from Verify 3D on the SAVES server and ProSA web gave a Z-score of-6.15 (Laskowski et al., 1993; Eisenberg et al., 1997; Wiederstein and Sippl, 2007). The crystal structure of ACE2 was retrieved from PDB 1D: 2AJF, pre-processed with Prime on Schrödinger, and used for docking into the RBD interface. The SDF structures of the selected FDA-approved drugs were downloaded from Selleck chemicals and PubChem. Ligand and protein preparation was performed using the Ligprep and protein preparation wizard tool on Schrödinger Maestro version 12.2. Structural-based docking to the RBD interface of the Spike and ACE2 was also performed using Schrödinger Maestro and BIOVIA Discovery Studio (Dassault Systems) for docking analysis and visualization.

RESULTS

We took an unbiased approach to screen 2,701 drugs approved by global regulatory agencies for the ability to block the interaction between recombinant, trimeric SARS2 spike protein (Wrapp et al., 2020), and latex-bead-conjugated recombinant human ACE2 using a previously published assay (Gniffke et al., 2020) (**Figure 1A**). The use of a cell-free system prevented potential cytotoxic effects of drugs inherent to cell-culture-based, live-virus assays, and allowed us to focus solely on inhibition of the Spike-ACE2 interaction. We quantified the amount of Spike-ACE2 co-association in the presence of high concentrations (200 μM–1 mM) of each drug, in duplicate, and calculated the percent inhibition using six replicates of vehicle control per plate (31 plates total). In this first-round screen, 114 drugs that exhibited 90% or greater inhibition were identified (**Figure 1B**; **Supplementary Table S1**).

We next performed serial dilutions of these 114 drugs to measure the IC_{50}s of the ACE2–spike interaction (**Figures 1C,D**). Fifty-eight of the drugs were revealed to be either false positive hits (they showed no inhibition upon re-screening), or showed inhibition only at the highest concentration tested, and were eliminated. The drug with the lowest (best) IC_{50} was Thiostrepton (IC_{50} = 3.95 ± 0.02 x 10^{-6} M), a cyclic oligopeptide used as a topical antibiotic in animals that interacts with the transcription factor FOXM1 to inhibit the growth of breast cancer cells *in vitro* (Hegde et al., 2011). Next was Oxytocin (IC_{50} = 4.10 ± 0.07 x 10^{-6} M), a peptide hormone that is administered to induce childbirth, and that may increase social cognition when administered intranasally (Keech et al., 2018). The next four best candidates were actually two closely related pairs of drugs, which demonstrates the robustness of our screen in identifying each compound twice. Nilotinib, which was identified as both a free base (IC_{50} = 4.21 ± 0.36 × 10^{-6} M) and an HCl salt (IC_{50} = 8.43 ± 1.18 × 10^{-6} M), is a selective tyrosine kinase inhibitor used to treat chronic myelogenous leukemia (Tokuhira et al., 2018). Hydroxycamptothecine (IC_{50} = 6.87 ± 0.77 × 10^{-6} M) and its stereoisomer S-10-Hydroxycamptothecine (IC_{50} = 7.22 ± 0.06 × 10^{-6} M) are DNA topoisomerase I inhibitors with anti-cancer activity (Fei et al., 2013). Interestingly, three derivatives that have also been approved for cancer therapy, Topotecan, Irinotecan, and Belotecan were included in the screening panel, but did not inhibit spike-ACE2 binding. The IC_{50}s of all 56 compounds are listed in **Supplementary Table S2**; given the decreased severity of COVID-19 in females (Peckham et al., 2020), it is notable that

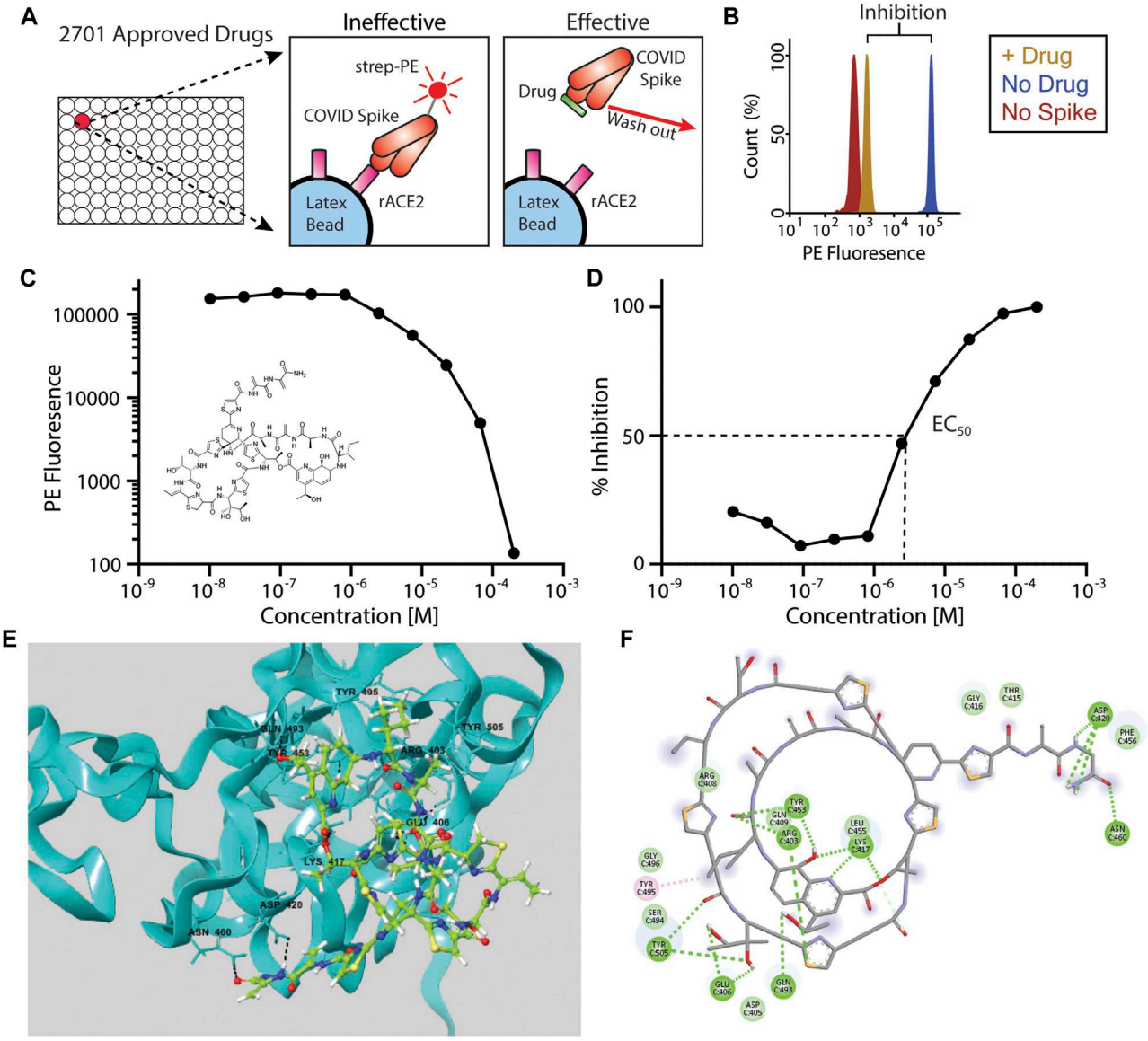

FIGURE 1 | Inhibition of Spike-ACE2 binding by repurposed drugs. **(A)** *In vitro* assay design showing inhibition of ACE2-Spike binding by "effective" drugs. Note that compounds may also bind to ACE2, in addition to the spike protein as illustrated here. **(B)** Example histogram from primary screen showing >90% inhibition of ACE2-Spike binding following drug addition. **(C, D)** IC_{50} data for the top candidate, Thiostrepton (structure inset) expressed as PE fluorescence **(C)** or percent inhibition **(D)**. **(E)** Three-dimensional and **(F)** two-dimensional computational rendering of Thiostrepton binding to the SARS-CoV-2 spike protein.

Estradiol Benzoate (a synthetic estrogen) inhibited the interaction (IC_{50} = 1.75 × 10^{-5} M), although the IC50 we measured is far greater than the physiological concentration of estrogen.

We next performed molecular docking studies on the top twelve screening hits with both the Spike and ACE2 proteins, focusing on the interface region between the receptor binding domain (RBD) of the Spike protein and ACE2, to provide mechanistic insight into our identified compounds' inhibitory activity. The Cryo-EM structure (PDB ID: 6VSB) was used for docking since the bioassay used the same trimeric spike protein plasmid construct as was used in determining that structure. The two most active screening hits, thiostrepton and oxytocin, were predicted to bind preferentially to the Spike protein based upon their Glide scores (**Table 1**), interacting with several key residues that mediate Spike-ACE2 binding (Hegde et al., 2011) (**Figure 1**; **Supplementary Figures S1, S2**; **Supplementary Table S3**). Thiostrepton in particular appears to bind extensively to Spike residues, but the OH-group in Thiostrepton was found to bind simultaneously with Lys417 of Spike and Asp30 of ACE2. Simultaneous binding of these two critical interface resides would likely disrupt the Spike-ACE2 interaction, resulting in the low observed EC50 value. Nilotinib and Hydroxycamptothecine exhibited higher Glide scores than thiostrepton and oxytocin in the Spike protein, but still within

TABLE 1 | Summary of the top 12 drug candidates. Computationally modeled glide scores for ACE2 and spike binding and IC_{50} values measured with the recombinant Spike-ACE2 binding inhibition assay are displayed.

	IC_{50}		Glide score		Prior evidence:
	Mean	St.Dev	Spike	Ace2	
Thiostrepton	3.95E-06	2.19E-08	−7.173	−4.819	-
Oxytocin	4.10E-06	7.14E-08	−7.024	−5.205	Computational Mamkulathil Devasia et al. (2021)
Nilotinib AMN-107	4.21E-06	3.66E-07	−5.669	−6.207	Cell culture inhibition Fan et al. (2020), computational Zhang et al. (2021)
Hydroxycamptothecin	6.87E-06	7.71E-07	−5.321	−5.679	Computational Ahsan and Sajib (2021)
S-(10)-hydroxycamptothecin	7.22E-06	5.52E-08	−5.335	−5.673	*see Hydroxycamptothecin*
Nilotinib HCl	8.43E-06	1.18E-06	−5.659	−6.209	*See Nilotinib AMN-107*
Selamectin	8.47E-06	3.68E-08	−4.207	−3.503	Cell culture inhibition Hanson et al. (2020)
Picropodophyllin	9.84E-06	3.99E-06	−4.072	−4.158	
Docetaxel	1.01E-05	1.66E-06	−4.737	−5.359	
Doramectin	1.28E-05	1.06E-07	−4.65	−3.964	Computational Leake et al. (1980)
Anidulafungin	1.32E-05	1.35E-06	−4.649	−5.846	Computational Zhang et al. (1998)
Estradiol benzoate	1.74E-05	5.11E-06	−4.003	−5.345	Cell culture inhibition Dyall et al. (2014), clinical Bojadzic et al. (2020)

TABLE 2 | Reported pharmacokinetic properties of top hits. All studies were performed on humans unless otherwise indicated.

	Indication	C_{max} (ng/ml)	MW (g/mol)	C_{max} (uM)	EC_{50} (uM)	Elimination $T_{1/2}$	Dosing Regimen	References
Thiostrepton	Topical antibiotic, vetrinary		1,664		4.0			
Oxytocin	Modify social behavior (expeirmental)	0.005	1,007	0.005	4.2	>1 h	Intranasal Single dose, 44 ug	Gossen et al (2012)
	Induction of labor	0.005	1,007	0.005	4.2	>>1 h	IV infusion 6.7 ng/min	Leake et al. (1980)
Nilotinib	Kinase inhibitor, Cancer treatment	1,360	529	2.57	4.2	16 h	Oral BID 300 mg	Tian et al. (2018)
Hydroxycamptothecin (in Rats)		15,930	364.4	43.7	7.3	428 min	RATS 10 mg/kg IV	Zhang (1998)
Selamectin (in Dogs)		86.5	770	0.11	8.5	266 h	DOGS topical 24 mg/kg	Sarasola et al. (2002)
		7,630	770	9.9	8.5	45.7 h	DOGS oral 24 mg/kg	Sarasola et al. (2002)
Picropodophyllin (PPP)	IGF inhibitror, Cancer treatment (aka AXL1717)	207–1,035	414	0.5–2.5	10.0	2 h	Oral 390 or 520 mg BID	Ekman et al. (2016)
Docetaxel	Microtubule inhibitor, Cancer treatment	933	808	1.23	10.3	25.4 h	IV infusion 30–36 mg/m^2	Gustafson et al. (2003)
Doramectin	Antiparasitic, vetrinary	12.2	899	0.0135	12.8	10 days	CATTLE topical 500 ug/kg	Gayrard et al. (1999)
Anidulafungin	Antifungal	2,500	1,140	2.2	13.1	27 h	IV infusion 100 mg	Wasmann et al. (2018)
Estradiol Benzoate	Contraceptive	0.75	376	0.002	17.3	3 days	IM injection, 5 mg	Oriowo et al. (1980)

reasonable ranges. They were, however, predicted to have slightly more favorable binding with ACE2. Interestingly, both interacted with Arg393 on ACE2, a critical spike-binding residue, and both also interacted with the Spike receptor binding interface (**Supplementary Figures S3–S6**; **Supplementary Table S3**). The remaining compounds generally gave poorer glide scores, especially in the Spike protein. Docetaxel, Anidulafungin, and Estradiol, however, gave Glide scores in ACE2 that were comparable to the hydroxycamptothecin analogs, suggesting they may bind there. Some of the compounds such as Selamectin, Picropodophyllin, and Doramectin were predicted to bind poorly to both Spike and ACE2 (see **Supplementary Figures S7–S12**; **Supplementary Table S3**, and **Supplementary Discussion**). It is possible that these compounds bind to either Spike or ACE2 in a non-competitive manner outside the interface region that could result in conformational changes to the protein and thus disrupt the Spike-ACE2 interaction, as has been suggested for estradiol benzoate (Yang et al., 2021).

DISCUSSION

Overall, this study identified 56 approved drugs that show some efficacy in blocking the interaction between the COVID spike protein and its receptor, ACE2. Many of the identified drugs are already approved for clinical use in humans (**Table 2**). Moreover, several of the identified drugs have already shown promising results in computational modeling or cell-based studies (**Table 1**;

Supplementary Table S2). Nilotinib (Hit #3 and #6 **Table 1**) has been reported to inhibit SARS-CoV-2 infection *in vitro* with an IC_{50} of 1.5–3 μM (Cagno et al., 2021), similar to our IC50 of 4 μM. The related Abl tyrosine kinase inhibitors Dasatinib and Imatinib were reported to inhibit SARS-CoV-1 and Middle Eastern Respiratory Syndroms (MERS) coronaviruses (Dyall et al., 2014). In our screen, three different formulations of Dasatinib inhibited > 90% in the first round, but only Dasatinib HCl (Hit #40 **Supplementary Table S2**) yielded an IC_{50} value, and Imatinib failed to inhibit in the first round screen. Similarly, Dasatinib, and Imatinib failed to inhibit SARS-CoV-2 in cell-culture-based studies (Cagno et al., 2021). Selamectin (Hit #7, **Table 1**) was one of three compounds identified in a screen of 2,406 clinically approved drugs that used a SARS-CoV-2-related pangolin corona virus in cell culture (Fan et al., 2020). This study also identified Cepharanthine (Hit #42 **Supplementary Table S2**), and Mefloquine (failed round 1, with 77% inhibition). Estradiol benzoate (Hit #12 **Table 1**) has also been reported to inhibit SARS-COV-2 replication in culture (Yang et al., 2021), possibly *via* binding to Spike in an area outside of the RBD. In humans, a retrospective study of women over 50 years old taking hormone therapy showed a 50% reduction in COVID-19 fatality that was not present in women 15–49 years old (Seeland et al., 2020). However, estrogen has a wide range of effects, including anti-inflammatory effects, and the concentration of estrogen *in vivo* is orders of magnitude less than that our observed IC_{50}, so these data should be interpreted with caution.

We also note that at least two additional studies have screened compounds for the ability to block the receptor binding domain (RBD) of the Spike protein from binding to ACE2. Hanson et al.(2020) identified a single confirmed hit, Corilagin, which was not included in our drug panel. A second study (Bojadzic et al., 2020) screening organic dyes identified Methylene Blue as a potential inhibitor, which failed our screen in the first round (44% inhibition). Methodological differences, as well as our use of a stabilized prefusion trimeric spike protein, which behaves differently from the smaller RBD construct in our assay (Gniffke et al., 2020), may account for the differences between these studies.

Aiming towards clinical translation, there are several obvious issues with our identified compounds. First, many of the drugs are chemotherapy agents, and have toxic side-effects that would not be tolerable in COVID-19 patients. For example, Nilotinib inhibits a kinase important in B cell signaling (Tian et al., 2018; Tokuhira et al., 2018), and may prevent normal immune function, while Picropodophyllin (Ekman et al., 2016) and Docetaxel (Gustafson et al., 2003) both produce moderate to severe side effects when used in the context of chemotherapy. Secondly, several of the drugs have peak plasma concentrations that are orders of magnitude lower than the concentration required to inhibit spike-Ace2 binding in the *in vitro* assay, for example Oxytocin (Gossen et al., 2012) and Doramectin (Gayrard et al., 1999) (0.005 vs 4.2 μM and 0.014 vs 12.8 μM, respectively). Finally, Oxytocin's clearance rate would necessitate continuous infusion, which seems impractical.

In light of known side-effects and pharmacokinetic data of many of our high-ranking drug candidates, Selamectin (Hit #7, **Table 1**) may be the top candidate for further study. Selamectin is an anti-parasitic used in dogs and cats to prevent infestation with nematode and arthropod species, and is structurally related to Ivermectin. Oral Selamectin is well tolerated in dogs and can achieve a peak plasma concentrations (9.9 μM) (Sarasola et al., 2002) comparable to both our measured IC_{50} (8.5 μM), and the 10 μM dose that inhibited the cytopathic effect of a SARS-CoV-2-related Pangolin corona virus on Vero E6 cells in culture (Fan et al., 2020). However, we were not able to identify any studies using Selamectin in humans, since Ivermectin is the standard alternative. Given recent controversy over Ivermectin as a treatment for COVID-19 (Ivermectin (2021)), it is worth noting that a different Ivermectin derivative, Doramectin, was our #10 hit, and Ivermectin itself just missed our first round cut-off with 89% binding inhibition. Thiostrepton may also be a top candidate, but pharmacokinetic data are lacking.

An advantage of our recombinant approach, using a cell-free system, is that the effects of drug toxicity on cell growth do not confound the readout of Spike-Ace2 binding. However, our results would need to be replicated in a cell or animal model using live virus to ensure that anti-SARS-CoV-2 effects appear at drug concentrations low enough to prevent toxicity. In addition, we did not confirm if the compounds bound directly to ACE2 or spike protein, only that their presence inhibited the binding of the two proteins. The relatively weak (micromolar) binding kinetics of the drugs identified here, as well as their known toxicities, bioactivities and/or high clearance rates, suggest that many would currently be unlikely to be viable for treating acute disease or for prophylactic use. However, they could serve as starting points for future medicinal chemistry optimization efforts to rationally design derivatives that are both less toxic and bind to the COVID spike with higher affinity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SS conceived the study. KT, CA, EG, JW, and SS planned experiments. DS provided recombinant COVID proteins for the inhibition assay. KT and EG performed microsphere-based experiments, CA performed modeling studies under the supervision of JW. KT, CA, JW, and SS, analyzed the data. KT, CA, JW, and SS wrote the manuscript, and all authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.685308/full#supplementary-material

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Conflict of Interest: Seattle Children's Research Institute has filed a provisional patent on the use of the inhibition assay for drug screening described herein.

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OPINION
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Serratiopeptidase, A Serine Protease Anti-Inflammatory, Fibrinolytic, and Mucolytic Drug, Can Be a Useful Adjuvant for Management in COVID-19

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INTRODUCTION

The COVID-19 pandemic, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a public health emergency with high mortality and disability rates. Given its high mortality rate, there is a serious need for possible effective medications to eliminate the virus, limit the severity, and improve the prognosis (Altay et al., 2020). The management of COVID-19 has continued to rely on drugs repurposed based on their pharmacological effects, including antiviral, antibiotic, anti-inflammatory, and or immunomodulatory, along with availability of numerous vaccines against SARS-CoV-2 in past few months (Fan et al., 2020). Repurposing of drugs has gained enormous attention over identifying novel drug candidates, due to known safety, potency, and multi-targeted pharmacological action as an immunomodulatory, anti-inflammatory, and antimicrobial agent. Studies report that after fever, cough is one of the major symptoms in about 76% patients and sputum production in 28% patients along with 55 and 44% of patients showing dyspnea and myalgia, respectively (Huang et al., 2020). In a study determined the prevalence of asymptomatic cases of COVID-19 and characterized the symptoms of patients with mild COVID-19 report that of the 213 individuals with COVID-19, 19.2% were asymptomatic until admission (Kim et al., 2020). Among the remaining patients with mild COVID-19, cough (40.1%) was the most common symptom followed by hyposmia (39.5%) and sputum (39.5%). In individuals with hyposmia, 90% had accompanying symptoms such as hypogeusia, nasal congestion or rhinorrhoea (Kim et al., 2020). Sputum or productive cough seem a significant symptom in asymptomatic as well as symptomatic (Kim et al., 2020). Cough was observed most common symptom followed by hyposmia and sputum, while fever (>37.5°C) was only observed in 11.6% (Kim et al., 2020). Another study reported that nasal congestion (62%) was the most common symptom in individuals with mild COVID-19 (Chang et al., 2020).

The role of mucolytic and bronchodilator administration and tracheal suctioning have been observed beneficial in airway hygiene by reducing the mortality rate of COVID-19 (Farooqi et al., 2020). Therefore, the role of mucolytics, in particular, has been suggested to protect the body from respiratory pathogens ascribed to their expectorant action, and are considered important as an adjuvant in the management of COVID-19 (Esam, 2020). In the purview of the pharmacological basis of therapeutics, we hypothesize that a proteolytic drug of natural origin, serratiopeptidase

(SEPD), also known as *Serratia E-15 protease* or serralysin, serratiaprotease and serrapeptase (Bhagat et al., 2013). SEPD (EC number 3.4.24.40), a serine protease super is derived from the non-pathogenic enterobacteria, which exists in the intestine of the silkworm and facilitates disruption of the cocoon to free the silk moth (Maeda and Morihara, 1995). The forms used in pharmaceutical preparations are isolated from *Serratia marcescens* or *Serratia* sp. *E 15* based on fermentation or the recombinant production using *Escherichia coli* (Srivastava et al., 2019).

Enzyme drugs are reputed in therapeutics due to their strong target binding and specificity and catalytic behavior to change many target molecules into the desired effectors (Reshma, 2019). Proteolytic enzymes can be useful in the treatment of nosocomial, viral, and resistant infections, especially in pediatric and geriatric age groups due to its relative safety, less tolerance and resistance and its synergic effects (UmaMaheswari et al., 2016). Several proteolytic enzymes act in an orchestrated manner to control and coordinate the entry of virus, replication and diffusion in the host cells. Thus, the proteolytic enzymes could be important in interfering with virus machinery in the host cells and suggested useful in COVID-19 (Gioia et al., 2020). Recently, SEPD has been suggested to be considered in integrative management of COVID-19 (Holloway et al., 2020). One of the case report suggested the role of immunostimulants and proteolytic including SEPD in the treatment of COVID-19 (Kobakova et al., 2020).

Our proposition is to repurpose a drug that possesses not only mucolytic property but also potent anti-inflammatory, and antimicrobial properties with a long history of safe clinical use. Herein, we present the possibilities of repurposing SEPD, a mucolytic that could be advantageous over others in COVID-19 treatment due to its wide range of therapeutic effects, including anti-inflammatory, antimicrobial, atheroprotective, antithrombotic, and fibrinolytic properties. Based on these properties, we opined that these properties may provide better therapeutic benefits in limiting the severity and progression of the disease, by reducing the risks of respiratory complications and related death.

Serratiopeptidase as A Mucolytic Drug Can Be Useful in COVID-19

In individuals with COVID-19, sputum production, nasal congestion and cough are reported one of the common symptoms after fever (Chang et al., 2020; Huang et al., 2020; Kim et al., 2020). As cough is a major symptom of SARS-CoV-2 infection, the caseinolytic and mucolytic actions of SEPD on the sputum believed to be beneficial. Recently, one of the mucolytic drugs, bromhexine, has been suggested to be repurposed for the possible treatment of COVID-19 (Maggio and Corsini, 2020). Mucolytics either enhance bronchial mucus secretion or reduce mucus viscosity and further facilitate its removal by coughing. The mucus secreted by the goblet cells is an adhesive viscoelastic gel containing high molecular weight mucous glycoproteins and water. The airway mucus is well-known as the first line of airway defense against pathogens, including coronaviruses. The hypersecretion of the airways mucus in a defensive response to the pathogens are believed to cause airway obstruction that leads to respiratory distress (Lu et al., 2021).

The mucus in airways traps and keep the microorganisms by a coordinated process of mucociliary clearance which involves release of mucus from the secretory cells controlling the transportation and viscoelasticity by motile cilia on multiciliated cells (Janssen et al., 2016). Mucus accumulation and increase in sputum viscoelasticity reduce mucociliary and cough clearance, thus retaining the sputum and obstructing the airways that enhance inflammation, infection, and progressive lung diseases by neutrophil infiltration (Maggio and Corsini, 2020). SEPD is shown to enhance mucociliary transportability (Maheshwari et al., 2006) and mucociliary clearance by decreasing neutrophils and modulating sputum viscoelasticity in patients with airway diseases (Nakamura et al., 2003). In addition to the mucolytic property, SEPD through oral administration in allergic conditions decreases the viscosity of the nasal mucus by improving rheological properties; thus, it plays a role in mucociliary clearance (Majima et al., 1988; Majima et al., 1990). SEPD has been found bioavailable in the nasal or tracheobronchial mucus, and it exerts proteolytic action even after oral intake (Majima et al., 1988; Majima et al., 1990).

Recently, the role of mucins glycoproteins, the structural components of mucus and its interaction with microorganisms particularly SARS-CoV-2 and its pathophysiological and therapeutic relevance has been presented to enhance mucosal defense and control respiratory infections (Chatterjee et al., 2020). The elevated levels of mucin has been reported in the airway mucus of critical ill COVID-19 patients (Lu et al., 2021). The higher levels of mucins are reported in the COVID-19 patients bronchoalveolar lavage fluid (BALF) and lungs of preclinical models of SARS-CoV-2 (Liu et al., 2020). Liu et al. (2020) suggested that during SARS-CoV-2 infection, the rise in the IFN-β and -γ leads higher expression of mucins in alveolar epithelial cells. The mucins stick with the blood-gas barrier and accumulated alveolar mucus affects the blood-gas barrier thereby impeding the gaseous exchange of O_2 and CO_2 and causing hypoxia, a key factor that initiates COVID-19-induced mortality. Following progression in the diseases, increase in barrier thickness, along with raised inflammatory exudates causes impediment in exchange of O_2 and CO_2 that leads to the critical illness and complications (Liu et al., 2020).

Additionally, SEPD has shown useful in chronic respiratory diseases (Nakamura et al., 2003), chronic sinusitis (Majima et al., 1988), ear, nose and throat disorders (Mazzone et al., 1990), secretory otitis media (Bellussi et al., 1984) and chronic airway disease with troubled expectoration (Nagaoka et al., 1979). Based on the role of SEPD on mucociliary clearance, relieving cough and promoting airway hygiene, it may be useful in delaying pulmonary complications and improving quality of life in COVID-19.

Serratiopeptidase as an Anti-inflammatory Drug Can Be Useful in COVID-19

The anti-inflammatory effects of SEPD were reported in the late 1960s, and since then, it has been popularly used in therapeutics

for inflammatory diseases in Japan and many European and Asian countries (Gupte and Luthra, 2017; Tiwari, 2017; Jadhav et al., 2020). Currently, it is available in United States, Canada and European countries as a natural health supplement or dietary ingredient, rather as a drug (Jadhav et al., 2020). It has been widely used in the management of pain and inflammation related to joints, sports-related chronic muscular swelling, sprain, scar, ruptured ligaments, chronic swelling and injuries, sinusitis, bronchitis, carpel tunnel syndrome, tooth extraction, breast engorgement, and post-surgery inflammation (Mazzone et al., 1990; Klein and Kullich, 2000; Tiwari, 2017).

SEPD has been shown to exert anti-inflammatory effects by reducing inflammatory cytokines and adhesion molecules, thus regulate inflammatory cells movement to the site of inflammation (Tiwari, 2017). It has been reported safer than conventional nonsteroidal anti-inflammatory drugs in terms of safety and efficacy and showed synergistic with them as well as with metal ions like zinc and manganese (Tiwari, 2017). SEPD has been shown to exert anti-inflammatory, antiedemic and fibrinolytic activity in resolving inflammation in patients with acute or chronic ear, nose or throat disorders in a multicenter, double blind, placebo-controlled study (Mazzone et al., 1990).

SEPD has been demonstrated to reduce neutrophil count and altering the viscoelasticity of sputum in patients with airway diseases (Nakamura et al., 2003). A reduction in the neutrophil count is believed to reduce elastase, a serine protease released from activated neutrophils in host defense response to attack proteins of pathogens; facilitate protein hydrolyzation in the host extracellular matrix, particularly collagen IV and elastin; ensue inflammation; and increase virus multiplication (Thierry, 2020). Elastase in the lungs can cause excessive water absorption that dehydrates the mucus and causes inefficient mucociliary clearance. Elastase also promotes the generation of ROS, alters the permeability of lung barriers, and triggers pro-inflammatory cytokines. Thus, elastase inhibition by SEPD could be useful in suppressing cytokine storm, causing acute lung injury in COVID-19. Inhibition of elastase by SEPD in the airways may also suppress airway inflammation characterized by reduced bronchial injury, improved ciliary beating, and reduced mucus hypersecretion (Thierry, 2020).

Additionally, the elevated levels of inflammatory cytokines, including interleukin (IL)-6 play vital role in pathogenesis and progression of complications, severity and mortality in COVID-19 (Cummings et al., 2020; Hojyo et al., 2020; Wang J. et al., 2020). The clinical manifestations of COVID-19 can range from mild to severe with widespread involvement of the lungs, beginning from pneumonia to acute respiratory distress, involving extensive alveolar damage along with progressive lung dysfunction, and leading to respiratory failure that may result in death (Yang et al., 2020).

Acute respiratory distress, which cause acute lung injuries characterized by infiltration of neutrophils, vasculitis, and secretion of proinflammatory cytokines, particularly results in a massive increase in IL-6 level, which has been found to be related to the severity of the disease, prognosis, and mortality (Giamarellos-Bourboulis et al., 2020; Han et al., 2020). Increased IL-6 levels also contribute to acute lung injury in murine models (Goldman et al., 2014), similar to those observed in patients with severe acute respiratory syndrome in COVID-19; thus, inhibition of enhanced IL-6 level seems to mitigate acute lung injury (Goldman et al., 2014; Pelaia et al., 2020). In a recent study, SEPD and curcumin nanoparticles (NPs) are shown to exert potent IL-6 inhibitory activity as evidenced by the reduction in IL-6 level ranging from 47 to 80% in lipopolysaccharide-stimulated human macrophages (Jaiswal and Mishra, 2018). The NPs of SEPD and curcumin showed potent synergetic immunomodulatory and anti-inflammatory properties (Jaiswal and Mishra, 2018). SEPD also found to inhibit IL-6, transforming growth factor-β (TGF-β) expression, chemokines (Selan et al., 2017), in the brain tissues of rat model of aluminum chloride-induced Alzheimer's disease (Fadl et al., 2013) and blood (Iie, 2013) after oral administration. SEPD has been demonstrated to attenuate proinflammatory cytokines in pulmonary tissues following liposomal delivery (Gupta et al., 2017).

Furthermore, the hyperinflammatory responses also involved the overproduction of bradykinins, which in turn determine disease severity, progression and mortality (Henderson et al., 2020). Bradykinin is one of the potent components of the vasopressor system that is degraded by angiotensin converting enzyme (ACE) and upon induction causes hypotension, vasodilation and natriuresis (Hofman et al., 2016). The increased bradykinin level from serine protease kallikrein has been determined to contribute to vasodilation, hypotension, and altered vascular permeability and can further lead to excessive formation of hyaluronic acid in the bronchoalveolar space of the lungs, which impairs lung function and plays a role in the onset of inflammation and pain (Garvin et al., 2020). The downregulation of the enzymes which degrade bradykinin are reported in bronchoalveolar lavage fluid (BALF) of patients with severe/critical COVID-19 infection (Garvin et al., 2020). The decrease in the enzymes is believed to shift the renin angiotensin system to produce Ang mediating ACE2. The upregulation of ACE2 and reduced degradation of bradykinin by ACE is believed to cause "bradykinin storm" which induces leakage of fluid into the lungs and it combines with hyaluronic acid forms a Jello-like material. This sticky formation obstructs exchange of O_2 and CO_2 and leads to the severe complications in COVID-19 (Garvin et al., 2020).

Additionally, SEPD has been showed to exert anti-inflammatory effects by inhibiting the release of serotonin and histamine. The anti-inflammatory activity of SEPD at the systemic and cellular level is suggestive of its potential in limiting cellular injury in different organs by inhibiting inflammation. Therefore, it can be suggested that SEPD may reduce acute respiratory distress and limit complications in COVID-19 ascribed to its inhibitory effect on bradykinin, serotonin, and histamine (Malshe, 2000).

Serratiopeptidase Potential in Coagulopathy and Thrombosis Complications

In addition to inflammatory cytokines, higher bradykinin levels with increased growth factor levels exhibit a strong association

between inflammation and coagulation (Hofman et al., 2016). Further, histamine and bradykinin, the vasoactive mediators are implicated in mucosal swelling. The neutrophil and mast-cell activation along with fibrinolytic system activation (i.e. plasminogen activation) are functionally linked to bradykinin production and considered to play role as one of important inflammatory product of the coagulation system (Hofman et al., 2016). Higher fibrinogen and lower antithrombin levels were reported in patients with COVID-19 and associated with the severity of infection, mortality, and prognosis in survivors (Tang et al., 2020). The development of thrombosis characterized by a significant increase in D-dimer and fibrin/fibrinogen-degradation products with coagulopathy is one of the major causes of cardiovascular complications in patients with COVID-19 (Connors and Levy, 2020).

Additionally, enhanced degradation products of fibrin have been identified to play a role in intravascular coagulation, a manifesation of viral coagulopathy following arterial, venous, and microvascular thrombosis and endothelial damage in the lungs that leads to acute respiratory distress syndrome (ARDS) (Kipshidze et al., 2020).

Many fibrinolytic therapies and tissue plasminogen activators based on serine protease known for their benefits in vascular disorders have been suggested to aid in COVID-19 treatment (Lechowicz et al., 2020). SEPD has been reported to holds extensive substrate affinity and fibrinolytic property (Kotb, 2013). SEPD possesses the ability to degrade blood clots, cysts, and arterial plaques, therefore being useful under the conditions of increased risk of stroke, atherosclerosis, and thrombophlebitis (Mazzone et al., 1990). The fibrinolytic activity of SEPD coupled with multiple properties, including proteolytic, caseinolytic, antifibrotic, anti-inflammatory, antiatherosclerotic, and antioxidant activity, suggests its potential benefits in reducing the severity of vascular complications involving thrombosis or coagulopathy in COVID-19.

Serratiopeptidase Potential in Countering Oxidative Stress

The extrapulmonary complications of COVID-19 are acute liver injury, acute cardiac injury, acute intestinal inflammation, and acute neurological manifestations, which may further lead to sepsis and multi-organ failure with poor prognosis (Wang et al., 2020; Xu et al., 2020). The pathogenesis of acute complications of different organs involves an abrupt disruption in antioxidant defense against oxidative stress subsequent to systemic hyperinflammatory response (Henderson et al., 2020). Further, serine protease enzymes showed to exert free radical scavenging activity that also help in its therapeutic benefits (Davies, 1986). SEPD conjugated with folate and superoxide dismutase has been considered useful in inflammatory conditions by enhancing retention and localized delivery of the conjugate along with augmentation of proteolytic activity and free radical scavenging activity against reactive oxygen species (ROS) generated from macrophages (Srivastava et al., 2017). Thus, the antioxidant activity may also contribute to tissue protective effects and explain therapeutic benefits of SEPD in reducing organ complications.

Serratiopeptidase Synergizes Antibacterial Drugs and Corticosteroids

In COVID-19, the increased risk of secondary bacterial infections in critically ill patients contribute to the cumulative inflammatory burden in addition to viral pneumonia and has been reported to cause complications and death (Fu et al., 2020). SEPD exerts synergistic antimicrobial activity with drugs belong to the antibiotic family of penicillins, cephalosporins, fluoroquinolones, and tetracyclines (Maheshwari et al., 2006). SEPD was found to eradicate implant related periprosthetic infection in an *in vivo* animal model of staphylococcal infections (Mecikoglu et al., 2006). It has also been showed a valuable agent in combination with antibiotics and anti-inflammatory agents in the treatment of periimplantitis (Sannino et al., 2013).

SEPD has also been shown to enhance the absorption of antibiotics and prevent biofilm formation in pulmonary tissues in patients undergoing thoracotomy (Koyama et al., 1986). The pulmonary delivery of SEPD with levofloxacin in liposomes exerts potent antimicrobial activity against *Staphylococcus aureus* infections in rats and reduces bacterial resistance by inhibiting biofilm formation. This combination was found bioavailable and synergistically effective in respiratory infections and has further reduced the doses of levofloxacin for bacterial infections (Gupta et al., 2017). SEPD in preclinical studies showed to increase the levels of cefotiam in plasma and lungs in pleuritis and only in lungs in pneumonitis (Ishihara et al., 1983), in subacute bronchitis (Kase et al., 1982) and synergizes the efficacy of ciclacillin, ampicillin, cephalexin and minocycline in gingival infections caused by staphylococci (Aratani et al., 1980).

Additionally, SEPD has been reported to synergize corticosteroid drugs methylprednisolone and dexamethasone (Murugesan et al., 2012), which received attention for their potential use in COVID-19 (Tomazini et al., 2020). In acute respiratory distress, corticosteroids, mainly methylprednisolone, improve oxygenation, lessen the requirement of mechanical ventilation, and decrease mortality risks (Steinberg et al., 2006). However, high doses or prolonged use of corticosteroids may result in excessive immune suppression and related mortality. Hence, when the pathogenesis progresses from inflammation to fibrosis, the adverse effects of anti-inflammatory drugs likely outweigh any potential benefit. SEPD does not directly interfere with lipoxygenase enzymes, which are a major target of non-steroidal anti-inflammatory drugs (NSAIDs), therefore being devoid of numerous adverse effects and exhibiting synergistic effect in combination with NSAIDs. The synergistic and comparable action of SEPD with methylprednisolone and dexamethasone is suggestive of its potential in limiting respiratory distress and delaying the requirements of mechanical ventilation (Murugesan et al., 2012).

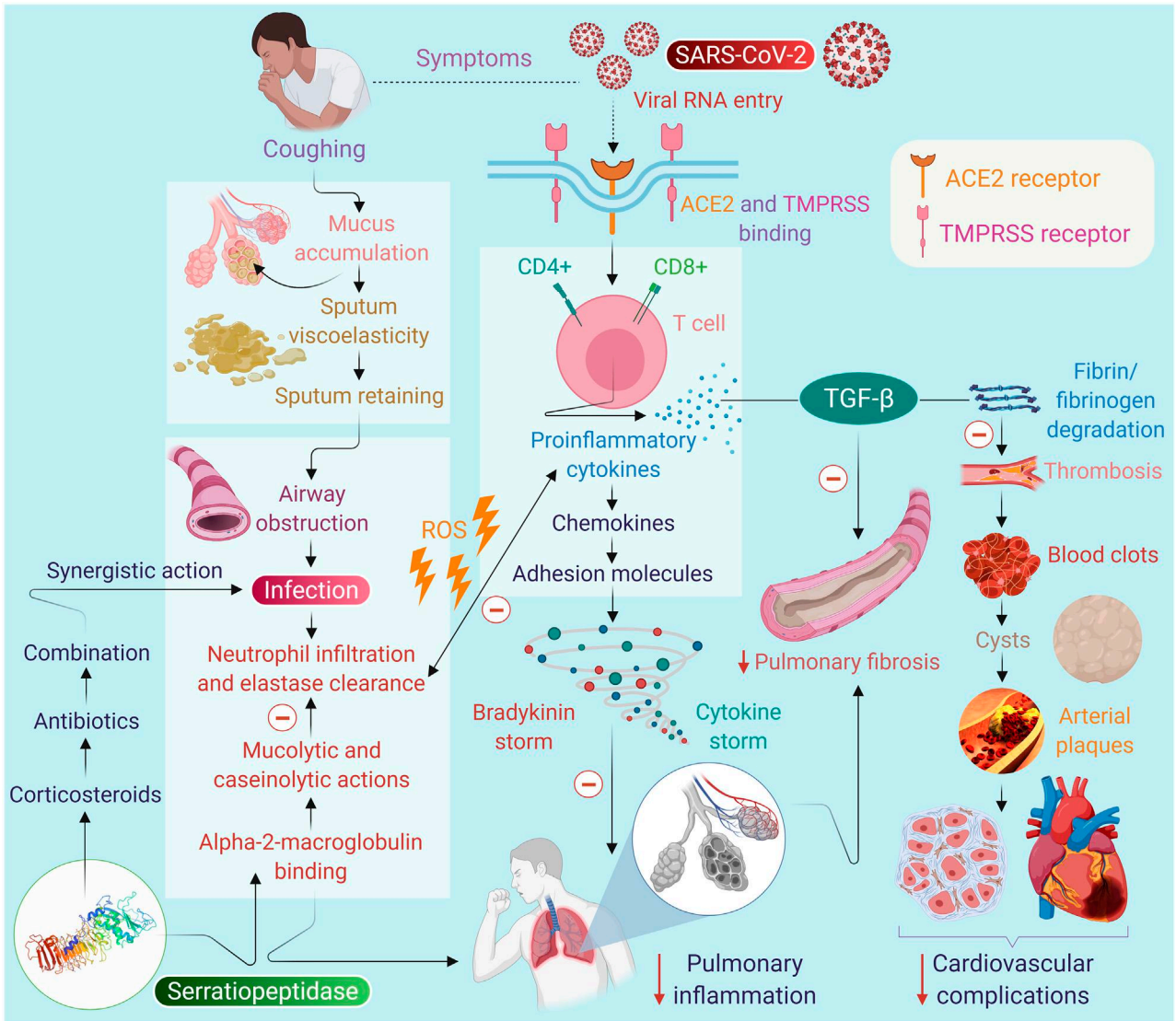

FIGURE 1 | The proposed scheme on the potential of serratiopeptidase on infection, immunity and inflammation in context to SARS-CoV-2 and on the symptoms of COVID-19.

Serratiopeptidase May Be Useful in Pulmonary Fibrosis in COVID-19

There are reports that in some COVID-19 survivors, pulmonary fibrosis develops as a post-infection sequela (Lechowicz et al., 2020). Pulmonary fibrosis is often characterized by activation of TGF-β and matrix metalloproteinase, fibroblast proliferation mediated by accumulation of collagen and extracellular matrix, and injury to alveolar epithelium and parenchyma and capillaries that may lead to difficulty in breathing and may cause acute respiratory failure (MacLaren and Stringer, 2007). TGF-β1 is one of the major contributors to fibrosis and ROS production. Excessive production of ROS that induces oxidative stress and overexpression of cytokines contributes to pulmonary fibrosis. The proteolytic activities are considered as a secondary antioxidant defense in oxidative conditions, along with regulation of inflammatory cytokines and migration of immune cells from the lymph node to the inflamed and injured tissues (Tiwari, 2017). The ability of SEPD to suppress growth factors, particularly TGF-β along with inhibiting oxidative stress and expression of pro-inflammatory cytokines, chemokines, adhesion molecules (Fadl et al., 2013; Gupta et al., 2017; Jaiswal and Mishra, 2018), plausibly indicates its possible potential in the treatment of lung fibrosis.

Serratiopeptidase Doses, Safety, and Adverse Effects

SEPD is generally well tolerated with few exceptions of rare adverse effects. It is available alone or in combination with

anti-inflammatory agents as tablet, mostly as enteric-coated tablets or capsule. SEPD is distributed to the tissues and bioavailable in plasma and lymph following binding to alpha-2-macroglobulin in the blood thus devoid of allergenicity and retains its enzymatic activity at the systemic and cellular level within 1 h.

The usual doses of SEPD in a majority of the human studies range from 10 to 60 mg/day in divided doses, with the most preferred dose of 10 mg, thrice daily on an empty stomach. Usually, it is used for 2–4 weeks depending on the aim of therapy and outcome. The dose of 10 mg is considered equal to 20,000 units of enzyme activity. Therefore, we propose that the dose of 10 mg thrice daily could be examined as an adjuvant in COVID-19. Using SEPD can be virtuously justified, being safe and effective and devoid of side effects that commonly develop with the use of conventional mucolytics that may cause sedation, euphoria, gastrointestinal disturbances, respiratory irritation, and constipation probably due to the absence of any interaction with receptors. A scheme is presented in **Figure 1** to depict the possible mechanisms and effect of SEPD on mucus production, infection, inflammation, and immunity in the context of SARS-CoV-2.

CONCLUSION

SEPD may be a promising therapeutic candidate for repurposing due to its immunomodulatory, anti-inflammatory, mucolytic, antifibrotic, antithrombotic, antiviral, and fibrinolytic properties. SEPD, being an age-old, inexpensive, natural, and tolerated drug, may be a better alternative over other mucolytics or adjuvant with other drugs particularly in individuals with symptoms of sputum or mucus or productive cough. Recently, the animal models of COVID-19 become available that may facilitate preclinical evaluations to distinguish whether these candidate compounds are likely to become effective drugs. Though, the suggestion on the use in COVID-19 remains inconclusive until the proof of concept preclinical and clinical studies undertaken. But the potential of SEPD can't be overlooked ascribed to its promising possible benefits in COVID-19. It may be able to limit fatal complications, including pulmonary and cardiovascular diseases, and improve the prognosis of COVID-19. However, it is important to highlight that, to date, no studies have demonstrated the experimental or clinical effects of SEPD in COVID-19.

AUTHOR CONTRIBUTIONS

SO conceptualized the hypotheses. MFNM ideated the scheme. NKJ draw the schemes and drafted the artwork. CS, SNG, CRP, and SO contributed in editing and revisions of the manuscript. All authors read, edited and approved the manuscript.

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Tocilizumab in COVID-19: Factors Associated With Mortality Before and After Treatment

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Tocilizumab (TCZ) has been administered in SARS-CoV-2 pneumonia but the factors associated with mortality before and after treatment remain unclear. Cox regression models were used to estimate the predictors of time to death in a cohort of hospitalized patients with COVID-19 receiving TCZ. In addition, the mean differences between discharged and deceased patients in laboratory parameters measured before and 3, 6 and 9 days after TCZ administration were estimated with weighted generalized estimation equations. The variables associated with time to death were immunosuppression (Hazard Ratio-HR 3.15; 95% confidence interval-CI 1.17, 8.51), diabetes mellitus (HR 2.63; 95% CI 1.23–5.64), age (HR 1.05; 95% CI 1.02–1.09), days since diagnosis until TCZ administration (HR 1.05, 95% CI 1.00–1.09), and platelets (HR 0.27; 95% CI: 0.11, 0.69). In the post-TCZ analysis and compared to discharged patients, deceased patients had more lactate dehydrogenase (p = 0.013), troponin I (p = 0.013), C-reactive protein (p = 0.013), neutrophils (p = 0.024), and fewer platelets (p = 0.013) and lymphocytes (p = 0.013) as well as a lower average PaO_2/FiO_2 ratio. In conclusion, in COVID-19 diagnosed patients receiving TCZ, early treatment decreased the risk of death, while age, some comorbidities and baseline lower platelet counts increased that risk. After TCZ administration, lower platelet levels were again associated with mortality, together with other laboratory parameters.

Keywords: COVID-19, immunosupression, tocilizumab, mortality, risk factor, platelet

INTRODUCTION

More than one year after the identification in December 2019 of a cluster of atypical pneumonia cases in Wuhan (China) caused by a new type of Coronavirus (SARS-CoV-2), the so called Coronavirus disease 2019 (COVID-19) pandemic is not under control despite the efforts of massive vaccination protocols. Therefore, the clinical management of non-immunized patients is still a big priority for the research community (Thoguluva Chandrasekar et al., 2021).

Male sex (Huang et al., 2020a), older age (Imam et al., 2020; Wu et al., 2020) and comorbidities (Imam et al., 2020) such as hypertension (Pranata et al., 2020), cardiovascular diseases (Aggarwal et al., 2020) and diabetes mellitus (Huang et al., 2020b) are risk factors for hospitalization and/or

mortality in COVID-19 patients. Moreover, several analytical markers have been associated with severe COVID-19 disease and/or poor prognosis: elevated C-reactive protein (CRP), ferritin, procalcitonin, D-dimer, interleukin (IL)-6 and white blood cell levels as well as decreased albumin, lymphocyte and platelet levels (Zhang L. et al., 2020; Huang et al., 2020c; Henry et al., 2020; Yang et al., 2020). Of note, the alterations in D-dimer and platelet levels (together with other markers) may reflect hemostatic abnormalities similar to those occurring in the disseminated intravascular coagulopathy associated with sepsis (Huang et al., 2020c; Lippi et al., 2020; Tang et al., 2020).

In the severe stage of COVID-19 (Siddiqi and Mehra, 2020), shock, and respiratory and systemic organ failure may manifest secondary to a surge of proinflammatory cytokines (cytokine storm) which include IL-6, IL-1β, IL-2, granulocyte colony stimulating factor, macrophage inflammatory protein 1-α and tumor necrosis factor (Huang et al., 2020a; Siddiqi and Mehra, 2020; Wu et al., 2020). These cytokines increase vascular permeability facilitating the entrance of a large amount of fluid into the alveoli, thus causing dyspnea and respiratory failure (Zhang C. et al., 2020). IL-6 seems to have a prominent role in this stage. On the one hand, IL-6 binds to membrane IL-6 receptor (IL-6R) and induces the production of acute-phase proteins such as CRP and fibrinogen, biomarkers associated with poor COVID-19 outcomes (Siddiqi and Mehra, 2020). On the other hand, IL-6 binds to soluble IL-6R forming hyper-IL-6 which can activate all kind of cells, presenting a central role in the cytokine storm (Chastain et al., 2020). Thus, the use of IL-6R antagonists has been suggested as a potential therapy for severe COVID-19-related pneumonia cases.

Tocilizumab (TCZ) is an IL-6R antagonist that can effectively block the IL-6 signal transduction pathway. In observational studies, administration of TCZ to patients with pneumonia due to SARS-CoV-2 has been associated with higher survival rates (Gokhale et al., 2020; Rodríguez-Baño et al., 2020), and/or significant clinical improvement, including laboratory parameters like CRP (Madenidou and Bukhari, 2020; Toniati et al., 2020; Xu et al., 2020). Moreover, while some clinical trials did report no association of TCZ with clinical improvement (Roche Group Media Relations., 2020; Stone et al., 2020; Hermine et al., 2021; Salvarani et al., 2021; Veiga et al., 2021), those with a higher sample size did report a better COVID-19 outcome after TCZ treatment compared to the control group or null hypothesis (Perrone et al., 2020; Abani et al., 2021; Salama et al., 2021). Among the latter, it may be highlighted the RECOVERY trial, with 4,116 hospitalized COVID-19 patients, which showed that TCZ improved survival and other clinical outcomes regardless of the level of respiratory support (Abani et al., 2021). Thus, despite some initial controversy, the latest results seem to point TCZ as an efficacious treatment in severe COVID-19. Therefore, it is crucial to study the factors associated with better/worse outcomes as well as early markers of prognosis in COVID-19 patients under TCZ treatment and other therapies.

The aims of this study were to analyze the baseline predictors of hazard of death as well as the mean differences between discharged and deceased patients in several laboratory parameters measured in four consecutive tests before and after TCZ administration in a cohort of hospitalized patients with severe pneumonia or respiratory failure due to SARS-CoV-2 infection in Granada, Spain.

MATERIALS AND METHODS

Study Design and Participants

This was a retrospective observational evaluation of all patients diagnosed with COVID-19 who received TCZ and were 18 years of age or older, admitted at Hospital Universitario Virgen de las Nieves (HUVN) in the city of Granada (southern Spain) between 13 March and November 5, 2020, coinciding with the peak of the second COVID-19 wave. COVID-19 diagnosis at admission included a positive polimerase chain reaction (PCR) test or a radiological and analytical suspicion. Patients were followed-up until hospital discharge or death.

The HUVN criteria to administer TCZ changed as the epidemic progressed and more knowledge was acquired. In March, TCZ was prescribed to patients with a severe hyperinflammatory syndrome, defined by severe bilateral pneumonia with criteria for acute respiratory distress syndrome (ARDS), or by the presence of two of the following criteria, fever >38.4°C, respiratory rate >24/min and $Pa0_2/Fi0_2$ <300 mmHg, and at least one analytical criterion of the following IL-6 >40 ng/L, D-dimer >1 mg/L and ferritin>300 μg/L. As of april, the criteria were two possible: 1) severe pneumonia with CRP >100 mg/L plus ARDS or $Pa0_2/Fi0_2$ <200 mmHg; 2) pneumonia in radiological progression, with progressive respiratory failure and/or progressive increase in CRP, D-dimer or ferritin, or progressive decrease in lymphocytes or presence of elevated IL-6. As of April 04, 2020, a single dose of TCZ of 600 mg in patients of ≥75 Kg and 400 mg in patients of <75 Kg was indicated. Previous protocols allowed administration of up to three doses in 72 h; thus, some patients of our cohort received more than one dose. Patient consent was obtained for the off-label use of TCZ.

All admitted patients received prophylactic doses of enoxaparin or bemiparin, adjusting for weight and renal function. In case of renal insufficiency, half of the weight-adjusted dose was used. This might be further adjusted according to anti-Xa levels. Anticoagulation with low molecular-weight heparin was started at intermediate doses (1 mg/Kg/day) if the patient had a high risk of thrombosis. Finally, if the patient had clinical suspicion of pulmonary embolism, low molecular-weight heparin was started or increased at therapeutic doses.

All data were fully anonymized before the analyses. The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the Research Ethics Committee of Granada, with waiver of informed consent due to the retrospective design and emergency of the research question.

Variable Measurement and Definitions

The primary end point was time to death, defined as the time from administration of the first dose of TCZ until death.

Censured data included hospital discharges. All the data used in this study were collected from electronic medical records for each patient.

General Information on Patient's Admission

Sex; age (analyzed as continuous variable); COVID-19 wave (first or second); presence of comorbidities (hypertension, diabetes mellitus, dyslipidemia, previous pulmonary diseases such as asthma or chronic obstructive pulmonary disease, cardiovascular disease, immunosuppression like oncohematological tumor with active chemotherapy or immunosuppressive therapy); clinical findings on admission analyzed as dichotomic (yes/no) variables (fever, cough, fatigue/asthenia, dyspnea, headache, diarrhea, acute respiratory distress syndrome, acute cardiac injury, thrombosis, and acute renal injury); and smoking status (never smoker, current smoker, or ex-smoker).

Physical Examination and Laboratory Tests During Hospitalization

Information was collected before administration of TCZ (same or previous day) and 3, 6 and 9 days after TCZ administration. When a patient did not have a laboratory test in those specific dates, data from ± 1 day were used instead. The variables analyzed were: temperature (continuous variable), PaO_2/FiO_2 ratio, leukocyte count, lymphocyte count, neutrophil count, platelet count, total serum proteins, albumin, alanine transaminase, aspartate transaminase, γ-glutamyl transferase, lactate dehydrogenase (LDH), ferritin, CRP, procalcitonin, troponin I, D-dimer and fibrinogen. In addition, pre-TCZ X-ray findings were translated into a radiological scale for evaluation of patient admission (ERVI) (Catalá-Forteza, 2020).

Additional Pharmacological Treatment

Patients received the pharmacological standard treatment at the time of hospital admission, which changed throughout the pandemic: hydroxychloroquine, lopinavir/ritonavir, azithromycin and/or systemic methylprednisolone. Some patients received an additional pulse of methylprednisolone. Additionally, few patients received colchicine or cyclosporine or a dose of anakinra.

Other Variables

Place of hospitalization while administration of TCZ (general ward vs. ICU); time since symptoms onset until TCZ first dose administration; time since COVID-19 diagnosis until TCZ first dose administration; confirmed (by PCR) or suspicious diagnosis of COVID-19 with a negative PCR test on admission; and presence (yes/no) of a positive blood culture for secondary infection after TCZ administration.

Statistical Analysis

A description of the baseline characteristics of the study participants was performed, reporting separately the patients who survived and those who died.

The variables alanine transaminase, aspartate transaminase, γ-glutamyl transferase, LDH, troponin I, CRP, procalcitonin, albumin, ferritin, leukocyte count, lymphocyte count, neutrophil count, platelet count, IL-6, D-dimer and PaO_2/FiO_2 ratio were log-transformed before regression analyses in order to reduce the skewness and the influence of extreme values.

Cox proportional hazard regression models were estimated in order to quantify the magnitude of associations between instantaneous death rate (measured as hazard ratio-HR-) and patients' baseline characteristics. Because of the low ratio participant: independent variables, we used a three-step modeling process (Rivera-Izquierdo et al., 2020). First, univariate models were estimated for each predictive variable. Second, we defined subgroups of baseline variables (demographic, smoking status, COVID-19 wave, comorbidities and physical examination, pharmacological treatment, symptoms on hospital admission and laboratory values before TCZ administration). Then, we used a stepwise process to build multivariate models for each group, including all the variables with a p-value <0.2 in the univariate analyses except variables with >10% of missing values that could compromise the statistical power. Third, the variables retained in each group model were incorporated in a new stepwise regression to build a final model. In all stepwise regressions performed, those variables with p-value <0.05 were sequentially retained in the model and those with p-value ≥0.10 were excluded from it. We calculated for each HR the 95% confidence intervals (CI).

The mean change in the 18 parameters along the four laboratory tests (baseline, and days 3, 6 and 9) was analyzed, considering the death during follow-up (yes/no) as the independent variable. For this analysis, weighted generalized estimation equations were calculated using the *xtrccipw* command in the statistics software Stata, which allowed the truncation of deaths along the follow-up (Daza et al., 2017). An adjustment of the p-value because of multiple comparisons was performed by means of the Benjamini-Hochberg method.

A p-value ≤ 0.05 was set for the level of statistical significance. Statistical analyses were performed using the statistics software Stata v.15 (Stata Corp, 2017) and graphs were built using Graph Pad v.8.4.3.

RESULTS

A total of 120 patients diagnosed with COVID-19 received TCZ at HUVN during the recruitment period. By the end of the follow-up period, 86 (72%) had been discharged and 34 (28%) had deceased with a mean time to death (from TCZ administration) of 15.9 days and a standard deviation (SD) of 16.6 days. Baseline demographic, clinical and pharmacological characteristics, as well as laboratory parameters, are shown in **Table 1** for the whole cohort as well as for the groups of patients that died and survived respectively. On the one hand, median LDH, albumin, CRP, IL-6 and D-dimer levels were above normal values on both deceased and discharged patients, and median troponin I level only in the group of patients that died. Of those patients with high Troponin-I levels (above 20 pg/dl), only four had records of clinical cardiologic affectation: 2 with arrhythmia, 1 with ST segment depression and 1 with a hyperdynamic left ventricle.

TABLE 1 | Baseline demographic, clinical, pharmacological and laboratory data of patients diagnosed with COVID-19 receiving tocilizumab.

	Total (*N* = 120)	Deceased (*N* = 34)	Survivors (*N* = 86)
Sociodemographic variables			
Age, mean (SD)	63.0 (13.8)	68.2 (14.3)	61.0 (13.1)
Sex (men), *n* (%)	86 (71.7)	28 (82.4)	58 (67.4)
COVID-19 wave (first), *n*(%)	59 (49)	15(44)	44 (51)
Smoking status, *n* (%)*			
Non-smoker	50 (52.1)	9 (40.9)	41 (55.4)
Smoker	6 (6.2)	3 (13.6)	3 (4.1)
Ex-smoker	40 (41.7)	10 (45.5)	30 (40.5)
Comorbidities and physical examination, *n* (%)			
Hypertension	65 (54.2)	22 (64.7)	43 (50.0)
Dyslipidemia	50 (41.7)	20 (58.8)	30 (34.9)
Cardiovascular disease	49 (40.8)	18 (52.9)	31 (36.1)
Diabetes mellitus	23 (19.2)	11 (32.4)	12 (13.9)
Previous pulmonar disease	25 (20.8)	10 (29.4)	15 (17.4)
Immunosuppression	13 (10.8)	8 (23.5)	5 (5.8)
Diagnosis by PCR			
Confirmed	98 (81.7)	26 (76.5)	72 (83.7)
Suspicion	22 (18.3)	8 (23.5)	14 (16.3)
Pharmacological treatment, *n* (%)			
Lopinavir/ritonavir	61 (50.8)	15 (44.1)	46 (53.5)
Hydroxychloroquine	59 (49.2)	15 (44.1)	44 (51.2)
Azytromicine	62 (51.7)	14 (41.2)	48 (55.8)
Methylprednisolone	107 (89.2)	30 (88.2)	77 (89.5)
Pulses of methylprednisolone	86 (72.9)	20 (60.6)	66 (77.6)
Cyclosporine	2 (1.7)	1 (2.9)	1 (1.2)
Colchicine	3 (2.5)	1 (2.9)	2 (2.3)
Anakinra	13 (10.8)	9 (10.5)	4 (11.8)
TCZ characteristics			
Days since symptoms until TCZ, mean (SD)*	10.9 (4.6)	9.5 (6.0)	11.4 (3.8)
Days since diagnosis until TCZ, mean (SD)	4.8 (7.6)	6.7 (13.5)	4.1 (3.0)
Second dose of TCZ, *n* (%)	28 (23.3)	9 (26.5)	19 (22.1)
Third dose of TCZ, *n* (%)	2 (1.7)	1 (2.9)	1 (1.2)
Hospitalization when TCZ administration			
General ward, *n* (%)	91 (75.8)	23 (67.6)	68 (79.1)
ICU, *n* (%)	29 (24.2)	11 (32.4)	18 (20.9)
Clinical findings on admission, *n* (%)			
Fever	99 (82.5)	28 (82.4)	71 (82.6)
Dry cough	90 (75.0)	25 (73.5)	65 (75.6)
Fatigue	67 (55.8)	17 (50.0)	50 (58.1)
Myalgia	45 (37.5)	6 (17.6)	39 (45.3)
Dyspnea	82 (68.3)	21 (61.8)	61 (70.9)
Headache	15 (12.5)	4 (11.8)	11 (12.8)
Diarrhea	14 (11.7)	2 (5.9)	12 (13.9)
ARDS	24 (20.0)	9 (26.5)	15 (17.4)
ACI	3 (2.5)	2 (5.9)	1 (1.2)
Thrombosis	4 (3.3)	1 (2.9)	3 (3.5)
Secondary infection	10 (8.3)	3 (8.8)	7 (8.1)
ARI	9 (7.5)	5 (14.7)	4 (4.6)
ERVI Scale*	5.5 (1.7)	5.6 (1.6)	5.4 (1.7)
Laboratory findings pre-TCZ, median (IQR) [N] except when indicated			
Total serum proteins (gr/dL), mean (SD) [N]	6.5 (0.8) [99]	6.3 (0.8) [28]	6.6 (0.8) [71]
Albumin (gr/dL)	3.4 (3–3.8) [91]	3.3 (3–3.5) [25]	3.4 (3–3.9) [66]
AST (U/L)	38 (26–54) [116]	37 (25–54) [33]	38 (26–54) [83]
ALT (U/L)	36 (23–66) [118]	28.5 (22–52) [34]	37.5 (25–71) [84]
GGT (U/L)	67.5 (47–110) [86]	54.5 (44–78) [26]	73 (51–129) [60]
LDH (U/L)	476 (392–573.5) [120]	490 (409–574) [34]	473 (391–566) [86]
Troponin I (pg/ml)	6.4 (2.7–17.6) [80]	18.8 (6–82.7) [26]	4.6 (2.4–9.5) [54]
C-reactive protein (mg/L)	119.9 (67.2–182.7) [118]	133.3 (80.3–182.7) [33]	107.1 (67.2–182.5) [85]
Procalcitonine (ng/ml)	0.2 (0.0–0.5) [80]	0.2 (0.1–0.5) [26]	0.2 (0.1–0.4) [54]
Ferritin (ng/ml)	1,415.7 (812.9–2,475) [114]	1748.9 (865.1–2,811.8) [32]	1,322.1 (794.6–2,202.6) [82]
Leukocyte count (/μL)	8,480 (6,390–11,330) [120]	8,255 (5,560–12,420) [34]	8,545 (6,760–11,200) [86]
Neutrophil count (/μL)	7,050 (4,900–9,930) [119]	6,760 (4,800–9,780) [33]	7,690 (5,020–9,930) [86]
Lymphocyte count (/μL)	690 (430–990) [119]	660 (380–970) [33]	695 (460–990) [86]
Platelet count (/μL)	228,500 (179,000–293,500) [120]	193,500 (140,000–242,000) [34]	245,500 (199,000–343,000) [86]
Interleukin-6 (pg/ml)	74.6 (39.6–133.3) [80]	78.4 (37.8–240.2) [23]	65.8 (40–111.7) [57]

(Continued on following page)

TABLE 1 | (*Continued*) Baseline demographic, clinical, pharmacological and laboratory data of patients diagnosed with COVID-19 receiving tocilizumab.

	Total (*N* = 120)	Deceased (*N* = 34)	Survivors (*N* = 86)
D-Dimer (mg/L)	1.0 (0.6–2.3) [113]	1.1 (0.6–3.0) [32]	1.0 (0.6–2.3) [81]
Fibrinogen (mg/dl), mean (SD) [N]	725.9 (274.8) [85]	752.0 (305.5) [28]	726.4 (261.2) [57]
PaO_2/FiO_2 ratio	180 (163–252.5) [120]	171 (138–200) [34]	193.5 (172–263) [86]
Temperature (°C), mean (SD) [N]	36.8 (1.1) [60]	36.1 (0.9) [14]	36.9 (1.1) [46]

ACI, acute cardiac injury; ARDS, acute respiratory distress syndrome; ACI, acute cardiac injury; ARI, acute renal injury; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; ERVI, scale for assessment of hospital admission; GGT, γ-glutamyl transferase; IQR, interquartile range; LDH, lactate dehydrogenase; SD, Standard deviation.
**Variables with missing values: Smoking status* N = *96,* $N_{deceased}$ = *22,* $N_{survivors}$ = *74; Days since symptoms until TCZ* N = *118,* $N_{deceased}$ = *32,* $N_{survivors}$ = *86; ERVI Scale* N = *116,* $N_{deceased}$ = *33,* $N_{survivors}$ = *83.*

On the other hand, mean PaO_2/FiO_2 ratio as well as lymphocyte count were below the normal range in both groups. Mean platelet count was much lower in the patients that died compared to those that remained alive, but in both case values entered into the normal range. Central tendency and dispersion values together with sample size for the analytical parameters measured at day 3, 6 and 9 are provided in **Supplementary Table S1**.

The univariate analyses revealed a statistically significant positive association of age, diabetes mellitus, immunosuppression, troponin I levels, and days since diagnosis until TCZ administration with hazard of death (**Table 2**). Instead, myalgia on admission, temperature, platelet count and total serum proteins were significantly related with a lower hazard of death (**Table 2**).

In Cox stepwise regression models within each group of factors, diabetes mellitus, immunosuppression, days since diagnosis until TCZ administration, myalgia and platelets were the variables retained. In the final stepwise regression model, the variables associated with a higher hazard of death were age (for each year of increase in age, HR 1.05; 95% CI 1.02–1.09), diabetes mellitus (HR 2.63; 95% CI 1.23–5.64), days since diagnosis until TCZ administration (for each more day, HR 1.05, 95% CI 1.00–1.09) and immunosuppression (HR 3.15; 95% CI 1.17–8.51). The immunosuppressed group included five patients with haematological neoplasms and five with other type of neoplasms under active treatment with chemotherapy, two transplant recipients with immunosuppressive treatment and one patient with an autoimmune disease and under treatment with biological therapy and methotrexate. Furthermore, for every logarithmic unit increase in platelet count there was a 73% decrease in the instantaneous death rate (HR 0.27; 95% CI 0.11–0.69). Survival curves illustrating the variables associated with time to death in the final regression model are represented in **Figure 1**. For continuous variables, population was divided in groups. In days from diagnosis until TCZ treatment, two groups are showed: ≤7 days and >7 days. For age, two groups were built with individuals below/above the median (63 years). And for platelets, three groups: ≤ 200,000, 200,000–400000, ≥400,000/μL.

With regards to the changes in biochemical and hemogram parameters after TCZ administration and comparing with participants who remained alive during the follow-up, participants who died had a significant positive mean difference (higher mean values along the four measurements) in LDH, troponin I, CRP, procalcitonin, neutrophils, D-dimer, IL-6 and leukocytes (**Table 3**). After adjustment for multiple comparisons, the parameters that remained significant were LDH, troponin I, CRP and neutrophils, while a borderline non-significant association was retained for IL-6. The mean difference was negative (lower values in the deceased patients) for PaO_2/FiO_2 ratio, and lymphocyte and platelets counts. The statistically significance was kept for the three parameters after adjustment (**Table 3**).

Kinetics varied depending on the parameter (**Figure 2**). On the one hand, IL-6, PaO_2/FiO_2 ratio, lymphocyte and neutrophil counts, CRP and LDH showed differences between survivors and deceased patients that were amplified over time. Thus, IL-6 and neutrophil counts increased only in the deceased population, reaching pathological median levels in the case of neutrophils by day 3. Lymphocyte count and PaO_2/FiO_2 ratio values improved only in the survivors, entering the physiological range by day 6 in the case of the lymphocytes. CRP decreased in both groups but at a higher rate in the survivors, such as that the median level reached the physiological levels (<5 mg/L) by day 6. For LDH an increase was observed in the deceased and a decrease in the survivor group. On the other hand, troponin I and platelet counts presented baseline differences between both groups of comparisons that were kept over the four measurements. Of note, platelet counts increased at day 3 and 6 and then decreased both in deceased and discharged patients; however, levels were always lower in the patients that died. Contrary, troponin I levels were higher in deceased patients at all four time points.

Finally, as one adverse effect of TCZ treatment is the risk of bacterial infections, we hypothesized that immunosuppressed individuals receiving TCZ may be at higher risk of death precisely because of secondary co-infections. In our cohort, 38 patients (31.7%) presented a secondary systemic infection after TCZ administration, of which 18 (47%) died. Post-TCZ coinfections were associated with mortality (χ^2 test, p-value = 0.002) and were more common in the ICU than in the general ward (χ^2 test, p-value<0.001). However, the percentage of post-TCZ coinfections was similar between immunosuppressed and non-immunosuppressed individuals (Fisher's test, p-value = 0.481). Therefore, we also searched actively for any record of *Aspergillus* spp. growth in broncho-alveolar aspirates in patients hospitalized at the ICU. None of them was positive for this pathogen.

DISCUSSION

There were more men than women in our cohort, and the percentage skewed even more in the deceased group, a common observation in COVID-19. However, in agreement

TABLE 2 | Association between baseline variables and time to death.

Group variables	Variable	HR[c]	95%CI	*p*-value	HR[d]	95%CI	*p*-value	HR[e]	95%CI	*p*-value
Demographic variables	Male	1.68	0.70–4.08	0.248						
	Age (years)[a]	**1.04**	**1.01–1.07**	**0.015**				**1.05**	**1.02–1.09**	**0.001**
COVID-19 wave	Second	1.26	0.64–2.50	0.503						
Smoking habit	Smokers	2.24	0.60–8.34	0.231						
	Ex-smokers	1.04	0.41–2.63	0.930						
Comorbidities and physical examination	Hypertension	1.60	0.79–3.24	0.196						
	Dyslipidemia	1.70	0.86–3.38	0.128						
	Cardiovascular disease	1.51	0.77–2.98	0.230						
	Diabetes mellitus	**2.26**	**1.09–4.68**	**0.029**	**2.25**	**1.08–4.69**	**0.030**	**2.63**	**1.23–5.64**	**0.013**
	Previous pulmonar disease	1.44	0.68–3.03	0.340						
	Immunosuppression	**4.85**	**2.15–10.95**	**<0.001**	**4.87**	**2.15–11.07**	**<0.001**	**3.15**	**1.17–8.51**	**0.024**
	Confirmed diagnosis by PCR	0.59	0.26–1.33	0.204						
Pharmacological treatment	Lopinavir/ritonavir	0.75	0.38–1.48	0.407						
	Azytromicine	0.85	0.43–1.69	0.640						
	Anakinra	0.99	0.35–2.83	0.993						
	Methylprednisolone	0.93	0.33–2.66	0.899						
	Ciclosporine	2.59	0.35–19.36	0.354						
	Pulses of methylprednisolone	0.85	0.42–1.72	0.644						
	Colchicine	1.27	0.17–9.35	0.814						
	Hydroxycloroquine	0.79	0.40–1.57	0.507						
	More than one dose of TCZ	1.03	0.46–2.29	0.941						
	Days since symptoms until TCZ[a]	0.93	0.86–1.02	0.117						
	Days since diagnosis until TCZ[a]	**1.06**	**1.03–1.09**	**<0.001**	**1.07**	**1.03–1.10**	**<0.001**	**1.05**	**1.00–1.09**	**0.032**
Symptoms and signs pre-TCZ	Dry cough	1.00	0.47–2.16	0.991						
	Fatigue	0.75	0.38–1.48	0.411						
	Myalgia	**0.35**	**0.15–0.86**	**0.021**	**0.35**	**0.15–0.86**	**0.021**			
	Dyspnea	0.69	0.35–1.38	0.294						
	Headache	0.70	0.25–2.00	0.511						
	Diarrhea	0.68	0.16–2.87	0.602						
	Acute Respiratory distress syndrome	0.68	0.30–1.53	0.353						
	Acute cardiac injury	3.22	0.76–13.64	0.113						
	Thrombosis	0.88	0.12–6.43	0.897						
	Acute renal injury	1.95	0.75–5.08	0.172						
	Scale ERVI[a]	1.02	0.83–1.25	0.858						
	Hospitalization when TCZ administration	0.64	0.30–1.35	0.243						
	Fever	0.62	0.25–1.53	0.301						
Laboratory findings pre-TCZ	**Total serum proteins (gr/dL)**[b]	**0.53**	**0.31–0.92**	**0.025**						
	Aspartate transaminase (U/L)[b]	0.78	0.39–1.56	0.481						
	Alanine transaminase (U/L)[b]	0.55	0.30–1.00	0.052						
	γ-Glutamyl transferase (U/L)[b]	0.67	0.38–1.18	0.161						
	Procalcitonin (ng/ml)[b]	0.97	0.74–1.26	0.804						
	Albumin (gr/dL)[b]	0.40	0.05–3.50	0.409						
	Interleukin-6 (pg/ml)[b]	1.08	0.79–1.48	0.611						
	Lactate deshydrogenase (U/L)[b]	1.87	0.55–6.29	0.312						
	C-reactive protein (mg/L)[b]	0.93	0.66–1.31	0.669						
	Ferritin (ng/ml)[b]	1.24	0.82–1.88	0.302						
	Leukocyte count (/μL)[b]	0.69	0.31–1.54	0.368						
	Neutrophil count (/μL)[b]	0.69	0.32–1.48	0.343						
	Lymphocyte count (/μL)[b]	0.88	0.47–1.65	0.695						
	Platelet count (/μL)[b]	**0.20**	**0.10–0.40**	**<0.001**	**0.20**	**0.10–0.41**	**<0.001**	**0.27**	**0.11–0.69**	**0.006**
	Fibrinogen (mg/dl)[b]	1.00	1.00–1.00	0.440						
	Troponin I (pg/ml)[b]	**1.35**	**1.08–1.69**	**0.007**						
	D-dimer (mg/L)[b]	1.15	0.86–1.52	0.342						
	PaO_2/FiO_2 (mmHg)[b]	0.79	0.30–2.25	0.663						
	Temperature°C[a]	**0.39**	**0.21–0.76**	0.005						

PCR: polymerase Chain Reaction; Scale ERVI: X-ray scale for assessment of hospital admission; TCZ: Tocilizumab; HR: hazard ratio; CI: confidence interval. For dichotomous variables, the reference category was "no" except hospitalization when TCZ administration that it was "general ward" (comparison ICU) and confirmed diagnosis-PCR that it was "suspicious diagnosis with negative PCR" (comparison confirmed positive PCR result). For smoking habit the reference was "never smoker". Highlighted in bold if p < 0.05.

[a]*Hazard ratios are expressed per unit increase in the variable.*

[b]*Log-transformed quantitative variables.*

[c]*Obtained with Cox's univariate proportional hazard regression models.*

[d]*Obtained with stepwise regression model within groups of variables, including variables with* p*-value <0.2 in univariate analysis, except total serum proteins (N = 99), troponin I (*N = *80) and temperature (*N = *60) which were excluded from multivariate models because of presenting >10% of missing values.*

[e]*Obtained with stepwise regression including variables retained in models by group.*

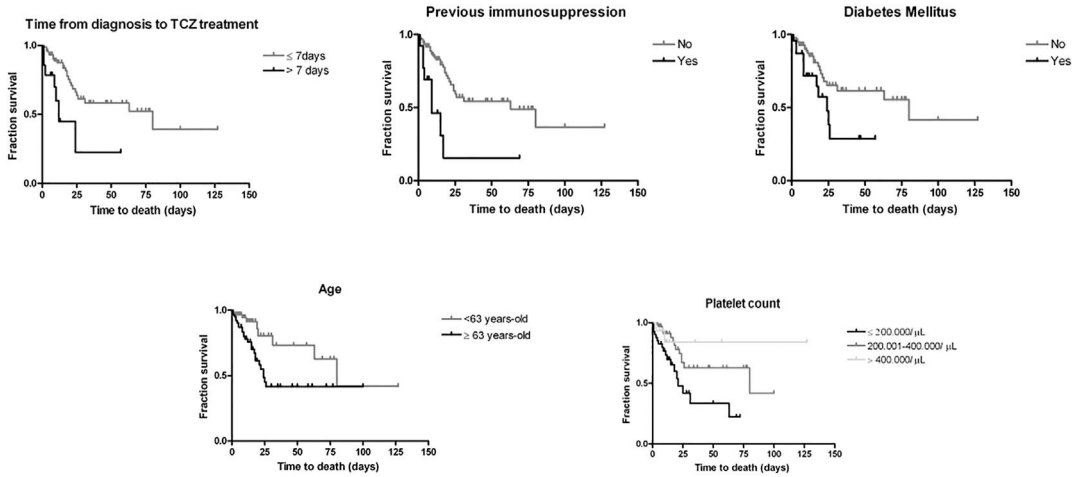

FIGURE 1 | Survival curves of the variables associated with time to death. Censored (discharged by the end of follow-up) subjects are indicated on the curve as tick marks.

TABLE 3 | Mean differences in the biochemical markers and hemogram parameters between individual with COVID-19 according to the vital status.

Variable	Mean difference (95% CI)[a]	*p*-value	*q*-value
Lactate Dehydrogenase (U/L)[b]	0.26 (0.13, 0.39)	**<0.001**	**0.013**
Troponin I (pg/ml)[b]	1.58 (0.78, 2.37)	**<0.001**	**0.013**
C Reactive protein (mg/L)[b]	0.76 (0.31, 1.22)	**0.001**	**0.013**
Procalcitonin (ng/ml)[b]	0.77 (0.18, 1.36)	**0.010**	0.100
Neutrophils (/µl)[b]	0.27 (0.10, 0.45)	**0.002**	**0.024**
D-dimer (mg/L)[b]	0.59 (0.10, 1.09)	**0.019**	0.152
Interleukin-6 (pg/ml)[b]	1.41 (0.37, 2.44)	**0.008**	0.088
Albumin (gr/dL)[b]	−0.05 (−0.09, −0.00)	0.058	0.406
Platelet count (/µl)[b]	−0.30 (−0.48, −0.12)	**0.001**	**0.013**
Aspartate transaminase (U/L)[b]	0.12 (−0.07, 0.32)	0.213	0.679
Alanine transaminase (U/L)[b]	−0.13 (−0.42, 0.16)	0.386	0. 679
γ-Glutamyl transferase (U/L)[b]	−0.12 (−0.52, 0.28)	0.556	0. 679
Ferritin (ng/ml)[b]	0.37 (−0.03, 0.77)	0.070	0.420
Leukocyte count (/µL)[b]	0.23 (0.05, 0.41)	**0.013**	0.117
Lymphocyte count (/µL)[b]	−0.38 (−0.58, −0.18)	**<0.001**	**0.013**
Total serum proteins (gr/dL)	−0.19 (−0.44, 0.05)	0.124	0.577
PaO_2/FiO_2 ratio[b]	−0.27 (−0.37, −0.17)	**<0.001**	**0.013**
Fibrinogen (mg/dl)	17.56 (−65.48,100.60)	0.679	0.679

[a]*Mean difference between survivors and deceased along four laboratory measurements (days 0, 3, 6 and 9 after TCZ administration) using generalized estimating equation;*
[b]*Parameters analyzed in logarithmic units; CI: confidence interval;* q-*value:* p-*value adjusted by multiple comparisons with Benjamini-Hochber method. In bold if* p-*value < 0.05.*

with previous TCZ-cohorts of COVID-19, sex was not associated with the risk of death (Moreno-Pérez et al., 2020; Morrison et al., 2020; Desai et al., 2021). On the contrary, risk death did increase with age, while literature shows contradictory results (Moreno-Pérez et al., 2020; Morrison et al., 2020; Desai et al., 2021).

In our study, the comorbidities associated with a higher hazard of death were immunosuppression and diabetes mellitus. Diabetes mellitus has been largely associated to poorer outcomes in COVID-19 patients [reviewed in (Huang et al., 2020b)], while contradictory results have been observed in cohorts under TCZ treatment (Moreno-Pérez et al., 2020; Morrison et al., 2020; Desai et al., 2021). Obesity has been related to a higher risk of death in COVID-19 patients (Demeulemeester et al., 2021) and may act as a cofounder or modifier variable for this finding. Unfortunately, as we collected the information retrospectively from clinical records where obesity was not codified, we could not analyze its effect in our death risk estimation. With regards to immunosuppression, it was not associated with mortality in a large COVID-19 cohort ($N = 1,305$) in the United States (Imam et al., 2020). However, the effect of this condition in COVID-19 risk/mortality may depend on the type of immunosuppression. Thus, while cancer and solid organ transplant patients seem to present higher rates of mortality (Belsky et al., 2021) and autoinmune diseases's

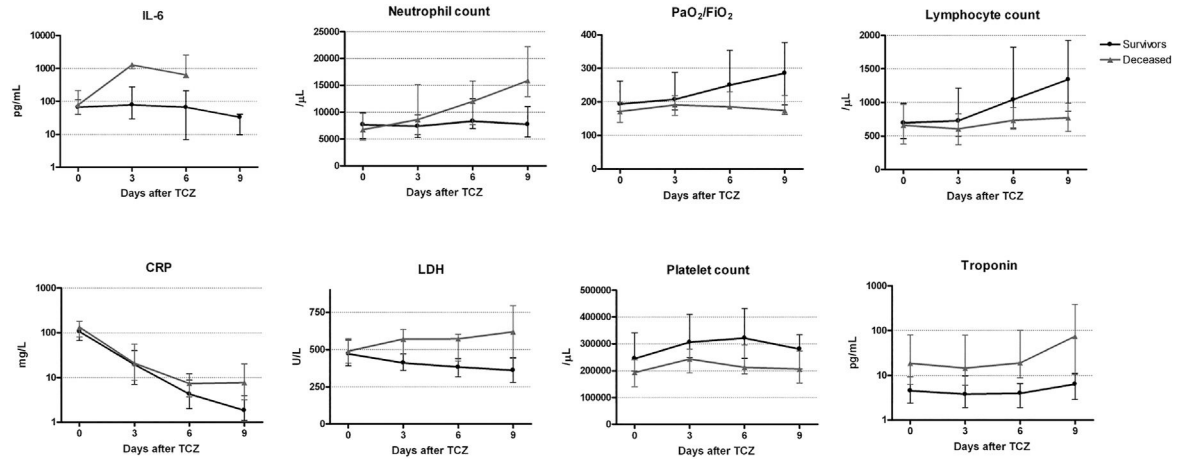

FIGURE 2 | Changes in laboratory parameters after tocilizumab administration and their comparison between survivors and deceased patient. LDH: Lactate dehydrogenase; CRP: C-reactiveprotein; IL-6: interleukin-6. Values are represented as median (circle) and interquartile range (25th and 75th percentiles error bars).

patients have a higher risk of COVID-19 infection (Akiyama et al., 2021), people living with Human Immunodeficiency Virus were not found to be at higher risk of poorer COVID-19 outcomes (Lee et al., 2021). In TCZ-COVID-19 cohorts, the effect of immunosuppression was not estimated (Moreno-Pérez et al., 2020; Morrison et al., 2020) or the variable was included in the group of comorbidities, precluding a specific analysis (Lohse et al., 2020; Galván-Román et al., 2021). Nevertheless, a publication recently reported a higher risk of death among cancer patients receiving TCZ due to COVID-19, but not among patients with previous rheumatology/infectious diseases (Desai et al., 2021). Due to our study design, we cannot conclude whether the TCZ-induced immunosuppression acted as an added risk factor for death in previously immunocompromised patients. However, it seems unlikely as coinfections were not more frequent in immunosuppressed individuals in our cohort. Further studies are necessary to confirm our finding and to provide knowledge about a potential underlying mechanism.

Time elapsed from COVID-19 diagnosis to TCZ was positively associated with the risk of death, as previously reported (Morrison et al., 2020). Of note, when we stratified our cohort into patients with a PaO_2/FiO_2 ratio lower and higher or equal to 200, the effect of time to treatment on mortality was only observed in those with PaO_2/FiO_2 ratio <200 (data not shown), so timing seems specially important in moderate/severe disease. In a similar direction, Galván-Román et al. reported that early TCZ administration improved the PaO_2/FiO_2 ratio (Galván-Román et al., 2021). The effect in our study was clearly observed when we classified patients in early (within a week of diagnosis) and late (after 7 days) TCZ treatment. This time window is in consonance with the studies referenced above, that established it in 11–12 days since symptoms onset (Morrison et al., 2020; Galván-Román et al., 2021), which usually occurs days before diagnosis. In our study, time from symptoms to TCZ did not show significant differences, probably as the first day of symptoms is often not recorded properly as they are usually mild and vague. Nevertheless, our results emphasize the importance of the appropriate timing to administer TCZ, which may explain the contradictory results about its efficacy reported by observational and experimental studies from the literature.

Our cohort included patients hospitalized in the general ward (less severe disease) and in the ICU at the time of TCZ administration. Surprisingly, mortality risk was similar between both groups, suggesting that initial clinical differences were not related to a poorer prognosis. Nevertheless, post-TCZ coinfections were much more likely to occur at ICU as expected (Quartuccio et al., 2020), and they tended to be associated with mortality in contrast to a previous study with a TCZ-cohort (Morrison et al., 2020). In COVID-19 TCZ-cohorts, percentages of patients who developed secondary infections have ranged from 10 to 40% (Alattar et al., 2020; Moreno-Pérez et al., 2020; Pérez-Sáez et al., 2020; Quartuccio et al., 2020), what covers the 32% presented here.

Baseline levels of ferritin, CRP and procalcitonin have been related to mortality or poor outcomes in hospitalized COVID-19 patients (Bonetti et al., 2020; Huang et al., 2020c) but not in our final regression model, in agreement with other COVID-19 –TCZ cohorts (Conrozier et al., 2020; Knorr et al., 2020). However, after TCZ administration, the longitudinal laboratory test analysis showed that CRP decreased differentially in survivors and deceased patients, indicating that TCZ was more effective controlling inflammation in those patients that remained alive at the end of the study. This is in consonance with other studies (Alattar et al., 2020; Antwi-Amoabeng et al., 2020; Conrozier et al., 2020; Knorr et al., 2020; Morrison et al., 2020; Pérez-Sáez et al., 2020) and suggests that CRP may be used as a prognostic biomarker after TCZ administration in COVID-19 severe patients.

IL-6 has been recognized as another key inflammatory marker in COVID-19 and a meta-analysis has shown elevated levels in patients with complicated COVID-19 (Coomes and Haghbayan, 2020). Here, it was not found an association between baseline IL-6 levels and death, probably because these levels were already elevated in all patients, suggesting an adequate used of its antagonist TCZ. Interestingly, we report that IL-6 levels

increased massively in the following days after TCZ administration only in the patients that subsequently deceased, but not in those that remained alive where we observed just a small spike at day 3. Other authors have found a similar trend for IL-6 concentration differences between deceased and discharged patients but with no statistical analysis for between-group comparison (Luo et al., 2020; Madenidou and Bukhari, 2020; Toniati et al., 2020). However, it is unclear whether IL-6 represents a marker and/or mediator of COVID-19 severe progression (Chastain et al., 2020). This finding was accompanied by an increased on neutrophils few days after the IL-6 peak, as it is well recognized that IL-6 stimulates neutrophil production in the bone marrow (Abbas et al., 2018). Indeed, while there were no baseline differences in neutrophil numbers between comparison groups and levels were within physiological values, their median value reached pathological values after TCZ only in the deceased group. The neutrophil count is probably a more easily measurable, available and cost-effective parameter than IL-6 and therefore may be used as a prognostic IL-6 proxy factor.

LDH is a well-known marker of tissue damage, and in our study it was another of the parameters showing differences between discharged and deceased patients early after TCZ administration, in consonance with a previous study (Morrison et al., 2020). Of note, baseline levels were higher than normal and very similar in both groups, and no association with time to death was observed. However, 3 days after TCZ administration, an increase in LDH levels was observed in the patients that subsequently died, suggesting further tissue damage could be occurring in these patients. The PaO_2/FiO_2 ratio is a marker of severity of acute respiratory distress syndrome, a common and severe complication of COVID-19 (Badraoui et al., 2020), considered moderate if the values range between 100 and 200, and severe if <100. There were basically no differences in the baseline values between patients that died and survived on our cohort and no association with mortality at this stage. However, after TCZ, a progressive increase in the ratio values was observed only in the survivor group, suggesting a pulmonary improvement in agreement with the better clinical outcome. Similarly, median lymphocyte levels were below the normal range in both groups before TCZ treatment, but after the treatment, only the survivors increased their counts.

Troponin I showed an association with risk of death in the univariate analysis. However, because of having many missing values, this variable was excluded from the multivariate analysis, precluding the opportunity to study its effect in the global regression model. Nevertheless, differences between deceased and survivors were also observed along the four measurements after TCZ treatment. Plasma troponin I is a marker of cardiac muscle damage and/or myocarditis and its levels have been related to poor COVID-19 outcomes [reviewed in (Alzahrani and Al-Rabia, 2021)]. Its role in our cohort is probably independent of the effect of TCZ.

The variable most clearly (inversely) associated with mortality was platelet count, as baseline as well as the mean longitudinal change post-TCZ was associated with mortality. Corticosteroid treatment was not associated to differences in baseline platelet levels (data not shown). Unfortunately, we did not collect information about concomitant treatment with other potential drugs altering platelet levels such as anticoagulants because at the time of the study design evidence for the role of coagulation in COVID-19 was not so strong. We cannot rule out that this was a bias in our study, as the patients at risk of thrombosis were more likely to die but also more likely to receive anticoagulant treatment that may decrease platelet count. Nevertheless, in agreement with our results, thrombocytopenia as well as lower platelet count has been repeatedly related to poor COVID-19 outcomes, in general hospitalized cohorts [reviewed in (Lippi et al., 2020)] as well as a TCZ-cohort (Conrozier et al., 2020). Thus, an increase in platelet counts after any clinical or pharmacological intervention might be understood as a positive sign. However, here we report that regardless of the health outcome (live or death), an early increase in the platelet count occurred 3 and 6 days after TCZ administration followed by a decrease, in consonance with a longitudinal analysis of a similar cohort (Conrozier et al., 2020). This temporarily increase may mislead practitioners about the disease outcome and suggest that total platelet count rather than progression should be taken into account when interpreting this parameter in relation to COVID-19 progression. Other hemostasis alterations reflecting intravascular or consumption coagulopathies are common in COVID-19 (Bonetti et al., 2020; Huang et al., 2020c; Tang et al., 2020). In contrast with some of these studies, we did not find an association between baseline D-dimer or fibrinogen concentration and mortality. And while significant mean post-TCZ differences were observed between deceased and discharged patients for D-dimer, the significance was lost when we adjusted for multiple comparisons.

Our study has some limitations: 1) A possible lack of statistical power due to the relatively low sample size and the presence of missing values for some laboratory parameters. Nevertheless, to allow the analysis of all the variables recorded despite the small sample size, we did a three-stage modeling process grouping predictors and reducing the number of variables included in a regression model at a time. 2) The absence of a control group as patients with no TCZ treatment would not be clinically comparable (less severe disease). 3) In this retrospective study, the information source were the clinical records and therefore the effect of possible relevant variables, e.g. the obesity, could not be evaluated due to not having been routinely registered.

This study has also important strengths. Although few similar articles in COVID-19 patients under TCZ treatment have been published (many of them referenced along the manuscript), our statistical approach was different as it allowed the analysis of multiple variables resulting in a model with those that contributed most to mortality. Furthermore, while most studies focused on the baseline predictors of mortality, we also analyzed the laboratory parameter evolution early after TCZ administration and how this evolution differed between discharged and deceased patients. Finally, our larger follow-up period allowed us to observe the final outcome (discharge or death) of the whole cohort.

As conclusions, our results show that in a cohort of COVID-19 diagnosed patients under TCZ treatment, early treatment

decreased the risk of death, while age, immunosupression, diabetes mellitus and baseline lower platelet counts increased that risk. Lower platelet levels were also associated with mortality after TCZ administration, while increased troponin I values were observed in the deceased patients. Moreover, IL-6, neutrophil and lymphocyte count, PaO_2/FiO_2 ratio, LDH and CRP evolved differently in deceased and discharged patients after TCZ treatment, and may be used as prognostic factors in these patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Granada province. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization, LS and PR; Data curation, LS, JA-U, MN-F, MF-G, and VR; Formal analysis, LS, IS-B and RB-R; Investigation, LS, JA-U, MN-F, MF-G, VR, IS-B, RB-R, RC-C and PR; Supervision, RC-C and PR; Writing—original draft, LS, IS-B, RB-R, and PR; Writing—review and editing, LS, JA-U, MN-F, MF-G, VR, IS-B, RB-R, RC-C and PR. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.620187/full#supplementary-material

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Ten Rules for Conducting Retrospective Pharmacoepidemiological Analyses: Example COVID-19 Study

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Since the beginning of the COVID-19 pandemic, pharmaceutical treatment hypotheses have abounded, each requiring careful evaluation. A randomized controlled trial generally provides the most credible evaluation of a treatment, but the efficiency and effectiveness of the trial depend on the existing evidence supporting the treatment. The researcher must therefore compile a body of evidence justifying the use of time and resources to further investigate a treatment hypothesis in a trial. An observational study can provide this evidence, but the lack of randomized exposure and the researcher's inability to control treatment administration and data collection introduce significant challenges. A proper analysis of observational health care data thus requires contributions from experts in a diverse set of topics ranging from epidemiology and causal analysis to relevant medical specialties and data sources. Here we summarize these contributions as 10 rules that serve as an end-to-end introduction to retrospective pharmacoepidemiological analyses of observational health care data using a running example of a hypothetical COVID-19 study. A detailed supplement presents a practical how-to guide for following each rule. When carefully designed and properly executed, a retrospective pharmacoepidemiological analysis framed around these rules will inform the decisions of whether and how to investigate a treatment hypothesis in a randomized controlled trial. This work has important implications for any future pandemic by prescribing what we can and should do while the world waits for global vaccine distribution.

Keywords: drug repurposing, retrospective analyses, observational study, COVID-19, pharmacoepidemiology

INTRODUCTION

Imagine we are only halfway through 2020; the COVID-19 pandemic is raging, and widespread vaccination is thought to be at least a year away. Treatment ideas abound for COVID-19, and around the world more than 2,000 clinical treatment trials have been initiated to begin testing a wide variety of drugs hypothesized to help infected patients. Unfortunately, constrained resources can only fund some subset of the investigator-initiated trials; hence, trials resourced to begin patient enrollment must be chosen judiciously based on the soundness of the medical hypothesis, the availability of preclinical evidence, and the trial's feasibility, cost, and potential impact. It is in this environment that you have arrived with a novel idea for an effective pharmaceutical intervention for COVID-19 (or the next pandemic).

The gold-standard way to evaluate your hypothesis is a randomized controlled trial (RCT), but that takes time and resources you (and the world) may not have at the moment. In fact, the window to pursue your trial is limited as interest (and resources) will increasingly focus on progress in vaccine development. Assuming your trial would be ethically permissible and otherwise feasible (e.g., reasonable follow-up periods and realistic recruiting goals), is there anything you can do right now to investigate your hypothesis and determine the priority of testing it in an RCT? There are three common types of retrospective studies to consider, each of which uses observational data: cross-sectional studies, case-control studies, and cohort studies. This paper provides a framework for investigating your pharmaceutical hypothesis carefully and responsibly using a retrospective cohort study. Beyond just advocating for a clinical trial, your investigation can inform many of the decisions regarding the details of a clinical trial (e.g., which drugs and dosage levels to test), as well as who is most likely to benefit from your treatment; all of this may influence how stakeholders choose to prioritize your trial. A retrospective analysis focused on today's disease (even after widespread vaccination) can also improve our understanding and preparedness for a novel disease we encounter in the future; completed studies targeting readily available treatment options in a related disease could help save countless lives when the next pandemic strikes and the world is again waiting for a vaccine.

Countries around the world have defended themselves against SARS-CoV-2 using travel restrictions, national lockdowns, facemask policies, and other non-pharmaceutical interventions to stop the spread of SARS-CoV-2, and evaluating these population-level actions requires different tools than what we present in this paper (i.e., there is no path to an RCT for some public health measures). Here, we use the tools of pharmacoepidemiology, a field spanning clinical pharmacology and epidemiology, to study the effects of drugs in large numbers of people in order to estimate probabilities of beneficial and/or adverse effects. We introduce this body of knowledge as 10 rules for retrospective pharmacoepidemiological analyses designed to evaluate a treatment hypothesis (see **Figure 1** for the 10 rules and **Table 1** for common vocabulary). These rules are the result of a community effort, including academic, health care, nonprofit, and industry contributors, to establish a set of best practices for retrospective analyses. A retrospective analysis aims to estimate the comparative effectiveness of one treatment vs. another (e.g., a new treatment vs. the standard care) using real-world evidence (Office of the Commissioner, 2020) obtained from preexisting data such as electronic health records (EHR), insurance claims databases, or health care registries. We embark on a retrospective analysis knowing that it should not stand alone as the sole evidence supporting adoption of a new treatment; observational study evidence should be considered *suggestive* rather than *conclusive*. A retrospective analysis can contribute a body of real-world evidence as a supplement to the medical theory supporting the treatment and any preclinical studies conducted *in vitro* and/or *in vivo*, all of which combine to inform decisions about whether and how to pursue a randomized trial.

COVID-19 STUDY

Here we introduce a potential COVID-19 pharmaceutical treatment to discuss the 10 rules more concretely. Prior work indicates that certain alpha-1 adrenergic receptor antagonists (alpha blockers) disrupt cytokine storm syndromes, a pathological hyperinflammatory response associated with respiratory infection and other diseases (Staedtke et al., 2018; Koenecke et al., 2021; Thomsen et al., 2021). Subsequently, others determined that hyperinflammation is implicated in morbidity and mortality in COVID-19 patients (Mehta et al., 2020; Li et al., 2021). Many COVID-19 patients were already taking alpha blockers prior to infection for unrelated, chronic medical conditions. Consistent use of doxazosin (a particular alpha blocker) prior to COVID-19 diagnosis is the exposure of interest, and the goal is to estimate its effectiveness for preventing in-hospital death.

We are now ready to dig into the 10 rules. Rules 1–3 describe three guiding principles for a retrospective pharmacoepidemiological analysis. Rules 4–7 discuss key preparations for the analysis. Rules 8–9 address how to develop and refine the analysis plan. Rule 10 concludes with executing, summarizing, and reporting the results to facilitate replicating and extending them. Each rule could have its own paper or book chapter (and in many cases they do), and we expand the discussion of each rule considerably in the supplementary material to explain the concrete, actionable steps the rules require.

GUIDING PRINCIPLES: BUILD AND FOCUS THE TEAM

Rule 1: Form a Multidisciplinary Team

Get the right people involved at the start, in the middle, and at the end. Every step of the way you are going to need to make decisions about the medical rationale for the proposed exposure, treatment practices in clinics and hospitals, the nuances of relevant data stores and common coding practices,

FIGURE 1 | The first phase of the 10 rules involves building the right team to envision the perfect trial and then consider the limitations of an observational study. The study then enters a preparation phase in which the details of the study are specified: hypotheses, which population to target, essential confounders to observe, and which data sets might support the study criteria. In the analysis planning phase, the objective is to refine and validate the study definitions and selected methods without being influenced by real results. Finally, the study concludes when the study is run, carefully summarized, and reported accurately.

the study design, and the statistical analyses and interpretation of results. Specifically, high-quality retrospective analyses depend on input from committed individuals with different domain expertise: medical, data sources, epidemiology, and causal analysis.

COVID-19 Study

Clinicians provide insights into the differences between exposed (those prescribed doxazosin) and unexposed groups; understanding the conditions that lead to treatment is critical in designing the study. Clinical experience working with patients diagnosed with COVID-19 is also helpful for gaining insight into the dynamics of COVID-19 testing and patient care. For example, the protocols for testing and admitting patients have varied over place and time, especially early in the crisis. In an evolving pandemic, these factors motivate accounting for changing patient populations; failing to do so could result in biased estimates of treatment effects.

A COVID-19 study presents unique challenges. First, there is an urgency to rapidly (and comprehensively) assess a proposed exposure. Second, the landscape changes while the study is underway: new datasets emerge and published results change attitudes for different treatments. Third, near-constant sharing of ideas and work products is crucial, but the study team members are likely isolated. Getting feedback early and often from all parties is crucial for reducing time-to-iterate without sacrificing research quality (London and Kimmelman, 2020). While still ensuring HIPAA protections are appropriately observed, tools like Slack, GitHub, and Google Docs for conversing, collaborating on code, and writing, respectively, facilitate the kind of rapid progress that is otherwise hard to achieve.

Rule 2: Emulate a Randomized Controlled Trial

Design your observational study to mimic — as closely as possible — a randomized controlled trial with similar goals, an approach known as *trial emulation* (Rubin, 2004; Rosenbaum, 2010; Hernán and Robins, 2016; Dickerman et al., 2019). Carefully consider what you measure, when you measure it, and in whom you measure it. Draw a CONSORT diagram of the ideal RCT you wish you could run (Begg et al., 1996). Emulating an RCT should ideally include preregistration of the study and analysis plans (described in Rule 9).

COVID-19 Study

Our retrospective analysis should emulate the desired RCT investigating doxazosin as a prophylactic treatment for severe symptoms among patients with COVID-19 (Konig et al., 2020). The trial would target older adults, a group who appears to have the greatest risk of adverse outcomes from COVID-19 (D-19 Provisional Coun, 2020). Emulating this trial requires focusing on the same patient group in our retrospective analysis. Without random exposure assignment, the retrospective study must identify people taking doxazosin prior to a COVID-19 diagnosis. In the United States, many older adults take doxazosin for conditions including hypertension and benign prostatic hyperplasia (BPH). Thus, emulating a trial in older

TABLE 1 | This table of common terms provides working definitions for vocabulary appearing in the following 10 rules.

Term	Definition
causal effect	a difference between two potential outcomes, one where the individual is exposed and one where the individual is unexposed (or exposed to a different treatment)
cohort	a group of people with some defining characteristic (e.g., a disease)
comorbidity	a co-occurring medical condition in addition to the primary condition
comparison group/control group	groups that identify individuals who have not received the treatment of interest and have instead received either no treatment or a different treatment; often denoted as unexposed
confounders	variables satisfying three properties: they are associated with the outcome (i.e., risk factors), they are associated with the exposure (i.e., they are unequally distributed among the exposure groups), and they are not effects of the exposure
confounding	a bias in the measure of a treatment effect resulting from treatments and outcomes sharing a common cause
confounding by indication	when the condition or indication prompting exposure also affects the outcome (e.g., if the exposure of interest in a drug-repurposing study is a diabetes drug, individuals with prior prescriptions for this drug likely have diabetes and might be expected to have worse outcomes)
directed acyclic graph (DAG)	a tool for depicting assumptions and selecting variables to include in the analysis using directed arrows representing cause-effect relationships
exposure	the treatment or experience that defines the intervention under investigation (e.g., takes a drug, undergoes physical therapy, etc.)
external validity	how generalizable the finding is beyond the study population
internal validity	the degree to which the observed result is believed to be attributable to the observed treatment and not unseen factors
outcome	a clearly defined, measurable indicator of health status (e.g., blood pressure level, disease recurrence within a specified timeline, or in-hospital death)
pharmacoepidemiology	a field spanning clinical pharmacology and epidemiology focused on studying the effects of drugs in large numbers of people in order to estimate probabilities of beneficial and/or adverse effects
potential outcomes	what an individual would have counterfactually experienced when either exposed or not exposed (e.g., received a drug vs. no drug)
preregistration	registering the details of a study -- hypotheses, methods, analysis plans -- before it is conducted
retrospective analysis	an estimation of the comparative effectiveness of one treatment vs. another (e.g., a new treatment vs. the standard care) using real-world evidence obtained from preexisting data such as electronic health records (EHR), insurance claims databases, or health care registries
selection bias	a distortion of the treatment-outcome association principally resulting from the lack of randomized treatment assignment
sensitivity analysis	analyses conducted to observe the study result's sensitivity to a change in population/definition/method/assumption
surrogate outcomes	synthetic or permuted outcomes used to blind investigators to the real study results until various code and definition validations are complete
trial emulation	designing an observational study to mimic a randomized controlled trial with similar goals

adults would be both meaningful (by studying the impact on a group at risk for adverse outcomes from COVID-19) and feasible (since observing doxazosin use in this group is likely). There is a cost, however, to targeting a subset of the population; the study can lose external validity for other patient groups (Holdcroft, 2007).

Rule 3: Realize That Rule 2 Is Impossible and Proceed Carefully

In an observational study, our choices of what to measure and in whom to measure it are limited by what data already exists. Even more concerning, our inability to randomize exposure assignment introduces categories of variables that we worry less about in randomized controlled trials, most notably confounders. Confounders satisfy three properties: they are associated with the outcome (i.e., risk factors), they are associated with the exposure (i.e., they are unequally distributed among the exposure groups), and they are not effects of the exposure (Jager et al., 2008). If not observed and sufficiently addressed, confounders lead to confounding, which is a bias in the measure of a treatment effect resulting from treatments and outcomes sharing a common cause (Hernán and Robins, 2020). Review the different kinds of covariates that can exist in a causal analysis of observational data and how each can impact causal estimates (see Rule 5). Confounding by indication is likely to occur in observational data, and the primary concern in your observational study is the identification and mitigation of potential confounders. Your analysis will therefore need to address confoundedness as evidenced by observed differences in the covariate distributions of the various exposure groups, and you can conduct descriptive analysis characterizing observed differences between treatment and control groups to complement qualitative information gathering about the treatment assignment process in order to guide your thinking about what variables will be necessary to include in the data to mitigate confounding.

COVID-19 Study

Expanding on our previous observation that older people are more likely to be taking doxazosin, we now consider how confounding can emerge in an observational study and the importance of addressing it. Without the deliberate recruitment and randomization of an RCT, doxazosin use will be concentrated among the older individuals eligible for our study because both hypertension and BPH prevalence increase with age (Partin et al., 1991; AlGhatrif et al., 2013). COVID-19 outcomes appear to be worse with increased age, suggesting that age is a confounder we must address. Even if doxazosin is effective at reducing all-cause mortality, doxazosin is disproportionately

prescribed to older people who disproportionately have worse outcomes. Unless we account for age, a truly beneficial treatment effect could be estimated with negative bias (possibly making the treatment appear harmful). This example from our COVID-19 observational study highlights the reasoning required to identify important covariates to consider in our analyses.

PREPARATION PHASE: ESTABLISH THE HYPOTHESES AND ACQUIRE RESOURCES TO EVALUATE THEM

Rule 4: Formalize the Research Goal

Specify the exposure in terms of quantity, duration, frequency, and recency. Define the comparison groups of interest (e.g., define *unexposed*). Bias (e.g., *selection bias*) can arise from many sources in an observational study, but it fundamentally stems from the lack of randomized exposure assignment, resulting in the construction of a control group having different concerns than the treated group with regard to censoring, missing data, self-selection, or even eligibility for treatment (Hernán et al., 2004). While confounding by indication is almost guaranteed to be present in non-experimental pharmacoepidemiology research and will be addressed in other rules, we highlight the importance now of identifying comparison groups in which every individual theoretically has some probability of receiving the proposed treatment. An example of questionable comparison group construction could be comparing two groups with the same disease but where the two groups take different drugs based on significant differences in disease severity (e.g., metformin for less advanced type 2 diabetes mellitus vs. insulin for more advanced type 2 diabetes mellitus). Next, define an outcome that is specific, measurable, and sufficient to answer the research question. Finally, formalize your hypotheses (i.e., specify the null and alternative, sidedness, primary vs. secondary exposures and outcomes).

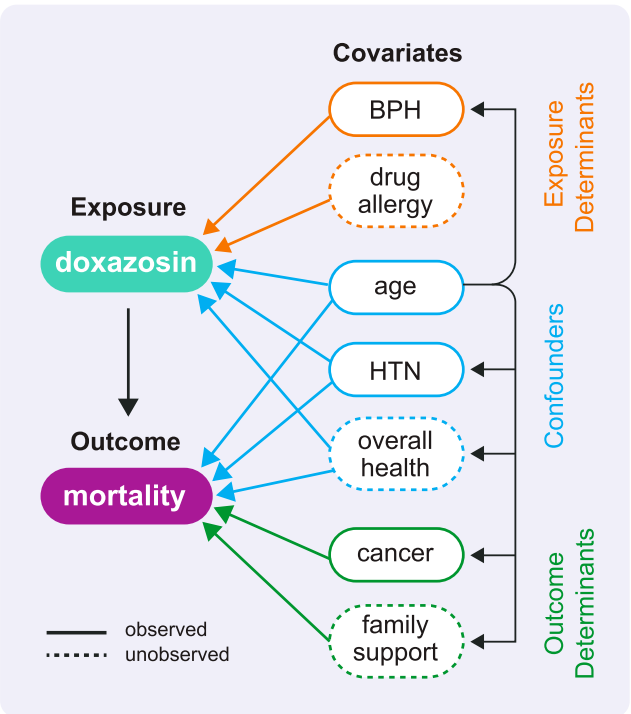

FIGURE 2 | This directed acyclic graph (DAG) shows the types of variable relationships described in Rule 3 using the example COVID-19 study. A DAG has no cycles, which means no variable can cause itself, either directly or through one or more other variables. In our effort to estimate the causal effect of doxazosin on mortality, this DAG helps us identify which variables will be important to adjust for in our analyses (in reality, this diagram would include many more variables of these same types). It is the set of confounders that has the ability to distort the association between exposure and outcome as revealed by the arrows leading from each confounder to both the exposure and the outcome. We highlight two observed confounders: the demographic confounder age and the comorbidity confounder hypertension (HTN). We also depict the unobserved confounder overall health, which we might attempt to measure using indicators of overall health like frequency and duration of recent inpatient stays.

COVID-19 Study

A pharmaceutical study considers a particular drug, dosage, recency, and duration by using prescription records to qualify a patient as either exposed or unexposed to the medication under investigation (e.g., doxazosin, ≥4 mg daily, prescription valid through COVID-19 diagnosis date, continuous use reflected by total days' supply covering 80% of the previous 3 months — a quantity known as the medication possession ratio or MPR (Andrade et al., 2006)). When quantifying duration and recency, multiple filled prescriptions for a drug better indicate continued use than a single fill that may have gone unused. Prescriptions lasting until some key date (possibly allowing for skipped doses) provide better evidence that the drug was in use on the date of interest. Unfortunately, researchers are usually unable to confirm the medication was consumed as intended. Some patients deviate from the prescribed drug regimen, and this is often unobservable; we therefore conduct *intent-to-treat* analysis by grouping patients according to inferred exposures revealed in prescription records (Gupta, 2011). The comparison group might include anyone who does not meet the exposure definition, only people who have not taken the proposed drug for a specified length of time, or perhaps only people who have never taken any alpha blocker. Importantly, the comparison group should not be made up of people who cannot take alpha blockers for reasons that could relate to their health outcomes.

As COVID-19 was entering its first peak, many countries' chief concerns were ventilator resources and anticipated deaths. Outcomes related to ventilator dependence or mortality may be of particular interest. We found that using ventilator dependence as an outcome is often problematic for two reasons. First, ventilator usage depends on the standard of care with respect to administering ventilator resources at a particular time and

place, and the severity of patients in the data as well as treatment protocols differed substantially by time and place during the pandemic. Second, insufficient ventilator availability and inconsistent ventilator coding practices makes ventilator dependence a complicated outcome in some places. All-cause mortality is not completely unaffected by the changing practices related to ventilators, but mortality proves to be the more clearly defined outcome of ultimate importance. Since we cannot quantify the exact role of COVID-19 in hospital deaths, the best practice is to use all-cause mortality as the primary outcome of interest.

Rule 5: Identify and Reason About Potential Confounders

Confounders will be present; make every effort to observe these confounders and adjust for them appropriately. Include standard demographic variables, relevant comorbidities, and a comorbidity index and/or other indicators of overall health. Note that identifying confounders before you have data will help you better assess the utility of candidate datasets. Organize your understanding of the key variables with a causal diagram (see **Figure 2**). A directed acyclic graph (DAG) is a powerful way to depict the causal relationships in your analysis (Greenland et al., 1999; Pearl, 2009) and examine potential biases your analysis might permit (VanderWeele et al., 2008). Bias might result from an unobserved confounder that is not measured in the data and therefore cannot be adjusted for in the analysis; a significant unobserved confounder can invalidate all results obtained from the study. Thinking through each variable and the corresponding existence and direction of arrows (representing both observed and unobserved cause-effect relationships) helps prevent unknowingly inviting bias into your analysis and mitigate potential sources of bias that you do include. Following procedures for identifying a minimally sufficient adjustment set (MSAS) of confounders in a DAG (VanderWeele et al., 2008) can eliminate adjustment-induced bias. Ultimately, a DAG provides an excellent visual representation of the known or assumed relationships between variables and helps identify the necessary variables to adjust for to minimize confounding in a multivariable analysis. Know that no matter what you do, you will likely still have unobserved confounding (we describe sensitivity analyses to quantify the magnitude of this issue in the Rule 9 supplement).

COVID-19 Study

Several alpha blockers (doxazosin included) have an FDA indication for hypertension, so we expect the exposed population will have higher rates of hypertension, a condition that might lead to worse outcomes. Relevant comorbidities that serve as confounders per clinicians' expertise include sex, age, diabetes mellitus, hypertension, cardiovascular disease, and chronic obstructive pulmonary disease. For the doxazosin hypothesis, patient location has significance as prescription practices and the standard of care for relevant conditions vary around the world. Even with these considerations, unobserved confounding can still affect a study's results. Unobserved confounding is one reason why the results of observational studies of hydroxychloroquine have differed from those of RCTs (Hernandez et al., 2020).

Rule 6: Operationalize the Target Population

Select the target population for your observational study to reflect the intended RCT population. Refine the potential study population by setting the inclusion and exclusion criteria to minimize confounding. Consider the impact of refining the target population on both internal validity (focused on groups the study includes) and external validity (focused on groups to which the findings might extend).

COVID-19 Study

In a COVID-19 retrospective cohort study, the defining characteristic of patients in the cohort is a COVID-19 diagnosis. In our observational study, the exposure was administered prior to the COVID-19 diagnosis. Using a post-treatment variable to define the cohort can introduce post-treatment bias, so choosing to select the sample on the basis of a post-treatment variable (COVID-19 diagnosis) implies we believe the exposure has no impact on one's susceptibility to infection and likelihood of diagnosis. We are aware of no evidence that taking doxazosin changes one's susceptibility to SARS-CoV-2 infection; doxazosin could, however, affect whether a person is diagnosed by mitigating symptoms to a degree that a patient self-treats rather than seeing a doctor to receive a formal diagnosis. Early in the pandemic, COVID-19 tests were only available in inpatient environments and were reserved for the sickest patients. Individuals were urged to stay home until they truly needed hospital resources. This led to many unobserved, undiagnosed patients. We cannot estimate the treatment effect in this population as we do not observe the qualifying condition: a COVID-19 diagnosis. Later in the pandemic, we face the same problem, but for a different reason; widespread community testing facilitates diagnoses, but these test results and diagnoses may not enter a patient's health records or claims history (both common data sources for retrospective studies). We could again lose visibility of milder cases where a patient recovers at home, limiting our assessment to the severe cases warranting hospitalization. This is a notable limitation of defining the cohort by a COVID-19 diagnosis.

We focus the doxazosin study on older patients because this group is at high risk of adverse outcomes from COVID-19. Older men in the United States take doxazosin at a far higher rate than women, primarily because doxazosin is a treatment for BPH. Compared to other men of the same age, a prior BPH diagnosis is not expected to have any impact on COVID-19 outcomes. We now make the consequential restriction to focus the study on older men, allowing us to capture many exposed individuals with no above-average risk for negative outcomes. This target patient population attempts to minimize the impact of unobserved confounding. While this may be appealing, the exclusions have important implications. Pragmatically, reducing the population under consideration may reduce statistical power by limiting the sample size. Societally, focusing the study exclusively on older men limits the study's internal validity to older men. It will take

additional assumptions and/or further analyses to extend the study's findings to women and young people.

Rule 7: Get the Best Data for the Study

Invest time in getting access to the best possible data for your study such that your desired study definitions can be realized. Know what your data source contains, where it originated, and how it was assembled. Know the biases and limitations of candidate datasets. Identify the target population using carefully selected, standardized diagnosis and/or procedure codes. Identify chronic comorbidities using standard condition code sets (Chronic Conditions Data Warehouse, 2020) and sufficient patient histories.

COVID-19 Study

Identifying COVID-19 patients can be difficult because of the nonexistence of COVID-19-specific International Classification of Diseases (ICD) codes early on in the pandemic. It was only on April 1, 2020 that ICD-10 U07.1 was introduced for a confirmed diagnosis of COVID-19, and adoption of this code for billing purposes remained variable and inconsistent for some time. Using an established, community-derived definition for the COVID-19 population is recommended (e.g., as provided by the National COVID Cohort Collaborative - N3C (National COVID Cohort Collaborative, 2020)). COVID-19 population definitions often divide into two groups: *COVID-narrow* includes confirmed COVID-19 diagnoses while *COVID-broad* adds suspected COVID-19 patients who have not been tested but exhibit multiple COVID-19 symptoms. Large hospitals that treated thousands of COVID-19 patients and performed in-house testing (e.g., Mount Sinai Hospital in New York City) are best situated to precisely construct a COVID-19 cohort (Wang et al., 2020).

In the early stages of a pandemic, finding a well-curated, sufficiently sized data set to test your hypothesis on the novel disease may be impossible. Expert clinical input may identify a suitable substitute for COVID-19 that reflects the same symptoms and disease progression your treatment is theorized to target (e.g., cytokine storm syndrome resulting from acute respiratory distress or pneumonia). Identifying such a disease with established coding and extensive patient records can jumpstart your research while the data practices surrounding an emerging pandemic stabilize.

The hypothetical doxazosin study requires access to each individual's inpatient, outpatient, and prescription drug history for at least the year leading up to COVID-19 diagnosis. Clinical data from the U.S. Veterans Health Administration (VHA) is an ideal candidate data set for this type of study for several reasons. Older adults are well represented in the VA health care system, typically with extensive patient histories. This reduces the likelihood of having the insufficient patient histories that sometimes accompany individuals in a claims database who have recently changed employers. In addition, the VA health system would have comprehensive records: diagnoses, procedures, prescription drug use, doctors' notes, in-hospital medications received, and lab results.

ANALYSIS PLANNING PHASE: DEVELOP AND REFINE THE ANALYSIS PLAN

Rule 8: Explore and Model Your Data With Surrogate Outcomes

Use permuted outcomes or synthetic data (Koenecke and Varian, 2020) as you build and test your analysis code to prevent being influenced by any premature results. First, examine the univariate and pairwise distributions of the covariates that will be used in the analysis. Second, examine all covariate distributions after stratification by exposure group and/or time period, compute each individual's propensity for treatment (i.e., estimate a propensity score), and obtain better empirical overlap using propensity trimming (Lee et al., 2011). A propensity score reflects the probability that an individual would receive treatment (i.e., belong to the exposed group) on the basis of observed covariates. To counter confounding by indication, a variety of analytical techniques employ propensity scores to balance the exposed and unexposed groups by matching or weighting using propensity scores, which assign greater weight to the unexposed individuals who appear more similar to the exposed individuals in terms of the observed covariates. Third, begin modeling with an unadjusted modeling approach (e.g., simple logistic regression) to establish a baseline treatment effect estimate. Finally, use additional modeling approaches that adjust for confounders (e.g., doubly robust methods (Bang and Robins, 2005) employing propensity scores and covariate adjustment in the outcome models), favoring methods that seek covariate balance.

COVID-19 Study

Examining the covariate distributions of the exposed and unexposed groups will likely reveal that doxazosin users are generally older and have more comorbidities than non-users. Unadjusted models with no consideration of age would likely compare a younger, healthier unexposed group to an older, less healthy exposed group. We addressed this problem by including age as an observed confounder and by establishing inclusion/exclusion criteria that ensured anyone in the study could reasonably have been exposed to doxazosin. Now, we further exclude observations exhibiting extremely high or low propensity for treatment (on the basis of all covariates, not just age); this could include the extremely young, old, healthy, sick, etc. Extreme propensities indicate that almost all similar units share the same treatment assignment, such that there is limited information in the data about how similar individuals would have fared if their treatment assignment had been different.

Rule 9: Augment the Main Analysis With Extensive Sensitivity Analyses

Plan a thorough assessment of the robustness of your results to the many choices made along the way to estimating a treatment effect. Start by conducting supplementary analysis designed to illustrate clearly the role of observed confounders for both treatment assignment and outcome modeling, as this can build intuition about what factors are likely important in these

processes (Athey et al., 2017). Quantify the extent of unobserved confounding required to change your conclusions (Rosenbaum and Rubin, 1983; Rosenbaum, 2010; VanderWeele and Ding, 2017) (i.e., determine how correlated an unobserved variable must be with the exposure and outcome to nullify any perceived treatment effect). Assess the robustness of your results to different modeling techniques, hyperparameters, outcome definitions, exposure definitions, inclusion/exclusion criteria, and other aspects of the study design. Explore additional sets of covariates, including different comorbidities and indicators of temporal health trends. Conduct negative outcome experiments and treatment control experiments (Lipsitch et al., 2010). Refine, lock in, and preregister your formal analysis plan before examining any real model outputs using the true outcome data.

COVID-19 Study

Robustness checks for a doxazosin study assess the impact of making adjustments to the treatment, outcome, and population definitions. We can test our hypothesis on both a COVID-narrow cohort and a COVID-broad cohort. Our confidence in the treatment will also be tied to how well our results hold up to changing the medication possession ratio and changing the post-diagnosis window we are monitoring for all-cause mortality. We can explore additional covariates beyond chronic comorbidities that may indicate increased health concerns closer to the COVID-19 diagnosis (e.g., other inpatient stays within 2 months of diagnosis).

EXECUTION PHASE: EXECUTE THE ANALYSIS PLAN AND REPORT THE RESULTS

Rule 10: Execute, Summarize, and Share (With Caveats)

Execute your analysis plan with the true outcome data once you are satisfied with the quality of your data set and have sufficiently tested your code. If necessary, make the smallest possible refinements to your analysis plan and execute again, always ensuring you report deviations from your preregistered plan. Give your reader something that looks like what they are used to seeing (i.e., conventional measures of treatment effect, standard tables and figures). Explicitly describe the limitations of your study. Provide all the necessary method descriptions and code to facilitate replication.

COVID-19 Study

We include a CONSORT diagram to show the split of doxazosin users and nonusers in the dataset, followed by their respective outcome counts, to help visualize the study like an RCT. We are targeting a clinical research-savvy audience including clinical trialists, so we present the treatment effect as an odds ratio (OR), which is a familiar metric for the likely readers. We define our null hypothesis as OR = 1 (i.e., the exposure does not change the odds of the outcome occurring). We then assess doxazosin to be beneficial if we find OR < 1. We present the associated confidence interval (CI) to convey the precision of our treatment effect estimate. Together, the OR and CI indicate the strength of evidence supporting further investigation of the doxazosin hypothesis.

CONCLUSION

As the pandemic is far from over, especially in lower resource countries and communities, we see the value both now and in future pandemics of responsibly investigating the efficacy of inexpensive, repurposed drugs as early treatment options while we wait for vaccine development, mass production, and global distribution. The primary benefits associated with conducting these investigations with retrospective analyses lie in reducing costs and increasing speed relative to running an RCT (assuming the RCT would be feasible and ethical). Moreover, retrospective pharmacoepidemiological analyses can be run even when no patients are available (e.g., after everyone is vaccinated) to learn more about potential treatments for future pandemics. Retrospective analyses make it easier to explore a variety of treatments with limited time and other resources, setting the stage for an RCT to test the most promising interventions. In the COVID-19 era, these are valuable benefits, but they come with a cost. The challenges facing retrospective analyses arise from the requirement to use data generated without a particular study in mind. Unlike an RCT, where researchers are able to decide exactly who will be recruited to participate, which exposure(s) will be assessed (e.g., drug, dosage, frequency, duration, etc.), and which outcome(s) will be measured, the observational study approach described here limits the researcher to only those definitions of exposure, outcome, confounders, and sample population that can be realized with available data. This places a significant burden on the researcher to determine whether the desired retrospective analysis is possible to conduct with available data. When the time and cost savings of performing a study with observational data outweigh the costs of constrained data collection and study design, using these 10 rules as a guide will support the execution of a rigorous retrospective pharmacoepidemiological analysis that speeds the time to clinical trials and, hopefully, proven effective treatments for patients.

SUPPLEMENT: HOW TO FOLLOW THESE 10 RULES

This supplement serves to explain in detail the many recommendations made in the 10 rule paragraphs in the main text. Individual sentences in the rule paragraphs generally correspond to one or more paragraphs in this supplement explaining why the recommendation was made and how to satisfy its requirements.

Guiding Principles: Build and Focus the Team

Rule 1 Supplement: Form a Multidisciplinary Team

The main text states we require continuous input reflecting different kinds of domain expertise: medical, data sources,

epidemiology, and causal analysis. Medical expertise ensures the study remains medically coherent while decisions are made throughout the design of the study. Data source expertise (including medical terminologists) can expedite the process of finding, accessing, and understanding relevant data sources and corresponding coding conventions, while also making known their potential limitations. The expertise in epidemiology that comes from working with observational health data ensures the study design and study definitions meet accepted standards in the literature (e.g., defining treatments, conditions, and other health indicators with observational data). Causal inference expertise ensures the use of appropriate analysis methods to support making a causal claim. The degree to which each expert contributes in each successive rule varies, but it is difficult to underestimate the value of assembling this group at the start.

Rule 2 Supplement: Emulate a Randomized Controlled Trial

Design your observational study to mimic — as closely as possible — a randomized controlled trial with similar goals, an approach known as *trial emulation* (Rubin, 2004; Rosenbaum, 2010; Hernán and Robins, 2016; Dickerman et al., 2019). To start down this path, we must first clearly state the research objective. Most likely the clinician(s) on the team will be the source of the medical hypothesis. What is the pathophysiological mechanism this study seeks to understand? Which exposure(s) might reasonably affect this mechanism? Which subset of the population do we think the exposure(s) will benefit? Who could reasonably be eligible to receive the proposed exposure? Which measurable outcome(s) will reveal the efficacy of the proposed exposure(s)? Which analyses will be needed to do the appropriate comparisons? These details will continue to be refined as we think through the remaining rules, and we will rely on the team's clinical expertise to ensure any refinements continue to support the primary research objective.

Carefully consider what you measure, when you measure it, and in whom you measure it. It can be helpful to lay out key aspects of the study design just as would be done in an RCT using a CONSORT flow diagram (Begg et al., 1996) and other observational study reporting standards (Benchimol et al., 2015; Langan et al., 2018). For example, a person considered for trial participation must be deemed eligible for the trial at the time of exposure group assignment, which must then occur before any follow-up periods begin or outcomes are observed. Suppose your ideal trial has an exclusion criterion barring participation of anyone with a history of heart problems. Heart problems that surface at some point after a person receives the exposure might be visible in observational data; since post-exposure health problems could not have been observed for the purposes of RCT enrollment, we ignore them when deciding the eligibility of patients for observational studies (Dickerman et al., 2019).

Preregister your study and analysis plan just like an RCT. Before an RCT begins, the individuals running the trial will have already amassed a corpus of information about the relationship between the exposure and outcome (e.g., in preclinical data). They have used this information to design the trial and get approval from an institutional review board (IRB). Given this information, the study plan is fixed prior to collecting any patient information in the actual trial phase. The trial emulation proposed in this paper similarly promotes an exploratory data analysis and modeling phase that uses surrogate outcome data to refine the analysis plan before committing to a final outcome analysis to be run on actual outcome data (discussed further in Rules 8–10). Preregistering the study and documenting a final analysis plan avoids several pitfalls associated with the recent replication crisis: questionable research practices (John et al., 2012), HARKing -- hypothesizing after results are known (Kerr, 1998), gardens of forking paths (Gelman and Loken, 2014), and p-hacking (Schuemie et al., 2018). Avoiding these pitfalls is particularly important in a pandemic study since even preliminary results from individual studies can have profound policy and public health implications, as well as implications for ongoing clinical trials (Piller and Travis, 2020). While the idea of preregistration in observational studies continues to grow in popularity, the effectiveness of the practice has notable limitations. For example, often the data has already been collected and been available for research prior to a study's preregistration, making it hard to verify whether preregistration actually preceded the reported analysis.

Recall the assumptions necessary in order to make a causal claim. A key premise of an RCT is that the exposure assignment is random; in particular, exposure assignment is independent of factors that affect patient outcomes. To facilitate random exposure assignment, the study inclusion/exclusion criteria in an RCT must be designed to ensure that every trial participant can reasonably be assigned to any exposure group. Random exposure in an RCT is then accomplished by arbitrarily assigning people to either of the exposed or unexposed groups using a coin flip, or in the case of a stratified RCT, a coin flip that depends only on observed pretreatment factors. Our inability to achieve random exposure in an observational study means we must make some assumptions to estimate treatment effects when we do not observe all of the patients' potential outcomes (e.g., both the exposed outcome and the unexposed outcome for each patient when there are two exposure groups). Here we state one of the acceptable sets of assumptions for conducting a retrospective analysis. First, theoretical *overlap* ensures that for any possible set of values of pretreatment traits (i.e., patient characteristics), there is a non-zero probability of being in either group. Lack of overlap might occur in practice if patients with certain characteristics are either excluded from the exposure group or always assigned to the exposure group (e.g., the exposed group only contains adults while the unexposed group contains both children and adults). Second, the property of *unconfoundedness* (also known as *strong ignorability*) ensures that exposure assignment is independent of the potential outcomes given the observed covariates. Of these assumptions, overlap can be verified empirically, but there is no test to prove we have satisfied the unconfoundedness assumption.

Finally, we assume (both in observational studies and RCTs) that the specific exposure assigned to one individual does not interfere with the exposure or potential outcomes of any other individual in the study. For example, interference may occur when one patient in an RCT receives the exposure and is cured,

which may then free up hospital resources to the benefit of an unexposed patient in an adjacent room. Furthermore, the exposure must be the same for everyone in an exposure group (e.g., identical drug regimen). Together, these two criteria comprise the Stable Unit Treatment Value Assumption (SUTVA) (Imbens and Rubin, 2015).

A gold-standard randomized controlled trial satisfies all of these assumptions by construction; however, the lack of randomized exposure assignments in an observational study means there is significant work associated with emulating an RCT as closely as possible. It is almost certain that meaningful differences exist between the exposed and unexposed groups, and that the factors that differ are also related to outcomes. Confounding by indication is likely to occur in observational data, and the primary concern in your observational study is the identification and mitigation of potential confounders, which is the basis of Rule 3.

Rule 3 Supplement: Realize That Rule 2 Is Impossible and Proceed Carefully

Recall the different kinds of covariates in a causal analysis and how each can impact causal estimates. The lack of randomized exposure assignment in an observational study forces us to address the pretreatment variables that we observe in our data. Given that we are seeking to determine the causal effect of an exposure on an outcome, there are three types of observed variables that can exist in relation to this study. The first, outcome determinants, affect the outcome but do not directly affect the exposure. While you can include outcome determinants in your analysis to improve the precision of your causal effect estimate, a causal analysis can proceed without them. The second, exposure determinants, affect the exposure but do not directly affect the outcome. Exposure determinants will also not affect our analysis because there will be zero covariance between the outcome and the exposure conditional on these variables. A note beyond the scope of this paper: econometric analysis can reveal whether any of these exposure determinants is a *strong instrumental variable*. In this case, a separate instrumental variables analysis (Hernán and Robins, 2006) is preferable for studying the effect of the exposure on the outcome by exploiting the fact that the instrumental variable's effect on the outcome definitionally only exists via the exposure. The third type of variable affects both the exposure and the outcome; these are known as *confounders* and are the essential variables to identify for your study.

Think hard (and then think harder) about confounders for your study. As defined in the main text, confounders satisfy three properties: they are associated with the outcome (i.e., risk factors), they are associated with the exposure (i.e., they are unequally distributed among the exposure groups), and they are not effects of the exposure (Jager et al., 2008). Identifying important confounders requires collaborating with specialists who can make appropriate clinical recommendations; for example, one might learn that there exists a *comorbidity* (an additional, simultaneously occurring disease or condition) for which patients would be taking the exposure drug. This comorbidity would be considered the indication or reason for prescribing the drug (as listed in the US prescribing information, though clinicians may prescribe for other reasons). Perhaps this comorbidity typically leads to worse outcomes given the worse overall health of these patients. Such a comorbidity would be a confounder; other common confounders include demographic variables such as age and sex.

Make a plan to address non-overlap and confoundedness. First, we must recognize that we only have data for observed confounders (as opposed to unobserved confounders, for which we have no data, and which in general lead to bias in estimates of causal effects). To address non-overlap, we must ensure that for any observed combination of confounder values, there are patients with very similar observed combinations of confounder values in each of the exposed and unexposed groups, even if presence in one group is more likely than another. If there are any combinations of confounder values for which the probability of exposure is either zero or one, it is impossible to estimate the treatment effect for patients with those confounder values. As a practical matter, the associated observations should be excluded to achieve overlap; the target population for which we estimate the treatment effect is correspondingly narrowed. To deal with confounders, we must mitigate the non-random exposure assignment in our data by ensuring similar distributions of confounder values between exposed and unexposed groups. There are two main approaches to doing so: outcome modeling and covariate balancing; when combined, the approaches may be doubly robust in that they are still valid if errors are made in either modeling or balancing (but not both), as discussed in more detail in Rule 8. Outcome modeling builds a model of the relationship between covariates and outcomes, allowing the analyst to adjust for the impact of differences in covariates across groups on differences in outcomes. Covariate balancing attempts to reweight or subsample from data such that the exposed and unexposed groups are comparable in terms of covariates, so that the covariates are no longer associated with exposure in the new, reweighted data; this can be accomplished, for example, through sample restriction with inclusion/exclusion criteria, reweighting by inverse propensity scores (probability of assignment), stratification, or matching (Stuart, 2010) on confounders. Note that almost certainly there exists unobserved confounding in any observational study, and unobserved confounding distorts our view of the exposure-outcome relationship. If we believe there is an important unobserved confounder, it may be appropriate to abandon the study or use a different approach (e.g., instrumental variables analysis). We will address unobserved confounding in greater detail in Rule 5 and how to account for it with sensitivity analyses in Rule 9.

Preparation Phase: Establish the Hypotheses and Acquire Resources to Evaluate Them

Rule 4 Supplement: Formalize the Research Goal

Specify the exposure in terms of quantity, duration, frequency, and recency. The study's purpose is to evaluate the efficacy of this

exposure, and this should dictate your first step in formalizing the research goal. The proposed exposure in a pharmaceutical-based hypothesis involves identifying a set of drugs for testing. At a minimum, this requires labeling each patient in the study as exposed or unexposed to one of the drugs in question; doing so requires completing two tasks. The first task is for the clinician team to specify the precise list of drugs and corresponding dosages they wish to include as the exposure drug set based on the pathophysiological mechanism they wish to target. The second task is to determine the timing of the observed drug exposure. For example, does it matter if the patient is a current, recent, or historical user of the drug at the time of the patient's diagnosis (Pazzagli et al., 2018)? How long must a patient have used the drug to be part of the exposed group? These questions directly relate to the pathophysiological mechanism the proposed treatment aims to target, and the answers to these questions may have implications for the degree to which the study can truly emulate an RCT. Note that every consideration above also applies to analysis of a non-pharmaceutical exposure. Investigating the effectiveness of a non-pharmaceutical therapy requires the same attention be given to defining the precise list of qualifying therapies as well as the quantity, duration, frequency, and recency of any treatment a patient received.

Define the comparison groups of interest (e.g., define unexposed). If you could do a randomized experiment, what other exposure groups would you randomly assign people to for comparison? In a pharmaceutical study, this could include taking a placebo, taking an active comparator (an alternative treatment known to be effective), or even taking the same drug according to a different regimen. Defining a comparison condition requires the same level of detail required for the exposure definitions. Most likely the comparison condition represents the existing standard of care, and the purpose of the study is to see if the hypothesized exposure provides an improvement over the standard care. As you define the exposure and comparison conditions, it may well be the case that some individuals meet none of these group definitions and must accordingly be excluded from the study. For example, some patients may fall just short of qualifying as exposed (e.g., too few days on the proposed drug treatment, too small a dosage), but their classification as unexposed would be inappropriate as well.

Define an outcome that is specific, measurable, and sufficient to answer the research question. Defining an outcome includes clearly stating exactly what will be measured, when it will be measured, and how it will be measured for all patients in the study. The outcome must be observable in a consistent manner for all patients in your study. Thoughtful consideration should be given to the followup time required to observe the outcome in both exposed and unexposed patients. Additionally, for outcomes other than mortality, competing risks may prevent observing the outcome of interest (e.g., loss to follow-up in a lengthy study).

Formalize your hypotheses. At this point in the team's preparation for the study we have clearly defined the exposure(s) and outcome(s) and are ready to articulate the causal effect of interest. This involves clearly stating the specific null and alternative hypotheses your analysis will test; determine if a one-sided or two-sided test is more appropriate for your medical hypothesis. Commit to the primary and secondary exposure and outcome definitions, target population, and outcome-focused results you believe will produce a credible analysis. Note that the hypothesis is based on definitions that reflect what you hope to observe, and they may not be what you can actually find in an available data set (discussed further in Rule 7).

Example Application of Rule 4 to the COVID-19 Study

This retrospective study estimates the causal effect of baseline use of doxazosin (daily dose ≥4 mg with prescriptions covering the day of COVID-19 diagnosis and at least 80% of the previous 3 months) compared to nonuse (no prescriptions for any alpha blocker in the previous year) on reducing all-cause mortality in adults over 45 years old who have been diagnosed with COVID-19. We state the following hypotheses for the odds ratio (OR) associated with the treatment effect on all-cause mortality:

$$H_0 : OR \geq 1, \quad H_A : OR < 1.$$

Rule 5 Supplement: Identify and Reason About Potential Confounders

Confounders will be present; make every effort to observe these confounders and adjust for them appropriately. Consider a study wherein patients are prescribed a drug to treat a certain disease with varying degrees of severity. A high dosage tends to be prescribed for patients with a more severe case of the disease, whereas a low dosage tends to be prescribed for patients with a less severe case of the disease. It would be no surprise to find that patients with severe cases have worse outcomes as a group - even if the drug (and dosage) they are taking is the best option for their individual situations. In observational data, dosage level is inherently related to severity of illness. Hence, severity of illness is a confounder because it affects the exposure-outcome relationship; if left unobserved, severity of illness could irreparably confound any study results. The circumstances surrounding the administration of an exposure can also make observing confounders challenging. For example, suppose we are studying the efficacy of a drug for preventing death from an acute condition, and the drug is typically given as a last resort to patients who are nearing death from that condition. Then it may be difficult or impossible to observe the factors that affect both exposure and outcome, since not all factors that lead a physician to believe that the patient is at high risk of death will be recorded. During some time periods in the COVID-19 pandemic, different drugs (such as hydroxychloroquine) were given off-label to the sickest patients. In such circumstances, receiving the drug is an indication that the patient was very ill. In contrast, if we study exposure to a drug that was prescribed for a chronic condition long before a patient developed COVID-19, then exposure will not be determined by the patient's severity of symptoms from COVID-19. For example, some underlying factor such as hypertension might be related to both drug exposure and risk of poor outcomes from COVID-19, so it will still be important to carefully adjust for all such factors.

Include standard demographic variables. Common demographic covariates such as sex and age (including nonlinear transformations

like age-squared) are standard confounders to consider, appearing in nearly all epidemiological models. Another variable to consider is the time or location of the sample-defining diagnosis (e.g., a positive lab test or clinician diagnosis). Diseases like influenza often change from year to year in terms of which strains are more prevalent, and the geography of outbreaks may not be uniform. Depending on how fast a disease mutates or the standard of care changes, capturing the year, month, or even week of diagnosis, and/or hospital or patient location, may be important covariates when examining observed outcomes.

Include relevant comorbidities. A confounding comorbidity is one that impacts both exposure assignment and outcomes. Other comorbidities may be unrelated to the proposed exposure but could still be helpful as proxies for confounders by identifying which patients are already at higher risk for severe outcomes based on components of their health beyond basic demographics (e.g., cancer or heart failure). Still other comorbidities might serve as proxies for the proposed treatment; running an analysis that includes these comorbidities may lead to "post-treatment bias" because the comorbidities would appear as concurrent treatments, hence reducing the estimated treatment effect of the actual treatment. Post-treatment bias can also result from considering post-treatment traits. For example, controlling for emphysema when examining the causal effect of smoking on lung cancer would likely transfer some of the treatment effect from smoking to emphysema, which we might assume to have resulted from smoking. Choosing to consider a confounder that was observed post-treatment requires a deliberate assessment of the potential causal relationship between the exposure and the observed trait. For example, if an observed comorbidity is of a chronic nature, it may be unlikely that a recent exposure caused the comorbidity; most likely the unrelated condition prompting the exposure led to the healthcare encounter where the comorbidity was first diagnosed. Another class of variable to avoid is known as a collider. A collider is a variable that can be considered an effect of both the exposure and the outcome; controlling for such a variable introduces bias in the effect estimate.

Include a comorbidity index and/or other indicators of overall health. The Elixhauser comorbidity score (Elixhauser et al., 1998) and Charlson comorbidity index (D'Hoore et al., 1993) are two established measures combining various observed medical conditions in order to serve as more general indicators of overall health than an individual, disease-indicating covariate. The potential for unobserved, general health problems can also be addressed by looking at a patient's recent health care encounters and prescription data. Encounter-related covariates may include the number of inpatient or outpatient visits occurring in the year preceding the relevant diagnosis, the duration of inpatient stays (i.e., the number of days the patient had been in the hospital in the previous year), and indicators for whether the comorbidities listed above were observed closer in time to the relevant diagnosis (e.g., within two months prior rather than within one year prior). Considering the recency of documented health concerns is useful for establishing whether a declining health trend exists both at the individual level and at the level of comparing different exposure groups. You may also want to consider certain procedures in addition to diagnoses (e.g., colonoscopies, flu shots (Jackson et al., 2006)), which can also serve as indicators of overall health and/or access to health care. As with all of our confounders, remember to ensure that any indicators of overall health only capture pretreatment health conditions.

Know that no matter what you do, you will likely still have unobserved confounding. Failing to include unobserved confounders in an analysis leads to omitted variable bias, which violates the unconfoundedness assumption. As indicated above, the missing confounders we are most concerned with relate to unobserved indications of poor or declining health; however, these may not always be available. If you determine a set of critical confounding variables and find that some are unobservable (either directly or via a proxy variable), we can investigate the potential magnitude of this unconfoundedness violation (in some cases, your proposed study may be too flawed to justify pursuing it). There is certainly a bit of tension here as we perform analysis under the assumption of unconfoundedness while simultaneously acknowledging the likelihood of unobserved confounding. We address this tension with sensitivity analyses described in Rule 9.

Example Application of Rule 5 to the COVID-19 Study

This retrospective study considers the following confounders: sex, age, diabetes mellitus, hypertension, cardiovascular disease (acute myocardial infarction, ischemic heart disease, heart failure), chronic obstructive pulmonary disease, patient location, Elixhauser comorbidity score, inpatient stays in the prior year, inpatient stays in the prior 2 months, inpatient days in the prior year, and inpatient days in the prior 2 months.

Rule 6 Supplement: Operationalize the Target Population

Select the target population for your observational study to reflect the intended RCT population. Patient selection is a key task in RCTs, and an observational study emulating an RCT should implement the same inclusion and exclusion criteria as the RCT. Given that an RCT likely excludes individuals with certain comorbidities, one benefit of an observational study is the opportunity to conduct a subanalysis of individuals that the RCT would exclude.

Refine the potential study population by expanding the inclusion and exclusion criteria to minimize confounding. In Rule 5 we described many types of potential confounders; in Rule 6 our objective is to find a subset of the population who may receive the exposure of interest for reasons that have minimal expected impact on the outcome of interest (i.e., minimal confounding); importantly, these individuals should also include candidates to remain unexposed. There is no rule of thumb for this, but rather it is through the creative efforts of your team that you can specify a target population refinement that can still potentially answer the research question while significantly reducing confounding. Note that changing the sample inherently changes the estimand, and there is often a tradeoff between studying the population that is of greatest interest and studying the population where estimates are most credible.

Consider the impact of refining the target population on internal and external validity. Minimizing confounding is desirable as it increases the internal validity of the study, but excluding certain groups from the study may limit the external validity of the results to only the refined population under study (Imai et al., 2008; Rudolph et al., 2014). Consider again a scenario where a drug is administered in some cases for conditions with serious health risks and in other cases as more of a lifestyle drug. If we exclude from our study any patients with the more serious condition, we can likely achieve more similar exposed and unexposed groups, which is important for attributing any difference in expected outcome to the exposure under investigation. The cost is not knowing how those with the more serious condition fare with the exposure versus without the exposure. Additionally, there is an important emerging literature on demographic fairness with regard to clinical studies (Holdcroft, 2007). Be careful in your efforts to minimize confounding so that you do not unintentionally or unnecessarily exclude a portion of the population that also requires study.

Example Applications of Rule 6 to the COVID-19 Study

1) This retrospective study focuses on adults over 45 years old to maintain internal validity for all older adults. 2) This retrospective study focuses on adult men over 45 years old to minimize confounding by focusing on a large group of people that use doxazosin for a condition unlikely to affect COVID-19 outcomes (BPH).

Rule 7 Supplement: Get the Best Data for the Study

Invest time in getting access to the best possible data for your study. Above all else, this means the target patient population is sufficiently represented in the dataset. Recognize that data access and sharing may be challenging; any health care data you use will often have data access restrictions due to legal and/or privacy concerns, proprietary interests, or other competitive barriers (Byrd et al., 2020). Typically, IRB approval, an IRB waiver for de-identified data, or business associate agreements enable data access and permit its use for your specific research objective.

Know what your data source contains, where it originated, and how it was assembled. Having someone on the team who knows the data source well helps the team avoid the early stumbles that inevitably happen while working with new data. The best data sources will capture data on the population, exposure, outcomes, and covariates relevant for a study. Once you acquire access to potential datasets, consider the reliability of the data collection (e.g., provenance, missingness, measurement error, trends over time, and sampling or representativeness of the target population). While we recommend defining your ideal exposure(s), outcome(s), and target population first, you may have to revise some of these definitions to be compatible with the existing dataset or combination of data sources (e.g., claims data, labs, or electronic health records from multiple participating hospitals).

Know the biases and limitations of candidate datasets. It is likely the case that no single data source is sufficient to represent the broader population. The ideal data source would have extensive electronic health records with thorough patient histories documenting inpatient and outpatient encounters, diagnosed conditions, and drug prescription and fill data. Outside of national healthcare systems or other integrated systems such as the US Veterans Health Administration (VHA) and Kaiser Permanente, obtaining all relevant information about a specific patient from a single source is rare. Often, hospital data will not have extensive pre-hospitalization data (if any), and claims databases will lack the rich details of hospital records (e.g., clinicians' notes and lab results). Further, observed outcomes in patient groups from different data sources may not always be indicative of what is expected in the broader population. Certain types of hospitals (e.g., tertiary care centers) may handle more advanced cases of a disease and have higher rates of certain outcomes in their electronic health records data. Some insurance claims databases may only represent the portion of the population that is employed, has healthcare insurance, and has demonstrated access to healthcare services. Each data source may also be idiosyncratic according to varying standards of care and coding practices for the time, location, and patient groups it represents. The information that appears in health data can also reflect payment systems and incentives; for example, minor hospital procedures may not appear in claims databases because insurers may not pay for them directly. It is important to know and understand these issues before trying to run your models across different datasets, only to be confused by the inconsistent results. The best approach is to evaluate your hypothesis using as many appropriate data sources as possible and look for consistently observed effects across data sets.

Obtain a sample of the target population using carefully selected, standardized codes. The typical way of identifying patients for a cohort study involves selecting patients with a documented record of a particular disease or medical procedure, most often by means of an International Classification of Diseases (ICD) code (e.g., ICD-10-CM Clinical Modification). Many diseases and procedures have a large number of codes delineating the various subtypes of the disease (e.g., pneumonia) or procedure (e.g., mechanical ventilation), so a careful inspection of the potential list of qualifying condition codes is necessary to properly define the intended sample. If possible, attempt to validate the cohort by also checking for confirmatory lab tests and/or prescribed medications, which may or may not be available in your data.

Identify chronic comorbidities using standard condition code sets and sufficient patient histories. The data you will need for a cohort study must contain some mechanism for observing the confounders you identified in Rule 5. Diagnoses for comorbidities, much like the diagnoses used to define our target patient population, can include a broad range of ICD codes for each disease or condition. Identify comorbidities by using a standard set of ICD codes that medical researchers generally agree encompass the common comorbid conditions, such as the Chronic Conditions Data Warehouse (CCW) (Chronic Conditions Data Warehouse, 2020) produced by the Centers for Medicare & Medicaid Services (CMS). You will need reasonably long-duration patient histories (e.g., 12+ months of

inpatient and outpatient records preceding the diagnosis meriting inclusion in your study's cohort) to ensure adequate opportunity to observe relevant comorbidities in patient records. As a general rule for most chronic conditions, we recommend considering a patient to be positive for a given chronic condition if any of the listed condition codes in a standard code set is referenced as a diagnosis on any inpatient or outpatient record in the 12 months preceding the qualifying diagnosis. In turn, researchers should exclude any patient that cannot be tracked in the data for that entire lookback period (e.g., in insurance claims data, if the patient was not continuously enrolled during that time). The clinicians and data source experts on the team should determine whether any alternate criteria should be considered (e.g., multiple codes, multiple occurrences, different lookback period, lab values, and procedure codes).

Make your study definitions realizable in your data. It should be expected in database-facilitated research that not all desired quantities may be available. For example, rarely can we know what medication a person actually consumed; instead, we observe what was prescribed and filled. An insurance claims database does not generally record indicators of a patient's lifestyle such as body mass index (BMI), alcohol use, and smoking status (though they could be very useful); they may not record certain demographic and socioeconomic data (also relevant for many diseases and hypotheses). Instead, an insurance company needs to know which diagnoses were given and which procedures were administered for claims reimbursement purposes. As you look for data that allow you to operationalize your study definitions for exposure, outcome, confounders, and target population, you may be forced to adjust those definitions to reflect what is in the data. You must carefully assess whether what you do observe is close enough to what you wish you could observe to be sufficient for the research question.

Example Application of Rule 7 to the COVID-19 Study

This retrospective study uses Veterans Health Administration data with patients identified according to the National COVID Cohort Collaborative's COVID-broad criteria. Pretreatment comorbidities are identified by searching each patient's inpatient and outpatient records (electronic health records or insurance claims) for the presence of a qualifying ICD code for each of several comorbid conditions according to the comorbidity-specific ICD code sets provided by the Chronic Conditions Data Warehouse.

Analysis Planning Phase: Develop and Refine the Analysis Plan

Rule 8 Supplement: Explore and Model Your Data With Surrogate Outcomes

Use permuted outcomes or synthetic data as you build and test your analysis code. In an RCT, blinding prevents patients and clinicians from knowing exposure group assignments, which might affect their respective actions. In observational studies, the concept of blinding relates to only seeing what you have to see to accomplish a certain task. Research team members can be blinded to the exposure, the outcome, and potentially even the hypothesis (Berman and Parker, 2016). We start this rule by blinding ourselves to the outcome because all code goes through a debugging phase, and there is a risk that, at least subconsciously, you might be influenced by frequently seeing a range of results from different methods, confounder/covariate sets, etc. As you proceed with your analysis, you may discover that certain covariates are either sufficiently sparse or so highly correlated with other covariates that issues of numerical stability arise with certain modeling approaches. As you encounter these issues and fine-tune your list of covariates, it is best that these modifications be made without subjective bias arising from prematurely observing any effect estimates. Remember, the purpose here is to specify the details of the analysis plan and to implement working code, not to produce a final causal effect estimate just yet. If a step can be performed with surrogate outcome data for the purpose of testing, it should be.

Examine the univariate and pairwise distributions of the variables (or covariates) that will be used in the analysis. This serves to assess any issues with missingness, data entry errors, and the accuracy of any constructed variables. Also important is the opportunity to assess these distributions for their adherence to known or believed attributes of the population under study.

Examine all covariate distributions after stratification by exposure group and/or time period. A key claim in any retrospective analysis, as mentioned in Rule 3, is that the exposed and unexposed groups either have similar covariate distributions or that the authors have done something to address the fact that the distributions are meaningfully different. The difference in the exposed and unexposed groups' covariate distributions is typically referred to as "covariate balance," which should be calculated and visualized before and after employing certain types of models (Austin, 2009).

Achieve better empirical overlap using propensity trimming. Propensity scores quantify each patient's likelihood of receiving the exposure conditional on the observed covariates. There may exist observations in your data that possess combinations of covariate values that are only ever observed in either the exposed group or the unexposed group, but not in both (leading to uncommonly high or low propensity scores). This violates the overlap assumption we required in Rule 2 (while this statement applies as written to categorical variables, a relaxed version still applies to continuous variables where exact matches are unlikely). A standard technique to maintain overlap is to remove such observations from the data by trimming on the basis of propensity scores (i.e., restricting the sample to areas with propensity score overlap). There are many common approaches to calculating propensity scores; the R packages *grf*, *twang*, and *MatchIt* calculate propensity scores using honest forests, generalized boosted models, and logistic regression, respectively (Ho et al., 2011; Athey et al., 2019; Ridgeway et al., 2020) (note that some machine learning models are characterized by bias or inconsistency in estimates of propensity scores, and so properties such as honesty as implemented in *grf* may be important if machine learning methods are used in propensity score estimation). The distributions of propensity scores in the exposed and

unexposed groups are then used to identify and trim (remove) observations that are in the extremes of these distributions and have few or no counterparts in the other exposure group with a similar propensity score. This process ensures that in every region of the preserved covariate distribution, there exist observations in both the exposed and unexposed groups. Thus, overlap ensures we are estimating a causal effect over regions of the covariate distribution supported by data rather than through extrapolation. Achieving this overlap is how we most closely emulate the RCT reality in which every patient has some positive probability of assignment to each exposure group. Note that the groups as a whole could still look quite different (e.g., in terms of comorbidity prevalence).

Use an unadjusted modeling approach to establish a baseline treatment effect estimate. Assuming two exposure groups and two potential outcomes, start with any method operating on 2-by-2 contingency tables; you could use Fisher's exact test, the chi-square test for association, or a basic logistic regression model to evaluate the exposure-outcome association with no adjustment for any confounders. Importantly, you want to obtain point estimates and confidence intervals (CI) from these methods as we are concerned with the magnitude and precision of the treatment effect estimate. Despite our repeated emphasis on identifying and accounting for confounders, having an unadjusted model result that is compatible with the adjusted model results (described next) demonstrates that you have not reached your final treatment effect estimate simply by selecting a favorable set of covariates. When unadjusted and adjusted results disagree, one explanation could be dissimilarities in the covariate distributions of the exposed and unexposed groups. For example, if certain ages or comorbidities are not approximately equally represented in all exposure groups, controlling for such covariates could potentially change the sign of the estimated treatment effect. This could be evidence that your inclusion/exclusion criteria do not by themselves go far enough to yield similar exposed and unexposed groups.

Adjust for confounders, favoring methods that both adjust for outcomes and seek covariate balance. Methods that adjust for outcomes build a model mapping covariates to expected outcomes and then adjust for these differences when estimating treatment effects. Ordinary least squares or logistic regression are common methods for outcome adjustment; machine learning methods can also be used, but caution must be exercised, as there is a danger that regularization might omit or insufficiently adjust for confounders, creating bias (Belloni et al., 2014). Covariate balance goes beyond ensuring overlap: now the exposed and unexposed groups must resemble each other in their covariate distributions. More simply, observed values in the exposed group should occur with similar frequency in the unexposed group (either by weighting or excluding observations). Methods that accomplish this include inverse propensity-weighted (IPW) average of outcomes and matching (Rubin, 2001; Stuart, 2010; Jackson et al., 2017).

There are many choices of regression methods that adjust for confounders; among these are a set of methods known as doubly robust methods. A doubly robust estimator is one that employs both a propensity score model and an outcome regression model in such a way that if either model is correctly specified, the resulting causal effect estimator is statistically consistent (Bang and Robins, 2005). An example of a doubly robust method is inverse propensity-weighted (IPW) regression. Inverse propensity score weighting seeks covariate balance by weighting unexposed observations in the regression according to the inverse of their propensity scores (Austin and Stuart, 2015). Thus, observations that do not resemble exposed observations contribute less to the treatment effect estimate, and unexposed observations resembling exposed observations count more. This type of weighting has the effect of attempting to achieve covariate balance by weighting observations rather than excluding observations. Other examples of doubly robust methods include augmented inverse propensity weighting or AIPW regression and causal forests (Bang and Robins, 2005; Athey et al., 2019). We note that if machine learning techniques are used to estimate outcome models and propensity scores in AIPW methods, it is important to use cross-fitting, where the outcome adjustment and propensity score model for a given observation is estimated excluding that observation. When out-of-bag estimates are used with random forest methods, this will happen automatically, but with other methods, the analyst must estimate multiple versions of these models on different folds of the data.

As an alternative to the above doubly robust methods, one can employ matching methods to stratify the sample into one group per exposed observation. Groups or "matched pairs" are sized such that each exposed observation has a corresponding number of unexposed observations according to a specified match ratio. Importantly, the matching process should only retain the exposed observations for which an acceptable number of unexposed observations serve as good matches. This is the nearest you can get to seeing how a person's potential outcomes might be different on the basis of exposure. Matching can be accomplished many ways, including on the basis of propensity score or Mahalanobis distance (Stuart, 2010). To estimate the causal effect of the exposure on the outcome in the matched pairs, one might use the Cochran-Mantel-Haenszel test (Mantel and Haenszel, 1959) to evaluate the collective evidence presented by a series of 2×2 contingency tables documenting the exposure-outcome counts in each matched pair. The process of matching could produce a potentially much smaller data set that attempts to achieve covariate balance by excluding observations.

For methods that rely on covariate balance as part of the approach to adjust for confounders, it is critical to conduct appropriate diagnostics to see if these approaches achieved acceptable covariate balance. If you are unable to achieve reasonable covariate balance between exposed and unexposed individuals, you have likely discovered fundamental differences in the two groups that no modeling approach can reliably overcome (Glynn, 2017).

Example Application of Rule 8 to the COVID-19 Study

We first create a permuted copy of the outcome variable representing in-hospital death. We use the R package *grf* to estimate propensity scores (i.e., real exposure assignments as a function of the pretreatment traits identified in Rule 5). We then

trim the sample to retain the overlapping region of the exposed and unexposed propensity score distributions by keeping scores above the maximum of the two distributions' first percentiles and below the minimum of the two distributions' 99th percentiles. With the remaining sample, we perform an unadjusted analysis of the exposure-outcome relationship with Fisher's exact test (OR, CI, and *p*-value obtained with base R Fisher exact test). We conduct an adjusted analysis using the same pretreatment traits in an inverse propensity-weighted (IPW) logistic regression (OR, CI, and *p*-value obtained with the R package *survey*). We use the R package *MatchIt* to execute 5:1 Mahalanobis distance-based matching (identify five unique, unexposed matches for each exposed patient) on the same pretreatment traits (OR, CI, and *p*-value obtained with base R Cochran-Mantel-Haenszel test). Finally, we assess the covariate balance achieved by IPW and matching by calculating and visualizing standardized differences of means for included covariates. Executing all of these steps with permuted outcomes helps us debug code, identify potential incompatibilities with our data and selected methods, and conduct meaningful diagnostics for covariate balancing methods — all with zero awareness of the impact on our treatment effect estimates.

Rule 9 Supplement: Augment the Main Analysis With Extensive Sensitivity Analyses

Plan a thorough assessment of the robustness of your results to the various choices you made on the way to calculating an estimated treatment effect. Maybe you left something out that could explain everything (i.e., an unobserved confounder). Do alternative design and analysis approaches yield similar results? A secondary set of analyses could include adjusting for covariates with nonlinearities or time lags; you could also try different regression or propensity estimation methods. There could be many reasonable specifications for your model; to avoid tying your results to a set of arbitrary decisions, one way to evaluate a collection of reasonable models is to observe the distribution of resulting effect estimates using specification curve analysis (Simonsohn et al., 2019). Exploring different exposure or outcome definitions, covariates, designs, and analysis techniques also helps measure the sensitivity of your results to the specific choices you made along the way. Assessing robustness is by itself a comprehensive analysis.

Quantify the extent of unobserved confounding required to change your conclusions. If you are using observational health data to perform your study, you should expect that unobserved confounding exists; the difficulty lies in estimating how serious it is. There is no test for unobserved confounding (neither its existence nor its impact, given that it is unobserved), yet it likely exists in nearly all observational studies. This reality is what makes having domain experts carefully reason through confounder specification so critical. Starting with (Rosenbaum and Rubin, 1983), numerous approaches have been proposed that generally aim to estimate how strongly correlated an unobserved confounder would have to be to either the exposure, the outcome, or both, to move the estimated treatment effect to the null (Rosenbaum, 2010). Then you can reason about how likely it is that such a confounder might exist and is either unknown or unmeasurable. One such method for assessing unobserved confounding is the E-value (VanderWeele and Ding, 2017).

Assess the robustness of your results to choices regarding specific modeling techniques, hyperparameters, etc. One way to accomplish this involves trying a range of estimation approaches. Compare the treatment effect estimates from a range of doubly robust methods, for example. Use a variety of machine learning methods to estimate propensity scores and outcome models in doubly robust methods such as AIPW, or use approaches such as residual balancing (Athey et al., 2018) that do not rely on having an easy-to-estimate propensity model. The reason to augment your analysis by testing multiple approaches is to see if the obtained results were sensitive to the specific methods you chose to employ. While the methods introduced so far are designed to estimate average treatment effects for a population or some subset of the population, knowing whether the treatment effect is generally constant across the considered group can be very important. To explore this, one can construct causal trees to estimate heterogeneous treatment effects or HTE (Athey and Imbens, 2016).

Assess the robustness of your results to modifications in the study definitions and study design. You can make small changes to the definitions of the exposed and unexposed groups as well as the outcomes and confounders. For example, to identify a patient as a user of a particular drug, adjust the aforementioned medication possession ratio or look-back period in the exposure definition (i.e., ensuring a medication supply of more than 50, 70, or 90% of days within a look-back period of 90, 180, or 365 days). You can consider different recency requirements such as whether the most recent prescription spanned the inpatient admission date of interest. For an outcome like all-cause mortality, you could explore all-cause mortality in the hospital or within 7, 14, 30, or 60 days of diagnosis. Comorbidity identification could employ different code sets and/or a different look-back period. You may also consider adjusting for additional (or only a subset of) potential confounders within your models, to observe the extent to which confounder choice matters. The objective here is to see whether or not any observed treatment effect is simply a chance result stemming from a very specific set of definitions. Some of these changes are sufficient to change the study design. For example, defining the unexposed group to only include users of a different, comparable drug is known as the *active comparator design*, which can be an effective approach for minimizing confounding as the exposed and unexposed groups will be more similar (Yoshida et al., 2015). If we define the exposed group to only include new users of a drug, thus ensuring observed comorbidities existed before exposure and eliminating concerns over prevalent user bias, we are implementing a new user or incident user design. There are many study designs to choose from (e.g., prevalent user, incident user, active comparator, etc.), and each design deserves thoughtful consideration regarding the implications it has for the study in question and physiological mechanism under investigation. While investigating robustness to changes in study design can provide more evidence for the hypothesis, it can also help identify potential sources of unobserved confounding when different designs lead to different conclusions.

Explore additional sets of covariates, including different comorbidities and indicators of temporal health trends. Covariate sufficiency is the notion that no other covariate can meaningfully supplement what we have learned from the already identified covariates (Stone, 1993; VanderWeele and Shpitser, 2013). We can explore the sufficiency of our identified confounders by observing how results are impacted by the inclusion of other comorbidities. We can also explore the impact of differing time trends in the health of the exposed and unexposed populations. If one exposure group was observed to be getting sicker faster in the months before the target inpatient admission, that could warrant different expectations for outcomes in the exposed and unexposed groups. Your confounder definitions may have difficulty addressing not only the presence of a condition, but also its recency and its severity. Many comorbidities have their own severity indices (e.g., Diabetes Complications Severity Index), but viewing all the data required to compute these scores may not always be possible in certain data sets (e.g., claims data lacks lab results). Observing health decline is thus challenging; consider examining recent inpatient stays and other medical encounters as signs of declining health that may not otherwise be captured in existing confounder definitions.

Conduct negative outcome experiments and treatment control experiments. In a negative outcome experiment (Lipsitch et al., 2010), your goal is to assess whether the hypothesized exposure has an apparent benefit that extends to an outcome it could not reasonably impact (i.e., no medical theory connecting the exposure to the outcome). A negative outcome experiment is run to study the effect of the proposed treatment on an outcome not associated with that treatment. Here, we should expect to find no favorable treatment effect; otherwise, there is likely unobserved confounding contributing to better outcomes for the exposed group. A treatment control experiment is run to study a different treatment with no known connection to the outcome of interest; you should observe no protective effect of this different treatment on your original outcome. Again, if you see a benefit where there should be no benefit, the logical conclusion is the presence of unobserved confounding.

Refine, lock in, and preregister your formal analysis plan before examining any real model outputs using the true outcome data. Preregistration for observational studies involves uploading a detailed analysis plan to a study registry like the ones supported by the US National Library of Medicine (*clinicaltrials.gov*) and the Center for Open Science (*cos.io/initiatives/prereg*). While we encourage preregistration, in some cases it may not be possible to preregister an analysis plan before ever seeing the data; your understanding of the data prior to working with it may be too limited to make preregistration worthwhile. Preregistering your analysis plan is an attempt at transparency regarding what is exploratory and what is confirmatory in your final analysis. You may discover some things while exploring your data and testing your proposed statistical methods that require you to refine prior decisions. Maybe your set of confounders and outcome determinants is incompatible with a method you've chosen because one variable is too rarely observed or is too highly correlated with another variable. This is fine; you can make the necessary changes to your analysis plan with no fear of p-hacking because you were not using real outcomes (due to outcome permutation or synthetic data generation per Rule 8) and have not seen an effect estimate yet. Your preregistered analysis plan may include a range of exposures, outcomes, and modeling approaches you intend to evaluate, but you must clearly articulate from among these which combination you commit to reporting as your primary result. Define your primary result with a clear statement of the hypothesis, details of the modeling approach, and definitions for the cohort, treatment, outcome, and confounders.

Example Application of Rule 9 to the COVID-19 Study

We assess robustness to unobserved confounding with the E-value. We estimate the treatment effect with different exposure definitions, specifically combining 50, 70, and 90% MPR with 90-, 180-, and 365-days exposure windows. We estimate the treatment effect using AIPW and heterogeneous treatment effect with causal trees as supplementary methods. We consider mortality within 30 days of diagnosis as an alternative to in-hospital mortality. We perform a negative treatment control experiment with triptans as the exposure. We perform negative outcome control experiments using accidental injuries and non-prostate cancer as alternate outcomes.

Execution Phase: Execute the Analysis Plan and Report the Results

Rule 10 Supplement: Execute, Summarize, and Share (With Caveats)

Execute your analysis plan with the true outcome data once you are satisfied with the quality of your data set and have sufficiently tested your code. A significant responsibility of your team at this point is to stick to the proposed analysis plan. Other outcomes and exposures may appear to have a stronger effect than what is observed for the primary outcome and exposure, but there was significant thought and clinical expertise applied to these decisions in the planning phase of the study. There is danger in evaluating a host of different outcomes and only reporting the most favorable outcome(s); this greatly increases the potential for a Type I error, meaning that you could be reporting a treatment effect that does not actually exist.

If necessary, make the smallest possible refinements to your analysis plan and execute again. Even with all your planning, there is a chance that your analysis plan cannot be executed as-is. For example, you may discover that a rarely observed confounder in your data is perfectly predictive of the outcome in one of your exposure groups. This perfect separation of the data could cause your preferred method to fail, leaving you no choice but to change one of your selected methods or your selected confounders or both. If this happens, all is not lost. Simply make the minimal possible change necessary to conduct your analysis, and then note in your publication how you had to amend your analysis plan and what potential impacts your change may have had on your results.

Give your reader something that looks like what they are used to seeing. If your retrospective analysis has the stated purpose of motivating a clinical trial, write your results like a clinical trial

paper. Include a CONSORT flow diagram to help the reader visualize important properties of your sample. Understand how the intended audience expects to see results reported for the selected outcomes. The clinician audience you are writing for is accustomed to seeing odds ratios with corresponding confidence intervals to describe treatment effects. Presenting results in a conventional way eliminates one potential obstacle your audience may face when evaluating your work. While much attention is given to your primary result, your results in total are more than just an OR and a confidence interval; report the results of your sensitivity analyses as well to convey the robustness of your finding.

Explicitly include in your reporting the limitations of your study. You have not just completed an RCT; instead, you performed an observational study modeled after an RCT, but with many limitations and assumptions. Your biggest enemy is unobserved confounding, and it might be the case that it has seriously affected your results; however, if done well, your retrospective analysis may be just what is needed to generate the momentum and funding required to evaluate your idea in a clinical trial (Vandenbroucke, 2004). Alternatively, your analysis may actually provide evidence against the hypothesized exposure. Reporting negative results is just as important; your work can help ensure limited resources are spent on more promising treatments.

Provide all the necessary details to facilitate replication. You took great care in constructing and executing a comprehensive analysis plan; as you prepare to disseminate your findings, sharing those details matters. More than just your results, some readers will want to know everything necessary to reproduce your analysis. This means you should expect to provide details about the data used, including source and provenance as well as the codes (e.g., ICD) used to define the target patient population, inclusion/exclusion criteria, the exposure(s), the outcome(s), and any confounders. It can't be assumed that a reader will be able to guess your definitions without having them explicitly written out. Other researchers could sensibly reach many different definitions of what they believe you meant by the various outcomes, exposures, and confounders listed in your retrospective analysis. Providing text definitions, formulas, and ICD-code lookup tables ensures that any other attempts to implement your definitions are able to accurately do so. Providing all of this information in the standard organization of a clinical trial paper will help your clinical audience find the key pieces of information they need to be able to envision the trial you are emulating.

Facilitate replication by providing analysis code. You may also want to create an open-source software package (e.g., R/Python) for dynamic exploration of a data set and/or to facilitate replication of your analysis on other data sets. It is likely the case that other entities (e.g., a hospital, an insurance company, or a country) cannot legally share their data set with you; you likely have the same restrictions preventing sharing your data outside your own institution. To get around these restrictions and make replication as easy as possible, you can share instructions and code for building the data set and running your desired analysis. Whether you provide a well-documented collection of scripts in an online Git repository or a more formal software package, if you want to see replication of your results (e.g., to support an RCT you aim to start), you have an incentive to provide a reusable codebase that can facilitate rapid replication of results in other data sets as well as provide a means of quickly exploring alternate hypotheses.

Future Directions

These 10 rules are intended as introductory guidelines to one small piece of the complicated world of observational studies; there is much more to learn and consider than is offered here. Perhaps most importantly, we acknowledge this paper's role in summarizing a framework for retrospective pharmacoepidemiological analyses, not as a template for all types of retrospective studies (e.g., investigating lockdowns and facemask policy effectiveness against the spread of COVID-19). Several other ideas came up in the course of establishing these 10 rules that fell just short of earning their own rules. Some are not yet standard practice but are growing in popularity, and others are even more aspirational. Among these are notions of sample splitting (Fafchamps and Labonne, 2017) and model pooling. Sample splitting in the world of machine learning is standard practice, but typically the machine learning problem is one of prediction where there exists validation data, making it possible to know how correct a model's predictions are and therefore tune the model. The causal inference framework differs on both those counts: prediction is not the goal, and there exists no validation data to help us see if we have missed any unobserved confounders. While sample splitting may not always be necessary, when doubly robust techniques are used and machine learning methods are used to estimate outcome models or propensity scores, cross-fitting is needed to apply existing theory (Chernozhukov et al., 2018; Athey et al., 2019); we recommend that approach as discussed in Rule 8. There is still interest, however, in using synthetic data generation techniques such as generative adversarial networks (Beaulieu-Jones Brett et al., 2019; Athey et al., 2021) and standard training/test splits for routine tasks like evaluating a constructed feature definition and validating code. Employing these or related techniques aims to facilitate completion of necessary tasks without being influenced by real-world results. Another growing area of interest is in the pooling of data and models from observational studies (Bareinboim and Pearl, 2016). Privacy concerns often restrict the pooling of data, but these concerns do not apply to the pooling of models. Pooling different linear models is nothing new, but combining nonlinear models shows promise for providing doubly robust causal estimates with lower variance, even when the source models have different covariates as inputs. As more research on these and other areas continues, it is likely we will see the associated advances make their way into some of the key ideas we have captured here.

AUTHOR CONTRIBUTIONS

MP, AK, AS, BC, SA, ES, and JV contributed to the initial formulation of the 10 rules framework. MP was the lead writer for this manuscript. All authors contributed key ideas related to their respective areas of expertise, reviewed

multiple drafts of the manuscript, and approved the final manuscript.

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