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Interventions for emerging infectious diseases

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Interventions for emerging infectious diseases

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The Value of Nasal and Oral Clinical Examination in Febrile Neutropenic Patients for Initiating Antifungal Therapy as a Preemptive Method

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Background: Invasive fungal infections (IFIs) are complications that lead to mortality and morbidity in hematologic malignancies. The time of starting antifungal therapy is vital. Preemptive antifungal therapy has appeared recently as a new policy for the management of IFIs based on noninvasive ways in neutropenic patients.

Methods: We enrolled leukemia patients with neutropenia after chemotherapy in Imam Khomeini Hospital Complex, Tehran, Iran. Patients who entered the neutropenic phase were divided into two categories (empirical and preemptive) for receiving antifungal agents. The patients were clinically examined in the preemptive group every day to find IFIs. As soon as clinical evidence of IFIs was observed, antifungal was prescribed. The empirical group patients received antifungals based on the ward protocol. Based on the data in each group, the diagnostic and therapeutic results of cases are followed-up to 3 months. To compare percentages between the two groups, the chi-squared test was used. And to compare two means between the two groups, the independent *t*-test was used. All the statistical analyses were done in the Statistical Package for the Social Sciences (SPSS) version 24 software (IBM Corporation, Armonk, New York, USA).

Results: We assessed 132 leukemic patients with inclusion and exclusion criteria. Eventually, 80 patients were enrolled. The mean age was 35.52 years. Demographics data and distribution of leukemia type show no significant differences between the two groups. Despite a higher percentage of IFIs discovered in the preemptive group than the empirical group (25 vs. 18.75%, respectively), but data show no significant differences. The average days of IFIs diagnosis since the beginning of neutropenia in the empirical group were 9.5 days while in the preemptive group, the average days were 5.4 days ($p < 0.05$). Totally, there were 15 patients with a proven IFI in each group (40% in the empirical group and 60% in the preemptive group). Results significantly show an increase

in surgical sinus debridement in the empirical groups (83.3%) vs. the preemptive groups (55.5%), ($p < 0.05$). The mortality rate differed significantly among the two groups; it was 7.5% in the preemptive group and 25% in the empirical group ($p < 0.05$).

Conclusion: Daily oral and nasal cavities examination to find the symptoms of IFIs and then start preemptive antifungal agents may be able to lead to accurate diagnosis, earlier treatment, and decreasing sinus surgery debridement in leukemia patients with neutropenia.

Keywords: invasive fungal infections, hematologic neoplasms, antifungal therapy, preemptive, empirical, neutropenia, nasal, oral

INTRODUCTION

Invasive fungal infections (IFIs) are severe complications that lead to mortality and morbidity in patients with an impaired immune system, such as acute or chronic leukemia (1–3). Nowadays, we are facing a general increase in IFIs (4, 5). New chemotherapy medication causes longer neutropenia, which raises immunocompromised patients. The diagnosis of IFIs in the early stages is challenging and it is crucial for a better prognosis of antifungal treatments (6–8). According to a previous study, almost 33% of newly diagnosed acute myeloid leukemia (AML) under chemotherapy has developed IFIs. A 30-day mortality rate of IFIs among patients with AML in another study was about 22.1% (7, 9). The remarkable point is that cytotoxic chemotherapy as a treatment for leukemia causes critical neutropenia (10–12). Studies have shown that prolonged neutropenia is the most significant risk factor for getting IFIs (13–16). Clinical manifestations of IFIs in immunocompromised hosts can be atypical and silent; therefore, the diagnosis of fungal infections is troublous (15–17). Some symptoms, such as fever, facial pain, and headache are nonspecific (3, 18). IFIs in the nasal cavity represent pallor and discoloration, ischemia, necrosis, ulcer in the septum, or eschar in the early stages (18, 19). In contrast, the air-crescent sign in CT scan emerges lately with the existence of advanced IFI, which is not pleasing for early recognition (6). Recent studies proved that the incidence of IFIs is higher in patients with no empirical antifungal therapies (20–22).

Candida spp. are responsible for the most prevalent fungal nosocomial infections (23–25). IFIs can affect a single organ or spread across the body. Invasive candidiasis commonly affects the bloodstream. The lungs and sinuses are the typical sites for invasive aspergillosis (26–29).

There is an association between a long time and profound neutropenia with impaired prognosis in patients undergoing antifungal treatments (30, 31). The time of starting antifungal therapy is vital in high-risk patients including chemotherapy patients (32, 33). Based on previous studies, different centers perform antifungal therapies in various ways: empirical, preemptive, and targeted therapy (13, 34). Empirical antifungal therapy is one of the most accepted ones for patients with febrile neutropenia after 3–7 days of constant fever, despite broad-spectrum antibiotics (13, 35). Considering clinical manifestation and the risk of the patient for IFIs, it leads to initiating antifungal (empirical) therapy, regardless of microbiological therapy (36, 37).

Empirical antifungal therapy mostly causes overtreatment, drug resistance, higher medical costs, and is not dedicated (38, 39). Doubt about the advantages and disadvantages of empirical antifungal therapy created the necessity for making another strategy to help the patients (3, 40). Preemptive antifungal therapy has appeared recently as a new policy for the management of IFIs in neutropenic patients. This method uses noninvasive ways, such as imaging (CT scan) or serological tests (galactomannan testing), for starting antifungal agents in suspected neutropenic patients in IFIs. However, preemptive strategies may lead to the delayed beginning of antifungal therapies, have no effect on higher mortality in patients, and decrease the medication costs (41–43). A Chinese survey comparing empirical therapy to preemptive therapy demonstrated the same survival rate, but fewer changes for preemptive patients (43). Thus, we designed this study to evaluate the benefits of initiating preemptive antifungal therapy based on physical examination of oral and nasal cavities for finding the clinical features of IFIs as a complementary method in leukemic patients with neutropenia.

PATIENTS AND METHODS

Study Population and Design

After evaluation of patients with acute leukemia, we enrolled hospitalized patients in Imam Khomeini Hospital Complex, Tehran, Iran from April 2018 to March 2020 who met the inclusion criteria in addition to none of the exclusion criteria and gave informed consent.

Inclusion Criteria

Diagnosis of acute leukemia, receiving chemotherapy, and neutropenia defined as an absolute neutrophil count (ANC) of < 500 cells/mm^3 were assumed as inclusion criteria. Peripheral blood smears of the leukemic patients were evaluated daily after chemotherapy for defining ANC.

Exclusion Criteria

We considered exclusion criteria as follows: age < 14 years, septic shock, diagnosis of hematologic malignancy except for leukemia, history of prior invasive fungal infections, reluctance

to participate in the project, and the impossibility of additional diagnostic tests for possible or definitive invasive fungal infection. Patients who entered the neutropenic phase were divided into two categories to have antifungal treatment approaches.

Group 1

In the preemptive group, patients who entered the neutropenic phase (ANC < 500 cells/mm^3) following chemotherapy without fever were daily examined to find a necrotic or ulcerative mucosal lesion, black scars, acute localized pain, purple discoloration in the nasal cavity, or similar lesions in the oral cavity, palate, and pharynx. We composed a datasheet that contains nasal and oral cavity examination features in the preemptive group. The daily examination was performed by a trained healthcare provider. The nasal speculum was used for nasal examination and evaluation of the mouth was performed by abeslang and a flashlight. As soon as discovering clinical evidence of invasive fungal infection (without starting fever), antifungal therapy with liposomal amphotericin B [3–5 mg/kg/daily/intravenous (IV)] was added to the medication regimen of the patient. Moreover, chest and paranasal CT scans, sinus endoscopy, and acquired samples were checked for pathology and culture and fungal smear. The serum galactomannan test was sent at the same time as seeing lesions in daily assessments. The endpoint for serial examination is recovery from neutropenia or the diagnosis of invasive fungal infection.

Group 2

The empirical group includes patients who experienced fever in their neutropenic period subsequent chemotherapy. According to specified fever and neutropenia guidelines in the hematology department of Imam Khomeini Hospital Complex, cases were managed. Based on the mentioned protocol, after 3–5 days of fever and neutropenia and no response to broad-spectrum antibiotics—without severe sepsis or septic shock condition—liposomal amphotericin B (3–5 mg/kg/daily/IV) was started and diagnostic measurements, such as imaging and galactomannan level of the serum, were done.

All the patients with neutropenia in both groups received fluconazole 400 mg/PO/daily for antifungal prophylaxis. The clinical indicators assessed in clinical examinations were recorded in datasheets. The diagnosis of IFIs in this study was based on the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC)/National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) criteria (44). The definitions determined 3 levels of probability to the diagnosis of invasive fungal infection in immunocompromised patients including "proven," "probable," and "possible" invasive fungal infection.

"Proven" invasive fungal infection is diagnosed only when the presence of invasion by fungus can be identified by histological diagnosis or culture of a specimen taken from the site of infection. In contrast, "probable" and "possible" invasive fungal infections consist of 3 factors. Probable IFI requires a host factor, a clinical criterion, and mycological evidence. Patients with a host factor and a clinical criterion but without mycological evidence are considered as possible IFI (44, 45). The diagnosis of proven cases in this study was based on histopathological evidence or tissue fungal culture.

Finally, based on the data in each group, the diagnostic and therapeutic results of proven and probable IFI cases were followed and recorded until 3 months. To compare percentages between the two groups, the chi-squared test was used and for comparing two means between the two groups, the independent t-test was used. All the statistical analyses were done in the Statistical Package for the Social Sciences (SPSS) version 24 software (IBM Corporation, Armonk, New York, USA).

RESULTS

In this study, we assessed 132 leukemic patients referring to Imam Khomeini Hospital Complex in Tehran, Iran. They suffered from neutropenia after chemotherapy with inclusion and exclusion criteria. Eventually, 80 patients were enrolled. The empirical (control) group included 40 patients that developed a fever after chemotherapy. They were treated by antibiotic, if the treatment did not stop fever; patients were immediately given antifungal therapy by an empirical method. The preemptive (case) group consisted of 40 patients that underwent daily clinical nasal and oral examination.

Among 80 patients, the age range of cases was between 15 and 59 years and the average of all was 35.52 years, with 10.25 years SD. The type of leukemia is shown in **Table 1**. The chi-squared test showed that there is no significant association between type of malignancy and the treatment method ($p > 0.05$). Demographics data and leukemia type distribution show no significant differences between the two groups.

Incidence of Invasive Fungal Infections

According to the EORTC/MSG criteria for the classification of IFIs (44), results are shown in **Table 2** divided by probable and proven cases in each group. The diagnosis of proven cases was based on histopathological evidence or tissue fungal culture. 12/15 (80%) cases were diagnosed based on histopathological evidence and 3/15 (20%) cases were diagnosed based on tissue fungal cultures as proven cases.

The average days of diagnosis of IFIs since the beginning of neutropenia in the empirical group were 9.5 days (with 2.01 days SD) while in preemptive patients, mean days of diagnosis were 5.4 days with 1.86 days SD and the difference in averages was statistically significant. The independent t-test showed a significant difference between the two means ($p < 0.05$).

Based on the results, nasal cavity symptoms were the dominant site (88.8%) of IFIs in this study, while 11.11% of patients had the symptoms of fungal infection in the oral cavity (change the color of the palate).

In total, out of 15 proven invasive fungal infections diagnosed in the two groups, 8 proven invasive fungal infections were mucormycosis and 7 proven invasive fungal infections were aspergillosis, while pathogen prevalence of IFIs is near the same in the two groups (**Table 2**). In each group, two patients had positive serum galactomannan levels (**Table 3**).

There were 15 patients with a proven IFI and 6 (40%) patients related to the empirical group and 9 (60%) patients

TABLE 1 | Distribution of empirical and preemptive therapy in 80 leukemic patients.

Type of malignancy, N (%)	Empirical	Preemptive	Total, N (%)
AML[a]	29 (36.25%)	30 (37.50%)	59 (73.75%)
ALL[b]	11 (13.75%)	10 (12.50%)	21 (26.25%)
Total (%)	40 (50.00%)	40 (50.00%)	80 (100.00%)

[a]*AML, acute myeloid leukemia;* [b]*ALL, acute lymphoblastic leukemia.*

TABLE 2 | Frequency of proven/probable fungal infections with regard to type of therapy.

		Empirical			Preemptive			Total (%)
Probable	IFIs positive, N (%)	9 (11.25%)			11 (13.75%)			
	IFIs negative, N (%)	31 (38.75%)			29 (36.25%)			
Proven	IFIs positive, N (%)	6 (7.50%)	Mucormycosis	3 (20.00%)	9 (11.25%)	Mucormycosis	5 (33.33%)	
			Aspergillosis	3 (20.00%)		Aspergillosis	4 (26.67%)	
	IFIs negative, N (%)	34 (42.50%)			31 (38.75%)			
Total (%)		40 (50.00%)			40 (50.00%)			80 (100%)

related to the preemptive group. In the course of the disease, 5 (83.33%) patients in the empirical group needed surgical sinus debridement. In contrast, among 9 preemptive patients, 5 (55.56%) patients needed debridement and 4 (44.44%) patients with IFIs did not need debridement.

In terms of the need for recurrent sinus surgery debridement, 83.3% of patients in the empirical group needed repetition, while this rate was 55.5% in the preemptive group. The chi-squared test significantly showed the increasing necessity of surgical sinus debridement of IFIs site in the empirical group vs. the preemptive group ($p < 0.05$).

The chi-squared test showed that all-cause mortality rate differed significantly among the two groups: lower mortality rate in the preemptive group against the empirical group. In the empirical group, 10 (25%) patients and in the preemptive group, 3 (7.5%) patients died in 3 months follow-up ($p < 0.05$).

DISCUSSION

Invasive fungal infection is one of the serious etiologies of chemotherapy-induced neutropenia (14, 45). As a common approach, empirical therapy has been used in treating IFI patients for years, but administering empirical antifungal agents may have certain problems, such as overtreatment or higher expenses and due to lack of trustworthy data, the efficiency of this method is still debatable (16, 38, 46).

With low specificity of clinical symptoms (e.g., fever, nodules, cough, hemoptysis, erythema, and maculopapular eruptions) and radiologic diagnostic tests, it has always been challenging to diagnose IFI in the early stages, but nowadays because of new diagnostic tools being employed for early identification of IFI, including the G-test, GM test, chest CT, and PCR, it is possible to define more exact starting points for antifungal treatment (4, 17, 18).

Studies comparing empirical vs. preemptive antifungal therapy in adult populations use different criteria including overall mortality, IFI-related mortality, percentage of patients with the final diagnosis of IFI, percentage of patients receiving antifungal therapy, and the number of days of antifungal treatment (19). To the best of our knowledge, this study is the first randomized controlled trial that recruits daily clinical nasal and oral examination before the manifestation of fever, as the inclusion criteria in the preemptive group and comparing with the empirical group. Patients with stem cell transplant and hematological disorders including neutropenic leukemia tend to have abrupt fatal courses of *Mucor spp.* or *Aspergillus spp.* sinusitis with a high mortality rate (47). Chen CY et al. reported 19 of 46 enrolled patients with invasive fungal sinusitis (IFS) died within 6 weeks indicating poor prognosis, especially in patients with prolonged neutropenia status in their study (ANC less than 500 cells/mm^3 for more than 10 days) (48). During the daily nasal and oral examination, any symptom of nasal discharge, stuffiness, epistaxis, periorbital swelling, and maxillary tenderness as nonspecified and nose ulceration, eschar of the nasal mucosa, black necrotic lesions, and perforation of the hard palate as a more specific manifestation of IFS can lead to an early-stage diagnosis and antifungal therapy (47, 49, 50).

In this study, although numerically, the detection rate of fungal infections was higher in the preemptive group; this method could not significantly increase the diagnostic rate of invasive fungal infection in the preemptive group, which was 25% in the preemptive group vs. 18.75% in the empirical group ($p > 0.05$).

Lower rates of surgical sinus debridement in the preemptive group compared to the empirical group were needed. Surgical sinus debridement was done in 5/9 (55.56%) cases in the preemptive group and 5/6 (83.33%) cases in the empirical group ($p < 0.05$). On the other hand, the mortality rate differed significantly among the 2 groups with a noticeable reduction of mortality rate in the preemptive group ($p < 0.05$).

TABLE 3 | Results of galactomannan test with regard to type of therapy.

Galactomannan Test, N (%)	Empirical		N (%)	Preemptive		N (%)	Total (%)
	Mucormycosis	Positive	0 (0.00%)	Mucormycosis	Positive	0 (0.00%)	0 (0.00%)
		Negative	3 (21.43%)		Negative	5 (35.71%)	8 (57.14%)
	Aspergillosis	Positive	2 (14.29%)	Aspergillosis	Positive	2 (14.29%)	4 (28.58%)
		Negative	1 (7.14%)		Negative	1 (7.14%)	2 (14.28%)
Total (%)			6 (42.86%)			8 (57.14%)	14 (100%)

As mentioned in this study, the preemptive method that we devised was based on diagnosing IFI in neutropenic patients prior to the onset of fever, which differed from other studies that defined preemptive antifungal therapy as a strategy initiated after at least 4 days of refractory fever (38, 51, 52). This study shows a significant decrease in the average days of diagnosis of IFIs in the preemptive group (5.4 $\pm$ 1.86 days) vs. the empirical group (9.5 $\pm$ 2.01 days) that can indicate more efficiency of this study design to define preemptive therapy method in comparison with other studies (38, 56).

The mean age of patients was 35.52 years in our randomized controlled trial; As reported by Carol A Kauffman, these range of ages indulge more in social interactions and have more exposure to fungal and bacterial infections, so we can observe a higher incidence of histoplasmosis, aspergillosis, and cryptococcosis in these patients more than others (53). As reported in this study, the distribution of mucormycosis and aspergillosis in our population of interest with an age range of 35.52 years, with 10.25 years SD, was 8/15 (53.3%) and 7/15 (46.6%), respectively; that was in accordance to the prevalence of mentioned fungal infections reported by Njunda et al. (54) and Dhooria et al. (55) in their studied population.

Costs associated with antifungal therapy as an important reason for implementing preemptive antifungal therapy were indicated in a previous study by Cordonnier et al. (56) to lower the expenses of patients by 35% using the preemptive method. Although, they stated an incidence rate of IFI of 3/143 (9%) patients in the preemptive group vs. 4/150 (3%) patients in the empirical group that was in accordance with our IFI incidence rate. Ko et al. (35) also indicated the cost-effectiveness of a diagnostic-driven (preemptive) approach as a novel method to treat patients of hematological malignancies with neutropenia following chemotherapy and hematopoietic stem cell transplant (HCST) recipients with severe graft vs. host disease (GVHD). They also stated that choosing the preemptive or empirical therapy should be individualized according to the hospital and patient factors (e.g., feasibility, the turnaround time of diagnostics, local epidemiology, and risk of IFI). They suggested using preemptive strategy in the patients with low risk of IFI due to epidemiological factors or diagnostic tools and empirical strategy in high-risk patients or patients with severe illness.

According to the systematic review published in 2015, including 9 studies comparing preemptive vs. empirical antifungal therapy, it states that the medical expenses of leukemic neutropenia patients infected with IFI received preemptive antifungal strategy have shown significantly lower antifungal exposure and less clinical expenses than patients in the empirical group without increasing both the overall and IFI-related mortality rate (57). In another retrospective study, to evaluate preemptive antifungal strategy, 348 neutropenia episodes in 234 patients were reviewed. The main elements of preemptive therapy included a weekly chest CT scan and GM test twice a week. Patients were under prophylactic therapy with fluconazole 400 mg and antifungal therapy started at the stage of prognosis of IFI. Chest CT scans in a 10-day interval in 81% of cases diagnosed 109 patients with IFI. Forty-nine patients passed away before day 100 and 2 IFI cases were found after their death that was not diagnosed in preemptive antifungal strategy.

As reported in studies and protocols, in the majority of cases, it takes 3–7 days of persistent fever to start antifungal therapy in empirical method and obviously, there is a high risk of disseminated infection, but in the preemptive approach with our novel study design, we can observe a significant decrease in IFI-related and overall mortality (13, 35, 57). This study shares the same results on the early-stage diagnosis of IFI but additionally, despite many previous studies, we can observe a decrease in mortality rate in the preemptive group compared to the empirical group.

This randomized study revealed that preemptive antifungal treatment guided by daily nasal and oral examination before the manifestation of fever in addition to imaging findings and the GM test was able to significantly decrease the mortality rate in the preemptive group with decreasing the need for surgical debridement in leukemia patients with neutropenia.

CONCLUSION

Daily oral and nasal cavities examination to find the features of IFIs and then start preemptive antifungal agents may be able to lead to accurate diagnosis, earlier treatment, decreasing sinus surgery debridement, and even mortality in leukemia patients with neutropenia.

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

The studies involving human participants were reviewed and approved by Tehran University of Medical Sciences, Tehran, Iran. The patients/participants provided their written informed consent to participate in this study.

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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Comparison of the Efficacy of Anatomic and Non-anatomic Hepatectomy for Hepatic Alveolar Echinococcosis: Clinical Experience of 240 Cases in a Single Center

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Background: Hepatic alveolar echinococcosis (AE) is a zoonotic parasitic disease. There are more than 16,000 new cases each year, approximately 60 million people are threatened, and the annual direct economic loss is RMB 3 billion. The prevalence of AE in some areas of the Qinghai–Tibet Plateau is as high as 6.0%. Radical resection, including anatomic and non-anatomic hepatectomy, for advanced AE can significantly prolong the survival time of patients. However, there is no literature compared the efficacy of anatomic and non-anatomic hepatectomy. Therefore, by comparing various clinical evaluation indices between anatomic and non-anatomic hepatectomy, this study explored the short-term and long-term efficacy of these two surgical methods for AE.

Methods: The clinical data of patients with AE who underwent radical hepatectomy at Qinghai Provincial People's Hospital from January 2015 to January 2021 were retrospectively analyzed. The patients were divided into two groups by surgical method, that were, non-anatomic hepatectomy group and anatomic hepatectomy group. We compared these two groups focusing on basic preoperative data, such as age, sex, lesion size, and liver function parameters; main intraoperative evaluation indices, such as operation time, intraoperative porta hepatis occlusion time, intraoperative blood loss, and blood transfusion; and postoperative recovery evaluation indicators, such as postoperative liver function, incidence of surgical complications, and AE recurrence.

Results: A total of 240 patients were enrolled in this study, including 123 in anatomic hepatectomy group and 117 in non-anatomic hepatectomy group. There were no significant differences ($P > 0.05$) between baseline characteristics. Anatomic hepatectomy group was advantageous than non-anatomic hepatectomy group regarding intraoperative blood loss ($P < 0.001$), blood transfusion ($P < 0.001$),

and porta hepatis occlusion time ($P < 0.001$). There were statistically significant differences in postoperative liver function (aspartate aminotransferase: $P < 0.001$; alanine aminotransferase: $P < 0.001$), surgical complications ($P < 0.001$), and AE recurrence rate ($P = 0.003$). The median survival of patients in the anatomic hepatectomy group was 66 months, compared to 65 months in the non-anatomic hepatectomy group ($\chi^2 = 4.662$, $P = 0.031$).

Conclusions: Anatomic hepatectomy was not only safe for AE but also showed better short-term and long-term superiority than non-anatomic hepatectomy.

Keywords: hepatic alveolar echinococcosis, non-anatomic, anatomic, hepatectomy, efficacy

INTRODUCTION

Hepatic alveolar echinococcosis (AE) is a zoonotic parasitic disease caused by the larvae of *Echinococcus multilocularis* that seriously endangers human health (1–3). It's treatments mainly include radical resection and medication (4, 5). Medication is mainly used for early-stage AE, while radical resection is the first choice for progressive cases (6–8). The best treatment strategy is combination of surgical and postsurgical medication therapy. However, in patients whose conventional resection is not possible, other curative therapeutic options includes *ex vivo* liver resection associated with autotransplantation and liver transplantation (9). Because AE lesions are most frequently located in the right liver lobe, especially in advanced cases the major bile ducts and vessels have been invaded, major hepatic surgery is often required (10). Palliative operations have been shown to be a cause of recurrence without improving patient survival and is not recommended nowadays (11, 12).

Hepatic AE shows a similar pattern to malignancies in terms of radiologic and clinical features. For this reason, oncological surgical principles should be applied during the resection of hepatic AE. Studies have shown that based on adequate preoperative evaluation of the feasibility, and knowledge about the intraoperative techniques such as hepatic blood flow control, liver anatomy, and portal vein and biliary reconstruction, radical surgical resection can improve the quality of life and extend the survival time of the patients (13). Both anatomic hepatectomy and non-anatomic hepatectomy are radical surgical resections. However, other studies have shown that anatomic hepatectomy for AE has the advantages of less intraoperative bleeding, low incidence of postoperative complications, and rapid recovery (14). Our clinical study on anatomic hepatectomy for AE found that it has the advantages of less liver function injury, low incidence of complications, and short postoperative hospital stay (15). However, due to the shortage of early-stage cases and short follow-up times, we mainly evaluated the short-term efficacy of anatomic hepatectomy for AE. To further study, we retrospectively analyzed the clinical data of 240 patients with AE who underwent hepatectomy in Qinghai Provincial People's Hospital from January 2015 to January 2021, to explore the effect of surgical methods in the long term in patients with HAE.

METHODS

Basic Patient Information

The clinical data of 513 patients with hepatic AE who underwent hepatectomy in Qinghai Provincial People's Hospital from January 2015 to January 2021 were retrospectively analyzed. Inclusion criteria: (1) Pathological diagnosed with AE; (2) Disease stages were I, II, or III according to the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) PNM classification (16); (3) Without previous surgical history; (4) There was no cirrhosis, and the patient's liver function was graded as A or B before operation according to Child-Pugh classification. For the patients whose liver function was grade B, reevaluation was performed after inteventions, and if it was grade A then, they were included in the study; (5) Open hepatectomies were performed. Exclusion criteria: (1) The porta hepatis and retrohepatic inferior vena cava were severely invaded and required revascularization or *ex vivo* liver resection; (2) The surgery was palliative.

Altogether 240 patients which met above criteria were enrolled. The patients were divided into non-anatomic hepatectomy group and the anatomic hepatectomy group according to distinct surgical methods. This study was approved by the hospital ethics committee.

Preoperative Preparation

After admission to the hospital, 240 patients underwent contrast-enhanced computed tomography and angiography (CTA) of abdomen; enzyme-linked immunosorbent assay (Diagnostic Kit for IgG Antibody to Hydatid, ELISA brand is HAI TAI and from Zhuhai special economic zone haitai biopharmaceutical Co. LTD) for the hydatid; and eight tests for infection (Including HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, HCV-Ab, HIV-Ag/Ab, and TPAb). Metastasis of AE to the brain, lung, and other organs was excluded before surgery based on relevant imaging examinations. The liver function, residual liver volume, and the relationships between the lesion and the blood vessels and bile ducts were evaluated.

There were 7 patients with jaundice in this study, including 3 patients in the anatomic hepatectomy group with total bilirubin levels ranging from 54.7 to 116.4umol/L. 4 patients in the non-anatomic hepatectomy group with total bilirubin levels ranging from 44.07-154.8umol/L. However, the preoperative

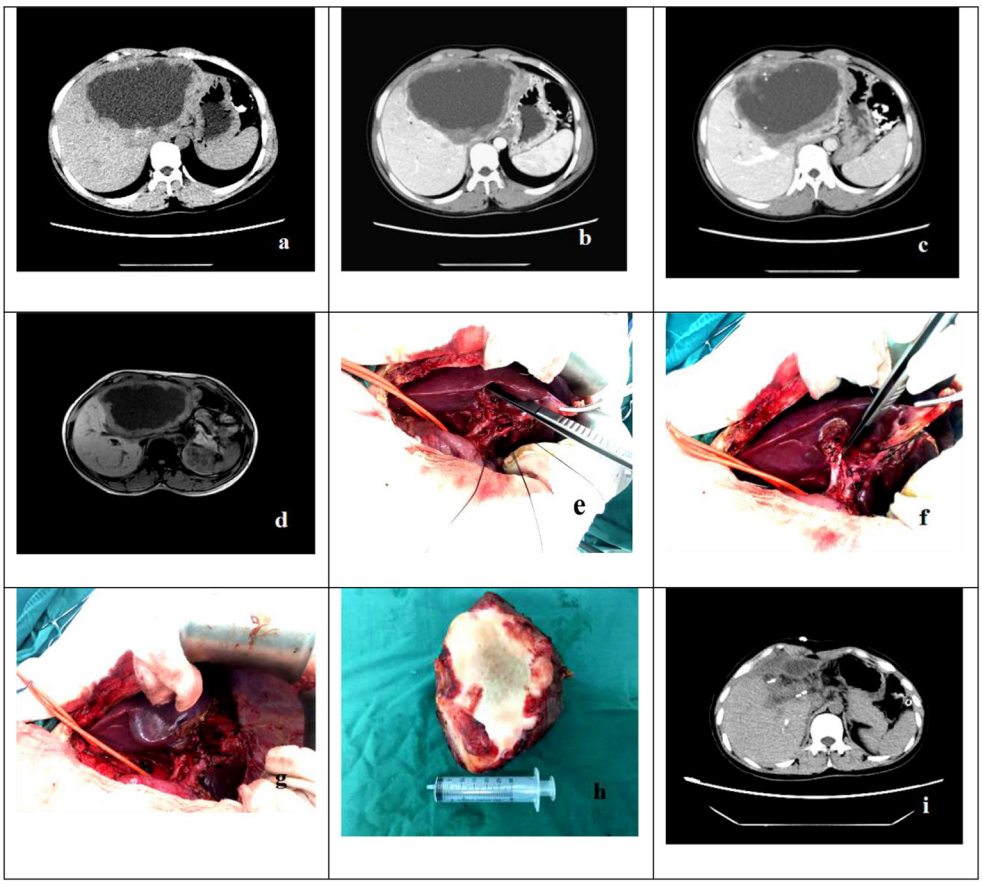

FIGURE 1 | The case is a 32-year-old female patient who was hospitalized due to the chief complaint of "intermittent right upper abdominal distension, pain and discomfort for more than 4 years". **(a–d)** Preoperative abdominal CT of patients showed that there was a lesions of echinococcosis in the liver with the size of about 12.0 × 7.0cm in S2-4 segments. **(e–h)** The intraoperative anatomy of the first hepatic portal and pathologic specimens. **(i)** Abdominal CT showed that there was residual liver compensatory and hyperplasia, but there was no recurrence of echinococcosis.

Child-Pugh grading of the above cases was grade B, and 3 patients underwent ultrasound-guided percutaneous liver puncture and bile duct drainage before surgery. The remaining 4 patients were treated with echinococcosis necrotic cavity puncture drainage after percutaneous liver puncture biliary drainage failed because intrahepatic bile duct dilation was not obvious. All 7 patients were treated with hepatoprotective medicine, and the liver reserve function was evaluated again after the total bilirubin level returned to normal, and the Child-pugh grade of all patients was A.

Surgical Methods and Indications

Anatomic hepatectomy: After the patient was successfully anesthetized, an inverted "L"-shaped incision was made in the upper abdomen, and the abdomen was examined layer by layer to detect whether there was metastasis of AE in the abdominal cavity. The first porta hepatis and second porta hepatis were routinely dissected and hung with ribbons to cut off the ligaments around the liver. When the resection range was large, a liver sling was placed behind the liver and in front of the vena cava. During the surgery, the central venous pressure was kept at 3–5 cmH_2O. The blood flow into and out of the liver was selectively blocked according to the scope of resection. The range of liver ischemia was observed, and a precut line was drawn along the ischemic line and the boundary of the lesion. R0 resection was performed when the resection margin was >1.0 cm from the lesion, and R1 resection was performed along the edge of the lesion to cut it. The liver parenchyma was transected with a water jet or ultrasonic scalpel. A duct with a diameter of ≤0.1 cm was cauterized with an electrotome, and an intrahepatic duct with a diameter of >0.2 cm was sutured and repaired with 5-0 Prolene. The cutting surface of the liver was sutured to stop the bleeding, a drainage tube was placed in the abdominal cavity, and the abdomen was closed (**Figure 1**).

The indications for anatomic hepatectomy were as follows: Anatomic hepatectomy should be preferred if AE lesions are at a certain distance from the porta hepatis and retrohepatic inferior vena cava or if it would not be difficult to dissect the first porta hepatis and second porta hepatis.

Non-anatomic hepatectomy: After successful general anesthesia, an inverted "L"-shaped incision was made, and the abdominal cavity was explored layer by layer. The ligaments

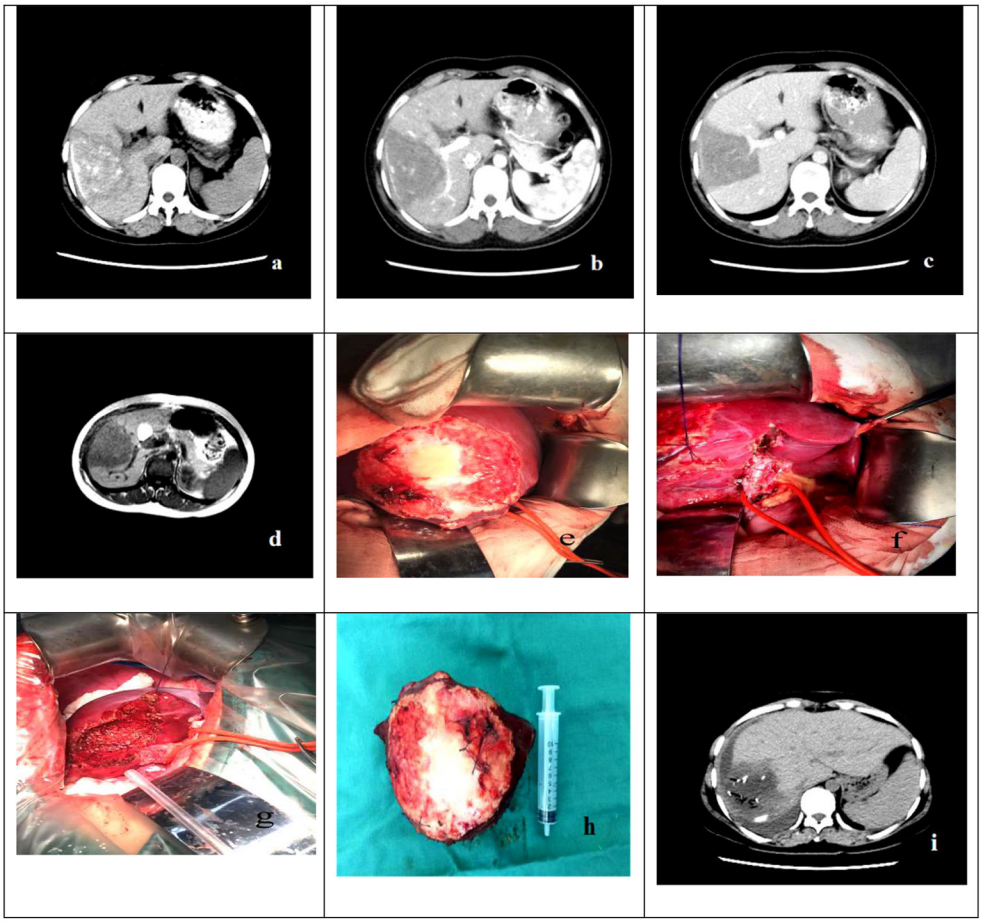

FIGURE 2 | The case is a 22-year-old male patient who was hospitalized due to the chief complaint of "liver space-occupying lesions found in physical examination for more than 1 year". **(a–d)** Preoperative abdominal CT and abdominal MRI of patient showed that there was a lesions of echinococcosis in the liver with the size of about 8.0 out.0cm in S5-6 segments. **(e–h)** The Intraoperative resection and postoperative pathological specimens. **(i)** The abdominal CT showed that there was residual liver compensatory and hyperplasia, but there was no recurrence of echinococcosis.

around the liver were dissociated according to the scope of the resection, and the precut line was marked according to the size and location of the lesions. The resection margins (R0, R1) were defined as above. The routine Pringle maneuver was used to block the first porta hepatis, and the liver parenchyma was transected layer by layer along the precut line. The duct structure with a diameter of >0.2 cm was ligated with silk thread or sutured and repaired with Prolene. Smaller blood vessels and bile ducts were cauterized with an electrotome. The cutting surface of the liver was treated with gauze dipped in hot saline for hemostasis. After confirming no active bleeding, a drainage tube was placed in the abdominal cavity, and the abdomen was closed (**Figure 2**).

The indications for non-anatomic hepatectomy were as follows: For the surgical safety of the patient, non-anatomic hepatectomy was selected if it would be difficult to dissect the first and second porta hepatis or the AE lesions were closely associated with important ducts inside and outside the liver.

Postoperative Management of Patients and the Administration of Albendazole

Patients in both groups were given an intravenous analgesia pump combined with subcutaneous injection of analgesics after surgery. The postoperative fluid volume was kept within 2,000–2,500 ml. The patients drank water after waking up from anesthesia and were encouraged to get out of bed as soon as possible. During the postoperative hospitalization, the patient did not take albendazole because the liver function was still recovering. Albendazole was prescribed for discharged patients in strict accordance with WHO guidelines for the diagnosis and treatment of echinococcosis (16).

Follow-Up After Discharge

The patient was re-examined every 6 months in the first 2 years after discharge and every 12 months thereafter. The follow-up examinations mainly included ELISA for the hydatid, liver and kidney function tests, abdominal color ultrasound or abdominal CT (CT of the whole abdomen every 12 months).

AE recurrence was diagnosed if imaging examination revealed new AE lesions in the liver and an ELISA for the hydatid was positive. AE recurrence was diagnosed the same way as the initial AE was, dividing it into resection margin recurrence and distant intrahepatic recurrence according to the recurrence site. Resection margin recurrence referred to recurrence when the edge of the new lesion was within 2 cm of the remaining cutting surface, and the distant intrahepatic recurrence referred to recurrence when the edge of the new lesion was >2 cm from the remaining cutting surface.

The treatments of AE recurrence, including drugs, reoperation, and comprehensive treatment, were based on the WHO guidelines for the diagnosis and treatment of echinococcosis (16). The treatment plan was chosen according to the lesion and the condition of the patient. The long-term efficacy of the patient's treatment was determined by follow-up, including over the telephone and face to face. The time between the date of surgery and the first recurrence diagnosis was defined as disease-free survival (DFS).

Statistical Methods

SPSS 22.0 software was used for data analysis. The non-normal measurement data are represented by median and quartiles [$M(Q1, Q3)$], and they were compared between groups by the rank-sum test. Count data were analyzed by the chi-squared test with four-fold tables or the $R \times C$ chi-squared test. Repeated measurements were used to compare the trend of indicators at different time points between the two groups. The Kaplan-Meier survival curves were plotted, and the differences in survival curves were analyzed by the log-rank test. $P < 0.05$ indicates that a difference was statistically significant.

RESULTS

Baseline Data

A total of 240 eligible patients with AE were enrolled, of whom 108 were males and 132 were females, with an average age of 34.20 ± 14.64 years (range: 5–79 years). Most (92.9%, 223/240) of the patients were Tibetans. Among the 240 patients, 123 underwent anatomic hepatectomy and 117 non-anatomic hepatectomy. There were no significant differences in age, sex, ethnicity, hydatid size or number, or liver function indices before the surgery between the two groups (**Table 1**).

TABLE 1 | Comparison of baseline data between the two groups.

Index	Groups		$t/\chi^2/Z$	P
	Anatomic hepatectomy	Non-anatomic hepatectomy		
Age (years)	33.93 ± 16.21	34.49 ± 12.85	−0.298	0.766
Lesion size (centimeter)	11.86 ± 3.42	11.71 ± 3.74	0.339	0.735
Alanine aminotransferase before surgery (U/L)	28.33 ± 14.39	30.32 ± 15.28	−1.035	0.302
Aspartate aminotransferase before surgery (U/L)	32.31 ± 10.63	32.78 ± 14.47	−0.285	0.776
Number of hydatids	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	−0.164	0.870
Child–Pugh score	5.00 (5.00, 6.00)	5.00 (5.00, 6.00)	−0.071	0.944
Sex				
Male	53	55	0.372	0.542
Female	70	62		
Ethnicity				
Han	7	6	1.147	0.563
Tibetan	115	108		
Hui	1	3		
Hepatitis B				
Yes	30	31	0.140	0.708
No	93	86		
Lesion location				
left lobe	34	27	0.814	0.666
right lobe	77	76		
middle lobe	12	14		
Surgical method				
Segmental hepatectomy	69	58	3.430	0.180
Hemihepatectomy	42	30		
Extended hemihepatectomy	12	29		

Age, hydatid size, alanine aminotransferase before surgery, aspartate aminotransferase before surgery comparison was performed using two independent samples t test. Numbe of hydatids and Child-pugh score were compared using the rank-sum test of two independent samples (Mann-Whitney U test). Sex and hepatitis B were compared by chi-squared test with four-fold tables. The R×C chi-square test was used to compare ethnicity, Lesion location and surgical method.

TABLE 2 | Comparison of intraoperative and postoperative data between the two groups.

Indices	Groups		$t/\chi^2/Z$	P
	Anatomic hepatectomy	Non-anatomic hepatectomy		
Occlusion time (minutes)	27.36 ± 11.30	48.38 ± 20.24	−9.869	<0.001
Intraoperative bleeding (milliliter)	300.00 (200.00, 600.00)	600.00 (400.00, 1,000.00)	−6.221	<0.001
Intraoperative blood transfusion (milliliter)	0.00 (0.00, 770.00)	600.00 (0.00, 1,200.00)	−3.196	<0.001
Duration of surgery (hours)	6.24 ± 0.86	6.34 ± 0.91	−0.877	0.381
Complication				
Yes	65	104	37.394	<0.001
No	58	13		
Recurrence				
R0	5	19	9.875	0.003
sR1	118	98		

Occlusion time and Duration of Surgery were compared using two independent sample T-tests. Two independent sample rank sum tests (Mann-Whitney U test) were used in the comparison of intraoperative bleeding and Intraoperative blood transfusion. The chi-squared test with four-fold tables was adopted for comparison between complication and recurrence.

TABLE 3 | Comparison of liver function indices between the two groups at different times before and after surgery.

Indices	Time				Sum	*F*	*P*
	Before surgery	1 day after surgery	3 days after surgery	5 days after surgery			
ALT (U/L)							
Anatomic hepatectomy	28.33 ± 14.39	327.02 ± 169.10	190.52 ± 77.81	98.35 ± 52.08	161.06 ± 147.82	274.307	<0.001
Non-anatomic hepatectomy	30.32 ± 15.28	439.46 ± 280.63	236.32 ± 135.86	115.64 ± 64.27	205.43 ± 221.12	182.380	<0.001
Sum	29.30 ± 14.83	381.84 ± 236.63	212.85 ± 112.14	106.78 ± 58.86	182.69 ± 188.39	411.767*	<0.001*
t	−1.035	−3.736	−3.183	−2.283	17.022*	10.475#	<0.001#
P	0.302	<0.001	0.002	0.023	<0.001*		
AST (U/L)							
Anatomic hepatectomy	32.31 ± 10.63	347.67 ± 173.31	100.91 ± 60.75	47.50 ± 16.96	132.10 ± 157.01	335.436	<0.001
Non-anatomic hepatectomy	32.78 ± 14.47	438.70 ± 255.10	127.46 ± 52.49	59.16 ± 32.61	164.77 ± 208.66	249.69	<0.001
Sum	32.54 ± 12.62	392.05 ± 221.35	113.85 ± 58.29	53.19 ± 26.40	148.01 ± 184.61	554.871*	<0.001*
t	−0.285	−3.217	−3.615	−3.448	16.747*	8.170#	0.003#
P	0.776	0.002	<0.001	0.001	<0.001*		

**indicates the F-statistic and P-value of the main effect; # indicates the F-statistic and P-value of the interaction. Two independent sample t-test was used to compare ALT and AST between different groups at the same time, and one-way repeated measurement analysis of variance was used to compare the changes of ALT and AST between the same group at different times.*

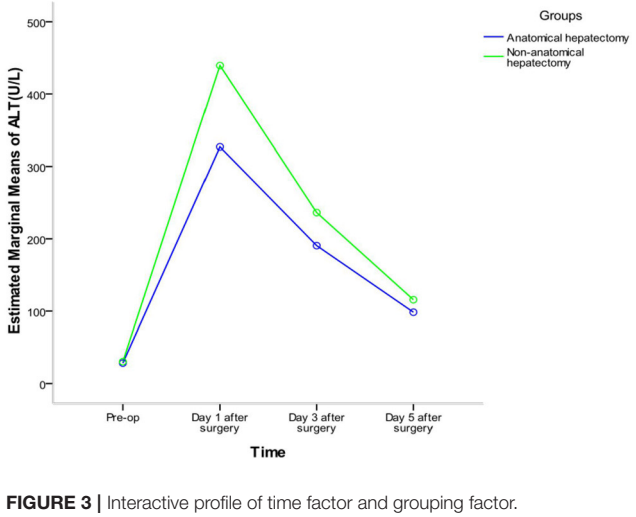

FIGURE 3 | Interactive profile of time factor and grouping factor.

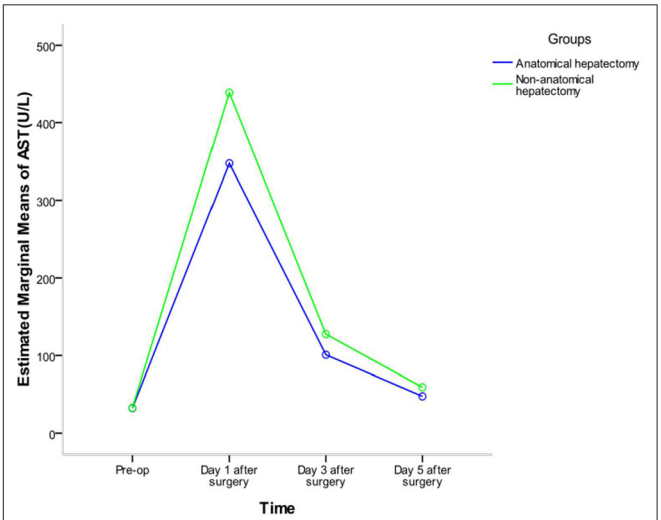

FIGURE 4 | Interactive profile of time factor and grouping factor.

Comparison of Intraoperative and Postoperative Indices

The comparison of the intraoperative and postoperative data of the two groups showed that the time of porta hepatis occlusion, intraoperative bleeding, intraoperative blood transfusion, complication rate, and AE recurrence rate in the anatomic hepatectomy group were significantly better than those in the non-anatomic hepatectomy group. The duration of surgery was not different. The results of the surgical margins showed that the recurrence rate of R0 margins was significantly lower than that of R1 margins ($\chi^2 = 175.135$, $P < 0.001$, **Table 2**).

Comparison of Liver Function Indices at Different Times

Repeated-measures analysis of variance of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of the two groups at different times showed that there were significant differences in ALT before and after surgery in the whole sample ($F = 411.767$, $P < 0.001$) and in each group (anatomic hepatectomy group: $F = 274.307$, $P < 0.001$; non-anatomic hepatectomy group: $F = 182.380$, $P < 0.001$). The trend of ALT in the two groups was the same: ALT was the lowest before surgery, peaked on the first day after surgery, and decreased gradually on the third and fifth days after surgery. The concentration of ALT in the anatomic hepatectomy group was significantly lower than that in the non-anatomic hepatectomy group ($F = 17.022$, $P < 0.001$). There was no significant difference in ALT between the two groups before surgery, but it was significantly lower in the anatomic hepatectomy group at all other time points. There was an interaction effect between

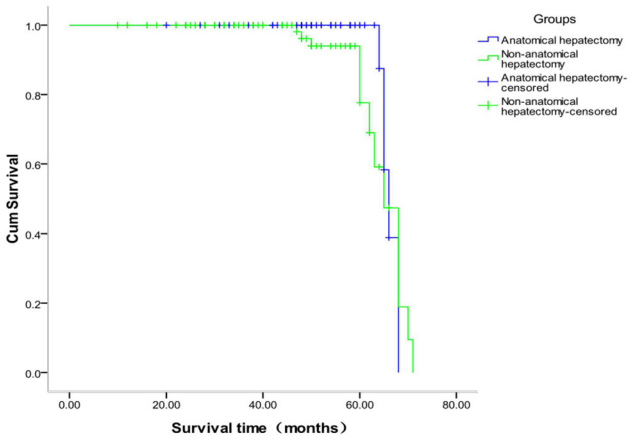

FIGURE 5 | Comparison of survival curves between the two groups with different surgical methods.

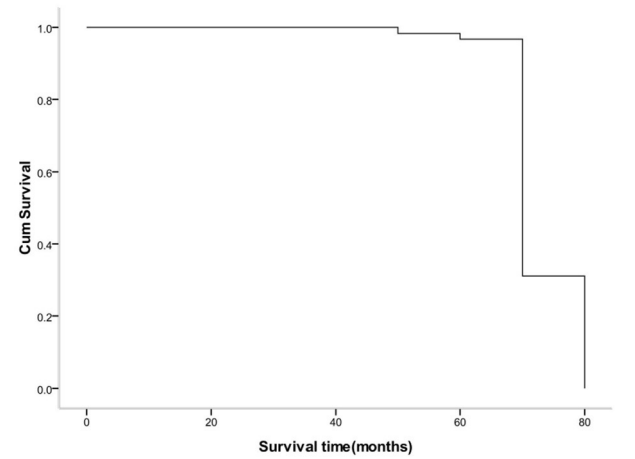

FIGURE 6 | Cumulative survival curve of hydatid patients.

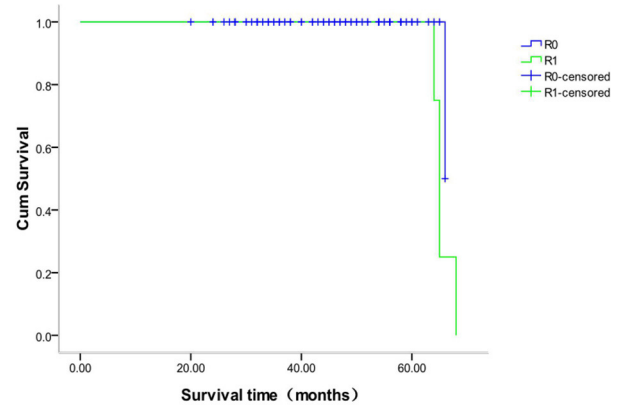

FIGURE 7 | Survival curve of patients with different surgical margins after anatomical hepatectomy.

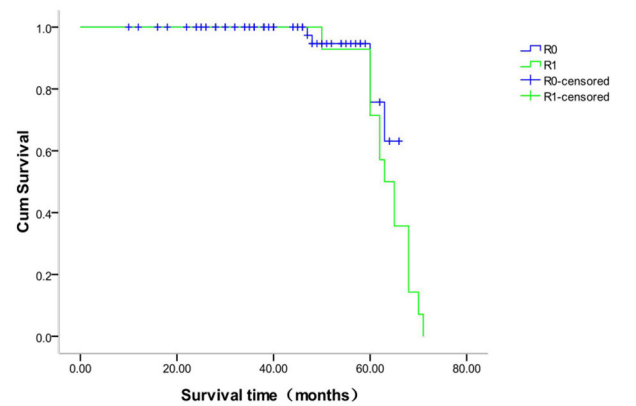

FIGURE 8 | Survival curve of patients with different surgical margins after non-anatomical hepatectomy.

ALT expression time and surgical method ($F = 10.475$, $P < 0.001$). The highest ALT expression was 1 day after surgery in the non-anatomic hepatectomy group, and the lowest was before surgery in the anatomic hepatectomy group. The changes in AST concentration in both groups were the same as those of ALT (**Table 3** and **Figures 3**, **4**).

Comparison of Survival Time Between the Two Groups

The survival analysis of the two groups showed that 118 cases were censored in the anatomic hepatectomy group, for a censoring rate of 95.9%, and 99 cases in the non-anatomic hepatectomy group, for a censoring rate of 84.6%. The median survival time of patients in the anatomic hepatectomy group was 66 months, compared to 65 months in the non-anatomic hepatectomy group ($\chi^2 = 4.662$, $P = 0.03$, **Figure 5**). From the survival curve in **Figure 3**, the prognosis of patients in the anatomic hepatectomy group was better than that in the non-anatomic hepatectomy group. The survival analysis of all patients showed that the median survival time of patients was 67.12 months, as shown in **Figure 6**.

Survival Time Analysis of Different Margins in the Anatomic and Non-anatomic Groups

The survival analysis of patients with different margins in the anatomic hepatectomy group showed that 118 patients with R0 margin were censored, for a censoring rate of 99.2%, and no patient was censored with an R1 margin. The median survival time of patients with R0 margins was 66 months and R1 margins 65 months ($\chi^2 = 1.561$, $P = 0.212$, **Figure 7**). The survival analysis of patients with different margins in the non-anatomic hepatectomy group showed that 98 patients with R0 margin were censored, for a censoring rate of 95.1%, and no patient was censored with R1 margins. There was no significant difference in the survival time between these two sub-groups ($\chi^2 = 0.947$, $P = 0.330$, **Figure 8**).

DISCUSSION

Hepatic AE is called "hydatid cancer" due to its special biological characteristics (17, 18). Radical resection of lesions is an effective treatment for advanced hepatic AE (19, 20). Anatomic hepatectomy is not only the basic method of precision liver surgery but also the ideal method of liver tumor resection (21, 22). Studies in China (23, 24) have shown that anatomic hepatectomy is safe and reliable for hepatic AE, with the advantages of fewer surgical complications and rapid recovery. The cited studies have mainly evaluated the short-term efficacy of anatomic hepatectomy for hepatic AE, with a postoperative follow-up time <1 year. Therefore, in this study, 240 patients with hepatic AE after hepatectomy were followed up for a long time, and the follow-up data were statistically processed to better evaluate the long-term efficacy of anatomic hepatectomy for hepatic AE.

This study mainly focused on the following three results. First, the short-term efficacy of anatomic hepatectomy was significantly better than that of non-anatomic hepatectomy in terms of rapid recovery of liver function and a low incidence of complications. Anatomic hepatectomy can best maintain the integrity of the residual liver structure and function by precise intraoperative methods, selective hepatic blood flow occlusion, and low central venous pressure, and these measures can effectively control intraoperative blood loss, since intraoperative blood loss and blood transfusion are closely correlated with poor outcomes (25). At the same time, selective hepatic blood flow occlusion can effectively reduce the ischemia–reperfusion injury of residual liver tissue (26–29). However, the focus of this clinical study was the long-term efficacy of anatomic hepatectomy for hepatic AE. Second, there was no difference in the overall survival time between the anatomic and non-anatomic hepatectomy groups, but the DFS time of patients in the anatomic hepatectomy group was significantly longer than that in the non-anatomic hepatectomy group. Similar results have been reported in studies of the prognosis of liver cancer (30, 31). Studies abroad have shown that radical resection for hepatic AE can significantly prolong the DFS of patients (6, 7). Studies in China (32, 33) have shown that some patients with hepatic AE have a high recurrence rate even after radical resection of lesions and regular oral administration of anti-echinococcosis drugs after operation. The main reason may be related to the range of surgical resection. Wen et al. (34) showed that the main factor for hepatic AE recurrence was the control of surgical margins. Shabunin et al. (35) reported more than 2 cm of that normal liver tissue around the lesion should be removed during radical resection for AE in order to reduce the postoperative recurrence rate. For this reason, the academic community in China has reached a consensus that during the thorough removal of echinococcosis lesions, the normal liver tissue more than 1 cm away from the lesion edge should be removed, aiming to eliminate the "infiltration zone" with active hyperplasia around lesions and reduce postoperative recurrence (36, 37). The infiltration zone is the location of actively proliferating cells, which is rich in microvessels and mainly includes the portal vein and hepatic artery. AE lesions are constantly infiltrating and growing into lesion microenvironment, which concept was firstly established by Dr. Aini et al. (38–40). This study found that the infiltration zone had not only active hyperplasia but also microvascular invasion of AE, which is similar to the microvascular invasion in the tissues adjacent to liver cancer. Microvascular invasion is closely related to the prognosis of liver cells (41, 42), but whether microvascular invasion of AE is related to postoperative recurrence is not known. Finally, in clinical practice, we often encounter irregular AE lesions, or lesions adjacent to the porta hepatis or retrohepatic inferior vena cava. In such cases, sufficient margin width (>1 cm) cannot be achieved, and only complete resection of the lesions and negative margins can be achieved. However, a recent research that studied AE lesion microenvironment proposed that different lesion categories had different infiltrative boundary, thus, tailored resection margin was strongly recommended (40). Therefore, any resection margin that do not consider lesion heterogeneity would not be appropriative in the era of precision management. These shortcomings may explain the high recurrence rate of patients in the non-anatomic hepatectomy group. Joliat also showed that when radical resection for AE was performed, the recurrence rate of patients with positive margins confirmed by postoperative pathology was as high as 41% at 7 years, even with the postoperative adjuvant albendazole treatment (43).

CONCLUSION

In conclusion, for hepatic AE, anatomic hepatectomy can achieve good long-term efficacy only on the premise of ensuring a large enough resection range. In addition to comparing the efficacy of the two surgical methods, this study examined the factors related to postoperative recurrence of hepatic AE, and we will continue to study this topic.

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 Ethics Committee of the Qinghai Provincial People's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JA, SZ, and HW: conceived and designed the study. JA, JZ, JC, and XA: collected the data. JA and JZ: contributed to data

analysis and interpretation. JA and JC: writing article. JY and XA: approved the study and this submission. All authors contributed to the article and approved the submitted version.

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Subcutaneous IL-6 Inhibitor Sarilumab vs. Standard Care in Hospitalized Patients With Moderate-To-Severe COVID-19: An Open Label Randomized Clinical Trial

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Background: The use of IL-6 blockers in COVID-19 hospitalized patients has been associated with a reduction in mortality compared to standard care. However, many uncertainties remain pertaining to optimal intervention time, administration schedule, and predictors of response. To date, data on the use of subcutaneous sarilumab is limited and no randomized trial results are available.

Methods: Open label randomized controlled trial at a single center in Spain. We included adult patients admitted with microbiology documented COVID-19 infection, imaging confirmed pneumonia, fever and/or laboratory evidence of inflammatory phenotype, and no need for invasive ventilation. Participants were randomly assigned to receive sarilumab, a single 400 mg dose in two 200 mg subcutaneous injections, added to standard care or standard care, in a 2:1 proportion. Primary endpoints included 30-day mortality, mean change in clinical status at day 7 scored in a 7-category ordinal scale ranging from death (category 1) to discharge (category 7), and duration of hospitalization. The primary efficacy analysis was conducted on the intention-to-treat population.

Results: A total of 30 patients underwent randomization: 20 to sarilumab and 10 to standard care. Most patients were male (20/30, 67%) with a median (interquartile range) age of 61.5 years (56–72). At day 30, 2/20 (10%) patients died in the sarilumab arm vs. none (0/10) in standard care (Log HR 15.11, SE 22.64; $p = 0.54$). At day 7, no significant differences were observed in the median change in clinical status (2 [0–3]) vs. 3 [0–3], $p = 0.32$). Median time to discharge (days) was similar (7 [6–11] vs. 6 [4–12]; HR 0.65,

SE 0.26; $p = 0.27$). No significant differences were detected in the rate of progression to invasive and noninvasive mechanical ventilation.

Conclusions and Relevance: Our pragmatic pilot study has failed to demonstrate the benefit of adding subcutaneous sarilumab to standard care for mortality by 30 days, functional status at day 7, or hospital stay. Findings herein do not exclude a potential effect of sarilumab in severe COVID-19 but adequately powered blinded randomized phase III trials are warranted to assess the impact of the subcutaneous route and a more selected target population.

Trial Registration: www.ClinicalTrials.gov, Identifier: NCT04357808.

Keywords: COVID-19, sarilumab, subcutaneous route, IL-6, IL-6 receptor inhibitors, IL-6 blockade, randomized controlled trial, subcutaneous

INTRODUCTION

Approaching the second year after WHO declared COVID-19 as a pandemic, many uncertainties persisted about the disease course, prognosis, and treatment. Vaccination has emerged as the real hope for the global threat, but global herd immunity will take months or even years to be reached (1). Therefore, thousands of patients will still require supportive and pharmacological treatment.

During the early days of the pandemic, the rapid spread of the SARS-CoV-2 coronavirus posed unprecedented challenges for health services to properly manage COVID-19 severe and critical manifestations affecting a wide population in a short period of time. Given the ineffective antiviral therapy on hospitalized patients (2), huge efforts were directed to abrogate the hyperinflammatory status that complicates the clinical course and eventually leads to death (3). Off-label use of plenty of immunomodulatory drugs emerged, targeting cytokines involved in COVID-19 acute respiratory distress syndrome (ARDS), where high IL-6 levels have a prominent role (4). Tocilizumab (TCZ), an IL-6 receptor (R) inhibitor, was the first anti-cytokine agent tested in the pandemic (5–7), based on the pathogenic role of IL-6 as a driver of hyperinflammation (4, 8) and high IL-6 levels as predictors of poor outcomes (9–11). Consistent with those results, an observational study conducted in our hospital during the first outbreak in Spain demonstrated that early IL-6 blockade with TCZ was associated with improvement of oxygenation and reduced the death rate in patients with IL-6>30 pg/ml, as this was the best predictor of invasive mechanical ventilation (IMV) (12). In those early days, intravenous IL-6R inhibitors began to be tested in several trials; however, no data on subcutaneous formulations were available.

Sarilumab (SAR) is a human monoclonal antibody that binds membrane-bound and soluble IL-6 receptors to inhibit IL-6 signaling, licensed in a subcutaneous route administration for the treatment of Rheumatoid Arthritis (13). At a moment where the health system was overrun, especially emergency and intensive care unit (ICU) facilities, with real concern about TCZ shortages, we conceived that subcutaneous administration of SAR could facilitate the administration of an IL-6 inhibitor in all settings, including wards and overloaded emergency rooms. Additionally, the safety and maximum pharmacodynamic effects of a single 200 mg dose of subcutaneous SAR are known through the results of two open randomized controlled trials (RCT) (14). Data were similar to those obtained with single doses of 4 and 8 mg/kg intravenous TCZ, with a longer effect of TCZ in the second week. Our hypothesis was that the use of 2 subcutaneous SAR injections and early intervention (window of opportunity) could prevent higher oxygenation requirements through non-invasive (NI) and invasive mechanical ventilation (IMV) and reduce death rate. Thus, we proposed an open pilot pragmatic RCT to evaluate the efficacy and safety of a single 400 mg subcutaneous dose of SAR, in patients with moderate to early severe COVID-19, compared to standard care (SC).

METHODS

Design

SARCOVID is an investigator-initiated open-label phase II RCT, conducted from April 13 (first patient enrolment) to December 4 (last patient's last visit), 2020, at Hospital Universitario La Princesa (HUP) during the first and second outbreak in Madrid, Spain. This design was a counterproposal from the Spanish Agency for Medicines and Health products (AEMPS) to our urgent proposal of an exploratory propensity score-matched observational study. The trial was approved by the AEMPS and the Research Ethics Committee of the HUP on April 9, 2020 (Reference number 4078) and was conducted in accordance with the principles of the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization.

The timeline of recruitment is illustrated in **Supplementary Figure 1**. Enrolment abruptly dropped following the decrease of COVID-19 incidence in Madrid. A formal amendment was submitted to the HUP Ethics Committee on May 7, 2020, for the inclusion of a positive serologic test (IgM/IgA by ELISA) as diagnostic confirmation of COVID-19 infection in the absence of a positive reverse-transcriptase–PCR (RT-PCR) assay for SARS-CoV2 in a respiratory tract specimen. After concomitant approval of the AEMPS, trial recruitment remained open until completion. A full version of the protocol and amendment, which includes the statistical plan, has been

published elsewhere (15). This study followed the Consolidated Standards of Reporting Trials (CONSORT) reporting guideline (See **Supplementary Material**).

Study Population

Patients ≥18 and <80 years attending the emergency room of HUP in need for hospitalization or those in hospital wards were eligible if they had confirmed pneumonia on chest imaging and a documented diagnosis of COVID-19 by RT-PCR assay or, in its absence, case definition of COVID-19 pneumonia as per local protocol and a positive IgM/IgA serologic ELISA test. For recruitment, at least 2 of the following additional criteria needed to be fulfilled: Fever ≥ 37.8°C; IL-6 in serum ≥ 25 pg/mL or PCR > 5 mg / dL; lymphocytes <600/mm^3; ferritin > 300 μg /L doubling in 24 h; ferritin > 600 μg /L in the first determination; and LDH > 250 or D-dimer > 1 mg / L. Exclusion criteria included requirements of IMV at inclusion; AST / ALT values more than 5-folds the upper normal limit; absolute neutrophil count <500/ mm^3; absolute platelet count <50,000/ mm^3; superimposed infection by pathogens other than COVID-19; complicated diverticulitis or intestinal perforation; immunosuppressive anti-rejection therapy; pregnancy or lactation; previous treatment with TCZ or SAR; contraindication to SAR or excipients; and comorbidities that can likely lead to an unfavorable result.

Randomization and Treatments

A total of 30 patients were randomly allocated in a 2:1 ratio to the intervention group, SAR (400 mg single dose in 2 subcutaneous injections 200 mg each in pre-filled syringe) plus SC, or current SC. Central telephone randomization was performed by the Clinical Research and Clinical Trials Unit (CRCTU) at the HUP using the program www.randomization.com with a 2:1 proportion and 5 blocks of 6 subjects. After checking that all entry criteria were met, the CRCTU communicated the assigned treatment to the recruiting investigator, who reported the correct allocation in the electronic clinical record (ECR). Patients in both arms received drugs, including corticosteroids, or full supportive care according to the best SC updated in the local protocol for COVID-19. Patients in the SC were given the option to receive intravenous TCZ after randomization if they worsened at the investigator's discretion, as this agent had become the SC in our center when the protocol was designed (12). Other immunomodulators or investigational drugs in trials were prohibited.

Outcomes

The primary endpoints were mortality by 30 days, mean change in functional status at day 7 on a 7-category ordinal scale as recommended by the WHO R&D Blueprint Group (16) (1. death; 2. hospitalized, requiring ECMO, IMV, or both; 3. hospitalized, requiring high-flow oxygen therapy, NIMV, or both; 4. hospitalized, requiring supplementary oxygen; 5. hospitalized, not requiring supplementary oxygen but in need of ongoing medical care; 6. hospitalized, not requiring ongoing medical care; and 7. not hospitalized), and time to discharge from randomization. Secondary outcomes included time to become afebrile during 48 h without antipyretics, mean change in 7-category ordinal scale at day 14, time to NIMV and IMV, time to oxygen supply independence, and adverse events (AE). As no events occurred in SC, the outcomes time to NIMV and IMV were changed to progression to NIMV and IMV.

As this trial has been included in a recent prospective meta-analysis of IL-6 inhibitors in hospitalized COVID-19 patients (17), mortality by 90 days and serious infections by 90 days were also assessed, although these outcomes were not included in the original protocol.

Procedures

This trial was intended to be carried out pragmatically according to the usual clinical practice in Spain during the first pandemic wave, avoiding any additional workload in treating physicians who assessed each patient several times a day. The study calendar and procedures are detailed in the protocol. Briefly, vital signs, targeted physical examination, supplementary oxygen requirements, and resting oxygen saturation were recorded daily and registered at admission and subsequent study visits at days 0, 1, 2, 5, 7, and 14 after randomization, and at discharge day. Efficacy assessments included an evaluation of clinical status by a 7-category ordinal scale at days 0 (randomization), 7, and 14 (through a phone call if the patient had been discharged).

Laboratory testing was performed locally according to clinical practice at established study visits when the patient continued to be at the hospital. IL-6 serum levels were determined at baseline, day 5, and on patients still admitted at day 14. Serum IL-6 was quantified in duplicate with the Human IL-6 Quantikine high-sensitivity ELISA from R&D Systems Europe Ltd (Abingdon, United Kingdom), following the manufacturer's instructions.

On day 30 after randomization and days 10–15 after discharge, the patient appraisal was performed through a phone call by a member of the research team. Screening for tuberculosis, Hepatitis B Virus, and HIV was also done on day 0, and safety and concomitant medication assessments were performed daily until discharge. Although not included in the protocol, a review of the ECR, including microbiological isolations and drug prescriptions, both in hospital and primary care settings, was done to assess mortality and infections by 90 days. Patients with no recorded data for this timeframe were interviewed through a phone call by the principal investigator.

All patient data were anonymized and recorded into a local database.

Data Quality Monitoring

Data quality on-site monitoring was performed by a dedicated staff of the CRCTU at the HUP, independent of the investigators' team, with 100% source data verification for all critical data points. All severe AE (SAE) was reviewed and evaluated by a qualified pharmacovigilance expert of the CRCTU, independent of the investigators' team.

Statistical Analysis

A formal calculation of the sample size was not performed, since the study was designed as a pragmatic proof of concept study

with a drug that had not been previously evaluated in COVID-19. Regulatory authorities (AEMPS) estimated that 30 patients (20 SAR: 10 control) might be sufficient for an initial evaluation of the study objectives.

Qualitative variables were described using a calculation of the proportions and due to the low number of patients in the SC group, the two-sided Fisher's exact test was used to compare categorical variables. Quantitative variables were represented as median and IQR and, considering the sample size, Mann–Whitney ***U*** test was used to analyze significant differences. Statistical significance was considered if $p < 0.05$.

The primary efficacy analysis was conducted on the intention-to-treat (ITT) population. To estimate the intervention effect size, hazard ratio (HR) was estimated when it was feasible. In these cases, follow-up was censored to 30 days, which was the longest duration of hospitalization since randomization. For the total length of hospital stay, follow-up was censored to 33 days. For HR estimation, dead patients were assigned 30- or 33-day follow-ups, respectively.

As three patients received TCZ in the SC group, sensitivity analyses for primary and secondary outcomes were performed excluding those patients.

To provide a more accurate assessment of our results, avoiding biases, we performed a multivariable linear regression using generalized linear models (*glm* command of Stata) with the primary outcome change in clinical status on the 7-category ordinal scale at day 7 as the dependent variable, and several independent variables that could be confounding factors (age, gender, baseline category in the ordinal scale, time from symptoms onset, comorbidities, cumulative glucocorticoid use …among others). Since there were 30 cases, we first tested the independent variables one by one, and then with those with a better performance we fitted the best model with 3 independent variables, namely, gender, cumulative glucocorticoid dose between baseline and day 7, and the allocated treatments.

Statistical analyses were performed using Stata 14.0 for Windows (Stata Corp LP, College Station, TX, United States).

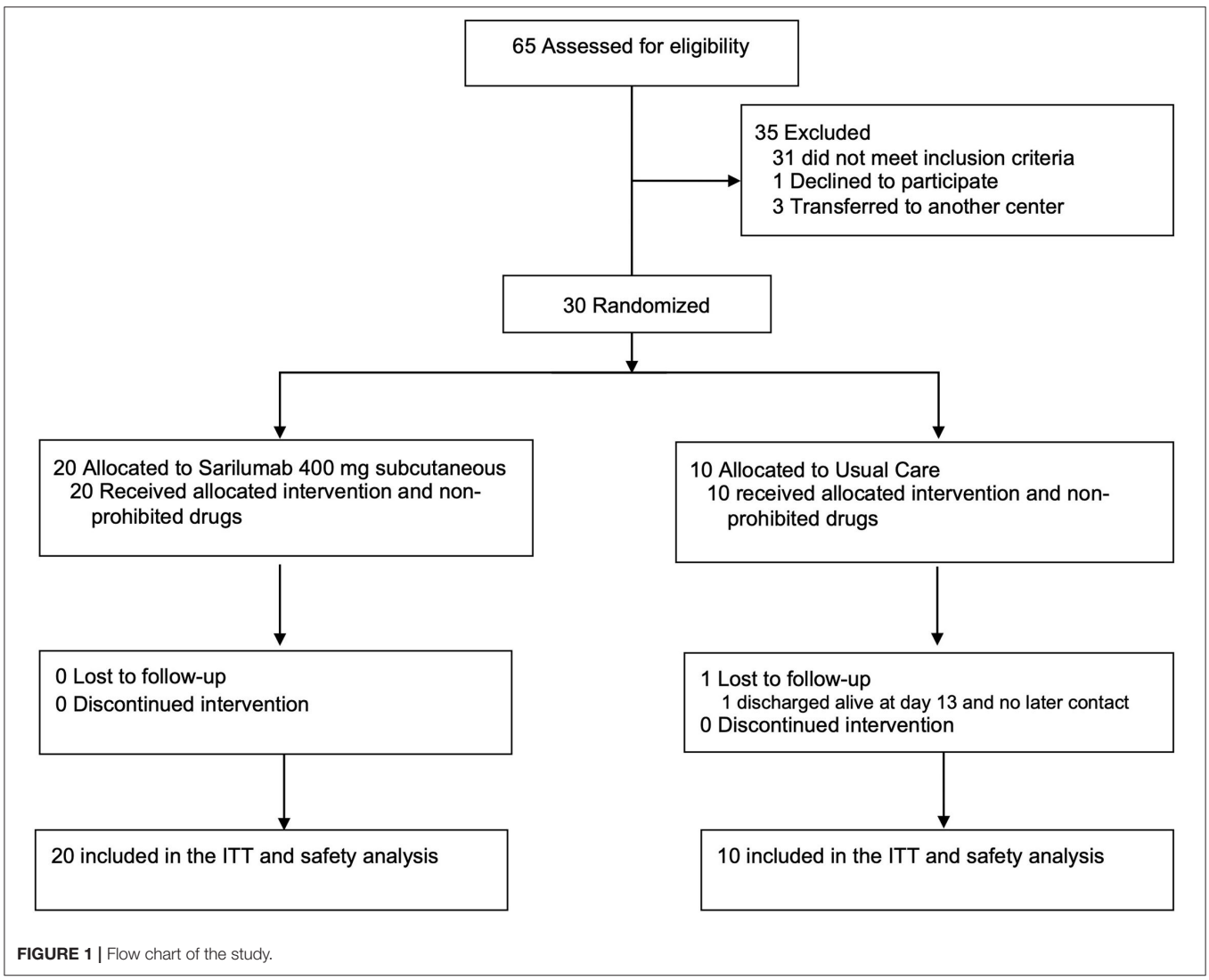

FIGURE 1 | Flow chart of the study.

RESULTS

Patients

Between April 13 and October 30, 2020, 30 of 65 screened patients underwent 2:1 randomization: 20 to SAR (400 mg single dose, subcutaneous) + SC and 10 to SC. The last patient's last visit was on December 4, 2020. All patients received the allocated intervention and completed the follow-up, except 1 in the SC arm, who was discharged alive on day 13, with no response to the study's post-discharge appointment (**Figure 1**). Of the 10 patients assigned to SC, 3 received late TCZ after randomization and were included in the ITT analysis on the arm they were originally allocated. The median age was 61.5 years (IQR 56–72), 67% were men (**Table 1**). All randomized patients had finally a documented diagnosis of COVID-19 by RT-PCR assay. Most clinical, demographic, and laboratory baseline data (**Tables 1**, **2**, respectively) were similar across treatment groups. However, a higher proportion of men, fever, and extended radiological pattern at admission was recorded in the SAR arm, along with a shorter duration from symptom onset to randomization. All patients receiving high flow oxygen therapy or NIMV at randomization were allocated to SAR while all patients under SC had low flow supplementary oxygen requirements. Conversely, 4/20 (20%) in the SAR arm had no supplementary oxygen requirements.

Median CRP levels were 9.28 (IQR 5.06–19.73) mg/dl without significant differences between allocations at randomization

TABLE 1 | Baseline demographics and clinical characteristics of the study population.

	n (%)		
	Total ($n = 30$)	**Sarilumab + SC ($n = 20$)**	**SC ($n = 10$)**
Median Age in years (IQR)	61.5 (56–72)	61.5 (50.5–72)	62 (58–71)
Male sex, n (%)	20 (67)	15 (75)	5 (50)
Race, ethnicity (%)			
White	14 (47)	10 (50)	4 (40)
Asian	1 (3)	0 (0)	1 (10)
Hispanic or latino	15 (50)	10 (50)	5 (50)
Coexisting Disorders, n (%)	19 (63)	14 (70)	5(50)
Hypertension	13(43)	8 (40)	5 (50)
Diabetes Mellitus	5 (17)	3 (15)	2 (20)
Obesity	3 (10)	2 (10)	1 (10)
History of Malignancy	2 (7)	2 (10)	0 (0)
COPD	2 (7)	1 (5)	1 (10)
Stage III Chronic kidney disease	4 (13)	2 (10)	2 (20)
Coronary artery disease	3 (10)	3 (15)	0
Median days from symptom onset to randomization (IQR)	11 (8-16)	10.5 (8-12.5)	16 (12-23)
Median days from admission to randomization (IQR)	2 (1-4)	2 (1-4)	3 (1-6)
Median body temperature at randomization (IQR), ˚C	37 (36.4-37.7)	37.1 (36.6-38.1)	36.5 (36.3-37.2)
Fever ≥37,5°C, n (%)	10 (33)	9 (45)	1 (10)
Oxygen support at randomization (7-category ordinal scale) n (%)			
5. No supplemental oxygen therapy	4 (13.3)	4 (20)	0 (0)
4. Supplemental low flow oxygen therapy[a]	22 (73.3)	12 (60)	10 (100)
3. High-flow supplemental oxygen therapy or NIV[b]	4 (13.3)	4 (20)	0 (0)
Median PaO_2/FiO_2 mmHg (IQR) at randomization	318 (233-358)	298 (223-348)	341 (261-404)
Additional treatment during hospitalization			
Hydroxychloroquine	6 (20)	4 (20)	2 (20)
Lopinavir/Ritonavir	5 (17)	4 (20)	1 (10)
Azithromycin	18 (60)	12 (60)	6 (60)
Interferon	0 (0)	0 (0)	0 (0)
Remdesivir at randomization	0 (0)	0 (0)	0 (0)
Corticosteroids at randomization[c]	25 (83)	17 (85)	8 (80)
Methylprednisolone bolus	17 (57)	14 (70)	3 (30)

COPD, Chronic obstructive pulmonary disease; IQR, interquartile range; NIV, noninvasive ventilation; $PaO_2/Fi0_2$, partial pressure of arterial oxygen/fraction of inspired oxygen; SC, standard care.

[a] O_2 flow ≤ 15 l/min e.g., by face mask, nasal cannula (NC).

[b] O_2 flow >15 l/min, e.g., by face mask, 'High Flow' devices (e.g., HFNC), CPAP or NIV including BiPAP and other devices.

[c] Corticosteroids: ≥ 30 mg Prednisone/d or equivalent; endovenous bolus of 6-Metilprednisolone 120–125 mg/d, except for 1 patient 80 mg/dl.

TABLE 2 | Baseline laboratory and radiologic findings of the study population.

	n (%)		
	Total ($n = 30$)	**Sarilumab + SC ($n = 20$)**	**SC ($n = 10$)**
Laboratory values (median, IQR)			
White Blood Count (cells/mm^3)	7,985 (5,160–11,140)	7,070 (4,975–12,310)	10,065 (6,750–10,460)
Lymphocyte Count (cells/mm^3)	830 (680–1,130)	825 (680–1,070)	865 (680–1,580)
Neutrophil Count (cells/mm^3)	5,910 (3,935–9,312)	6,215 (5,398–9,313)	5,850 (3,575–10,972)
Creatinine. mg/dL	0.80 (0.63–0.98)	0.83 (0.71–0.99)	0.65 (0.59–0.87)
Bilirubin. mg/dL	0.40 (0.32–0.52)	0.38 (0.32–0.53)	0.49 (0.34–0.52)
AST. U/L	33 (26-54)	40 (27-53)	32 (25-93)
ALT. U/L	46 (24-61)	48 (29-57)	28 (21-97)
GGT. U/L	56 (34-117)	41 (30-119)	71 (55–108)
LDH. U/L	297 (238–349)	317 (263–350)	263 (222–333)
Inflammatory markers			
serum IL-6. pg/mL ($n = 24$)	12 (3–21.5)	13.3 (7.5–24)	3 (1–16.5)
IL-6 $\geq$ 30 pg/mL, n (%)	4/24 (17)	3/16 (19)	1/8 (13)
Ferritin. ng/mL ($n = 29$)	1,179 (735–1,511)	1,048 (664–1,511)	1,265 (735–1,532)
CRP. mg/dL	9.28 (5.06–19.73)	8.59 (4.17–18.1)	9.94 (6.19–19.73)
PCT. ng/mL ($n = 13$)	0.11 (0.08–0.18)	0.11 (0.09–0.18)	0.12 (0.07–0.18)
D-dimer (μg/mL) ($n = 29$)	0.49 (0.37–1.14)	0.49 (0.36–1.28)	0.51 (0.37–1.09)
Baseline thorax radiologic findings (x ray and/or CT scan) [a]			
Alveolar pattern or ground glass opacities > 50%	14 (47)	11 (55)	3 (30)

AST, Aspartate amino-transferase; ALT, Alanine amino- transferase; CRP, C-reactive protein; GGT, Gamma-glutamyl transferase; IL-6, Interleukin-6; IQR, Interquartile range; LDH, Lactate Dehydrogenase; PCT, Procalcitonin. SC, Standard care.

[a] *All radiologic exams were assessed and reported by radiologists with pneumological expertise.*

(**Table 2**). Serum IL-6 levels were available in 24 patients at randomization, with a median of 13.3 (IQR 7.5–24) pg/ml on 16 SAR patients and 3 (IQR 1–16.5) pg/ml on 8 SC subjects. Only 3/16 patients in the intervention arm and 1/8 in SC had high IL-6 levels (**Table 2**), according to the threshold (30 pg/ml) previously established in our hospital population (12).

More than 80% of patients received glucocorticoids at randomization (**Table 1**), with no significant differences between arms in the median accumulated dose before randomization (156 mg [IQR 90–300] in SAR vs. 207 mg [IQR 80–550] in SC, $\boldsymbol{p} = 0.81$) and after allocation (105 mg [IQR 0–403] in SAR vs. 135 mg [100–200] in SC, $\boldsymbol{p} = 0.80$).

Primary Outcomes

No significant differences were seen in the median change [IQR] in clinical status on the 7-category ordinal scale at day 7 between SAR and SC (2 [0–3] vs. 3 [0–3], $\boldsymbol{p} = 0.32$) (**Table 3**). The proportion of patients in each category at this time point is shown in **Figure 2**. Regarding 30-day mortality, 2/20 (10%) patients died in the SAR arm while no events (0/10) were found in SC (**Table 3**). Those results were identical for in-hospital mortality. Two deaths occurred in patients with previous Grade III chronic kidney disease (CKD) and NIMV at randomization. Median days to discharge on SAR and SC were similar (HR = 0.65, SD = 0.26; $p = 0.27$).

We performed a multivariate analysis to determine which variables influenced the primary outcome "change in clinical status at day 7" that ranges from death (0) to discharge (7). Along with other confounding factors, age was not significantly associated with this outcome in the bivariate analysis and finally, the best multivariate model included 3 independent variables: sex/gender, cumulative glucocorticoid dose between baseline and day 7, and the allocated treatments. The results (**Table 4**) showed that higher requirements of glucocorticoids after randomization were significantly associated with a worse clinical evolution at day 7, likely reflecting a confounding by indication bias, as those patients that rapidly worsened were prescribed higher doses of corticosteroids. In addition, the female gender showed a trend to worse evolution. After adjustment by these variables, there were no significant differences between SC and SC plus SAR.

Secondary Outcomes

No significant differences were observed between arms for any of the secondary outcomes (**Table 3**). In SAR, 4/20 (20%) and 3/20 (15%) patients required NIMV and IMV, respectively, vs. none in the SC. Notably, 2/3 of patients progressing to IMV were not receiving corticosteroids at randomization day, although just one of these 2 patients died. The median time to oxygen withdrawal was similar between groups (**Table 3**).

Evolution of partial pressure of arterial oxygen/fraction of inspired oxygen (PaO_2/FiO_2) throughout study visits (**Figure 3A**) showed no significant differences between both allocated interventions at days 1, 2, and 7 after randomization, nor at discharge. Baseline high IL-6 levels ($\geq$ 30 pg/ml) appeared only in 3 patients allocated to SAR and 1 to SC. Along the study visits, patients with low baseline IL-6 levels (< 30 pg/ml)

TABLE 3 | Clinical outcomes in the intention-to-treat population.

Outcomes	Median (IQR)				
	Sarilumab + SC ($n = 20$)	SC ($n = 10$)	Hazard ratio (SE)	Log hazard ratio (Log SE)	*P*-Value
Primary outcomes					
Median change in clinical status (7-category ordinal scale [a]) at day 7,	2 (0–3)	3 (0–3)			0.32
Mean change on clinical status at day 7, (SD)	1.45 (1.93)	2.1 (1.45)			0.36
30-day mortality, n (%) [b]	2 (10)	0		15.11 (22.64)	0.54
Duration of hospitalization, days from randomization [c]	7 (6-11)	6 (4-12)	0.65 (0.26)		0.27
Secondary outcomes					
Median change in clinical status at day 14	3 (3)	4 (2-4)			0.36
Time to become afebrile for a minimum of 48 h, days[d]	3 (3-6)	4 (4-8)	1.60 (0.97)		0.39
Progression to NIMV, n (%)	4 (20)	0 (0)		15.09 (22.52)	0.27
Progression to IMV, n (%)	3 (15)	0 (0)		15.10 (22.52)	0.5
Time to supplemental oxygen withdrawal, days from randomization	5.5 (3−13)	4.5 (2-12)	0.83 (0.37)		0.83

IMV, invasive mechanical ventilation; IQR, interquartile ranges; NIMV, Noninvasive mechanical ventilation; SD, standard deviation: SC, Standard care; SE, Standard error.

[a] *Scale range: 1 = death to 7 = non hospitalized.*

[b] *One patient in the SC arm was lost to follow-up after discharge at day 13.*

[c] *Accounting for survival status by treating patients who died as having a 30-day hospital stay.*

[d] *Eleven patients in the SAR+SC arm and 5 in the SC arm were febrile at randomization.*

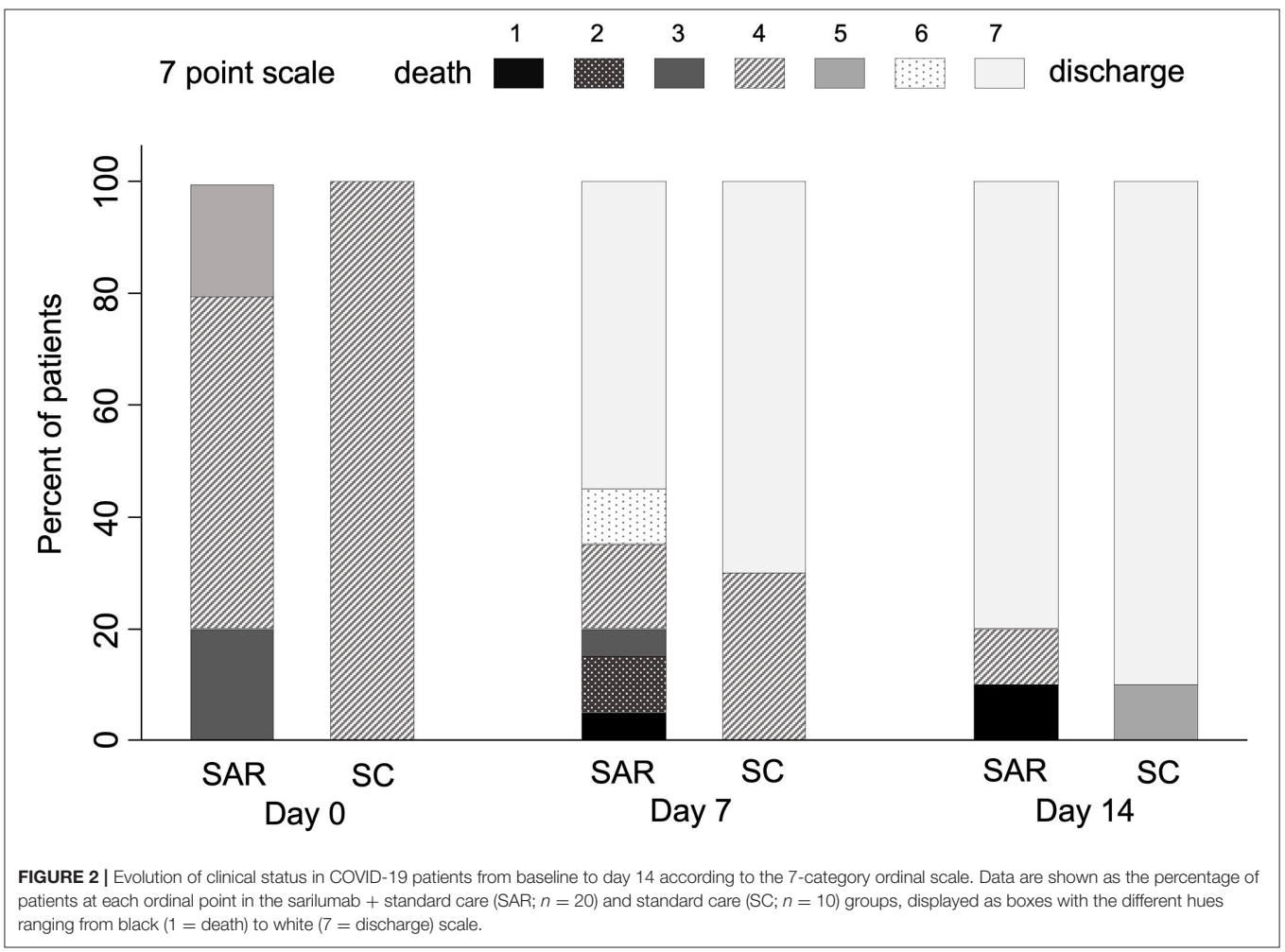

FIGURE 2 | Evolution of clinical status in COVID-19 patients from baseline to day 14 according to the 7-category ordinal scale. Data are shown as the percentage of patients at each ordinal point in the sarilumab + standard care (SAR; $n = 20$) and standard care (SC; $n = 10$) groups, displayed as boxes with the different hues ranging from black (1 = death) to white (7 = discharge) scale.

tended to present non-significantly higher PaO_2/FiO_2 than their counterparts (**Figure 3B**).

Regarding surrogate inflammatory markers and laboratory parameters, no significant differences were observed between arms at baseline nor throughout the study, except for significant reductions of LDH levels after day 2 from randomization (**Figure 4**, and data not shown) in patients allocated to SC. The time plot decline of median CRP levels was consistent with previously reported data for sarilumab after a single 200 mg subcutaneous injection (14) with a maximum decrease on day 7, but we did not observe a steeper decrease with 400 mg SAR on days 1, 2, 4, and 5 after randomization compared to the control group (**Figure 4A**, and data not shown).

To avoid a possible bias, we performed a sensitivity analysis excluding the three patients that received late TCZ as SC. Baseline characteristics of these populations are shown in **Supplementary Table 1**, with no significant differences between arms, except for a shorter disease duration to randomization in the SAR arm (10.5 days [8–12.5]) vs. SC (18 [12–24], $p = 0{,}013$), and lower median body temperature at randomization in SC (36.3 [36.2–37]) vs. SAR (37.1 [36.6–38.1] $p = 0.056$). No significant differences were observed for primary and key secondary outcomes between allocated interventions (**Table 5**), confirming the results of the ITT analysis.

Safety outcomes are reported in **Table 6**. Five SAE occurred in 4 patients allocated to SAR: 2 secondary respiratory bacterial infections by *Achromobacter xylosoxidans* and *Staphylococcus aureus*, 1 respiratory failure, and 2 fatal cases with failure of 2 organs (lung and kidney). The rate of AE of special interest was similar in SAR (50%) and SC (40%) (**Table 6**). Only one transient Grade III neutropenia on the SAR arm was considered as treatment related.

TABLE 4 | Variables associated with improvement in clinical status at day 7.

	β Coefficient	95% Confidence interval	*p*-value
Treatment			
SC	Reference	-	0.156
SC + Sarilumab	−0.89	−2.11 – 0.34	
Gender			
Male	Reference	-	0.085
Female	−1.07	−2.30 – 0.15	
Cumulative GC dose (by g of prednisone)[a]	−1.92	−3.27 to −0.58	0.005

SC, standard care; GC, glucocorticoids; g, gram.
[a]*Cumulative GC dose from randomization to day 7.*

Other Outcomes

No additional deaths or serious infections were recorded by 90 days in any of the allocated arms.

DISCUSSION

This pragmatic open pilot RCT in hospitalized patients with moderate-to-severe COVID-19 has failed to demonstrate the benefit of adding subcutaneous SAR to the SC for preventing high oxygen requirements, invasive ventilation, or death. Additionally, serious adverse events also occurred in the intervention arm, although no definite relationship with SAR could be demonstrated.

Limited evidence based on case series (18–20) and observational studies (21–23) suggested that SAR off-label use was safe and might be beneficial in the treatment of COVID-19 infection (24). However, a systematic review and

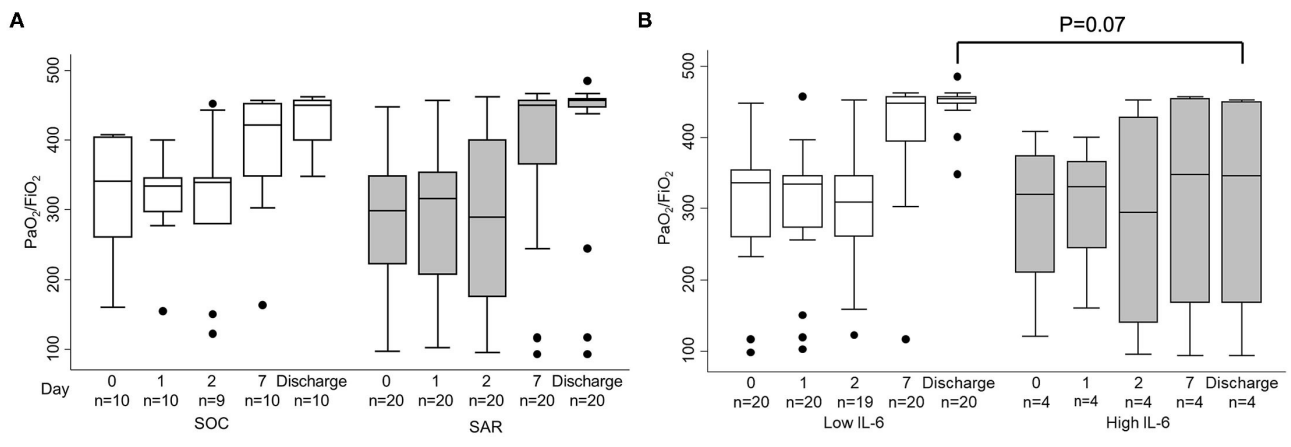

FIGURE 3 | Evolution of partial pressure of arterial oxygen/fraction of inspired oxygen (PaO2/FiO2) throughout study visits. Patients are grouped depending on **(A)** allocated interventions: standard care (SC) or sarilumab (SAR) and **(B)** level of serum interleukin-6 (IL-6) at randomization (cut-off for high levels ≥ 30 pg/ml). Two patients died and their last observed value was carried forward. IL-6 levels at randomization were available only in 24 patients; high IL-6 levels were observed in 3 patients from the SAR group and 1 patient from the SC group. Data are shown as interquartile ranges (p75 upper edge of the box, p25 lower edge, p50 midline) as well as the p95 (line above box) and p5 (line below). Dots represent outliers. Statistical significance was determined with the Mann–Whitney *U* test.

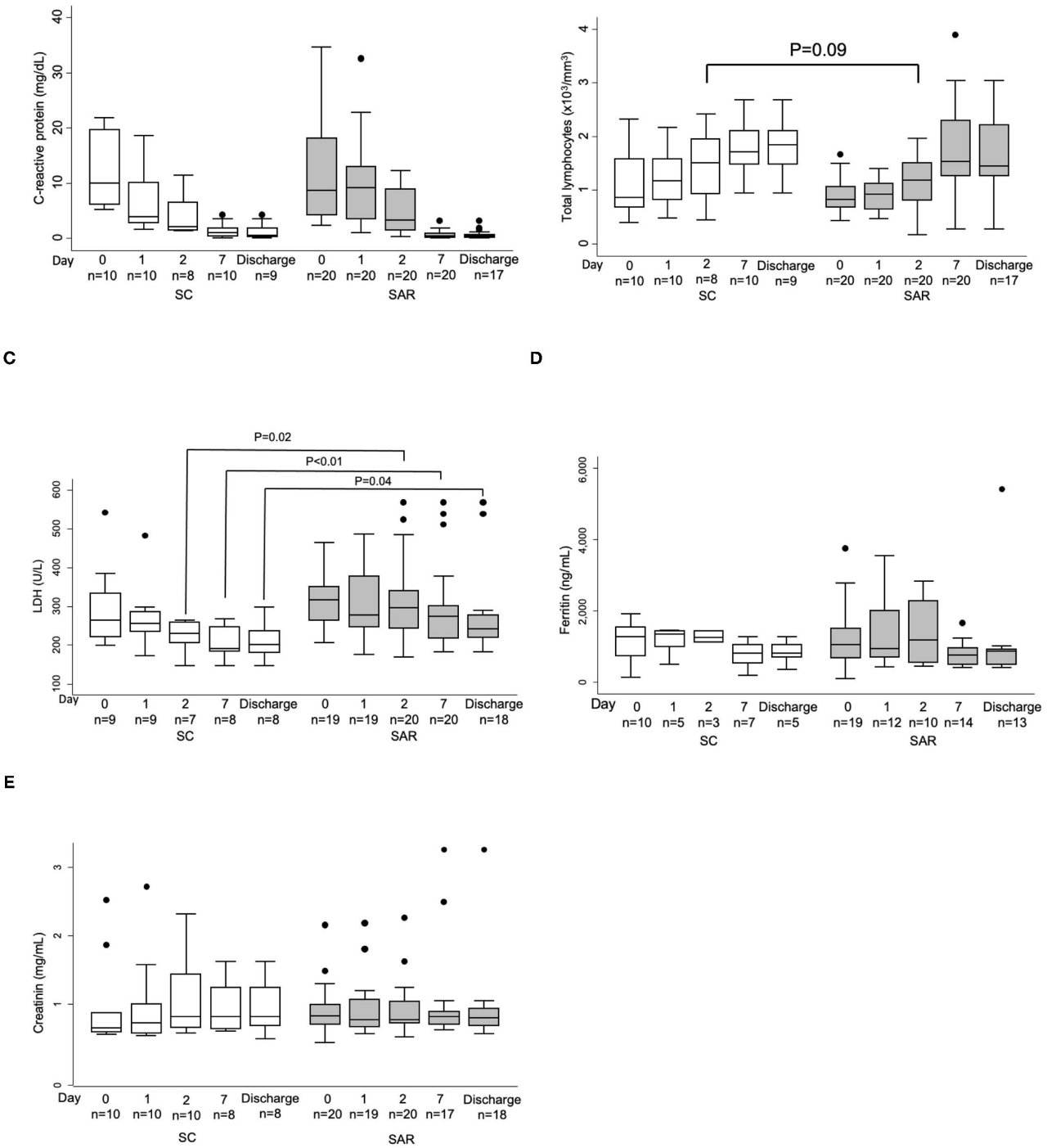

FIGURE 4 | Evolution of laboratory parameters throughout study visits. **(A)** C reactive Protein; **(B)** Total lymphocyte count; **(C)** Lactate dehydrogenase; **(D)** Ferritin; **(E)** Creatinin. Patients from standard care (SC; white boxes) and sarilumab (SAR; gray boxes). Only values available at each time point is shown and results are displayed as the interquartile range (p75 upper edge of the box, p25 lower edge, p50 midline) as well as the p95 (line above box) and p5 (line below). Dots represent outliers.

TABLE 5 | Clinical outcomes in the sensitivity analysis population.

Outcomes	Median (IQR)				
	Sarilumab + SC ($n = 20$)	SC ($n = 7$)	Hazard ratio (SE)	Log hazard ratio (Log SE)	*P* Value
Primary outcomes					
Median change in clinical status (7-category ordinal scale[a)] at day 7,	2 (0–3)	3 (0–3)	-	-	0.36
Mean change in clinical status at day 7, (SD)	1.45 (1.93)	2.14 (1.46)	-	-	0.40
30-day mortality, n (%)[b]	2 (10)	0	-	15.01 (22.60)	0.54
Duration of hospitalization, days from randomization[c]	7 (6–11)	5 (4-12)	0.54 (0.25)	-	0.12
Secondary outcomes					
Median change in clinical status at day 14	3 (2-3)	3 (3)	-	-	0.45
Time to become afebrile for a minimum of 48 h, days[d]	3 (3-6)	15 (8-22)	5.27 (5.70)	-	0.042
Progression to NIMV, n (%)	4 (20)	0 (0)	-	16.30 (-)	0.27
Progression to IMV, n (%)	3 (15)	0 (0)	-	15.01 (22.60)	0.5
Time to supplemental oxygen withdrawal, days from randomization	6 (4-15)	3 (2-8)	0.58 (0.28)	-	0.091

IMV, invasive mechanical ventilation; IQR, interquartile ranges; NIMV, Noninvasive mechanical ventilation; ES, standard deviation; SC, Standard care; SE, Standard error.
[a]Scale range: 1 = death to 7 = non hospitalized.
[b]One patient in the SC arm was lost to follow-up after discharging alive at day 13.
[c]Accounting for survival status by treating patients who died as having a 30-day hospital stay.
[d]Only 2 patients in the SC arm and 11 in the SAR+SC arm were febrile at randomization.

TABLE 6 | Safety outcomes.

Outcomes	N° (%)	
	Sarilumab + SC ($n = 20$)	SC ($n = 10$)
Any adverse event of special interest		
Number of patients	10 (50)	4 (40)
Number of events, n	11	4
Neutropenia Grade IV	0	0
Increased liver enzymes[a]	5 (25)	3 (30)
Steroid-induced hyperglycemia	4 (20)	1 (10)
Invasive bacterial or fungal infection	2 (10)	0
Serious adverse event		
Number of patients	4 (20)	0 (0)
Number of events, n[b]	5	0
Secondary bacterial infection[c]	2 (10)	0
One organ (lung) failure	1 (5)	0
Two organ (lung/kidney) failure	2 (10)	0

SC, Standard care.
[a]Increase in liver enzymes indicates an increase in serum levels of alanine and aspartate aminotransferases more than three times the upper limit of normal.
[b]One patient with respiratory and kidney failure under invasive mechanical ventilation also presented a respiratory bacterial infection by Achromobacter xylosoxidans.
[c]Refers to the same infection episodes described as AEs of special interest.

meta-analysis that included five prospective studies exploring outcomes in 389 patients who received SAR revealed that data are insufficient to establish conclusions about efficacy (25).

One retrospective case series study explored SAR in subcutaneous administration in severe and critical COVID-19 (18), suggesting a clinical benefit through early intervention before high levels of surrogate hyperinflammatory markers such as CRP or IL-6 become irresponsive. In the same way, an early observational case–control study in Italy reported survival advantage with the use of intravenous SAR when initiated in severe hyper-inflamed COVID-19 patients with a PaO2/FiO2 $\geq$ 100 mmHg, suggesting a potential therapeutic window of opportunity (26).

Restricted to critically ill patients, a metanalysis conducted by the international, adaptive platform trial REMAP-CAP has revealed beneficial effects of TCZ and SAR in-hospital mortality and prolonged organ support-free days in ICU (27). Those results were not validated in the first SAR RCT published in COVID-19, comparing intravenous SAR with SC in severe and critical patients (28). Neither the primary endpoint of time to improve clinical status 2 or more points on a 7-category ordinal scale nor the survival rate at day 29 showed the superiority of SAR over placebo, although a trend toward reduced mortality was observed in critically ill patients. Similar results have been reported in an early U.S. phase II/III trial, available in a non–peer-review publication (29), but the authors suggest that on patients with IMV, concomitant corticosteroids could increase the benefit of SAR.

In this regard, a recent prospective meta-analysis involving 10,930 hospitalized patients in 27 randomized trials concluded that the use of IL-6 antagonists, TCZ and SAR, was associated with a reduction in 28-day all-cause mortality, compared with SC or placebo, but this benefit was only found with concomitant administration of corticosteroids (17). SAR, mostly in the intravenous route, was assessed in nine trials, including the study reported herein, allocating 2,073 patients to SAR and 753 patients to usual care or placebo. Notably, the results were stronger for TCZ and in non–IMV-treated participants, maybe as more patients in the SAR group were under IMV and less number of patients received corticosteroids at randomization, compared to TCZ (17).

To date, no results from RCT are available supporting the use of SAR in early stages such as moderate-to-severe COVID-19.

Among clinical trials planned or initiated in those stages, only two are exploring SAR in subcutaneous administration (NCT04359901, EudraCT: 2020-001531-27) and their results are awaited.

Regarding our pilot study, we outline the limitations and propose explanations for several issues behind our results.

First, a limited sample size, especially on the SC arm with no events in key endpoints such as mortality at day 30 and the need for IMV or SAE, complicated the estimation of the effect size of the intervention.

Second, assigned treatment groups were not well-balanced with several data that point to higher baseline severity in SAR arm patients. Patients on SAR were randomized earlier after disease onset compared to SC participants, suggesting a more advanced or poor prognostic disease leading to meeting the inclusion criteria in a shorter time. A higher proportion was male, had a fever, needed high-flow oxygen requirements including NIMV, or presented larger radiological lung involvement compared to SC patients. In this regard, SAR has been associated with faster recovery than SC in a subset of patients showing minor lung consolidation at baseline (23). The limited sample size prevented us from performing stratified analysis and adjustments in the multivariable models could not include all the baseline potential confounding factors.

Third, unknown comorbidities led to randomize patients with a low probability of survival. Two deaths occurred in two 72-year-old patients with previous Grade III CKD and NIMV at randomization. In addition, one of them suffered from a previous chronic thromboembolic disease with right heart failure and the other one from a serious sleep apnea hypopnea syndrome, both discovered after randomization when further information was gathered.

Fourth, the trial was not blinded and might have influenced clinical decision-making. Likely, this can explain the introduction of TCZ after randomization in some SC assigned patients to prevent further deterioration, since in usual care clinicians were used to indicate off-label TCZ following the AEMPs criteria, quite similar to ours in the trial. Nonetheless, excluding those patients from the SC arm in the sensitivity analysis did not alter the results obtained in the ITT population.

Fifth, low baseline IL-6 levels in most SAR assigned patients might have halted the potential beneficial effect of IL-6 blockade. In our experience, besides prognostic information, IL-6 levels >30 pg/ml can also predict the response to IL-6R blockade (12). However, when the protocol was designed in March 2020, we were not aware of its discriminative value for therapeutic response. Thus, we did not include this threshold as a mandatory inclusion criterion. In our previous study, no benefit of TCZ was observed in severe or critical patients with low IL-6 levels (12), and similar findings have been reported with a baseline CRP cut-off of 15 mg/dL in patients requiring ICU support (30). On the other hand, the complex biology of IL-6 and the potential dysregulation of their activities in the context of SARS-CoV2 infection should be considered to understand the effect of IL-6 blockade across different disease outcomes (31). The use of IL-6 as a biomarker of disease severity does not identify IL-6 as a unique contributor to the distinct severe manifestations in COVID-19. In a systematic review and meta-analysis of COVID-19 studies, the estimated pooled mean for IL-6 concentrations in patients with severe and critical COVID-19 was 36.7 pg/ml (95% CI 21.6–62.3 pg/ml), far lower than those reported for IL-6 and other cytokines in patients with unrelated COVID-19 ARDS, sepsis, and CAR T cell-induced cytokine released syndrome (32). This distinct inflammatory profile prompted questioning the role of a cytokine storm in COVID-19-induced organ dysfunction and considering alternative models of organ failure (32).

Sixth, the limitations of using the initial ordinal scales for primary outcomes, endorsed by the WHO early in the pandemic, has been widely recognized (33). The question remains if a threshold level of respiratory support can guide the appropriate initiation of IL-6 inhibitors.

Seventh, the widespread use of glucocorticoids on both arms (85% SAR vs. 80% SC patients) might have affected the results. Corticosteroids were commonly used in our center from the first outbreak but became the rule after the publication of their beneficial effects in the RECOVERY trial (34) and the WHO REACT metanalysis (35). However, after adjusting for a cumulative dose of glucocorticoids at day 7 after randomization, no significant differences were detected between treatment groups in the primary outcome of change in clinical status at day 7. Additionally, a cumulative dose of corticosteroids before and after randomization was similar in both arms. Concerning the simultaneous use with SAR, robust evidence has been accumulated for a greater effect of IL-6R inhibitors in concomitant use with corticosteroids (17, 27, 36). In fact, in our study, 2 out of 3 patients progressing to IMV, early recruited and allocated to SAR, were not receiving corticosteroids at randomization day and were prescribed later, followed by one patient survival.

Lately, some concerns have arisen about the subcutaneous formulation and dosage in severe patients. In line with pharmacodynamic data provided for intravenous SAR in severe and critical COVID (28, 29), with a reported rebound of CRP after declining for 7 days, a single 400 mg dose of subcutaneous sarilumab could have been sub-therapeutic. The similar time plot decline of median CRP levels in both arms in our study does not support the use of the subcutaneous route, at least with a single 400 mg dose.

CONCLUSION

In our study of hospitalized patients with moderate-to-severe COVID who are not invasively ventilated at baseline, subcutaneous sarilumab added to standard care showed no additional benefit for preventing noninvasive and invasive ventilation or death by 30 days, early improvement of clinical status, or reducing hospital stay. Findings of this pilot study do not exclude a potential effect of sarilumab in moderate-to-severe COVID-19 and suggest that further blinded randomized phase III trials should be adequately powered with primary endpoint accuracy, testing higher or repeated doses, and selecting the population based on high baseline IL-6 levels. Questions remain open on subcutaneous administration and the appropriate

time of intervention pending the results of more powered ongoing RCT.

DATA AVAILABILITY STATEMENT

RG-V, FA-S, and IG-A have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the Hospital Universitario de la Princesa, IIS-Princesa, Madrid, Spain. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. All patients or their legal representatives provided oral informed consent according to the exceptional regulation applicable for COVID19 studies.

AUTHOR CONTRIBUTIONS

RG-V, IG-A, and FA-S contributed to the conception or design of the work. RG-V and SR-G contributed to the drafting of the manuscript. IG-A contributed to the statistical analysis. RG-V supervision. All authors contributed toward the acquisition, analysis, or interpretation of data, critical revision of the manuscript for important intellectual content, review and approval of the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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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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REVIEW
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Aspirin in COVID-19: Pros and Cons

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Since its emergence, the COVID-19 pandemic has been ravaging the medical and economic sectors even with the significant vaccination advances. In severe presentations, the disease of SARS-CoV-2 can manifest with life-threatening thromboembolic and multi-organ repercussions provoking notable morbidity and mortality. The pathogenesis of such burdensome forms has been under extensive investigation and is attributed to a state of immune dysfunction and hyperinflammation. In light of these extraordinary circumstances, research efforts have focused on investigating and repurposing previously available agents that target the inflammatory and hematological cascades. Aspirin, due to its well-known properties and multiple molecular targets, and ought to its extensive clinical use, has been perceived as a potential therapeutic agent for COVID-19. Aspirin acts at multiple cellular targets to achieve its anti-inflammatory and anti-platelet effects. Although initial promising clinical data describing aspirin role in COVID-19 has appeared, evidence supporting its use remains fragile and premature. This review explores the notion of repurposing aspirin in COVID-19 infection. It delves into aspirin as a molecule, along with its pharmacology and clinical applications. It also reviews the current high-quality clinical evidence highlighting the role of aspirin in SARS-CoV-2 infection.

Keywords: COVID-19, SARS-CoV-2, coronavirus, aspirin, salicylic acid

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INTRODUCTION

The latest pandemic caused by the novel SARS-CoV-2 virus has led to the emergence of coronavirus disease 2019 termed COVID-19. The disease first appeared in Wuhan, China in December 2019 as an outbreak of atypical pneumonia (Younis et al., 2020; Rana O; Zareef et al., 2020; Tsang et al., 2021; Younis et al., 2021). The virus has ever since spread at an unprecedented pace, exhausting the global health sector and endangering the economies (Arthi and Parman, 2021; Padhan and Prabheesh, 2021). SARS-CoV-2 belongs to the Coronaviridae family. Although the majority of Coronaviridae family members are implicated in mild upper respiratory tract illness, SARS-CoV-2 has caused a wide range of more serious illnesses (Morens et al., 2020). Overall, most COVID-19 patients exhibit mild to moderate disease (Landete et al., 2020). However, a small percentage of patients may display severe sickness and are placed at greater risk of experiencing mortality and morbidity (Landete et al., 2020). Studies have shown that some factors including obesity, older age, cardiovascular comorbidities, pre-existing pulmonary condition, and chronic kidney disease, among other factors, are associated with increased risk of hospitalization, mechanical ventilation and mortality (Attaway et al., 2020; Feng et al., 2020; Klang et al., 2020; Williamson et al., 2020; Wu and McGoogan, 2020; Phelps et al., 2021). In these cases, the disease has beleaguered multiple organ-

systems imparting significant irreversible damage. Unfortunately, the cardiovascular system is also embattled, with subsequent substantial complications has been reported (Magadum and Kishore, 2020; Zareef et al., 2020). Besides, a high rate of coagulopathy has been described in patients infected with COVID-19, suggesting a critical COVID-19 induced thromboembolic event. Such events are major cardiovascular complications and are associated with increased mortality (Zareef et al., 2020; Zhou et al., 2020; Aktaa et al., 2021). Studies have highlighted an astonishing rate of venous thromboembolism and pulmonary embolism in COVID-19 patients reaching 42 and 17% respectively in severe cases (Wu et al., 2021). Arterial thrombotic events at various sites including coronaries, brain, and extremities have also been described (Mehra et al., 2020; De Roquetaillade et al., 2021).

Vaccines developed against SARS-CoV-2 virus have shown effective reduction in transmission rate as well as the pattern of hospitalization, ventilation and mortality, as evident by the clinical trials (Polack et al., 2020; Voysey et al., 2021). However, even with the large-scale global vaccination programs, the virus has attained several remarkable mutations and produced new variants such as B.1.1.7, P.1, B.1.351, B.1.427, P.3, B.1.429, B.1.526, and B.1.617.2 (Baden et al., 2021; Chookajorn et al., 2021; Vasireddy et al., 2021; Voysey et al., 2021). This is particularly problematic as emerging variants might acquire the ability to transmit rapidly, and cause more severe disease, while escaping the host immune system (Vasireddy et al., 2021). They might as well challenge the previously proven vaccine efficacy (Bernal et al., 2021). Due to the rapid rise of events during the pandemic, and the high mortality and morbidity rates, the quest for therapeutic strategies has been ongoing. In this manner, drug repurposing has constituted an attractive mean for fighting the current crisis. Several regimens have been tried including steroids, azithromycin, ivermectin, hydroxychloroquine, tocilizumab, baricitinib, antivirals among others (Santos et al., 2020; Younis et al., 2020; Younis et al., 2021b). In light of the evidence of thromboembolic events and the noticeable hyperinflammatory state, several studies and investigators have suggested a possible role for aspirin in treating COVID-19 disease (Mohamed-Hussein et al., 2020). This paper discusses the potential therapeutic role of aspirin in COVID-19 disease through dissecting its pharmacology, cellular targets, clinical uses, as well as the current high quality clinical evidence.

PHARMACOLOGY OF ASPIRIN

Salicylic acid (Aspirin) is produced and administered via different routes in various doses and forms (Arif and Aggarwal, 2021). The usual therapeutic range of serum salicylate concentration is 10–30 mg/dl (0.7–2.2 mmol/L). Indeed, the dosing of aspirin is crucial as it dictates its mechanism of action. Traditionally, anti-thrombotic effects are achieved at low doses (75–81 mg/day), analgesic and antipyretic effects are achieved at intermediate doses (650 mg–4 g/day), while aspirin at high doses (between 4 and 8 g/day) is effective as an anti-inflammatory agent (Pillinger et al., 1998).

Aspirin intoxication can occur after ingesting 10–30 g in adults and as little as 3 g in children. Most patients exhibit signs and symptoms of intoxication if the serum concentration level of salicylate exceeds 40–50 mg/dl (2.9–3.6 mmol/L) (Hill 1973).

Pharmacokinetically

Acetylsalicylic acid is in general rapidly and completely absorbed by the gastrointestinal tract following oral administration (Awtry and Loscalzo, 2000). However, absorption may also be variable depending on several factors including the route of administration, the dosage, the rate of tablet dissolution, gastric pH, gastric contents, and emptying time (INC, 2017). It is mainly absorbed in the stomach and small intestine. The plasma concentration of salicylate peaks between 1 and 2 h following administration. It gets distributed to all body tissues shortly after administration, mainly to peritoneal, spinal, synovial fluids, milk, saliva, liver, kidneys, heart, and lungs. It is also known to cross the placenta (DrugBankonline, 2005). Aspirin is hydrolyzed in plasma to salicylic acid and its levels become undetectable 4–8 h after administration. The liver is the main site of metabolism for salicylate, although other tissues may also be involved. It then gets eliminated by the kidneys via glomerular filtration and tubular excretion processes (INC, 2017). An entire dose needs around 48 h for the salicylate to be completely eliminated. The half-lives of ASA versus salicylate is 15 min versus 4 h respectively, while the clearance rate of ASA is variable depending on several factors (DrugBankonline, 2005).

Pharmacodynamically

The pharmacodynamic aspect of aspirin is unique, as it doesn't interact with any surface or intracellular receptors. It exerts its activity through non-specific irreversible acetylation of molecules. The acetylation process instigates alterations at the level of macromolecules, and accordingly adjusts the function of the proteins. Due to the irreversibility of such modification, the duration of activity depends on the turnover rate of the target molecule irrespective of aspirin plasma concentration (Schrör, 2016).

MECHANISM OF ACTION OF ASPIRIN

Aspirin is one of the most commonly used drugs worldwide (Vane and Botting, 2003; Zhou et al., 2014). It is an anti-inflammatory, anti-pyretic, analgesic, and anti-platelet drug. Aspirin exerts its major activity by inhibiting the cyclooxygenase enzyme (COX), which exhibits two forms: COX-1 and COX-2 (Patrono et al., 2001) (Patrono et al., 2001). Subsequently, it blocks the conversion of arachidonic acid into prostaglandins and thromboxane, collectively called prostanoids. Its activity expands to target several other structures circumventing a set of inflammatory and thrombotic events.

Anti-Inflammatory Activity

Aspirin exerts its anti-inflammatory property through several mechanisms (**Figure 1**). At intermediate and high concertation,

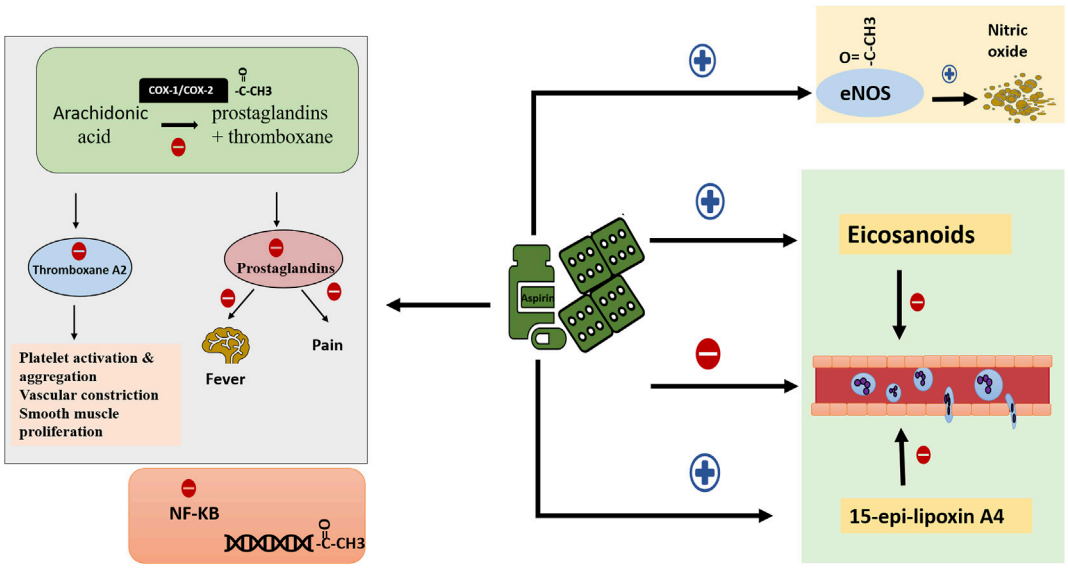

FIGURE 1 | Mechanism of action of aspirin. Aspirin possesses several targets through which it exerts its activity. First, it inhibits prostanoids synthesis thus employing anti-thrombotic, anti-inflammatory, anti-pyretic and analgesic effect. In addition, it acetylates multiple cellular proteins hence affecting DNA transcription and expression. It also constrains NF-KB production, limiting its pro-inflammatory effect. Furthermore, aspirin enhances the synthesis of eicosanoids and 15-epi-lipoxin A4. Combining all together, aspirin impedes PMNs interaction with platelets and endothelium, PMNs chemotaxis, adhesion, and migration. Finally, it acetylates and activates eNOS to maintain vascular homeostasis. (NF-KB: nuclear factor kappa B; PMNs: polymorphonuclear cells; eNOS endothelial nitric oxide synthase).

aspirin non-selectively acetylates and inhibits the activity of COX-1 and COX-2, hampering the biosynthesis of prostanoids and their subsequent inflammatory outcome. Indeed, COX-2 expression is induced by inflammatory cytokines, hormones, and growth factors, and it plays a role in cancer, acute stress, inflammation, and infection (Chiang and Serhan, 2009). Aspirin also interferes with innate immunity through the inhibition of thromboxane A2 production. Thromboxane A2 facilitates platelet-polymorphonuclear (PMNs) cells interaction and the subsequent migration of PMNs to the areas of inflammation. This also takes place at low doses of aspirin (Patrono et al., 1980; Cooper et al., 2004). Similarly, at low concentrations, aspirin stimulates the synthesis of certain eicosanoids that terminates the trafficking of PMNs (Claria and Serhan, 1995). Low dose aspirin also inhibits leukocyte adhesion and migration by stimulating the synthesis of 15-epi-lipoxin A4. 15-epi-lipoxin A4, known as aspirin-triggered 15-epi-lipoxin A4 (ATL), pathway alters leukocyte/endothelium interactions and limits leukocyte extravascular accumulation (Perretti et al., 2002; Serhan, 2002). In fact, the anti-inflammatory effect of aspirin doesn't stop at altering the biosynthesis of prostaglandins and thromboxane, it also interferes with various cellular pathways to intrude on the inflammatory response. Several studies have shown that aspirin disturbs intracellular signaling pathways including nuclear factor-kappa B (NF-K B) (Kopp and Ghosh, 1994; Grilli et al., 1996; Yin et al., 1998). NF-KB plays a role in the inflammatory response. Aspirin reduces the production of NF-KB, and at the same time inhibits the breakdown of its inhibitor (Kopp and Ghosh, 1994). Besides, aspirin exerts a protective effect at the cellular level as an anti-oxidant by induction of heme oxygenase-1 during inflammatory states (Grosser et al., 2003). At higher concentrations and over longer periods, aspirin can nonspecifically acetylate other proteins (Vane and Botting, 2003). Remarkably, one study highlighted the role of aspirin in gene regulation through acetylation of histones (Sabari et al., 2017). Endothelial nitric oxide synthase (eNO) is another target for aspirin. When aspirin acetylates the enzyme, it stimulates nitric oxide release thus maintaining vascular homeostasis (Taubert et al., 2004). Aspirin likewise acts as an anti-pyretic and analgesic agent. Prostaglandins potentiate the effect and sensitivity of pain receptors and other substances like histamine and bradykinin (Patrono et al., 2001). A decrease in prostaglandins production reduces pain and inflammation. Similarly, aspirin inhibits the production of brain prostaglandin E1 which is a potent fever-inducing agent, thus acting as an antipyretic (Vane, 1976).

Anti-Platelet Activity

Aspirin is a well-known potent anti-thrombotic (**Figure 1**). At low doses, the acetyl group of aspirin binds to serine 530 residue of COX-1 and irreversibly inhibits its activity (Funk et al., 1991). Therefore, prostaglandin H2 synthesis is inhibited (Patrono, 1994). Prostaglandin H2 is a substrate used by the enzyme thromboxane-A-synthase to produce thromboxane A2. Thromboxane A2 is a strong pro-thrombotic molecule that is synthesized and released by platelets; it stimulates platelets activation and aggregation (FitzGerald, 1991). It also promotes vascular constriction and smooth muscle proliferation. This inhibitory effect is irreversible. The synthesis of new thromboxane A2 depends on the synthesis of new platelets, a process occurring at a rate of 10% daily (Di Minno et al., 1983).

CLINICAL USES OF ASPIRIN

The medicinal history of aspirin dates back to more than 3,500 years ago, where the old Egyptians and Sumerians used the willow bark as anti-pyretic and analgesic agent. As medicine progressed, the first precursor of aspirin, salicylate, was described in 1763 by Reverned Stone as antipyretic, while aspirin was first described in 1897 by Felix Hoffman (Montinari et al., 2019). Today, aspirin is a well-known and widely used drug. It represents a famous well-established antipyretic and analgesic agent. It is extensively used in cardiovascular diseases, mainly in the acute settings of myocardial infarction, unstable angina, ischemic stroke, and for secondary prevention of recurrent coronary artery disease (Framework, 1998; De Gaetano, 2001; Members; Gibbons et al., 2003; Younis et al., 2021c) (Awtry and Loscalzo, 2000; Members; Antman et al., 2004; Pan et al., 2019). Perhaps, the most common off-label use of aspirin is for the primary and secondary prevention of atherosclerotic disease. However, the protective value of aspirin has not been well established in healthy individuals with no risk or previous occlusive events. (Collaboration, 2002; Dai and Ge, 2012). Aspirin is also the drug of choice for prophylaxis in revascularization surgeries including coronary bypass surgery (Members, Eagle et al., 2004; Smith et al., 2006). While being initially widely used, aspirin's use in rheumatic diseases such as rheumatoid arthritis has declined for two reasons: 1) its anti-inflammatory properties are established at relatively high doses, and 2) the desired effects are reached at lower doses with more efficacy with non-salicylate nonsteroidal anti-inflammatory drugs (NSAIDs) (Csuka and McCarty, 1989). Despite that, aspirin is still part of the treatment Kawasaki disease (Rife and Gedalia, 2020). Aspirin was also proved to be beneficial in preventing preeclampsia, and more ongoing trials are exploring the role of aspirin in reducing the risk of colorectal cancer (Roberge et al., 2018) (Dubé et al., 2007). In pediatrics, aspirin is used in patients with congenital heart disease and recently in treating multisystem inflammatory syndrome in children (Abani et al., 2021; Abi Nassif et al., 2021).

The use of aspirin is not without adverse effects. Increased risk of bleeding is possible, mainly secondary to decreased levels of thromboxane A2 (Arif and Aggarwal, 2021). In addition, aspirin, just like all NSAIDs, can potentially cause gastritis and ulcers, as cyclooxygenase is essential in maintaining the gastrointestinal mucosa (Awtry and Loscalzo, 2000). A rare side effect is tinnitus at high doses. Aspirin metabolite, salicylic acid, can alter cochlear nerve function, but tinnitus usually resolves after drug discontinuation (Edward et al., 2021). Reye's syndrome, which encompasses liver failure and encephalopathy, is associated with aspirin use in children [(MD) 2017]. Given this, aspirin is not generally used in kids except in the case of Kawasaki disease.

ASPIRIN AND COVID-19

COVID-19 complications have been linked to an immune dysregulation syndrome accompanied by cytokine storms (Coperchini et al., 2020; Mehta et al., 2020). This severe inflammation is known to trigger the coagulation cascade and inhibit fibrinolysis, disrupting blood homeostasis. On the other hand, COVID-19 has been found to infect vascular endothelial cells leading to endotheliitis (Varga et al., 2020). Endothelial inflammation and von-Willebrand exposure to sub-endothelial collagen, in turn, precipitate thrombus formation manifesting as arterial, venous, and microvascular thromboembolic events. Aspirin, with its anti-inflammatory and antithrombotic properties, could therefore protect against severe forms of COVID infection. High dose aspirin is in fact used in MIS-C for its anti-inflammatory properties. Aspirin is also commonly used in atherosclerotic CVD to stabilize diseased arterial endothelium. Its anti-platelet properties are mediated through COX-1 inhibition preventing platelet aggregation, while its anticoagulant properties stem from factor 8 activation inhibition. Acetylsalicylic acid also stimulates fibrinolysis and modifies thrombus architecture (Mekaj et al., 2015). Aspirin, through its pleiotropic effects, was therefore naturally hypothesized to prevent COVID's multifactorial complications and is the subject of the following review.

Pros

Pathologic examination of lung tissue in patients who died due to COVID-19 infection and its complications revealed morphological aspects of acute respiratory distress syndrome including diffuse alveolar damage, intra-alveolar edema, inflammatory infiltration by mononuclear and multinucleated cells, and vascular damage (Xu et al., 2020; Yao et al., 2020; Batah and Fabro, 2021). Further pathological examination revealed the formation of immune and fibrin microthrombi leading to intracapillary thrombosis (Colling and Kanthi, 2020; Batah and Fabro, 2021). Remarkably, it is suggested that such changes, specifically the edema and inflammatory infiltrates, develop before the pneumonia symptoms (Tian et al., 2020). Add to these the hyperinflammatory and hypercoagulable state (**Figure 2**), marked by laboratory aberrations namely increasing D-dimer and fibrinogen and decreasing platelet count with more severe disease (Lin et al., 2021). From these repercussions of the infection stems the utility of antiplatelet and anticoagulant agents specifically aspirin.

Because aspirin is a chronic medication for many patients, studies at first investigated the effect of chronic aspirin use on the course of COVID-19 infection (**Table 1**). Osborne's retrospective study included 35,370 patients with and without active aspirin prescription before acquiring SARS-CoV2 (Osborne et al., 2021). Aspirin users had a significantly decreased risk of mortality by 32% at 14 and 30 days after infection. After adjusting to confounding covariates [age, gender, comorbidities, and the Care Assessment Needs (CAN) 1-year mortality score] and propensity score matching, mortality dropped from 6.3 to 2.5% with aspirin use at 14 days and from 10.5 to 4.3% at 30 days in the propensity matched cohorts.

Other studies investigated in-hospital aspirin use irrespective of pre-infection use. A retrospective observational cohort study by Chow et al., studied the severity of the disease in patients who received aspirin within the first day of admission or a week before admission (Chow et al., 2021). Patients in both groups had similar

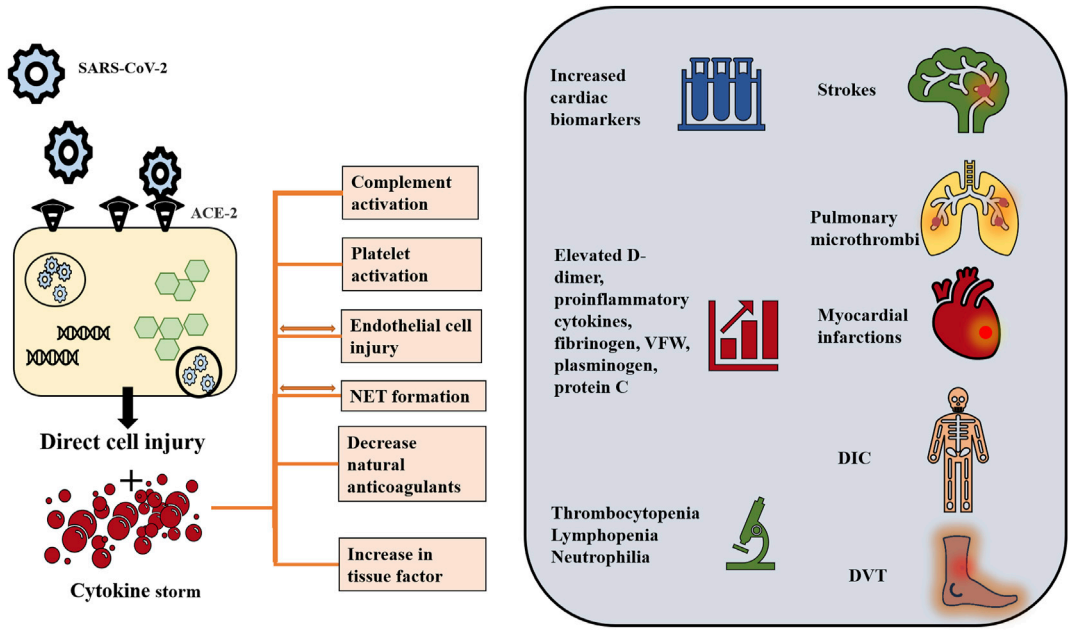

FIGURE 2 | COVID-19 induced hyperinflammatory and hypercoagulable states. Although the pathogenesis of COVID-19 induced coagulopathy has not been fully elucidated, interplay among immune dysregulation, hyperinflammation and thrombosis is proposed. SARS-CoV-2 virus, via its spike protein, interacts with the ACE-2 receptor to enter the cell through endocytosis. Endothelial cells as well as respiratory cells have high expression level of ACE-2. Inside the cell, the virus releases its genetic material and replicates using the cellular machinery. The viral effect is suggested to take place through two mechanisms: 1) Direct viral injury and 2) indirect cytokine-mediated injury. The viral cytopathic effect is implicated in direct damage and apoptosis of the host cell thus contributing to endothelitis. In turn, endothelial damage triggers platelet activation and aggregation. At the same time, the virus drives intense inflammation and immune dysregulation. It suppresses the lymphocytic activity and activates macrophages and polymorphonuclear cells, thus generating pro-inflammatory cytokines including IL-1, IL-2, IL-6, IL-10, IL-17, IL-18 and TNF-α, and leading to cytokine storm in severe illness. TF production, release of VWF and the initiation of coagulation cascade are triggered by cytokines and the injured epithelium. The cytokine storm also induces activation of complement system which contributes to coagulopathy by activating platelets, and increasing production of fibrin and thrombin. The cytokine storm is also associated with NETs, which in turn promotes VWF and TF activity and disables tissue factor inhibitor and thrombomodulin, therefore induing inflammation and microvascular thrombosis. These cellular processes are reflected on laboratory values, which ae usually remarkable for a combination of prolonged prothrombin time, normal to mildly prolonged activated partial thromboplastin time, thrombocytopenia, elevated D-dimer level, fibrinogen, fibrinogen degradation products, VFW, plasminogen, protein C, and factor VIII. Clinically, the hyperinflammatory response and endothelial dysfunction affect both venous and arterial systems. Venous thromboembolism includes pulmonary embolism and deep venous thrombosis. Arterial thromboembolism including myocardial injury and strokes have been also reported. Consumptive coagulopathy and ultimately DIC is also observed in critically ill patients (Lippi et al., 2020a; Iba et al., 2020b; Lippi et al., 2020b; Goshua et al., 2020; Guan et al., 2020; Levi and Thachil 2020; Mehta et al., 2020; Middleton et al., 2020; Tang et al., 2020; Varga et al., 2020; Zhang et al., 2020; Zhou et al., 2020; Tan et al., 2021). ACE-2: angiotensin converting enzyme-2; IL: Interleukin; VWF: von Willebrand factor; NET: neutrophil extracellular traps; TF: Tissue factor; DIC: disseminated intravascular coagulation.

vital signs and lab tests, except fibrinogen level, which was significantly lower in patients receiving aspirin. On initial crude analysis, there was no difference in in-hospital mortality between the two groups in spite of the improvement in other outcomes and no difference in rates of bleeding and overt thrombosis. However, after adjusting to age, sex, race, body mass index (BMI), comorbidities and home beta blocker use, patients receiving aspirin had reduced risk of mechanical ventilation, intensive care unit (ICU) admission and in-hospital mortality, these results persisted on subgroup analysis. In addition, sensitivity analysis was performed by stratifying patients relative to the timing of aspirin use: started in the first 24 h, in the 7 days prior to admission, or both; this analysis showed lower rates of ICU admission in patients who started aspirin in the first 24 h compared to the others.

Low in-hospital mortality was also deduced in other studies assessing different populations (Haji Aghajani et al., 2021; Liu et al., 2021). In those studies, patients were receiving aspirin during their hospitalization not withstanding prior chronic aspirin use, had similar baseline vital signs, inflammatory and infectious markers, and medications used during hospitalization.

To be able to assess the efficacy of aspirin in an acute COVID-19 setting, Meizlish et al. dissected the use of anticoagulants and antiplatelets in COVID-19 patients with documented abnormalities in D-dimer and fibrinogen levels (Meizlish et al., 2021). At first, propensity matched cohorts comparing patients receiving aspirin vs. patients receiving no aspirin were considered, adjusting to multiple factors including physicians' tendency to administer aspirin to critically ill patients. Multivariate analysis showed a lower cumulative incidence of in-hospital death in the aspirin cohort (Meizlish et al., 2021). After May 18, they released a recommendation to administer aspirin for all patients hospitalized for COVID-19, which showed similar favorable outcomes.

While considering the favorable outcomes that these studies revealed, one should consider many factors. All of these included

TABLE 1 | High quality clinical studies showing evidence of beneficial aspirin role in COVID-19 disease.

Author(s)	Country	Date	Study design	Dose	Mortality	Mechanical ventilation	Other outcomes
Osbourne et al.	United States	Feb-21	Retrospective database review	NA	Pre-existing aspirin use was associated with lower mortality at 14 and 30 days (adjusted OR: 0.38) Propensity matched cohort: drop in 14-day mortality from 6.3 to 2.5% and a drop in 30-day mortality from 10.5 to 4.3%		
Kow and Hasan	United States, United Kingdom, China, Italy, Germany	Apr-21	Meta-analysis	NA	Significantly reduced risk of a fatal course of COVID-19 with the use of aspirin in patients with COVID-19 relative to non-use of aspirin (pooled OR = 0.50 (0.32–0.77); pooled HR = 0.50 (0.36–0.69)		
Chow et al.	United States	Apr-21	Multicenter retrospective observational cohort	Low dose	In-hospital initiation of aspirin was independently associated with reduced in-hospital mortality (adjusted HR, 0.53, *p*-value = 0.02)	Aspirin was independently associated with a reduced risk for mechanical ventilation (adjusted HR, 0.56 (0.37–0.85), *p-value* = 0.007)	Aspirin was associated with a reduction in the risk of ICU admission (adjusted HR, 0.57 (0.38–0.85), *p-value* = 0.005)
Liu et al.	China	Feb-21	Single-center retrospective cohort	Low dose	In-hospital aspirin initiation had significantly lower 30-day and 60-day mortality compared to the non-aspirin group		No significant difference in the viral duration time (time from 1st positive PCR to 1st negative PCR) between the two groups
Meizlish et al.	United States	Apr-21	Multicenter retrospective	Low dose	In-hospital, aspirin initiation was associated with a lower cumulative incidence of in-hospital death, on multivariate regression and propensity score matching (HR = 0.52)		
Aghajani et al.	Iran	Apr-21	Retrospective cohort	NA	Aspirin (HR = 0.753 [0.573–0.991], *p*-value = 0.043) was associated with decreased risk of in-hospital mortality	16.07% of aspirin users and 90 13.74% of nonusers needed mechanical ventilation (*p*-value = 0.324)	Length of hospital stay was significantly longer in patients who received aspirin (*p*-value < 0.001)

studies were retrospective in nature. Some studies had an adequate number of patients but from a homogeneous sample enlisted in the Veterans Health Administration, which makes the results generalizable only to this population (Osbornes et al., 2021). Additionally, most studies failed to distinguish between the efficacy of chronic and acute aspirin uses. None of them have specified whether chronic aspirin use is superior to using aspirin only in the acute setting, and vice versa. Yet, the abovementioned studies indeed took into consideration the baseline factors that might affect patients' clinical course as well as eventual morbidity and mortality, including underlying conditions, demographics, vital signs, and lab values at presentation and others. Specifically, in Haji Aghajani et al., the initial bivariate analysis revealed higher in-hospital mortality in aspirin users (Haji Aghajani et al., 2021). After adjusting to the aforementioned factors, aspirin was found to be protective of mortality, although aspirin users were admitted for longer and needed more time on the mechanical ventilator.

A meta-analysis done by Kow and Hassan included 12 studies, six of which investigated antiplatelet use in COVID-19, and six others investigating aspirin use (Kow and Hasan, 2021). The pooled analysis showed a significant benefit to aspirin use in protecting the patients from a fatal course of COVID-19. To note, this benefit was not seen when considering antiplatelet use, suggesting that maybe the other effects of aspirin, specifically its anti-inflammatory properties, account for the favorable results.

Cons

Alongside publications supporting the use of aspirin in COVID-19 patients, there is also antipodal literature arguing against it (**Table 2**).

TABLE 2 | High quality clinical evidence displaying negative role for aspirin in COVID-19 disease.

Author(s)	Country	Date	Study design	Mortality	Mechanical ventilation	Other outcomes
Yuan et al.	China	Jan-21	Retrospective database review	No difference in mortality between CAD patients taking and not taking aspirin	No difference in need of mechanical ventilation between the 2 groups	No difference in severe disease, inflammatory markers, liver and kidney function and lung imaging between patients taking and not taking aspirin pre-hospitalization
Sahai et al.	United States	Dec-20	Retrospective database review	Neither aspirin nor NSAIDs affected mortality. They were associated with increased risk of MI, CVA, or VTE		
Salah and Mehta	United States, China, Iran	Mar-21	Meta-analysis	Mortality was not associated with the use of aspirin in patients with COVID-19 (RR 1.12, [0.84, 1.50])		
Son et al.	South Korea	Jul-21	Case control	Mortality was not associated with the use of aspirin. Adjusted OR = 0.92 (0.46–1.84)		No correlation between prior aspirin use and COVID-19 complications. Adjusted OR = 1.06 (0.66–1.69)
Abdelwahab et al.	Egypt	Jul-21	Retrospective cohort		No correlation between prior aspirin use and mechanical ventilation Adjusted OR = 1.095, *p*-value = 0.932	Decreased risk of thromboembolic events with prior aspirin use. Adjusted OR = 0.163, *p* = 0.02
Pan et al.	United States	May-21	Retrospective cohort	Mortality was not associated with the prior use of anti-platelets. Adjusted OR = 1.13 (0.70–1.82)		No correlation between prior anti-platelet use and the composite outcome (high oxygen need, invasive ventilation and death). Adjusted OR = 0.98 (0.65–1.46)
Tremblay et al.	United States	Jul-20	Retrospective cohort	Mortality was not associated with the prior use of anti-platelets. HR = 1.029 (0.723–1.466)	No correlation between prior anti-platelet use and mechanical ventilation. HR = 1.239 (0.807–1.901)	No correlation between prior anti-platelet use and either survival time, time to mechanical ventilation or hospital admission
Russo et al.	Italy	May-20	Retrospective cohort	In-hospital mortality was not associated with the prior use of anti-platelets. Adjusted RR = 0.51 (0.21–1.15) *p*-value = 0.110		No correlation between prior anti-platelet use and ARDS upon admission. Adjusted RR = 0.58 (0.38–1.14), *p*-value = 0.165
Banik et al.	Germany	Nov-20	Retrospective cohort	No correlation between prior anti-platelet use and the composite endpoint death or transfer for ECMO. Adjusted OR = 2.25 (0.0456–270)	No correlation between prior anti-platelet use and the need for mechanical ventilation. Adjusted OR = 0.781 (0.0253–17.0)	Prior anti-platelet use correlated with a positive chest CT. Adjusted OR = 12.1 (1.41–167), *p*-value = 0.0354 prior use of anti-platelet did not correlate with the length of hospital stay
Horby et al.	United Kingdom, Indonesia, Nepal	Jun-21	RCT	28-day mortality was not associated with aspirin treatment. RR = 0.96 (0.89–1.04) *p* = 0.35	Mechanical ventilation need was not associated with aspirin treatment. RR = 0.96 (0.9–1.03)	Rate of discharges before 28 days was slightly higher among patients in aspirin arm. RR = 1.06 (1.02–1.1) *p*- value = 0.0062 median time until discharge was 8 days in aspirin users versus 9 days in non-users. There was no correlation with successful cessation of mechanical ventilation or need for renal replacement therapy
Kim et al.	South Korea	Sep-21	Retrospective cohort	Increased risk of death among patients who took aspirin within the 2-weeks prior to COVID-19 diagnosis (40%) vs. those who did not (5%) *p*-value = 0.027; however, groups were not	Mechanical ventilation need was not associated with aspirin treatment either before (*p*-value = 0.141) or after (*p*-value = 0.173) diagnosis	People who received aspirin after diagnosis were at higher risk of needing oxygen therapy (46.7%) vs. those who did not receive aspirin (35.0%), *p*-value <0.0001. No correlation between oxygen

(Continued on following page)

TABLE 2 | (*Continued*) High quality clinical evidence displaying negative role for aspirin in COVID-19 disease.

Author(s)	Country	Date	Study design	Mortality	Mechanical ventilation	Other outcomes
				matched for prior CAD No correlation between mortality and aspirin treatment within 2 weeks after diagnosis		need and aspirin use before diagnosis. No correlation between COVID infection rate and prior aspirin use. No correlation between aspirin use before or after diagnosis and ICU admission

CAD, coronary artery disease; NSAIDS, non-steroidal anti-inflammatory drugs; MI, myocardial infarction; CVA, cerebrovascular accident; VTE, venous thromboembolism; ARDS, acute respiratory distress syndrome; ECMO, extra-corporeal membrane oxygenation; ICU, intensive care unit.

The RECOVERY trial is, to date, the only published randomized clinical trial (RCT) testing the benefits of aspirin therapy in COVID-19 patients. This trial is an open-label, platform RCT that recruited 14,892 inpatients with COVID-19. In this study, 7,351 patients were randomly allocated to receive 150 mg of aspirin daily alongside usual care, while 7,541 received usual care alone. It involved 177 hospitals in the United Kingdom, two hospitals in Indonesia, and two hospitals in Nepal. Authors did not find a correlation between aspirin intake and 28-day mortality, the study's primary outcome (rate ratio 0.96; 95% confidence interval 0.89–1.04; $p = 0.35$). There was also no significant difference in the composite outcome of mechanical ventilation or death within 28 days of admission between the two treatment arms (risk ratio 0.96; 95% CI 0.90–1.03; $p = 0.23$). On the other hand, a slightly higher but significant proportion of inpatients on aspirin was discharged alive before 28 days (75 vs. 74%; rate ratio 1.06; 95% CI 1.02–1.10; $p = 0.0062$) (Abani et al., 2021).

This study only looked at inpatients and excluded those on chronic aspirin therapy. This trial being a platform RCT, it also involved looking at many drugs simultaneously. For example, 90% of patients in this trial were taking corticosteroids while 93% were on low molecular weight heparin (LMWH). Authors suggest these high rates of antithrombotic therapy with LMWH and corticosteroid treatment must have decreased thrombo-inflammatory stimulation in the entire study population. Therefore, the treatment arm might not have significantly benefited from aspirin given that almost everyone was anticoagulated and taking corticosteroids. However, in a real-life scenario where a patient is admitted and given steroids and LMWH, it seems the addition of aspirin is not indicated and will not further improve outcomes. Aspirin might also be more beneficial among people with a higher risk of thrombosis, i.e., the patients on chronic antiplatelet therapy that were excluded from the study.

The remaining literature against aspirin use in COVID-19 disease stems from retrospective cohort and case control studies, none of which found a correlation between aspirin treatment and mortality despite adjusting for comorbidities such as cardiovascular disease and its risk factors (older age, hypertension, diabetes, hyperlipidemia, smoking . . .) (Pan et al., 2019; Russo et al., 2020; Tremblay et al., 2020; Banik et al., 2021; Sahai et al., 2021; Son et al., 2021; Yuan et al., 2021). These studies looked at patients with an underlying condition and who were prescribed aspirin before testing positive for COVID-19. The bias imparted by their comorbidities should be accounted for when adjusting for risk factors, however it cannot be excluded that a confounder might have been missed in the statistical analysis. Furthermore, no correlation was found between mortality and either aspirin use in specific (Sahai et al., 2021; Son et al., 2021; Yuan et al., 2021) or antiplatelet use more generally (Pan et al., 2019; Russo et al., 2020; Tremblay et al., 2020; Banik et al., 2021). The results were also similar between inpatient populations (Pan et al., 2019; Russo et al., 2020; Banik et al., 2021; Yuan et al., 2021) and mixed ambulatory and hospitalized patients (Tremblay et al., 2020; Sahai et al., 2021; Son et al., 2021). In addition, despite combining the findings of Russo et al. and Tremblay et al. in a meta-analysis, the effect of antiplatelets on mortality was still insignificant OR = 0.65 (0.40–1.06) $p = 0.498$ for a total of 3,964 patients (Russo et al., 2020; Tremblay et al., 2020). Similarly, but in a non-COVID setting, a meta-analysis done by Liang et al. including a pooled population of 6,764 patients showed that prior aspirin use was linked with a significantly lower incidence of ARDS in at-risk patients ($p = 0.018$) but had no effect on hospital mortality (OR, 0.88; 95% CI, 0.73–1.07; $p = 0.204$; $I^2 = 0\%$) (Liang et al., 2020). These findings were validated by Wang et al. in their 2018 meta-analysis on the same subject (Wang et al., 2018).

Microthrombi formation plays an essential role in COVID-19 pathophysiology, aspirin use could therefore be potentially beneficial in that regard. However, findings concerning aspirin's effects are contradictory. Sahai et al. noticed an increased risk of thromboembolic events among COVID-19 patients on chronic aspirin therapy despite adjustment for comorbidities like age, sex, smoking, hypertension, diabetes, and cardiovascular diseases (adjusted OR = 0.163, $p = 0.005$), however they failed to adjust for a history of MI, stroke or venous thromboembolism (VTE) (Sahai et al., 2021). Aspirin therapy may therefore simply be a coincidental signal of the increased baseline risk of thrombosis in these patients. However, it could also be an indication of a different mechanism of thrombosis in COVID-19. Manne et al. detected a deranged and altered platelet phenotype in SARS-COV-2 (Manne et al., 2020). While Elbadawi et al. found absolute neutrophil count to be an independent predictor of thrombotic events in patients with COVID-19

(Elbadawi et al., 2021). Several studies have also shown the role of neutrophil extracellular traps in thrombus formation (Skendros et al., 2020). These findings supporting an immunological trigger to thrombosis suggest platelets may be indirect mediators in this process and perhaps not the best direct targets for pharmacological intervention (Sahai et al., 2021).

Another retrospective trial conducted in Egypt by Abdelwahab et al. found that the risk of stroke or MI among patients on chronic aspirin therapy was significantly lower than among non-users after adjustment ($p = 0.02$), but anticoagulants were found to be even more effective (adjusted OR = 0.071, $p < 0.001$) (Abdelwahabet al., 2021). The essence of coagulopathy in COVID-19 is indeed hypothesized to be massive fibrin formation. Anti-platelets might therefore not work as well as anti-coagulants if microthrombi have more fibrin than platelets. In fact, 90% of hospitalized COVID-19 pneumonia patients have elevated D-dimer levels. D-dimer can also reach very high thresholds and it is associated with disease severity (Iba et al., 2020a).

Another studied outcome was the need for mechanical ventilation, and here too there was no clear benefit for aspirin among COVID-19 patients. Antiplatelets were not found to affect the need for or time to mechanical ventilation, nor the risk of acute respiratory distress syndrome (ARDS) upon admission among patients on aspirin or other anti-platelets (taken as primary or secondary cardiovascular prophylaxis or as treatment for thromboembolic disease) (Pan et al., 2019; Russo et al., 2020; Tremblay et al., 2020; Abdelwahab et al., 2021; Banik et al., 2021). Limitations within these studies include failure to adjust for smoking status and BMI, small sample size (Abdelwahab et al., 2021), inclusion of only inpatients (Abdelwahab et al., 2021), and lack of correction for the influence of post-admission treatments (Tremblay et al., 2020). Banik et al. were able to detect a positive effect of anti-coagulants on mechanical ventilation need but found no correlation between antiplatelets and intubation (Banik et al., 2021). Surprisingly, they found that a positive chest CT was correlated with antiplatelet intake despite adjustment (OR = 12.1 (1.41–167), $p = 0.0354$). While another retrospective cohort showed an increased risk of oxygen therapy in patients prescribed aspirin within 2 weeks after diagnosis (Kim et al., 2021); but here propensity matching failed to adjust for a history of CAD. SARS-Cov-2 was indeed found to infect pulmonary endothelial cells causing endothelial injury and thrombosis (Acosta and Singer, 2020). It also seems that an important feature of ARDS is platelet and/or neutrophil aggregation (Zarbock et al., 2006). Gongalves de Moraes et al. showed that aspirin treatment could increase neutrophil number in the bronchial alveolar fluid in a mouse model (Gongalves de Moraes et al., 1996) which could potentially explain the correlation between aspirin intake and a positive chest CT, even though this correlation had no prognostic value as noted by Banik et al.

CONCLUSION

The pandemic has imparted significant burdens on the global health and economic sectors, leaving behind substantial morbidity and mortality. While the scientific community merged efforts to obtain the vaccine at an unpreceded velocity, the search for a therapeutic agent is still ongoing. Aspirin with its various molecular targets and properties has been under clinical investigations. Gathering all the high-quality clinical evidence to date, it appears that the effect of aspirin is still not clearly delineated. Despite the large number of studies exploring aspirin role in COVID-19 disease, the evidence is still premature. Almost all studies are retrospective in nature, and many fail to consider baseline clinical status that might eventually alter the outcomes measured. More studies are needed to better define recommendations of clinical practice. The anti-inflammatory and anti-platelet properties of aspirin are appealing, yet future studies have to pledge to more objective designs. Multi-center placebo-controlled high-quality randomized clinical trials with plainly outlined baseline characteristics and outcomes are urgently needed to evaluate the efficacy of aspirin.

AUTHOR CONTRIBUTIONS

MA developed the idea and the review framework. RZ, MD, TA, and AM wrote the first draft of the manuscript. NY and FB did the final editing. All authors contributed to corrections and adjustment of subsequent iterations of the manuscript. All authors approve and agree with the content.

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Ontology-Based Classification and Analysis of Adverse Events Associated With the Usage of Chloroquine and Hydroxychloroquine

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Multiple methodologies have been developed to identify and predict adverse events (AEs); however, many of these methods do not consider how patient population characteristics, such as diseases, age, and gender, affect AEs seen. In this study, we evaluated the utility of collecting and analyzing AE data related to hydroxychloroquine (HCQ) and chloroquine (CQ) from US Prescribing Information (USPIs, also called drug product labels or package inserts), the FDA Adverse Event Reporting System (FAERS), and peer-reviewed literature from PubMed/EMBASE, followed by AE classification and modeling using the Ontology of Adverse Events (OAE). Our USPI analysis showed that CQ and HCQ AE profiles were similar, although HCQ was reported to be associated with fewer types of cardiovascular, nervous system, and musculoskeletal AEs. According to EMBASE literature mining, CQ and HCQ were associated with QT prolongation (primarily when treating COVID-19), heart arrhythmias, development of Torsade des Pointes, and retinopathy (primarily when treating lupus). The FAERS data was analyzed by proportional ratio reporting, Chi-square test, and minimal case number filtering, followed by OAE classification. HCQ was associated with 63 significant AEs (including 21 cardiovascular AEs) for COVID-19 patients and 120 significant AEs (including 12 cardiovascular AEs) for lupus patients, supporting the hypothesis that the disease being treated affects the type and number of certain CQ/HCQ AEs that are manifested. Using an HCQ AE patient example reported in the literature, we also ontologically modeled how an AE occurs and what factors (e.g., age, biological sex, and medical history) are involved in the AE formation. The methodology developed in this study can be used for other drugs and indications to better identify patient populations that are particularly vulnerable to AEs.

Keywords: chloroquine, hydroxychloroquine, COVID-19, drug, adverse event, ontology, FAERS database

INTRODUCTION

Generally, adverse events (AEs) are an undesirable experience associated with the use of a medical product, and can or cannot be causally related (see 21 CFR 314.80) (FDA, 2016). AEs can range from mild effects such as abdominal discomfort to more severe effects such as cardiac arrhythmias or acute neurological disorders. AEs may be detected during research or clinical studies or *via* postmarket surveillance. AEs can differ between patient populations, including patients taking the same drug for different indications (Yu et al., 2019). These differences can often be difficult to elucidate and may be overlooked in both traditional and newer pharmacovigilance methods.

To better study various AEs under different conditions, we can rely on the help from biomedical ontologies. A biomedical ontology is a structured vocabulary of computer- and human-interpretable terms and relations among these terms that represent entities in the biomedical world and how they relate to each other. Ontologies play a critical role in data science to facilitate biomedical data normalization, integration, processing, and analyses (Schulz et al., 2013; Hoehndorf et al., 2015). The Ontology of Adverse Events (OAE) is a community-based formal AE ontology designed to standardize and classify different types of AEs arising subsequent to medical interventions. In addition, OAE addresses AE properties and associated factors and supports computer-assisted reasoning (He et al., 2014). The OAE has been used to support drug and vaccine AE data analysis and could be used to identify AE differences between populations (Sarntivijai et al., 2012; He et al., 2014; Xie et al., 2016a; Xie et al., 2016b; Sarntivijai et al., 2016); additionally, OAE has demonstrated better performance in classifying AEs compared to the Medical Dictionary for Regulatory Activities [MedDRA, the default system for standardizing terms of AEs after medical interventions (Brown et al., 1999)] (Sarntivijai et al., 2012; Xie et al., 2016a; Xie et al., 2016b).

With the coronavirus disease 2019 (COVID-19) pandemic spreading worldwide, many FDA-approved drugs are being evaluated for their efficacy in COVID-19 treatment (Sultana et al., 2020). One class of drugs that has been evaluated is the quinoline antimalarials (Foley and Tilley, 1997), which include the FDA-approved drugs chloroquine (CQ) and hydroxychloroquine (HCQ). This drug class was hypothesized to treat COVID-19 based on findings in a related virus, SARS-CoV (Keyaerts et al., 2004). In late March 2020, the FDA issued an Emergency Use Authorization (EUA) for CQ and HCQ to treat certain patients with COVID-19 in a hospital setting (FDA, 2020b). However, on June 15, 2020, the FDA revoked the emergency use authorization of HCQ and CQ for treatment of COVID-19 patients due to lack of efficacy and a significant risk of AEs, including cardiotoxicity (FDA, 2020a). While CQ and HCQ were previously associated with cardiotoxicity, the prominent appearance of this AE in COVID-19 patients raises the possibility that AE profiles associated with these drugs depend on the disease being treated.

The aim of this paper is to develop a new ontology-based approach to evaluate different patient populations for AEs, using the various indications of CQ and HCQ as a use case. While the consensus is that CQ and HCQ are not effective for COVID-19 (Group et al., 2020), many clinical trials have been conducted, providing a significant amount of AE and other clinical data. In this study, we collected and analyzed AE reports from FAERS, literature, and product labels across and within the various indications and model the results in the OAE to identify similarities and differences in AEs. We also tested our hypothesis that CQ and HCQ would have different AE profiles from each other, and that these profiles would differ depending on the indication. The methodology developed in this study is scalable and can be used to identify similarities and differences in AEs between other drugs and their respective indications.

METHODS

General Workflow of Our Methodology

Figure 1 shows the workflow of our methodology, with **Figure 1A** providing the general workflow and **Figure 1B** providing the workflow for this evaluation of HCQ and CQ. Specifically, our research used three data sources: 1) the United States Prescribing Information (USPI), also known as package insert information, 2) literature databases including PubMed (Lu, 2011) and EMBASE (Wong et al., 2006), and 3) FAERS data. All data were processed and analyzed to extract drugs and their associated AEs under specific conditions (such as COVID-19 infection). Then the AE terms for one or more specific drugs were mapped to the OAE to generate OAE-based AE classifications. Alternatively, for the FAERS data, specific statistical tests, including proportional reporting ratio (PRR) (Evans et al., 2001), Chi-square, and minimal case number filtering tests were performed, and statistically significantly enriched AEs were identified. PRR is often used to measure the extent to which a particular AE is reported for individuals taking a specific drug, compared to the frequency at which the same AE is reported for patients taking other drugs in an AE case report system such as FAERS. These AEs under FAERS were tagged with MedDRA IDs and were then mapped to OAE terms. The OAE-based AE classifications were then used for further classification.

In this study, we used the above-described methodology to analyze the AE differences by indication for HCQ and CQ. The details about the HCQ and CQ AE analysis workflow (**Figure 1B**) are provided below.

AE Collection From US Prescribing Information Inserts and Literature

Three drug USPIs, commonly known as a package insert or label, databases were surveyed: Drugs@FDA, DailyMed, and RxDrugLabels, to find AEs associated with name brand and generic quinoline-containing drug products. The FDA USPIs available on September 30, 2020, of the name brand drugs of CQ and HCQ, Aralen® and Plaquenil® respectively, as well as two generic versions of the drugs, were surveyed. AEs listed under the "Adverse Reactions" section were extracted, mapped, and

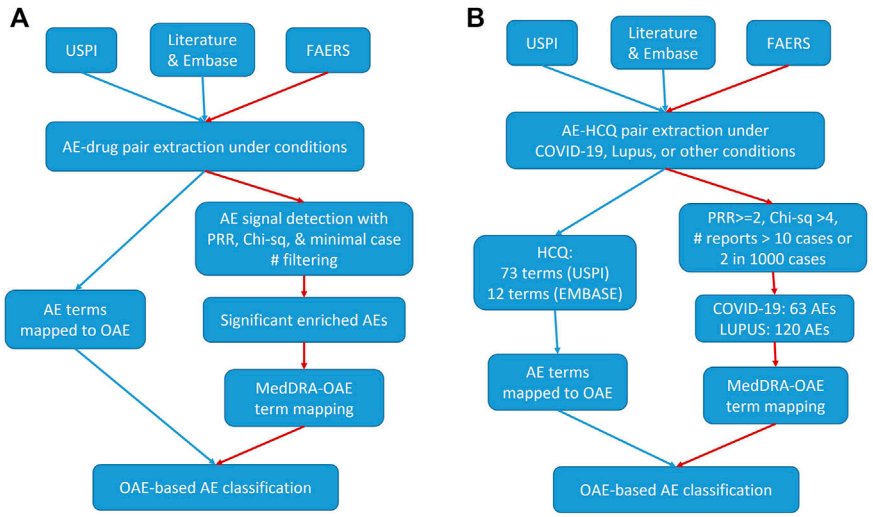

FIGURE 1 | Workflow for our drug AE analysis. **(A)** General workflow. **(B)** HCQ specific workflow. The red paths presented with red edges represent the workflow of FAERS-specific drug AE analyses. USPI, United States Prescribing Information. FAERS, FDA Adverse Event Reporting System. AE, Adverse Event. PRR, Proportional Reporting Ratio. OAE, Ontology of Adverse Event. HCQ, hydroxychloroquine.

catalogued using the OAE. Ontologies were created to visualize CQ and HCQ's specific AEs as well as their shared AEs. Meanwhile, the diseases treated by these drugs were also gathered.

AEs reported in published literature as of January 17, 2021, were found through the biomedical bibliographic database, EMBASE (Wong et al., 2006). The usage of CQ/HCQ was at its peak before June 15, 2020 when the EUA was revoked by the FDA due to safety issues and lack of efficacy (FDA, 2020a). The literature reports of their usage dramatically decreased after 2020. Each drug was searched under three disease states: COVID-19, systemic lupus erythematosus (SLE), and rheumatoid arthritis for HCQ, and COVID-19, systemic lupus erythematosus, and malaria for CQ. Results were then filtered by the "Drugs" category to select specific papers using CQ or HCQ. The key subheading, "Adverse drug reaction," was used to find specific AEs mentioned and provided the number of literature reports where these AEs were reported. The five most commonly reported AEs were collected, along with the number of papers mentioning those AEs and their respective percentages of the total, for each disease state. Results were then analyzed to exclude repeat data.

Additional clinical trials and case reports were found through PubMed using the search terms, "COVID-19", "chloroquine," or "hydroxychloroquine," and "adverse events", by the date of January 17, 2021. While over 100 clinical trials related to CQ/HCQ exist in clinicaltrials.gov, only five clinical trials were found to have AE results available (as of March 12, 2021).

AE Collection and Analysis From FAERS

FAERS is a database containing AEs and medication error reports that have been sent to FDA by industry, healthcare providers, patients, and other interested parties. To ensure these data are easily accessible, FDA has developed the FAERS Public Dashboard (https://www.fda.gov/about-fda/update-fda-adverse-event-reporting-system-faers-public-dashboard), a web-based interface that allows for user-friendly querying and organization of FAERS data. The dashboard sorts the data by individual AE or individual product (drug). AEs in the dashboard correspond to MedDRA Preferred Terms, and they are also grouped by "Reaction Group," which corresponds to the MedDRA System Organ Class (SOC) terms. The AE data for generic CQ, Aralen®, generic HCQ and Plaquenil® were downloaded from the dashboard as Excel files, and at the time of the analysis the last quarterly data update had been included on September 30, 2020. Additionally, the total number of reports in FAERS and the number of reports for each AE in FAERS were also recorded.

The Excel spreadsheets contained each AE reported for the individual drug and the number of cases reported for that AE-drug pair. The four products were analyzed separately and then compared. For both HCQ and CQ, the generic drug is more commonly used, and therefore used for further analysis. For HCQ (generic), the data was also sorted by reported indication to compare the incidence of AEs for COVID-19 and systemic lupus erythematosus. HCQ was chosen for further analysis as more cases have been reported in the FAERS database for all indications, which would reduce statistical error. The data was sorted by number of reported AE cases, and any AE with fewer than 10 reported cases or representing less than 0.2% of the number of case reports for that drug was deemed insignificant. Using the data in the spreadsheet, a Chi-square test and PRR test (Evans et al., 2001) were performed for each AE. PRR compares the individual case numbers for each AE to the overall cases in the FAERS system and the overall cases reported for the specific drug. Cases with a Chi-square result of less than 4 (corresponding with a p-value > 0.05) and a PRR result of less than 2 were determined to be insignificant (Sarntivijai et al., 2012). The resulting AEs were then sorted back into the high-level Reaction Groups/MedDRA

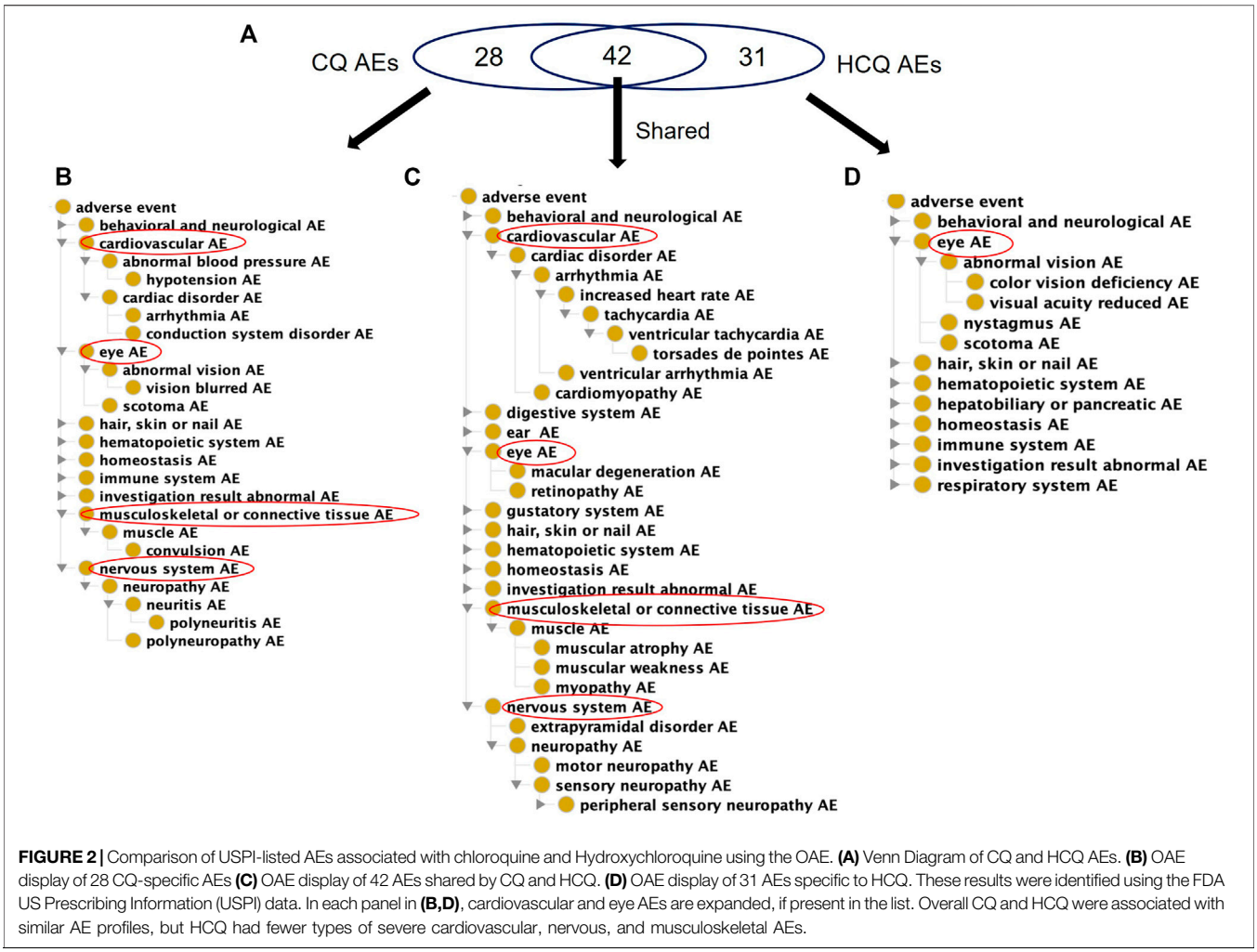

FIGURE 2 | Comparison of USPI-listed AEs associated with chloroquine and Hydroxychloroquine using the OAE. **(A)** Venn Diagram of CQ and HCQ AEs. **(B)** OAE display of 28 CQ-specific AEs **(C)** OAE display of 42 AEs shared by CQ and HCQ. **(D)** OAE display of 31 AEs specific to HCQ. These results were identified using the FDA US Prescribing Information (USPI) data. In each panel in **(B,D)**, cardiovascular and eye AEs are expanded, if present in the list. Overall CQ and HCQ were associated with similar AE profiles, but HCQ had fewer types of severe cardiovascular, nervous, and musculoskeletal AEs.

SOCs within Excel, and the number of unique cases for each group was recorded. This was then divided by the total significant AE instances and converted to a percentage to allow for comparison. The resulting lists for each drug were input into the OAE.

OAE Ontology Term Mapping, Extraction, and Visualization

An online biomedical ontology tool OntoFox (Xiang et al., 2010) was used to extract a subset of the Ontology of Adverse Event that includes the enriched AE terms identified in our data analysis as well as other high level AE terms related to these enriched AE terms. The OntoFox output was then visualized using the Protégè-OWL editor (Musen, 2015). This process develops a visual representation of all the AEs for a given drug and indication, and how they all relate to one another by placing them in groups. For example, "myopathy" is categorized under "muscle AE," which can then be categorized under the more general "musculoskeletal and connective tissue AE." This provides a way to directly compare specific types of AEs between different drugs and indications.

RESULTS

Comparison of CQ and HCQ AEs From USPIs

CQ and HCQ are generally considered safe molecules with low incidences of AEs (James et al., 2007). With their similar structures and both being 4-aminoquinolines, CQ and HCQ have similar AE profiles, with many AEs in common (**Figure 2**). There is evidence that HCQ, a derivative of CQ, is associated with fewer AEs, although no mechanism has been proposed (Felson et al., 1990; Ruiz-Irastorza et al., 2010). However, CQ has fewer AEs listed on the USPIs compared to HCQ. Although HCQ has more labeled AEs, it lacks specific cardiovascular, nervous system, and musculoskeletal/connective tissue AEs compared to CQ.

COVID-19 CQ/HCQ AE Profiles From Literature Mined Reports Using EMBASE

AEs from EMBASE, using the filters, "Drugs," and "Adverse drug reactions," were used to gather the number of published articles that report the specified AE for both CQ and HCQ, as

TABLE 1 | Five AEs most frequently discussed in papers describing chloroquine (CQ) use in various disease states (as discerned by a search of EMBASE).

COVID-19		Systematic Lupus Erythematosus (SLE)		Malaria	
Adverse Event	**Frequency (%[a])**	**Adverse Event**	**Frequency (%[a])**	**Adverse Event**	**Frequency (%[a])**
QT Prolongation	38 (43.7%)	Retinopathy	8 (34.8%)	General Side Effects	8 (32%)
Heart Arrhythmia	13 (14.9%)	Cardiomyopathy	6 (26.1%)	Headache	7 (28%)
Torsade des Pointes	13 (14.9%)	Eye Toxicity	5 (21.7%)	Retinopathy	7 (28%)
Retinopathy	11 (12.6%)	Heart Arrhythmia	4 (17.4%)	QT Prolongation	6 (24%)
General Side Effects	9 (10.3%)	Diarrhea	3 (13.0%)	Nausea	5 (20%)

[a]*Percentage of papers reporting this AE, as classified by the EMBASE database.*

TABLE 2 | Five AEs most frequently discussed in papers describing hydroxychloroquine (HCQ) use in various disease states (as discerned by a search of EMBASE).

COVID-19		Systemic lupus erythematosus (SLE)		Rheumatoid arthritis (RA)	
Adverse Event	**Frequency (%[a])**	**Adverse Event**	**Frequency (%[a])**	**Adverse Event**	**Frequency (%[a])**
QT Prolongation	133 (43.9%)	Retinopathy	39 (26.7%)	Retinopathy	23 (17.3%)
Diarrhea	47 (15.5%)	Cardiomyopathy	13 (8.9%)	Rash	13 (9.8%)
Nausea	47 (15.5%)	Eye Toxicity	12 (8.2%)	Infection	12 (9.0%)
Heart Arrhythmia	37 (12.2%)	Rash	12 (8.2%)	Nausea	12 (9.0%)
Torsade des Pointes	35 (11.6%)	Diarrhea	11 (7.5%)	General Side Effect	11 (8.3%)

[a]*Percentage of papers reporting this AE, as classified by the EMBASE database.*

summarized in **Table 1** and **Table 2**, respectively. The inclusion criteria for the papers screened were to be reporting results from a human randomized clinical trial and published during 2017–2021. The literature regarding COVID-19 were all published during 2019–2021, but because CQ and HCQ possess a long history in both autoimmune and infectious diseases, the inclusion criteria for literature regarding those diseases (rheumatoid arthritis, systemic lupus erythematosus, and malaria) was extended two additional years, back to 2017. This is to ensure the data is current and follows modern AE collection guidelines.

Of 87 published papers that were reviewed for CQ use for COVID-19, 38 (43.7%) reported the development of QT prolongation, 13 (14.9%) reported the development of heart arrhythmias, and 13 (14.9%) reported the development of Torsade des Pointes. Only 11 (12.6%) reported retinopathy, an AE commonly associated with CQ use. 23 published articles on CQ use for SLE were reviewed, of which 6 (26.1%) reported cardiomyopathy as an AE. The most prevalent AE associated with CQ use for SLE are eye AEs, including retinopathy (34.8%) and eye toxicity (21.7%). Additionally, 25 articles on CQ use for malaria were reviewed, of which the most common AEs include general side effects, headache, and retinopathy. QT prolongation was reported only in 6 (24%) articles.

This trend holds true when observing HCQ use for COVID-19 and SLE. QT prolongation is also the most prevalent AE reported in the 303 published articles on HCQ use for COVID-19, with other cardiac AEs being heart arrhythmias and Torsade des Pointes. HCQ use for SLE (lupus) resulted in similar AEs as CQ use for SLE, with cardiomyopathy being reported in 8.9% of the 146 published articles compared to retinopathy (26.7%) and eye toxicity (8.2%). As for the 133 published articles on HCQ use for RA, retinopathy was the most reported AE, followed by rash, infection, nausea, and general side effects.

COVID-19 CQ/HCQ AE Profiles From Clinical Trial and Observational Studies Reports

AEs associated with COVID patients treated with CQ and HCQ were collected. At the time of reviewing the related works, not many studies have been conducted. Although many reports focus on HCQ, only two studies focused on CQ. Therefore, these two studies were surveyed for CQ. Three representative HCQ studies were also surveyed. The results are summarized in **Table 3** and described below.

In a 2018 study in China, no serious AEs were reported (Huang et al., 2020). Out of ten COVID-19 patients treated with CQ, five reported AEs. The most frequent AEs were vomiting (50%), diarrhea (50%), nausea (40%), and cough (40%). Other non-serious AEs such as abdominal pain, rash, and shortness of breath were also reported (10%) (Huang et al., 2020). However, in a 2020 randomized clinical trial held in Brazil, high and low dosages of CQ were compared in patients with severe COVID-19 (Borba et al., 2020). The lower dose group (450 mg daily) showed fewer cases of AEs including decreased hemoglobin, increased creatinine, increased creatine kinase, and increased CK-MB, an isoenzyme of creatine kinase. Seven of thirty-seven patients (19%) receiving the high dosage reported a prolonged QTc interval compared to one patient receiving the low dosage, and two patients receiving the high dosage reported symptoms of ventricular tachycardia compared to zero patients receiving the low dosage (Borba et al., 2020).

In a 2020 study conducted in China, seventy-five COVID-19 patients were treated with HCQ with 30% of them reporting AEs.

TABLE 3 | AEs associated with CQ/HCQ administration were reported in two clinical trials and three observational studies where COVID-19 patients were given either CQ or HCQ in combination with standard of care drugs.

Drug/Location/References	Clinical Trial No./PubMed No	AEs Reported
Chloroquine (Rosenberg et al., 2020)	PMID: 32236562	Vomiting; abdominal pain; nausea; diarrhea; rash/itchiness; cough; shortness of breath
Chloroquine (Borba et al., 2020)	NCT04323527 PMID: 32330277	Decreased hemoglobin; increased creatinine; increased CK; increased CKMB; QTcF >500 m; ventricular tachycardia
Hydroxychloroquine (Tang et al., 2020)	PMID: 32409561	Diarrhea; vomiting; nausea; abdominal discomfort; thirst; sinus bradycardia; hypertension; orthostatic hypotension; hypertriglyceridemia; decreased appetite; fatigue; dyspnoea; flush; kidney injury; coagulation dysfunction; blurred vision; decreased WBC; increased alanine aminotransferase; increased serum amylase; decreased neutrophil count; disease progression*; upper respiratory tract*
Hydroxychloroquine (Rosenberg et al., 2020)	PMID: 32392282	Diarrhea; hypoglycemia; cardiac arrest*; abnormal ECG*; arrhythmia*; QT prolongation*
Hydroxychloroquine (RECOVERY Group, (Group et al., 2020)	NCT04381936	Atrial flutter/fibrillation, other supraventricular tachycardia, ventricular tachycardia, ventricular fibrillation, atrioventricular block requiring intervention

*Serious AEs, as defined by the respective study investigators, are marked with *.*

Three percent of patients reported serious AEs, including disease progression (1%) and upper respiratory tract infection (1%). The other 27% of patients who reported AEs reported non-serious AEs, the most common ones being diarrhea (10%) and vomiting (3%). Other AEs commonly associated with HCQ such as blurred vision, fatigue, and abdominal discomfort were only reported once each (Tang et al., 2020). In the second HCQ trial in New York (2020) involving 1438 participants, diarrhea was reported in 17% of patients treated with HCQ alone and 11.6% of patients treated with HCQ in conjunction with azithromycin, a broad-spectrum antibiotic used in treating infections present in COVID-19 patients. The New York study also reported serious cardiac AEs such as cardiac arrest (13.7%), abnormal ECG (27.3%), arrhythmia (16.2%) and QT prolongation (14.4%) in COVID-19 patients treated with HCQ alone (Rosenberg et al., 2020). In the impactful study done by the RECOVERY Group, who treated 1,561 COVID-19 patients with HCQ, 60 (8.2%) developed major cardiac arrhythmias compared to 90 of 3,155 (6.3%) of patients who received the usual care. The most common cardiac AEs reported were supraventricular tachycardias, with 56 (7.6%) patients from the HCQ group and 74 (5.2%) patients in the usual care group (Borba et al., 2020).

Differential FAERS AE Profiles by CQ and HCQ

In total, 1,998 AE case reports were collected for CQ and Aralen® with possibility for duplication. A total of 908 different AEs were reported for CQ and 351 different AEs were reported for Aralen®. After statistical analysis (see the Methods section for detail), 78 AEs met or exceeded the significance thresholds for CQ, and 63 AEs met or exceeded the significance thresholds for Aralen®. The three most frequent reaction groups among the significant AEs for CQ were Cardiac Disorders, Nervous System Disorders, and Eye Disorders. The two most frequent reaction groups among the significant AEs for Aralen® were Eye Disorders, and Nervous System Disorders. Under the source term there were 15 subheadings for CQ and 16 subheadings for Aralen®.

In total, 36,641 AE reports were collected for HCQ and Plaquenil®. A total of 3,486 different AEs were reported for HCQ, and 2,796 different AEs for Plaquenil®. After statistical data analysis, 353 AEs met or exceeded the significance thresholds for HCQ and 303 AEs for Plaquenil®. The three most frequent reaction groups among the significant AEs for HCQ and Plaquenil®, analyzed separately but with the same result, were General Disorders and Administration Site Conditions (top condition: drug ineffective AE), Musculoskeletal and Connective Tissue Disorders (top condition: rheumatoid arthritis AE), and Gastrointestinal Disorders (top condition: nausea AE). Under the top-level source term in OAE there were 23 subheadings for HCQ and 21 subheadings for Plaquenil®.

When considering FAERS reports across all indications, the percentage of significant AE cases for just CQ that falls in the FAERS reaction group of Cardiac Disorder is about 20.5% compared to 0.32% for HCQ. OAE's Cardiovascular AE (http://purl.obolibrary.org/obo/OAE_0000493) is highlighted in **Figure 2**. The number of AEs under the Cardiovascular AE heading for CQ (**Figure 3A**) is much larger than the number of AEs under the Cardiovascular AE heading for HCQ (**Figure 3B**). Compared with the results from USPIs (**Figure 2**), more cardiovascular AE term types were reported in both CQ and HCQ cases in FAERS. This could be a result of increased use after the EUA and stimulated reporting after news of cardiac complications. HCQ reports doubled in the year 2020, 10,362 compared to 5,042.

Differential FAERS AE Profiles in Systemic Lupus Erythematosus and COVID-19 Patients Treated With HCQ

The FAERS database had 1,002 case reports for COVID-19 patients and 1,527 case reports for SLE patients treated with HCQ. After analysis, there were 63 significant AE types for COVID-19 patients

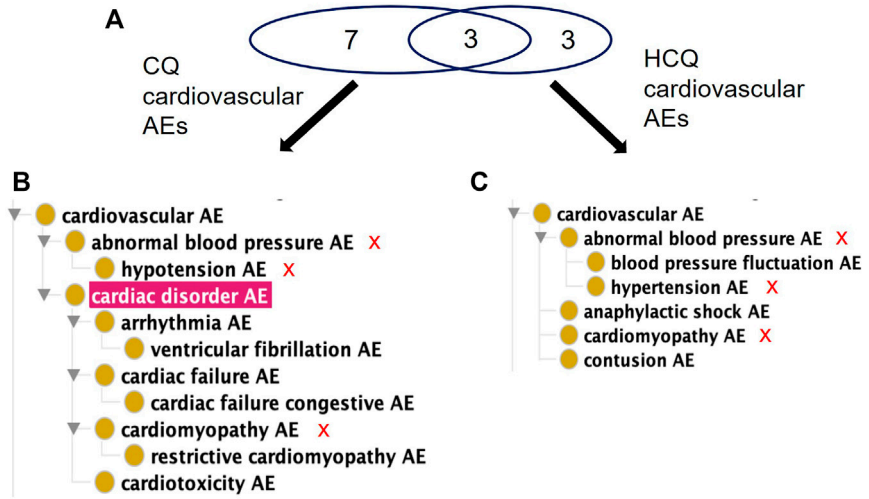

FIGURE 3 | Comparison of significant cardiovascular AEs for CQ and HCQ from the FAERS database. **(A)** Venn Diagram of significant cardiovascular AEs for CQ and HCQ. **(B)** OAE classification of 10 significant cardiovascular AEs for CQ. (C) OAE classification of 6 significant cardiovascular AEs for HCQ. The red sign of "X" next to the AE terms in **(B,C)** represent the shared AE for CQ and HCQ. Note that cardiac disorder AE passed the significance threshold for CQ **(A)** but not for HCQ **(B)**. Overall CQ was associated with more cardiovascular AEs.

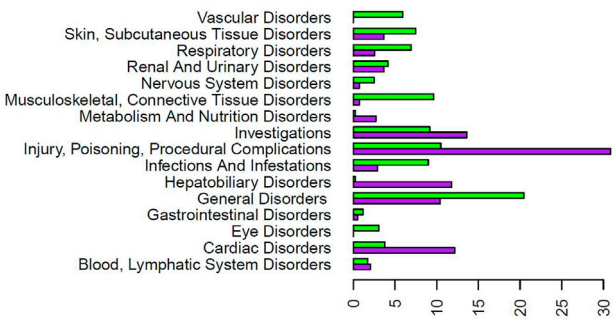

FIGURE 4 | Significant AE reaction group percentage comparison for COVID-19 and SLE patients treated with HCQ from the FAERS database. The purpose bars represent the percentage of the total significant count for COVID-19 for each reaction group. The green bars represent the percentage of the total significant count for SLE for each reaction group. The reaction groups of Ear and Labyrinth Disorders, Immune System Disorders, Pregnancy, Puerperium and Perinatal Conditions, Product Issues, Psychiatric Disorders, Social Circumstances, and Surgical and Medical Procedures were left off the graph due to small percentages for both patient types. Note that only the AEs that have passed the significance test were used here for all calculations). HCQ AE patterns appeared different depending on the diseases treated.

and 120 significant AE types for SLE patients (**Supplementary Table S1**). When broken into MedDRA higher level term percentages, of significant cases for each separate disease, the categories with the greatest differential were Cardiac Disorders, General Disorders and Administration Site Conditions, and Injury, Poisoning and Procedural Complications (**Figure 4**). Compared to HCQ, a greater percentage of the significant cases for both COVID-19 and SLE were cardiac disorders; however, cardiac disorders are markedly more reported in COVID-19 patients (i.e., 12.2%) than that in SLE patients (3.8%) taking the same drug (**Figure 4**).

Of the 63 significant AEs for COVID-19 patients treated with HCQ, 21 are classified by OBO as Cardiovascular AE (http://purl.obolibrary.org/obo/OAE_0000493), a concern FDA has previously highlighted in association with the treatment of COVID-19 patients with quinoline drugs (Arshad et al., 2020). Of the 120 significant AEs for SLE patients treated with HCQ, 12 fall under Cardiovascular AE (http://purl.obolibrary.org/obo/OAE_0000493). In addition, 14 AEs fall under the Cardiovascular AE sublevel Cardiac Disorder AE (http://purl.obolibrary.org/obo/OAE_0000084) for COVID-19 patients compared to 7 AEs for SLE patients (**Figure 5**). Notably, there were more reports of long QT syndrome, atrial fibrillation, and bradycardia in COVID-19 compared to SLE. This demonstrates that AE profiles for a drug may differ between indications.

Drug AE Condition Modeling and Classification

While our study has so far focused on the analysis of the difference between AE manifestation between SLE and COVID-19 patients following CQ/HCQ drug administration, AEs occur under specific conditions that also include many other factors than the disease type. **Figure 6A** provides a general OAE modeling and classification of how an AE occurs and what factors are involved in the generation of the AE. Basically, an AE of a patient occurs after a drug administration in the patient after its diagnosis of a specific disease with specific disease symptoms or signs. The patient's qualities (e.g., age and biological sex) and medical history are associated with and may affect the manifestation of the AE.

The general model shown in **Figure 6A** can be illustrated with specific patient cases. As part of the literature review, many case reports ($n = 1$) have been published as observational reports. We have selected one such case report in which a COVID-19 patient

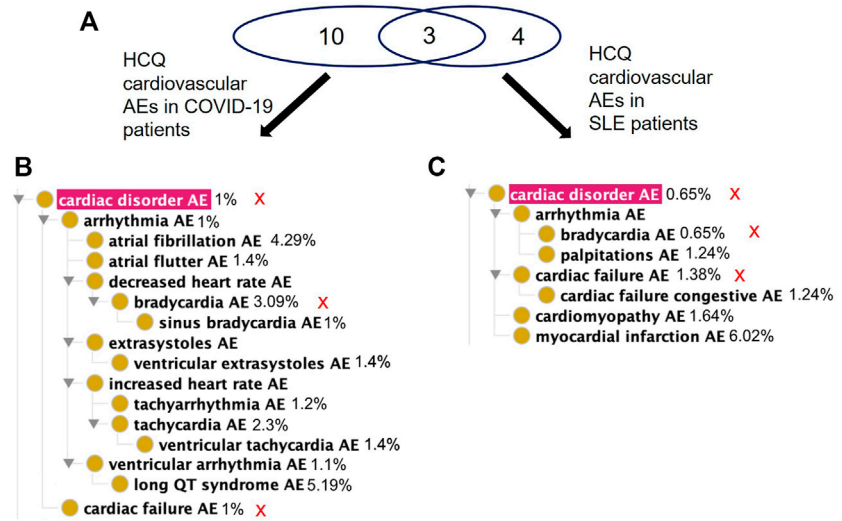

FIGURE 5 | Comparison of significant cardiac AEs for COVID-19 and SLE patients treated with HCQ from the FAERS database. **(A)** Venn diagram of significant cardiac AEs for COVID-19 and SLE patients treated with HCQ. **(B)** OAE classification of significant cardiac AEs for COVID-19 patients treated with HCQ. **(C)** OAE classification of significant cardiac AEs for SLE patients treated with HCQ. The percentages next to the AE terms represent the occurrence rates of these AEs under the specific condition. The red sign of "X" next to the AE terms in **(B,C)** represent the shared AE for the two types of patients. Different cardiac AEs were found to be associated with HCQ following its usage for treating COVID-19 and lupus.

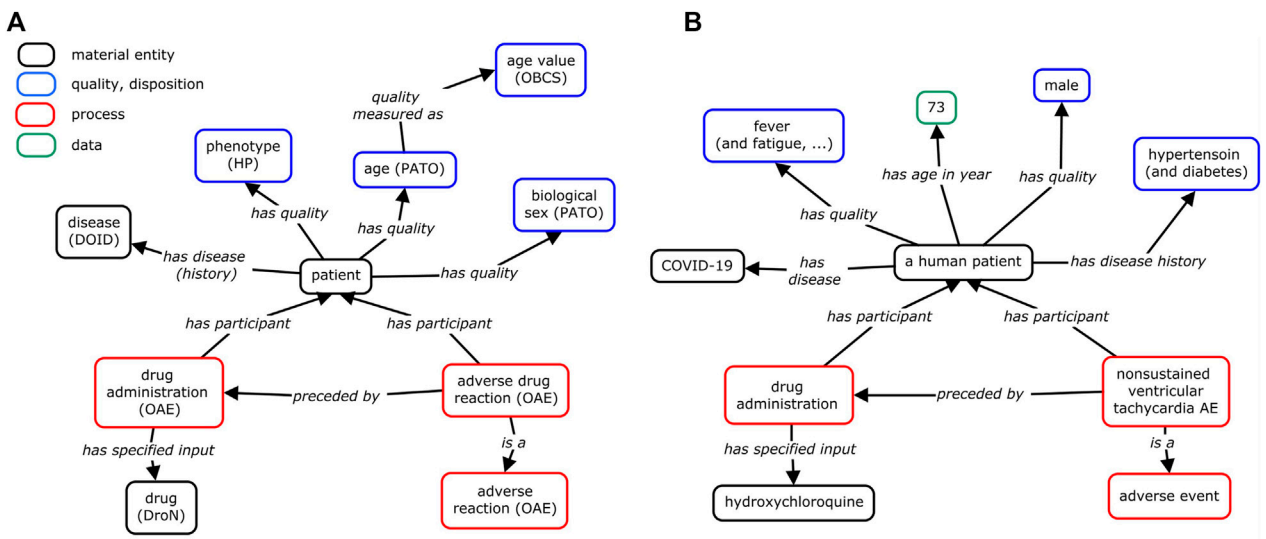

FIGURE 6 | Modeling of condition-dependent AE occurrence with an example published in PubMed. **(A)** General modeling. **(B)** HCQ AE case study example. In this example, a human patient with COVID-19 was treated with HCQ, and later suffered from unsustained ventricular tachycardia AE (Abdelmaseih et al., 2020). In addition to the positive COVID-19 diagnosis, the study of the HCQ AE needs to consider the patient's age (75 years old), biological sex (male), medical disease history (hypertension and diabetes), and the symptoms (e.g., fever, dry cough) before the usage of the HCQ drug. If these conditions had changed, the cardiac disease might have not occurred. To support ontology interoperability, OAE also reuses terms from other ontologies, including Human Phenotype Ontology (HP) (Kohler et al., 2021), Drug Ontology (DrON) (Hanna et al., 2013), disease Ontology (DOID) (Schriml et al., 2012), Phenotype And Trait Ontology (PATO) (Mabee et al., 2007), and Ontology of Biological and Clinical Statistics (OBCS) (Zheng et al., 2016). The relation "has age (in year)" is a shortcut relation of ("has quality" some (age and "quality measured as (in year)")). This method of ontology modeling allows us to better visualize the many factors that contribute to the manifestation of certain AEs.

developed cardiac AEs after beginning HCQ treatment (Abdelmaseih et al., 2020). The details of the case were mapped (**Figure 6B**) to help identify variables that could potentially affect disease state and AE outcomes. This is the modeling of the case study of a 75-year-old male presenting to the emergency room with worsening shortness of breath, dry cough, fatigue, and high fever (Abdelmaseih et al., 2020). The patient had a medical history of hypertension and diabetes. After testing

positive for COVID-19, the patient was treated with HCQ and subsequently developed episodes of unsustained ventricular tachycardia, which resolved after termination of HCQ. In this case, while the administration of HCQ in the COVID-19 patient was associated with a cardiac AE, a better understanding of this case requires our consideration of the patient's specific conditions including age, biological sex, and medical history.

The ontology modeling approach (**Figure 6**) facilitates the identification of variables that may affect the AE outcomes in COVID-19 or other disease patients given different conditions. In addition to the diseases to be treated, patient qualities such as age and biological sex, preexisting health conditions, other factors such as drug dosage can be included. OAE treats the AE as a pathological bodily process that is a dynamical process and thus may require dynamic monitoring. For example, given that the half-lives of CQ and HCQ are long (approximately 1–2 months), long-term monitoring for their safety profiles is also needed (Gevers et al., 2020). As a result, we could expect the generation of a metadata (i.e., the data that describe the representation of instance data) standard that standardizes these AE-associated variables and their measurement for better case reports and data analysis. Ontology modeling and standardization can provide support to such process.

DISCUSSION

In this study, we utilized a systematic methodology to analyze and classify AEs across the various indications of two drugs, CQ and HCQ. First, we developed a formal survey and analysis pipeline using three major resources (i.e., USPIs, literature resources including EMBASE and PubMed, and FAERS database) to consistently analyze AEs given difference conditions including the disease being treated. We have emphasized the role of ontology in the AE classification and modeling in our pipeline. Second, we have applied our methodology to study the profiles of CQ and HCQ AEs for treating diseases including COVID-19 and SLE. The findings in this study demonstrated that a systemic methodology leveraging complementary publicly available sources can assist with identifying differences in the number of and type of AEs between different indications of the same drug. For example, the FAERS and EMBASE analyses each demonstrated different AE types given different indications. OAE modeling of these results further supported these conclusions and enabled us to semantically represent different conditions under which an AE may be identified. In summary, although common AEs were found, our results identified many distinct AE results given various conditions, including different diseases treated by these two drugs.

One major focus of this paper was on the methodology for evaluating AEs (**Figure 1**). While AEs for multiple indications may be labeled on one USPI, there may be differences in AE rates between indications. Therefore, multiple sources were evaluated, including literature, USPIs, and FAERS. Beyond this analysis, ontology modeling facilitated the identification of similarities and differences in AE profiles. Ontologies have emerged to become important for standard data and knowledge representation, classification, and analysis. OAE provides a standard ontology method for classifying and analyzing various AEs. Previous studies demonstrated that OAE performed better than MedDRA (a commonly used AE presentation standard for AE case reporting) in ontology classification analysis (Sarntivijai et al., 2012; He et al., 2014; He et al., 2016; Xie et al., 2016a). In the current study, we used USPI data, FAERS data, and literature data in correlation to compare AE profiles of drugs in the same class and of one drug in different diseases. Our OAE-based AE classification method clearly shows the hierarchical structure of identified AEs and allows us to quickly group various AEs into specific categories. In addition, OAE can be used to model and represent AE formation processes in individual patients as shown in **Figure 6**, which can be further used to support AE data standardization and analysis (He et al., 2014; Wong et al., 2017).

This methodology demonstrated several strengths for evaluating AEs. First, using multiple sources allows for trend identification, signal strengthening, and can help reduce bias that may be present in one source. In this example, while FAERS may have biased reporting, cardiovascular events were also reported in the literature and labels, supporting the possibility that cardiovascular AEs are more prevalent in COVID-19 than SLE patients taking HCQ. Next, incorporating the information from these sources into OAE allows for easy visualization and analysis. In this example, we were able to identify AE differences between HCQ and CQ as well as SLE and COVID-19 *via* ontology modeling. This methodology can be utilized to evaluate other drug-drug pairs or drug-indication pairs for differences; other drugs have shown similar patterns, such as sirolimus (also known as rapamycin), which displays specific AEs (e.g., acne, stomatitis) that manifest when it is used to treat lymphangioleiomyomatosis, yet different AEs (e.g., anemia, hypertension) that manifest when used in renal transplantation (Yu et al., 2019).

Using the methodology described above, we analyzed different data sources related to CQ and HCQ AEs and made many interesting findings. First, our USPI data analysis found that while CQ and HCQ had similar AE profiles, HCQ lacked many cardiovascular, nervous, and musculoskeletal AEs found in CQ, including hypotension, arrhythmia, convulsion, and polyneuropathy AEs (**Figure 2**). While USPI results came from well-controlled randomized studies, the information about study size were limited in the USPI results. To complement the USPI reports, the EMBASE database includes a large number of studies and results, and the number of EMBASE papers citing specific AEs provides us a feasible way to rank the frequency of AE occurrences. EMBASE data mining found that CQ and HCQ were frequently associated with QT prolongation, heart arrhythmias, development of Torsade des Pointes, and retinopathy. QT prolongation was the most reported AE when treating COVID-19, and retinopathy was the most reported when treating lupus. The FAERS data was analyzed based on three methods: PRR, Chi-square test, and minimal case number filtering; the results of the analysis were then classified using the OAE. Our FAERS study found that HCQ was associated with 63 significant AEs (including 21 cardiovascular AEs) for COVID-19 patients and 120 significant AEs (including 12 cardiovascular AEs) for lupus patients, and different (**Figures 3–5**). These results supported our hypothesis that the disease being treated would

significantly affect the likelihood of certain CQ/HCQ AEs to be manifested and reported. Lastly, we developed an OAE-based ontological model for semantically representing different components involving drug AE generation, and we illustrated our model using an HCQ AE patient example reported in the PubMed literature database. The CQ/HCQ drug AE study provided in this paper illustrates the strengths of our newly proposed methodology.

This methodology does have several limitations that require careful investigation and addressing. First, FAERS does have several known biases, including under-reporting, duplicates, stimulated reporting, and confounding by comorbidities or other drug treatment (FDA, 2005). Our analysis demonstrated some of these limitations, as rheumatoid arthritis, a labeled indication for HCQ, was one of the top AEs for HCQ reported in FAERS. Additionally, by filtering out low FAERS case counts, we may have missed rare AEs. It's also possible that different dosages and exposure levels between indications could account for some of the AEs identified. Reported concentrations of HCQ vary widely, as concentrations may be affected by age, gender, comorbidities, and other confounding factors (Browning, 2014). Drug concentration levels could be evaluated in future iterations of this methodology. Additionally, this methodology does not take into account underlying population differences in event rates between indications, although this could also be evaluated in future iterations of this methodology. For example, both SLE and COVID-19 are known to be associated with cardiac complications (Kreps et al., 2018; Caforio, 2021; Murk et al., 2021), but the underlying event rates were not taken into account in this analysis. Similarly, age, gender, and other individual characteristics may play a role in AEs experienced, which were ontologically modeled (**Figure 6**) but not accounted for in the FAERS data analysis. In the future, we can further explore how the ontology modeling of these different characteristics can be applied for practical data standardization, sharing, and analysis of the FAERS AE data related to these characteristics. Finally, data extraction and compilation were performed manually; future iterations of this methodology will incorporate automatic data extraction. Ontology also supports data to be findable, accessible, interoperable, and reusable (Wilkinson et al., 2016; Wang and He, 2021; Xie et al., 2021). Ontology can be used to support automatic and FAIR data extraction and analysis.

CONCLUSION

To compare AEs between drugs (or indications) used for treating diseases under various conditions, a methodology was developed to apply ontological and statistical methods to analyze data from different sources including USPIs, EMBASE and PubMed literature resources, and FAERS database. As a use case, the AEs of CQ and HCQ following their usages for different diseases were systematically surveyed, represented, and analyzed. Our USPI study found fewer cardiovascular AEs associated with HCQ compared to CQ. Our EMBASE and FAERS data analysis showed that these two drugs have different AE profiles when they were used to treat different diseases including COVID-19 and lupus. An OAE ontology modeling with its usage on a HCQ AE example study further identified the semantic relations among components related to drug AE investigations. This study demonstrated that ontologies such as OAE are helpful and accessible tools to catalogue and identify AEs associated with drugs, allowing the public to further understand the correlation between various factors and drug AEs.

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

JN and MK collected the data, performed data analysis, and generated the results. JB provided result interpretation, comments, and suggestions for improvement as clinical COVID-19 domain expert. RR co-designed the methodology, guided the study as drug safety expert, and supported result interpretation and analysis. YH provided initial project design as COVID-19 researcher and ontology expert, performed ontology modeling and result interpretation, and managed the project. All authors contributed to the manuscript preparation and approved the final manuscript.

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Development and Validation of a Two-Step Predictive Risk Stratification Model for Coronavirus Disease 2019 In-hospital Mortality: A Multicenter Retrospective Cohort Study

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Objectives: An accurate prognostic score to predict mortality for adults with COVID-19 infection is needed to understand who would benefit most from hospitalizations and more intensive support and care. We aimed to develop and validate a two-step score system for patient triage, and to identify patients at a relatively low level of mortality risk using easy-to-collect individual information.

Design: Multicenter retrospective observational cohort study.

Setting: Four health centers from Virginia Commonwealth University, Georgetown University, the University of Florida, and the University of California, Los Angeles.

Patients: Coronavirus Disease 2019-confirmed and hospitalized adult patients.

Measurements and Main Results: We included 1,673 participants from Virginia Commonwealth University (VCU) as the derivation cohort. Risk factors for in-hospital death were identified using a multivariable logistic model with variable selection procedures after repeated missing data imputation. A two-step risk score was developed to identify patients at lower, moderate, and higher mortality risk. The first step selected increasing age, more than one pre-existing comorbidities, heart rate >100 beats/min, respiratory rate ≥30 breaths/min, and SpO_2 <93% into the predictive model. Besides

age and SpO_2, the second step used blood urea nitrogen, absolute neutrophil count, C-reactive protein, platelet count, and neutrophil-to-lymphocyte ratio as predictors. C-statistics reflected very good discrimination with internal validation at VCU (0.83, 95% CI 0.79–0.88) and external validation at the other three health systems (range, 0.79–0.85). A one-step model was also derived for comparison. Overall, the two-step risk score had better performance than the one-step score.

Conclusions: The two-step scoring system used widely available, point-of-care data for triage of COVID-19 patients and is a potentially time- and cost-saving tool in practice.

Keywords: prognostic score, two-step, time-and cost-saving tool, COVID-19, multicenter cohort study

INTRODUCTION

Coronavirus disease 2019 (COVID-19), the infectious disease resulting from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to morbidity and mortality in millions of people (1). A simple, reliable, point-of-care risk score to predict mortality could help clinicians triage patients and appropriately allocate resources. This is particularly important as health systems face shortages of hospital intensive care unit (ICU) beds that can lead to worse clinical outcomes (2).

Various prognosis scores have been proposed to achieve this goal (3–6). Several models have used varying combinations of demographic variables, laboratory tests, or imaging (7–10). Tools that provide accurate, low-cost risk estimates are needed, as estimates requiring extensive testing or imaging increase the burden on healthcare systems already operating at capacity. Prognostic tools based on data combined from different regions or countries (11–13) are problematic, as they ignore heterogeneity between populations that may increase the risk of bias (3). While the extent of this risk across all regions is not well elucidated, it has been demonstrated in one regional comparison by the ISARIC 4C Deterioration model (13).

We developed an easy-to-use, practical clinical prediction rule for mortality in patients with COVID-19, building on a conceptual framework of a two-step triage (14). With the proposed two-step procedure, early identification of lower- and higher- risk groups and accurate patient triage are possible while conserving limited resources. We validated our model on distinct external cohorts across various populations to fully characterize heterogeneity across settings and clinical presentation.

MATERIALS AND METHODS

Derivation Cohort and Validation Cohorts

Four universities with inpatient health centers including Virginia Commonwealth University (VCU), Georgetown University (GU), University of Florida (UFL), and University of California, Los Angeles (UCLA) participated in the study. Data were retrospectively extracted from electronic health records (EHRs) of each health system. The cohort from VCU, with the longest patient enrollment period (from March 2020 to June 2021) among centers, was used as the derivation cohort and the remaining three university health system cohorts were used for validation to assess model performance in heterogeneous populations.

Study Participants and Data Collection

Participants included from each center were hospitalized adults (18 years old and above) with a positive polymerase chain reaction (PCR) test for SARS-CoV-2 and a determined disposition (discharged or deceased) at the time of data extraction. The diagnosis of SARS-CoV-2 infection was based on World Health Organization interim guidance (15). The outcome of interest was in-hospital mortality, documented in each patient's EHR-based hospital disposition.

Data collection of the four cohorts all started in March, 2020. The derivation cohort VCU possessed the latest patient information by June, 2021. GU included data collection from March to August, 2020. Data of UFL was last updated by December, 2020, while the UCLA cohort enrolled patients until May, 2021. Demographic, clinical, and laboratory variables were extracted from the EHRs following the standardized approach to each variable definition (6). Those variables were divided into routinely available and laboratory available categories. Routinely available predictors included age, gender, vital signs, physical examination results such as heights and weight that generate body mass indexes (BMIs), and number of comorbidities. Comorbidities were defined using Clinical Classifications Software categories for diabetes mellitus (CCS 49), cardiovascular disease (CVD, CCS 101), asthma (CCS 128), and chronic obstructive pulmonary disease (COPD, CCS 127) (16), then these comorbidities were combined to create a count variable. Laboratory available predictors were commonly used laboratory test measurements (white blood cell count, neutrophil count, lymphocyte count, creatinine, platelets, blood urea nitrogen, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, C-reactive protein, and troponin-I). Only the first measured predictor variables available within 24 h of admission date/time were included.

Model Development

We developed a two-step risk score using an approach similar to that used by Fine and colleagues to develop the Pneumonia Severity Index (14). The study followed the Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) principles (17). The first

step was designed for rapid identification of lower- and higher-risk groups; the second step was for classification of the remaining patients using additional and more-difficult-to-obtain predictor variables.

Before model development, numerical variables were categorized according to their clinical normal ranges (18, 19). The neutrophil-to-lymphocyte ratio (NLR) was computed using extracted values (20, 21), and its dichotomous cutoff was derived from the max Youden index (22) of a univariable binary logistic model. Only categorical variables were used for model fitting. The multiple imputation (MI) method was applied for missing values of candidate predictor variables. Under the assumption of missing at random, a chained equations approach (23) carried out five imputations. We used Rubin's rules (24) to combine the model parameter estimates across the imputed datasets.

The developed algorithm involves two steps as shown in the flowchart in **Figure 1**. In the first step, only routinely available variables like demographics and vital signs were included as candidate predictors. Step 1 applied the MI-stepwise method (25) with a likelihood-ratio test statistic to select risk factors. We repeated the variable selection procedure 100 times and included those that were selected over 50 times. Then, a multivariate binary logistic model was employed with relaxed inclusion criteria ($P \leq 0.1$) to include more risk factors. After parameter estimation, each beta-coefficient was divided by the smallest one and subsequently rounded to the nearest integer to create a simple point score (18). The risk score was calculated additively. Patients with the lowest observed-cumulative mortality in Step 1 were classified into the lower-risk group, and those with observed-cumulative mortality >30% were classified into the higher-risk group. The corresponding observed mortality of the two groups was then used as the lower- and higher-risk cutoffs in the next step (11, 14). Patients who were not assigned to either the lower- or higher- risk group in the first step then participated in the second step. Both routinely available and laboratory available variables were taken into consideration for the second stage model. Step 2 conducted a similar procedure to develop its risk score as for Step 1, categorizing remaining individuals into lower-, moderate-, and higher-risk groups based on the corresponding observed cumulative mortality.

Model Validation

Complete datasets from each of the four health systems were used to evaluate the performance of the proposed risk score. The cohort from VCU was used for internal validation, while complete cases from the remaining health centers were used separately for external validation. The number of patients in each risk group and the corresponding mortality rate for each risk group were calculated for each health system cohort. Cochran-Armitage tests (26) were used to test for trends in mortality from an increasing number of points and classification categories. We also employed the Gaussian mixture model (GMM) (27) at the second step to assess the rationality of clustering and the consistency of risk group separation with the first step. Overall discrimination ability was assessed by C-statistics (28) with a corresponding 95% confidence interval. Calibration curves (29) and the Hosmer-Lemeshow test (30) were used to evaluate how well the predicted mortality matched the observed mortality. Sensitivity analysis was conducted using complete case data to assess the MAR missing assumption and to evaluate the goodness of MI-stepwise two-step method.

Comparison With Direct Risk Stratification

Traditional mortality predictive scores are often derived from direct logistic models to create single one-step risk scores (3–6). We used all risk factors available and employed the one-step model-fitting method on the derivation cohort ($P \leq 0.05$). After calculation of mortality scores, patients were classified into three groups according to the same observed cumulative mortality cutoffs of the two-step method. Model validation was also conducted on the complete cases for each cohort.

To compare the performance of the two methods, we assessed discrimination and calibration using C-statistics (28) and Brier scores (31), respectively. For those patients whose probability of death could not be evaluated due to missing variables needed for prediction, we also conducted MI-imputation using demographic variables and vital signs for mortality estimation. Decision curve analysis (32) was subsequently employed to compare the clinical utility of the two models at different risk thresholds. Briefly, by assuming a threshold probability for the higher mortality risk, we can derive the net benefit by weighing the benefit of the true-positive and the cost of the false-positive prediction. The net benefit curve obtained from different threshold probabilities reflects the clinical utility of a model. Two extreme strategies in which either all or none of the patients were classified to the higher-risk group served as reference points.

Ethics Approval

The overall study protocol was approved by the Institutional Review Board at the University of Georgia under approval number: PROJECT00002208.

RESULTS

Patient Demographic and Clinical Characteristics

The derivation cohort included 1,673 adults with PCR confirmed COVID-19, with 180 (10.8%) deaths. GU, UFL and UCLA had 558, 1,815 and 1,570 individuals, with 93 (16.7%), 269 (14.8%), 184 (11.7%) deaths, respectively. We summarized continuous variables as medians with interquartile ranges and categorical variables using proportions (**Supplementary Table S1**). The missing proportion of collected variables in the VCU cohort is shown in **Supplementary Table S2**.

Development of Predictive Risk Stratification of Two-Step Methods

In step 1, 63 (3.77%) individuals of the derivation cohort (VCU) had missing information for routinely available variables. The repeated MI-stepwise variable selection procedure identified age above 55, more than one pre-existing comorbidities, heart rate >100 beats/min, respiratory rate >30 breaths/min, and SpO_2 <93% as the most important predictors for mortality (**Suppmentary Tables S3**, **S4**). Individuals who scored zero,

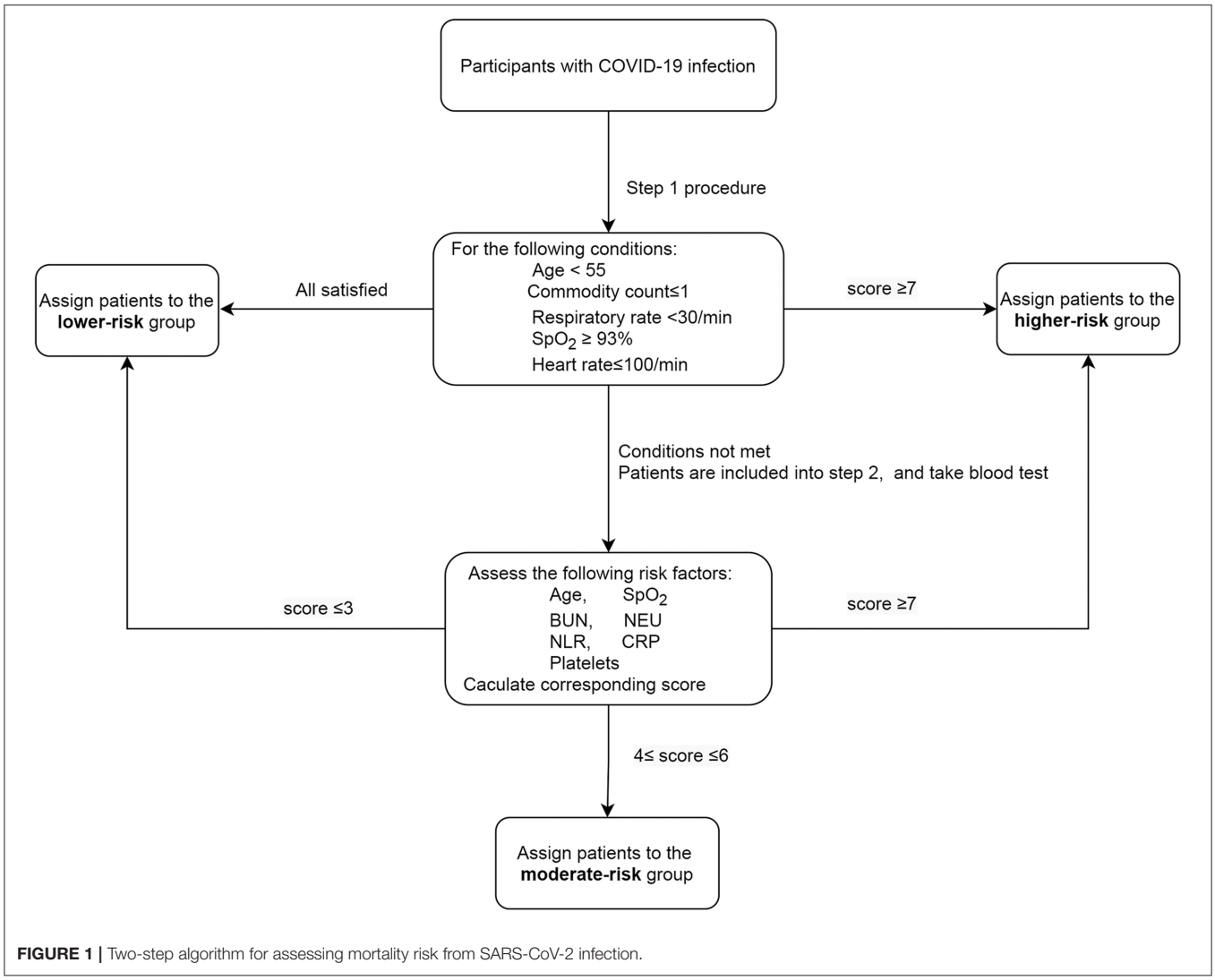

FIGURE 1 | Two-step algorithm for assessing mortality risk from SARS-CoV-2 infection.

without any of these risk factors, were classified into a lower-risk group. While patients with score ≥7 were considered as having relatively high risk of death, admitted into the higher-risk group (**Figure 1**). The corresponding observed cumulative mortality cutoffs was then used as the corresponding thresholds in the second step. In step 2, 1,155 patients from the remaining patients ($n = 1{,}220$) had missing information. Repeated MI-stepwise procedure showed that besides age and SpO_2, laboratory variables including blood urea nitrogen (BUN), neutrophils absolute count, C-reactive protein (CRP), platelets count and NLR also had significant influences on the mortality rate (**Supplementary Tables S5, S6**). The final risk score is shown in **Table 1**.

Independent Validation of Two-Step Rule

Internal Validation

When the derived predictive risk stratification was applied, mortality rates in step 1 were 2.0% and 30.1% in the lower- and higher-risk groups, respectively. Patients assigned to the lower-, moderate-, and higher-risk groups for step 2 had an observed mortality rate of 1.8, 7.6 and 35.5%, consistent with results from step 1. We merged patients of the two steps together to evaluate the overall death rates of each group, and the corresponding mortality rates were 1.9, 7.6 and 33.3% (**Table 2**; **Figure 2**), resulting in good separation among the risk groups. Mortality risk had an increasing trend ($P_{\text{trend}} < 0.001$) among groups. GMM on the score-based predicted probability of the second step indicated significantly different risk profiles between the three groups (**Figure 3B**). The C-statistic was 0.83 (95% CI, 0.79–0.88) with good overall discrimination ability. The calibration curve (**Figure 3C**) suggested that predicted and observed mortality matched well (Hosmer–Lemeshow test, $P = 0.995$).

GU as External Validation

The external validation in the GU cohort showed an overall good stratification. The mortality of the lower-risk group identified in the first step was 3.2%, while the higher-risk group had death count of 13 in total 19 cases (death rate: 68.4%). Risk probabilities

TABLE 1 | The proposed two-step risk score for coronavirus disease 2019 mortality.

Predictors of step 1	Points	Risk group	Points
Age (years)		Lower risk	0
< 55	0	Higher risk	≥ 7
55–64	2	Go to Step 2	1–6
65–74	3		
≥75	5		
Respiratory rate ≥30	2		
SpO_2 < 93%	2		
Commodity count ≥2	1		
Heart rate >100	1		
Maximum	11		
Predictors of step 2	**Points**	**Risk group**	**Points**
Age (years)		Lower risk	≤3
<55	0	Moderate risk	4–6
55–64	1	Higher risk	≥7
65–74	2		
≥75	3		
SpO_2 < 93%	2		
BUN > 20 mg/dl	2		
NLR > 3.7	2		
NEU >6.3	2		
Platelets ≥350	2		
CRP >10	1		
Maximum	14		

TABLE 2 | Validation of the two-step coronavirus disease 2019 risk score in 4 populations #.

Risk group	Internal validation	External validation cohorts			
	VCU	GU	UFL	UCLA	All External Validation
Lower	1.9% (7/362)	2.8% (5/180)	2.4% (12/499)	2.3% (5/220)	2.5% (22/899)
Moderate	7.6% (8/106)	7.2% (7/97)	10.3% (44/428)	9.4% (13/139)	9.6% (64/664)
Higher	33.3% (68/204)	49.5% (45/91)	31.0% (173/559)	33.1% (77/233)	33.4% (295/883)
AUROCC*	0.832	0.854	0.793	0.829	0.825

*AUROCC, Area under the receiver operating characteristic (ROC) curve.
#Numbers in parentheses were listed as deaths/total.

classified by the second step in lower-, moderate- and higher-risk groups were 1.2, 7.2, and 44.4%, respectively. Overall risk stratification demonstrated a similar trend (**Table 2**; **Figure 2**). An increasing trend was suggested by the Cochran-Armitage test ($P_{trend} < 0.001$). GMM curves (**Figure 3B**) also identified the existence of 3 groups of the remaining people. The C-statistic was 0.85 (95% CI, 0.80–0.91). Calibration curve showed a deviation (**Figure 3C**), yet the *P*-value of the Hosmer-Lemeshow test was 0.080.

UFL as External Validation

278 people were identified in the lower-risk group at the first step and 7 of them died (2.5%), while 149 individuals in the higher-risk group with 63 death cases (42.3%). Overall corresponding mortality rates were 2.3, 10.3, and 26.8% of the lower-, moderate-, and higher-risk groups ($P_{trend} < 0.001$) (**Table 2**; **Figure 2**). The GMM curves (**Figure 3B**) also supported three risk clusters among step 2-remaining patients. The validation in the UFL cohort showed slightly less differentiable observed risks among different groups, with a C-statistic at 0.79 (95%CI, 0.76–0.82). Calibration curve displayed satisfactory calibration. Corresponding *P*-value of the Hosmer-Lemeshow test was 0.197.

UCLA as External Validation

Mortality rates derived from the first step of the UCLA validation for the lower- and higher-risk groups were 3.5 and 46.8%. Observed probabilities of the lower-, moderate-, and higher-risk groups in step 2 were 0.9, 9.4, and 21.0%, respectively (**Table 2**; **Figure 2**). Stratification of overall risk was consistent with other cohorts (lower: 2.3%, moderate: 9.4%, higher: 33.1%). The UCLA cohort also presented an increasing trend of risk ($P_{trend} < 0.001$) across risk groups. GMM curve (**Figure 3B**) implied a 3-level risk stratification. C-statistic was 0.83 (95%CI, 0.79–0.87). *P*-value of the Hosmer-Lemeshow test was 0.968.

Sensitivity Analysis Using Complete Case Analysis

Our sensitivity analysis showed that both steps using complete cases selected similar variables to those selected by MI-stepwise procedure (**Supplementary Tables S7**, **S8**). Except for age, scores assigned to each level of selected risk factors remained the same as those assigned using the multiple imputation (MI) based two-step method. Besides, the two-step method using MI had better discrimination (**Supplementary Table S9**) and calibration (**Supplementary Figure S1**) abilities than the approach using only complete cases.

Comparison With the Direct Method

The one-step direct method identified age, SpO_2, blood urea nitrogen and C-reactive protein (CRP), white blood cell count, platelets count, and NLR as predictors. The score in each reference group was assigned to 0. Three older age groups (score: 1, 2, 2), SpO_2 below 93% (score: 1), above normal levels of laboratory variables including BUN (score: 2), CRP (score: 1), platelets count (score: 2), white blood cell count (score: 1), as well as NLR (score: 2) were associated with elevated death risk. A total score was obtained by summing all points each subject received, after which patients were directly classified into lower- (below 2 points), moderate- (3–5 points), and higher-risk (6 and above points) groups. More details are provided in **Supplementary Tables S10–S12**.

The two-step method (TS) had better C-statistics and brier scores than the one-step direct method (OS) (**Table 3**). Net

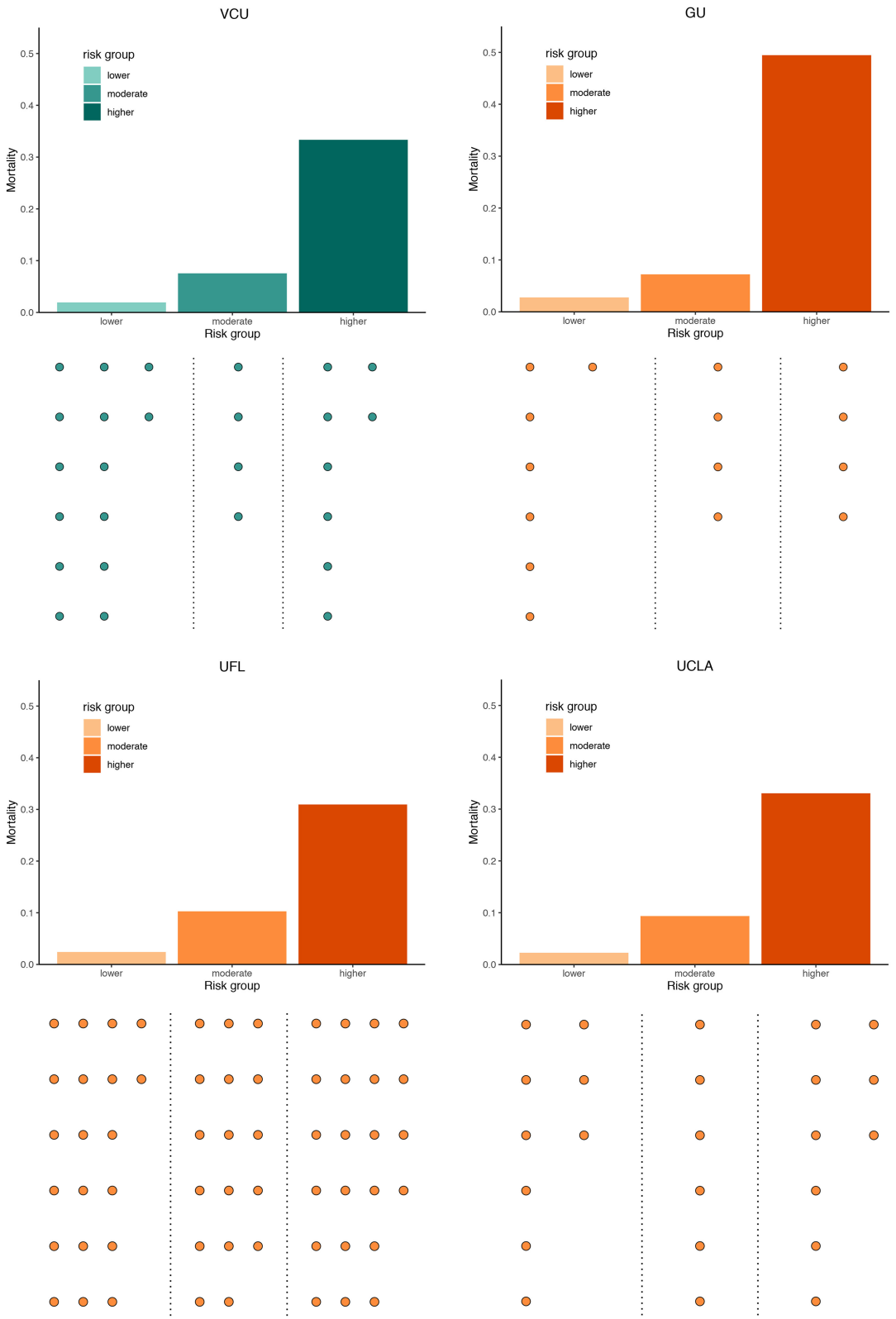

FIGURE 2 | Risk Stratification Among Derivation and Validation Cohorts. Bar plots represented mortality risk. A dot below each main plot represented five people within each corresponding group, and the number of dots suggests the approximate sample size in each group.

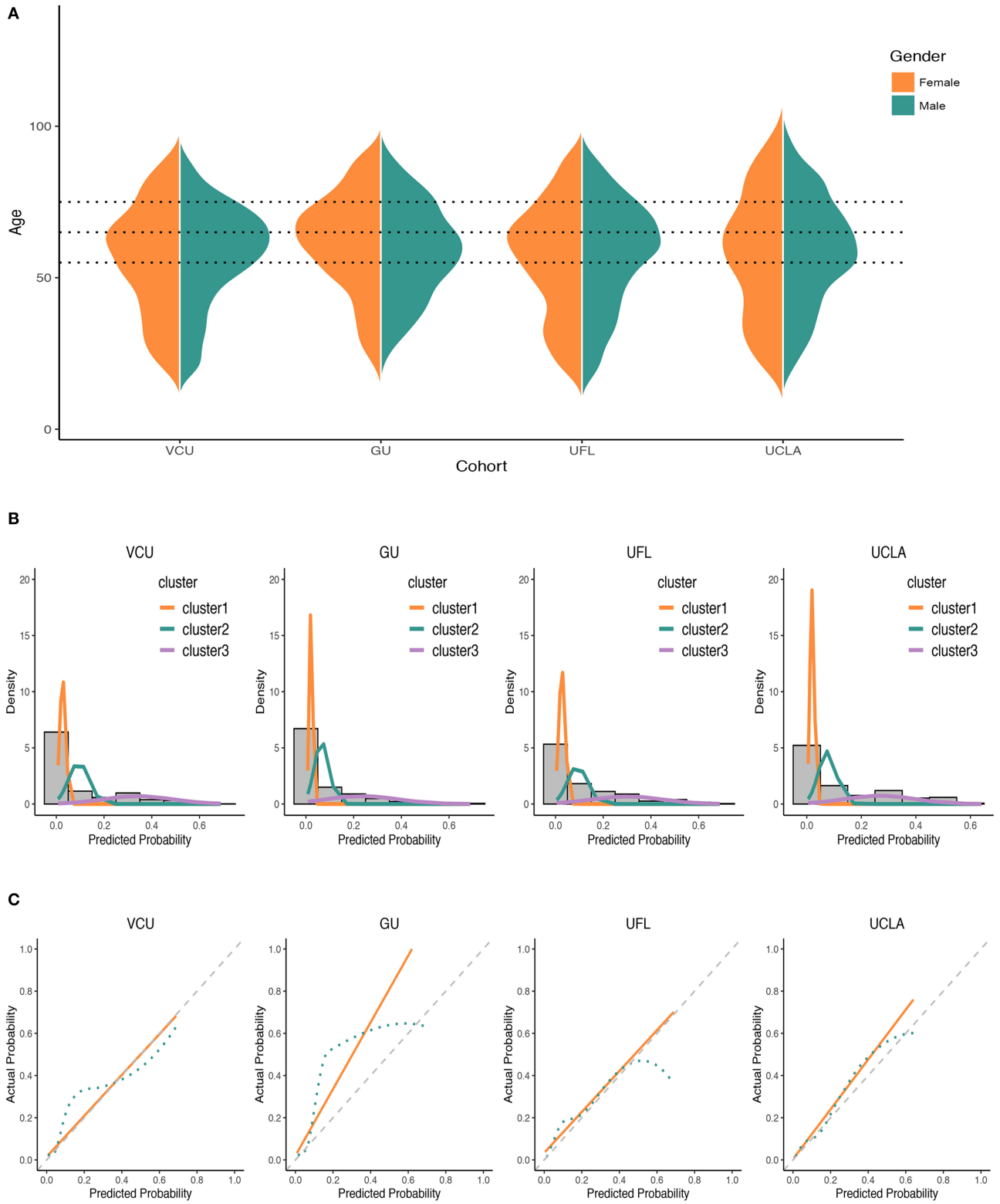

FIGURE 3 | Distribution of demographic variables, discrimination, and calibration ability of the two-step method in the derivation cohort. **(A)** Distributions of demographic variables. **(B)** GMM distributions among different cohorts. **(C)** Calibration curves for two-step method using logistic calibration and locally weighted scatterplot smoothing (lowess). The dot lines in **(A)** were age cutoffs. The first cluster line plot of VCU in **(B)** was truncated for convenient comparison with other cohorts. The actual peak of this line was at around 95.

benefit curves (**Figure 4**) were generated based on thresholds of score-derived probabilities to evaluate clinical utilities. The higher net benefits observed from the two-step method in VCU, GU and UCLA suggested that it benefits more people at the population level in these regions. In the UFL cohort, the two methods resulted in comparable net benefits. Compared with the one-step method, the two-step risk score classified additional 331, 77, 165, and 136 subjects into the lower- or higher-risk groups in VCU, GU, UFL, and UCLA cohorts, respectively. Over half of the individuals triaged in the first step of the two-step method would be uncategorized by the one-step method due to missing lab testing predictors (**Supplementary Table S13**).

TABLE 3 | C-statistics and brier score comparison between the two-step method (TS) and direct one-step (OS) method.

		VCU	GU	UFL	UCLA
C-statistics (95% CI)	TS	0.83 (0.79,0.88)	0.85 (0.80,0.91)	0.79 (0.76,0.82)	0.83 (0.79,0.87)
	OS	0.82 (0.77,0.88)	0.81 (0.74,0.87)	0.79 (0.76,0.82)	0.79 (0.74,0.84)
Brier Score	TS	0.09	0.11	0.11	0.11
	OS	0.12	0.12	0.12	0.11

DISCUSSION

SARS-CoV-2 has resulted in a growing number of deaths and a shortage of medical resources. Improved clinical prediction and decision support tools, feasible for implementation "at the bedside," are urgently needed. Various scoring methods have been proposed (3–6) to achieve this goal with additional testing including laboratory exams, CT imaging, amongst others, leading to increased time and costs for patients and hospitals. We developed a simple, quick, and practical two-step predictive mortality score system for adult COVID-19 patient triage. The first step uses only routinely available characteristics that are easily collected to identify individuals with lower and higher mortality risk. The second step assesses the remaining patients comprehensively using both routinely available and laboratory data. The score system was validated in cohorts from multiple regions in the United States and achieved overall satisfactory prediction. Those validation cohorts were also collected over different time courses. The relatively stable performance adds

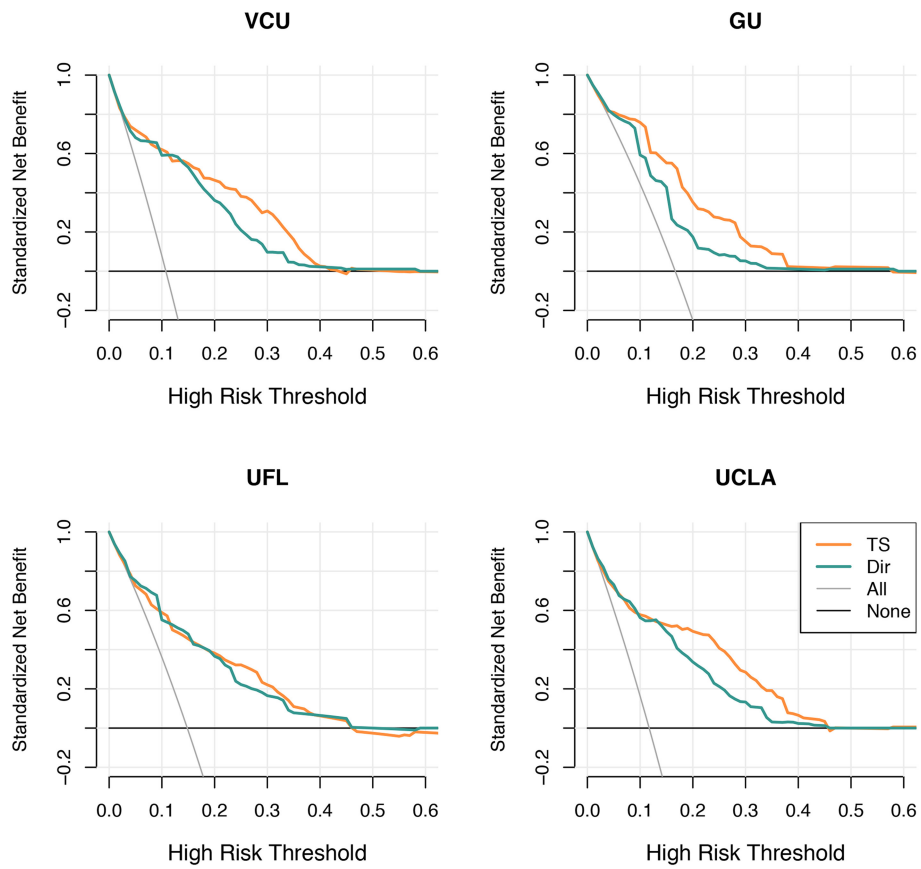

FIGURE 4 | Decision curve analysis plots for a comparison of net benefits at different risk thresholds between the two-step (orange line) method and the direct one-step (green line) method.

strength to the generalizability and future applications of the study findings.

In comparison, the two-step model had better overall discrimination and calibration than the direct one-step method (**Table 3**; **Supplementary Figure S2**). The primary strength of the two-step approach is the time and money saved by appropriately stratifying patients using only easy-to-collect and routinely available variables, e.g., no imaging information needed, and no lab tests needed unless you get to the second step. The first step in the two-step method also ensures a higher coverage of all SARS-CoV-2 infected patients, which eventually would benefit a larger population. Overall, more than half of the individuals identified as lower or higher risk in the first step of the two-step method would otherwise be left uncategorized by the one-step method due to missing laboratory testing predictors. The number of lower- and higher-risk individuals identified by the two-step method in the first step can be regarded as the "benefit" of using this two-step procedure. Rapid, accurate triage may improve timely decision making, particularly for those patients missed by the one-step method.

To assess the performance of the two-step method in heterogeneous populations, we validated the score system using multiple external cohorts. As expected, model performance varied. Across derivation and validation cohorts, UFL performed worse than other cohorts, possibly because of geographic variability and a surprising increase of mortality in that cohort in late 2020. Age and gender differences could have contributed to the observed heterogeneity, as the four cohorts showed disparities in age distributions stratified by gender (**Figure 3A**). Racial diversity and its associated social economic status, underlying health conditions, healthcare access, and care-seeking behavior may also be important factors influencing mortality (33–35). As a surrogate for racial heterogeneity across the cohorts, we obtained state-level racial diversity information for each site (36). The derivation cohort from Virginia had comparable racial distribution with Delaware (where GU is located). By comparison, Florida and California had distinct racial profiles potentially explaining the suboptimal validation performance from the UFL and UCLA cohorts. Overall, the results suggest that the two-step model is suitable for each of these regions, but also identified regional heterogeneity that should be further explored for model refinement. Prospective, regional studies are needed to assess heterogeneity bias more precisely.

There are several limitations to this study. Coronavirus mutations may alter the course of the disease, and the proposed two-step method needs further validation in patients infected with emerging SARS-CoV-2 variants. Variant information was not available in our datasets, though based on the timeframe of our data collection the majority of our enrolled patients were likely infected with the wild-type. Further validation of the proposed approach and possible development of new triage scores on cohorts with new and existing SARS-CoV-2 variants are warranted. Data on vaccination status was also unavailable in our cohorts, which precludes an assessment of the effect of vaccination. Based on our data collection period and the current knowledge that the vaccinated population is at a significantly reduced risk of hospitalization, we consider our study findings mainly apply to the unvaccinated population. In addition, only the first measured predictor variables available within 24 h of admission date/time were included in developing the prediction model. It is unknown at what point in the disease's course a patient was admitted. Early or late enrollment in the cohort could result in false negative or false positive results in the higher-risk group. However, subjects included in our study were hospitalized patients who likely had been infected beyond the incubation period before admitted to the hospitals. They were all sick enough to present symptoms to be initially admitted for inpatient care. As such, our primary purpose is to assist in initial triage when these patients present. We suspect including days from initial symptom onset as a potential predictor in the model may further improve the prediction accuracy and reduce the bias caused by false negative or false positive predictions. Unfortunately, our working datasets did not collect this information.

CONCLUSIONS

The proposed two-step score system for COVID-19-related in-hospital mortality among adults is time and cost-saving and may decrease health care burden in settings with high COVID-19 infection rates.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Medical records data. Requests to access these datasets should be directed to ebell@uga.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Georgia IRB. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YL, YK, and YS contributed to the conception and design of the study. RL, DT, AM, AZ, BB, W-JT, KM, MG, AK, and TG organized databases from each study site. ME, XC, and YK organized the combined database. YL, YK, and YS performed the statistical analysis. YL, YK, ME, LM, and YS wrote the first draft of the manuscript. M-HS, CL, and YJ provided technical supports to the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Pharmaceutical Prospects of Curcuminoids for the Remedy of COVID-19: Truth or Myth

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Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is a positive-strand RNA virus, and has rapidly spread worldwide as a pandemic. The vaccines, repurposed drugs, and specific treatments have led to a surge of novel therapies and guidelines nowadays; however, the epidemic of COVID-19 is not yet fully combated and is still in a vital crisis. In repositioning drugs, natural products are gaining attention because of the large therapeutic window and potent antiviral, immunomodulatory, anti-inflammatory, and antioxidant properties. Of note, the predominant curcumoid extracted from turmeric (*Curcuma longa* L.) including phenolic curcumin influences multiple signaling pathways and has demonstrated to possess anti-inflammatory, antioxidant, antimicrobial, hypoglycemic, wound healing, chemopreventive, chemosensitizing, and radiosensitizing spectrums. In this review, all pieces of current information related to curcumin-used for the treatment and prevention of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection through *in vitro*, *in vivo*, and *in silico* studies, clinical trials, and new formulation designs are retrieved to re-evaluate the applications based on the pharmaceutical efficacy of clinical therapy and to provide deep insights into knowledge and strategy about the curcumin's role as an immune booster, inflammatory modulator, and therapeutic agent against COVID-19. Moreover, this study will also afford a favorable application or approach with evidence based on the drug discovery and development, pharmacology, functional foods, and nutraceuticals for effectively fighting the COVID-19 pandemic.

Keywords: curcumin/curcuminoids, COVID-19, immunomodulation, nutraceuticals, inflammation, antioxidant, chemosensitizing, oxidative stress

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease that has rapidly spread throughout the world, leading to high mortality rates, and has become a epidemic from the end of 2019. Coronavirus disease (COVID-19), caused by the novel coronavirus—severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has surged across the globe, affecting 233 countries or territories, with greater than 337 million confirmed cases and over 5.56 million deaths till January

2022, with the World Health Organization (WHO, 2022) categorizing it as a pandemic (https://covid19.who.int). Infected patients manifest fever, cough, shortness of breath, and lost smell and taste, and critical cases might show acute respiratory infection and multiple organ failure. Probability of these severe indications is further enhanced by age and underlying comorbidities such as diabetes, cardiovascular, or thoracic problems, as well as due to an immunocompromised state (Adhikari et al., 2020). Coronavirus infection, including SARS-CoV, MERS-CoV, and SARS-CoV-2, causes daunting diseases that can be fatal because of lung failure and systemic cytokine storm. The development of coronavirus-evoked pneumonia is associated with excessive inflammatory responses in the lung, releasing extremely high amounts of cytokines known as "cytokine storms," which result in pulmonary edema, atelectasis, and acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). The pathophysiology of COVID-19 involves the activation of three main pathways: inflammatory, coagulation, and bradykinin cascades (Wiersinga et al., 2020).

SARS-CoV-2 is an enveloped virus belonging to the order Nidovirales, composed of a single-strand, non-segmented, and positive sense RNA genome. SARS-CoV-2 causes COVID-19 that is classified as beta-coronavirus, which primarily occurred and was recorded in December 2019 at Wuhan, China (Godeau et al., 2021). As one member of human coronavirus (HCoV) that has the largest RNA virus genome, the single positive-strand RNA genome of SARS-CoV-2 is approximately 30 kd with two untranslated regions (UTR) linked to the 3′-poly-A tail, and 5′-cap structure, which are crucial for gene transcription and RNA replication. The SARS-CoV-2 genomic structure comprises 5′UTR/NSP/S/3a/E/M/6/7a/7b/8/N/10/3′UTR organization. The non-structural protein (nsp) region of SARS-CoV-2 can be coded and translated as the non-structural proteins 1–16 (nsp1-16), the structural protein regions including spike protein (S), envelop protein (E), membrane protein (M), nucleoprotein (N), and accessory protein regions 3a, 6, 7a, 7b, 8, and 10. Two-thirds of the SARS-CoV-2 genome from the 5′-end contains two open reading frames (ORF), ORF-1a and ORF-1b, which are coded and translated into two long polypeptides, polyprotein 1a (PP1a) and polyprotein 1b (PP1b). The posttranslated cleavage of PP1a and PP1b by two virus-encoded proteases to form 16 NSPs: nsp1-16. Other one-third of the viral genome contains the distinct ORFs derived to form several single-guide RNAs (sgRNAs) that are translated into structural proteins and accessory proteins (Ciotti et al., 2019).

The Pathogenesis of SARS-CoV-2 Infection

The pathogenesis of the infection severity of SARS-CoV-2 encompasses several stages, the suppression of host antiviral and innate immune responses to increase the infected host cells (Shi et al., 2020; Oh and Shin, 2021), the viral replication-induced infected cell oxidative stress (Laforge et al., 2020; Saheb Sharif-Askari et al., 2020), and injury of infected cells expressing and secreting large amounts of various chemokines or cytokines, colony-stimulating factors, interferons (IFNs), interleukins (IL-1, IL-6, IL-8, IL-12), and tumor necrosis factor-α (TNF-α), which cause acute inflammation described as the "cytokine storm symptom" (CSS) (Junqueira et al., 2021; Kunnumakkara et al., 2021). The CSS of serious SARS-CoV-2 infection causes acute lung injury, tissue fibrosis, and pneumonia. Subsequently, CSS with hemophagocytic lymphohistiocytosis (HLH)/macrophage activation syndrome (MAS) may cause serious systemic hyper-inflammation, plasma leakage, peripheral tissue fluid accumulation, and hypotension. The serious pulmonary inflammation, edema, and tissue fluid accumulation in the lung such as ARDS, combined ARDS, HLH, and MAS will affect gas exchange that leads to systemic hypoxemia and multi-organ failure with disseminated intravascular coagulation (DIC) that have resulted in extreme morbidity and mortality (Horowitz and Freeman 2020; Kunnumakkara et al., 2021; Saad and Moussa 2021). Deficiency in red blood cells, serum, and alveolar glutathione levels has been published in the medical literature for ARDS, as well as viral and bacterial pneumonias, resulting from the increased levels of free radical/oxidative stress (Horowitz and Freeman 2020). Even the patients can recover from ARDS of SARS-CoV-2-infected pneumonia, the lung tissue remodeling and pulmonary fibrosis will continue to last and subsequently limit the pulmonary functional recovery (Bazdyrev et al., 2021; Giacomelli et al., 2021). Additionally, the pathophysiology of COVID-19 is highly heterogeneous, and the way of SARS-CoV-2 modulates the different systems of the host remains unidentified, despite recent discoveries such as viral nucleocapsid (N) protein can bind host mRNA to impair host stress response (Nabeel-Shah et al., 2022); viral infection-induced host hypoxia status can modulate ACE2 expression (Prieto-Fernández et al., 2021); and the overexpression of viral nsp9 can reduce the host's nucleoporin 62 expression to defective nuclear pore complex formation (Makiyama et al., 2021). Remarkably this deadly virus could affect multiple vital organs and systems (blood, lungs, heart, nervous system, and immune system); however, its exact mechanism of pathophysiology remains obscure. Usually depending on the viral load, infected and sick people commonly manifest fever, cough, shortness of breath, coagulopathy, cardiac abnormalities, fatigue, and death. This complex and multifactorial response of COVID-19 requires a comprehensive therapeutic approach, enabling the integration and refinement of therapeutic responses of a given single compound that has several action potentials. With comprehensive interaction (synergism), biosafety of multi-compounds or multiple treatments need to be taken into consideration and can also provide a promising strategy to cure COVID-19 infection. Currently, several available vaccines and drugs are in the process of evaluation of efficacy and safety, and the determination of dosage in the COVID-19 pandemic. Unfortunately, vaccines are on the market from Pfizer (BNT), Moderna, and AstraZeneca (AZ) with limited supply under an Emergency Use Authorization (EUA) by the WHO. In fact, several vaccines filed phase III clinical trials are still underway from other manufacturers (Srivastava et al., 2021). Intensely, BNT and Moderna vaccines have been currently approved by the FDA (Chilamakuri and Agarwal 2021). Within 2 years, there are several mutated variants of SARS-CoV-2 from the origin

strain, and their wide dispersion led to multiple waves of outbreaks, especially the mutation on the spike gene of new SARS-CoV-2 variants caused the alteration in several amino acid residues and change the structural conformation of spike protein, decreasing the titer of human antibodies and neutralizing antibodies induced by infection or vaccination (Khateeb et al., 2022; Ma et al., 2022; Tay et al., 2022). New antibody-resistant variants of SARS-CoV-2 in vaccine breakthrough infection can be seen even in people who have received two or three vaccinations within 6 months which shows that the vaccines have failed to fully protect the people from variants of SARS-CoV-2 infection such as Omicron (Khateeb et al., 2022; Servellita et al., 2022; Wang et al., 2022). On the other hand, exploring the repurposing of natural compounds and drugs may provide an alternative approach or strategy against COVID-19. Various repurposing phytochemicals are showing a broad range of antiviral activities, and its different modes of action have been identified (Kumar Verma et al., 2021; Kumar et al., 2022). Repurposing drugs such as Arbidol, hydroxychloroquine, chloroquine, lopinavir, favipiravir, remdesivir, hexamethylene amiloride, dexamethasone, tocilizumab, and INF-β that neutralize antibodies exhibit *in vitro* anti-coronaviral properties by inhibiting multiple processes in the virus life cycle. Plant-based antiviral compounds such as baicalin, calanolides, curcumin, oxymatrine, matrine, and resveratrol exhibit different modes of action against a wide range of positive-/negative-sense RNA/DNA virus, and future research needs to be conducted to ascertain their role and use in managing SARS-CoV-2 (Rai et al., 2021). Recently, several nutraceuticals have been proven to have an ability of immune-boosting, antiviral, antioxidant, and anti-inflammatory effects in COVID-19 infection (Adhikari et al., 2021; Isidoro et al., 2022), and this scenario will be addressed in the next section.

Prospective of Natural Compounds on COVID-19 Treatment

Remarkably, using herbal natural compounds is explored as a complementary approach to treating various diseases including COVID-19. Using the active constituents of medicinal plants has long been a well-accepted therapeutic treatment strategy, although understanding their complex pharmacological actions is a major challenge as they provide tremendous chemical varieties and frequently exhibit multi-pharmacological functions (Catanzaro et al., 2018). One report has demonstrated that crude extract or pure compounds isolated from several medicinal plants and/or herbs such as *Artemisia annua*, *Agastache rugosa*, *Astragalus membranaceus*, *Cassia alata*, *Ecklonia cava*, *Gymnema sylvestre*, *Glycyrrhizae uralensis*, *Houttuynia cordata*, *Lindera aggregata*, *Lycoris radiata*, *Mollugo cerviana*, *Polygonum multiflorum*, *Pyrrosia lingua*, *Saposhnikoviae divaricata*, and *Tinospora cordifolia* have shown promising inhibitory effects against coronavirus (Adhikari et al., 2021). Moreover, several phytocompounds including acacetin, amentoflavone, allicin, blancoxanthone, curcumin, daidzein, diosmin, epigallocatechin gallate, emodin, hesperidin, herbacetin, hirsutenone, iguesterin, jubanine G, kaempferol, lycorine, pectolinarin, phloroeckol, silvestrol, tanshinone I, taxifolin, rhoifolin, xanthoangelol E, and zingerol isolated from plants could also be considered as potential drug candidates against COVID-19 (Adhikari et al., 2021). In this article, we draw more attention to the ancient active substance, especially curcumin, to exploit all promising mechanisms of the antiviral property or applications in the treatment of COVID-19.

Literature Search

We reviewed all retracted studies up to Feb 2022 that have evaluated the medicinal benefits of curcumin for COVID-19 infection. The following databases were used: Web of Science, SciVerse, Sci-Hub, Google Scholar, Library Genesis, open access library, Public Library of Science, Scientific Research Publishing, Research Gate, Hindawi, ScienceDirect, Scopus, Medscience, Academia, JAMA, PubMed (NCBI), Springer, Directory of Open Access Journals (DOAJ), Elsevier, Wiley, Taylor and Francis, ProQuest, EBSCO, MEDLINE, and SciELO. Thus, case reports, clinical trials, original research, and review articles were also evaluated. We used free text and Medical Subject Heading (MeSH) terms "curcumin," "COVID-19," "ACE2," "Immunomodulation," "*In silico*," "*In vitro*," "*In vivo*," and "clinical trial." The search was done with no language restrictions. All cited articles related to COVID-19 and curcumin were categorized according to MeSH or keywords; the portion of each presenting item in this article is shown in **Figure 1**.

Data Extraction After Search

Data extraction was performed according to the following inclusion and exclusion criteria for the current study: studies examining the effects of curcumin on COVID-19 infection were included in this study. We also considered publications in English language including only English abstract. The authors independently obtained data from each selected publication including the type of study, study population (location, age, gender, and sample size), curcumin dose and measured outcomes, and study results.

Pharmaceutical Potential of Curcumin

For centuries, curcumin chemically known as (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) or diferuloylmethane, is a yellowish pigment and a phenolic component present in the rhizome of turmeric (*Curcuma longa* Linn) and other Curcuma spp., and has been consumed as a flavoring and therapeutic medicinal natural compound. Turmeric grows primarily in Asian countries, particularly India (Yousefian et al., 2019). Curcumin is a plant of the ginger family Zingiberaceae with the related compounds demethoxycurcumin, bis-demethoxycurcumin, and cyclo-curcumin, which are referred to as curcuminoids (Priyadarsini 2014). In *C. longa*, the crude extract curcuminoid makes up 1–6% of turmeric by weight, distributed in 60–70% curcumin (CUR), 20–27% demethoxycurcumin (DMC), and 10–15% bis-demethoxycurcumin (BDMC) (Nelson et al., 2017), whereas commercially available curcumin contains about 77% in curcuminoids (Esatbeyoglu et al., 2012). Curcuminoids vary in

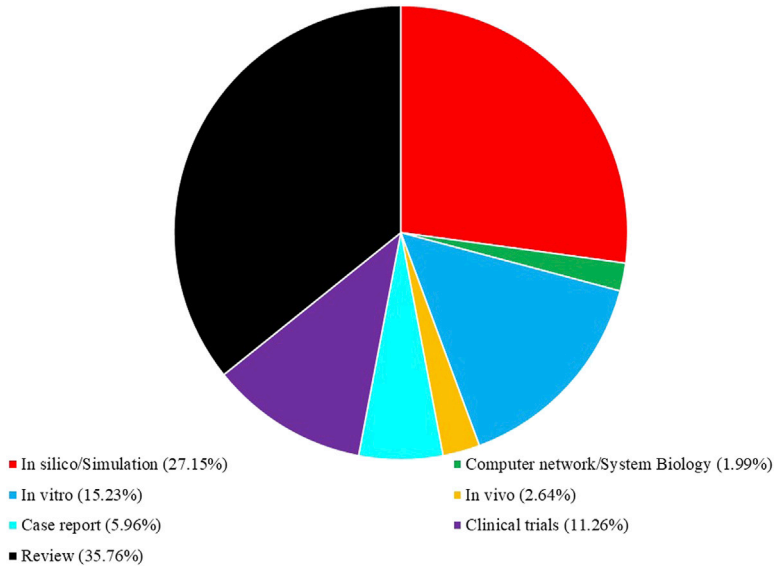

FIGURE 1 | Present proportion (%) of cited literature related to COVID-19 and curcumin according to their categories in this review article.

potency, effectiveness, and stability, that is, the relative potency for suppression of TNF-induced nuclear factor kappa B (NF-κB) activation was Cur > DMC > BDMC (Sandur et al., 2007). An extensive spectrum of pharmacological and physiological actions of curcumin according to an effective and safe substance has been previously documented in the literature. Numerous studies have shown that curcumin has a broad variety of medicinal functions, including anti-inflammatory (Ghandadi and Sahebkar 2017; Gorabi et al., 2021), antiangiogenic (Sahebkar 2010; Hewlings and Kalman 2017), antidiabetic (Panahi et al., 2018; Riyaphan et al., 2018; Huang et al., 2019), antimicrobial (Praditya et al., 2019; Zahedipour et al., 2020), and antitumor properties (Thiyagarajan et al., 2013; Lin et al., 2020a; Mohajeri et al., 2020). It has also been proven that curcumin confers the therapeutic benefits in inflammatory disorders, neoplasms, neurodegenerative disorders, rheumatologic diseases, and cardiovascular diseases (CVDs) (Aggarwal and Harikumar 2009; Velmurugan et al., 2018). Of note, there is scientific evidence of a beneficial impact on human health in functional foods produced by incorporating plant parts that have known or unknown bioactive components like flavonoids, phenolic acid, and alkaloids with various biological properties as aforesaid (Tsuda 2018; Xu et al., 2018; Roy et al., 2022). However, curcumin has some limitations in terms of physicochemical properties such as very low aqueous solubility, high degradation, and limited activity only in acidic pH, which leads to a decrease in bioavailability (Wan et al., 2012; Peng et al., 2018; Dei-Cas and Ghidoni 2019). Importantly, curcumin has been demonstrated to impede inflammation, oxidative stress, cancer cell proliferation, and cell death and abrogate infections caused by bacteria, fungi, and viruses (Omosa et al., 2017; Fu et al., 2021). Additionally, curcumin treatment supports total white blood cell counts and increases the levels of antioxidant indicators, circulating levels of antibodies to sheep red blood cells (SRBC), and plaque-forming cells (PFC) in the spleens of mice (Antony et al., 1999; Shakeri et al., 2017; Afolayan et al., 2018). Furthermore, it also enhances the phagocytic action of macrophages in various animal models including mouse (Wang et al., 2016; Sohn et al., 2021), ferret (Huang et al., 2021) and hamster (Lee et al., 2022; Seldeslachts et al., 2022). As mentioned before, curcumin is a polyphenolic compound, and the activity associated with its polyphenolic chemical structure varies depending on its concentration as well as the cell types and their status (sometimes it can act as a reactive oxygen species (ROS) scavenger, sometimes it can increase ROS production and cause apoptosis, etc.) (Balasubramanyam et al., 2003; Kim et al., 2016; Lin S.-R. et al., 2017; Larasati et al., 2018). From a molecular point of view, curcumin may mediate its pharmacological activities through Janus kinase/signal transducers and activators of transcription (JAK-STAT) (Rajasingh et al., 2006; Salehi et al., 2019), NF-κB (Marquardt et al., 2015; Mortezaee et al., 2019), protein kinase B (AKT or PKB) (Wang et al., 2019), transforming growth factor β (TGF-β) (Li et al., 2013; Thacker and Karunagaran 2015), and mammalian target of rapamycin (mTOR) (Lin J. et al., 2017; Thiyagarajan et al., 2018). In particular, the transcription factors nuclear factor E2-related factor 2 (Nrf2) and NF-κB can regulate 1) the inhibition of the transcription factor NF-κB mediates anti-inflammation, and 2) the induction of Nrf2 signaling pathways promotes antioxidant defense mechanisms and production of phase II enzymes (Dai et al., 2018; Esatbeyoglu et al., 2012). Anticarcinogenic effects of curcumin are also related to an increase in the p53 levels and thus in pro-apoptotic Bax and cytochrome C. The suppression of proliferation and a cell cycle arrest can also be modulated by curcumin through a p53-independent pathway such as the inhibition of NF-κB inhibitor α (IκBα), B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xl), cyclin D1, and IL-6. Moreover,

apoptosis can be initiated by curcumin by the increased cleavage of poly (ADP-ribose) polymerase (PARP) (Xu and Liu 2017; Esatbeyoglu et al., 2012). *In vitro* and *in vivo* studies of influenza A virus infection have shown curcumin treatment could suppress influenza A virus absorption and replication by activation of the Nrf2 pathway to suppress the NF-κB pathway and inflammatory cytokines to attenuate the oxidative stress and symptoms (Dai et al., 2018).

Challenges of Curcumin on COVID-19 Treatment

Several lines of evidence suggest curcumin as a talented prophylactic and therapeutic candidate for COVID-19 in the clinic and public health settings. First, curcumin exerts antiviral activity against many types of enveloped viruses, including SARS-CoV-2, through multiple mechanisms: direct interaction with viral membrane proteins, disruption of the viral envelope; inhibition of viral proteases, and induction of host antiviral responses. Second, curcumin protects from lethal pneumonia and ARDS *via* targeting NF-κB, inflammasomes, IL-6 trans-signal, and high-mobility group box 1 (HMGB1) pathways. Third, curcumin is basically recognized as safe and well-tolerated in both healthy and diseased human subjects (Miryan et al., 2021; Thimmulappa et al., 2021).

According to the aforementioned understanding about the COVID-19 pandemic and pharmacological merits of curcumin, the therapeutic use in SARS-CoV-2 infection remains to be explored. In this context, curcumin/curcuminoids have been shown to possess beneficial effects on the progression of inflammatory diseases including COVID-19 based on numerous action mechanisms including antiviral, anti-inflammatory, anticoagulant, antiplatelet, and cytoprotective (Babaei et al., 2020; Rattis et al., 2021). These investigations and many other effects of curcumin make it a promising agent in the adjuvant treatment of COVID-19; however, all current entities related to curcumin used for proposed treatment and prevention of COVID-19 infection are mostly based on *in vitro*, *in vivo*, *and in silico* studies, clinical trials, and new formulation designs. In fact, there is no direct use in the SARS-CoV-2-infected patients, so it is necessary to re-evaluate the efficacy of curcumin and provide deep insights into knowledge and strategy about curcumin's role as an immune booster, inflammatory modulator, and therapeutic agent against COVID-19.

Curcumin as the Anti-Inflammatory Drug Candidate and Immune Modulator

There is a great variation in the clinical symptoms of SARS-CoV-2 infection from asymptomatic infection, upper respiratory tract infection, pneumonia, ARDS to multi-organ dysfunction. Although the patients recover from ARDSs of serious COVID-19 pneumonia, their lung parenchyma tissue remodeling and pulmonary fibrosis will continue to suffer and limit the pulmonary functional recovery of the patients, indicating that anti-inflammation will be one of the most important focuses on the treatment of SARS-CoV-2 infection. As an inflammatory response, NAcht leucine-rich repeat protein 3 (NLRP3) inflammasomes induce the production of several cytokines, which have been confirmed to play major roles in the pathogenesis of several viral diseases, including COVID-19 (Freeman and Swartz 2020; Zhao M. et al., 2021; Junqueira et al., 2021; Pan et al., 2021). The key molecular mediators of inflammation include pro-inflammatory cytokines such as TNF-α; chemokines; inflammatory enzymes such as cyclooxygenase-1, and -2 (COX-1, COX-2), matrix metalloproteinase-9 (MMP-9), and 5-lipoxygenase (5-LOX); transcription factors such as signal transducer and activator of transcription 3 (STAT3) and NF-κB; and ILs, for example, IL-1, IL-6, and IL-8 (Ghasemi et al., 2019; Norooznezhad et al., 2020), and notably the inhibition on those proteins or pathways can be considered as the major therapy targets for anti-inflammatory treatments of COVID-19. On the other hand, the cellular oxidative stress could be attenuated by the Nrf2 and heme oxygenase-1 (HO-1) pathway, which helps in decreasing and maintaining the redox balance in cells thereby reducing inflammation (Yang et al., 2009; Ahmed et al., 2017; Zhang et al., 2021); the activation and increase in Nrf2 expression can suppress and downregulate NLRP3 inflammasomes (Hennig et al., 2018; Yarmohammadi et al., 2021); and the activation of Nrf2 relative pathways can be a strategy to attenuate the infective symptoms of COVID-19. Experimentally, *in vitro* curcumin treatment as an Nrf2 activator can stimulate the Nrf2 signaling pathway to deter the NF-κB pathway and suppress viral replication in the influenza A virus infection with an IC_{50} of 140.67 μg/ml. *In vivo*, rodents treated with curcumin (50 mg/kg) significantly promoted Nrf2 expression to scavenge ROS that protects cells against oxidative stress and lung injury (Dai et al., 2018; Lin and Yao, 2020). In *in vivo* experiments, gavage with curcumin (40–50 μg/ml) inhibited the activation of NLRP3 by triggering the SIRT1/Nrf2 pathway to abrogate the downstream cytokine expression, such as IL-1β, IL-6, IL-18, and TNF-α (Yin et al., 2018; Yin et al., 2020). For virus-induced inflammation, curcumin treatment can revoke pro-inflammatory cytokine production, such as IL-1β, IL-6, and TNF-α via the inhibition on the NF-κB signaling pathway to hinder NLRP3 expression (Sordillo and Helson 2015; Xu and Lin 2017; Saeedi-Boroujeni et al., 2021). Based on the anti-inflammatory dosage of curcumin in rodent experiments and the Guidance for Industry of the FDA (https://www.fda.gov/media/72309/download), we found that the effective dose for human adults (70 kg) is about 450–550 mg/day using the surface area-to-body weight relative relationship.

The anti-inflammation of curcumin suppresses the expressions of inflammatory mediators not only by triggering the SIRT1/Nrf2 pathway but also activating PPARγ, IL-10, and AMPK pathways. Through the activation of IL-10, AMPK, and PPARγ pathways, curcumin can downregulate NF-κB, COX-2, inducible nitric oxide synthase (iNOS) expression, and prostaglandin E2 (PGE2) levels (Zhai et al., 2015; Kumar et al., 2017), and through the activation of the PPARγ pathway, curcumin can obstruct NF-κB, STAT, activator protein 1 (AP-1), and mitogen-activated protein kinases (MAPK) (Carvalho et al., 2021). The inhibition of NF-κB can decrease the expression of several genes, including COX-2, MMP-9, IL-8, iNOS, and TNF-α (Bengmark 2006). Through the

activation of the AMPK pathway, curcumin can hinder mTOR, p38, p53, and COX-2 pathway (Thiyagarajan et al., 2018; Soltani et al., 2019), and through IL-10, curcumin can also increase the HO-1 expression and modulate the immune response (Naito et al., 2014). Based on the aforementioned studies, indirect anti-inflammatory investigations including signaling pathways are affected by curcumin, but very few results are directly pertaining to the SARS-CoV-2 treatment, and interestingly, there is still promising beneficial potential for treatment and prevention of COVID-19 in the future.

In Vitro and *in Vivo* Validations of Curcumin Use in SARS-CoV-2 Infection

According to the quick spread and high mortality of COVID-19, several clinical trials of the antiviral drugs or formulations applied for COVID-19 treatment were conducted or recruited without passing the *in vitro* and *in vivo* tests. Only very few *in vitro*-tested models of curcumin for COVID-19 treatment were investigated using Vero E6 cells or human Calu-3 cells that were infected with SARS-CoV-2 (Bormann et al., 2021). The data revealed that *in vitro* models treated with curcumin, nano-curcumin formulation, or turmeric root extracts could neutralize and decrease the SARS-CoV-2 viral RNA level at low subtoxic concentrations (Guijarro-Real et al., 2021; Pourhajibagher et al., 2021). Another biochemical and enzyme activity tests showed that the turmeric root extract can significantly constrain the main protease (M^{pro}) activity of SARS-CoV-2 (Dound et al., 2021). Within the past 3 months, the results of a new *in vitro* study by Bahun et al. (2022) showed the IC_{50} of curcumin on the SARS-CoV-2 protease $3CL^{pro}$ is 11.9 μM, but in another *in vitro* study by Marín-Palma et al. (2021), results illustrated that curcumin has the EC_{50} from 1.14 μg/ml to 6.03 μg/ml in the antiviral effects of several different SARS-CoV-2 variants, whereas the data revealed curcumin has a cytotoxic effects with the CC_{50} about 16.5 μg/ml, which is equal to 6.1 μM. The results of the two *in vitro* studies demonstrate that curcumin has the potential to inhibit the SARS-CoV-2 infection, but its effective and cytotoxicity doses are very close and have some overlapping that needs additional studies to evaluate its practical safe dose and treatment mode of its application for COVID-19 treatment. Due to urgent demands for curing or preventing COVID-19, there were several clinical trials based on the application of curcumin on COVID-19 treatment that are either under recruitment or completed, but only few non-human *in vivo* animal studies that are simulated to apply a curcumin formulation on COVID-19 therapy has been published. One study used human beta-coronavirus to mimic the SARS-CoV-2 infection (Dound and Sehgal 2021), and the result showed the application of a curcumin-based herbal formulation could significantly increase $CD4^+$ and $CD8^+$ cell count in blood and plasma IgG and IgM levels in virus-infected animals. Mounting evidence obtained from preclinical studies using animal models of lethal pneumonia shows curcumin exerts the protective effects by regulating the expression of both pro- and anti-inflammatory factors such as IL-6, IL-8, IL-10, and COX-2, promoting the apoptosis of polymorphonuclear leukocytes (PMNs) and scavenging ROS which exacerbates the inflammatory response (Liu and Ying, 2020).

In Silico Study of Curcumin on Viral Proteins

There are several major viral site targets for COVID-19 therapy or prevention simulated by *in silico* studies. The molecular docking results revealed that curcumin has multiple targets with potential to bind with five viral proteins of SARS-CoV-2, and those candidate proteins contain S protein, main protease (M^{pro}), RNA-dependent RNA polymerase (RdRp, nsp12), nucleic acid-binding protein (nsp9), and RNA uridylate-specific endoribonuclease (nsp15). *In silico* studies showed curcumin can bind and act on the host proteins that participate SARS-CoV-2 infection, such as angiotensin-converting enzyme 2 (ACE2), and several proteins applied for intracellular signal transduction pathways and modulate cytokines secretion (Noor et al., 2021).

There are two domains, S1 and S2, which consist the spike protein of SARS-CoV-2 (Walls et al., 2020), and 319–541 aa of S1 is known as the receptor-binding domain (RBD) (Trigueiro-Louro et al., 2020). Within the RBD of S1, 437–508 aa are known as an ACE2 receptor-binding motif (RBM) (Rath and Kumar 2020). The COVID-19 virus entry into host cells is initiated by binding of S protein to the host cell surface ACE2 as its target receptor (Yan et al., 2020), and the RBD is the virus–host binding spots (Veeramachaneni et al., 2021). *In silico* simulation data exhibited that curcumin has a high binding affinity to the RBD and ACE2 (Babaeekhou et al., 2021; Nag et al., 2021; Umashankar et al., 2021). The binding of amino acid residues to curcumin presented near the RBM of S1 protein, and some curcuminoids have stable interactions with key spot residues for the binding of ACE2 comprising the glycosylation site (Babaeekhou et al., 2021), suggesting the potential efficacy of curcumin/curcuminoids in hindering the formation of S protein–ACE2 complex (Jena et al., 2021). A recent genome sequencing study also indicated that the spread of double mutations at E484Q/L452R, T478K/L452R, and F490S/L452Q of RBD has the latent potential for the enhancement of viral mutated S protein (S^m) and host ACE2 to form S^m–ACE2 binding (Aggarwal et al., 2021), and concurrently, that may cause more and effective infection of SARS-CoV-2 mutants. Interestingly, the molecular docking results showed that curcumin and piperine have been demonstrated not only high binding potential with native S protein but also similar or more stability of binding potential with S^m or/and S^m–ACE2 complex (Nag et al., 2021).

The nsp12 is the RNA-dependent RNA polymerase (RdRp) of SARS-CoV-2, and its binding with nsp7 and nsp8 to form the nsp12–nsp7–nsp8 complex is the central component for viral replication and transcription (Gao et al., 2020; Peng et al., 2020). The inhibition of RdRp could abrogate SARS-CoV-2 replication; thus, this nsp12–nsp7–nsp8 complex is recognized as a potential target for COVID-19 treatment (Khan et al., 2020; Begum et al., 2021; Ruan et al., 2021). Compared with antiviral drugs (favipiravir and remdesivir), the results of molecular docking and molecular dynamic simulation showed curcumin/curcuminoids have a good binding affinity and stability with RdRp–RNA complex of SARS-CoV-2 (Kumar Verma et al., 2021;

Singh et al., 2021) and also showed they have higher potential to be developed as viral replication inhibitors of COVID-19.

The genome of the COVID-19 virus is encoded in two protease enzymes, main protease ($3CL^{PRO}$ or M^{pro}-nsp12) and papain-like protease (PL^{pro}, nsp3), which are involved in the proteolytic processing viral polyproteins into functional proteins for viral replication and genomic expression within the host cells (Das et al., 2021). The proteolytic cleavage by viral proteases at the posttranslational stage plays the crucial role in the life cycle of SARS-CoV-2. M^{pro} as a main protease can catalyze more than 11 proteolytic cleavage sites for nsp generation and without human homologs (Dai et al., 2020); therefore, M^{pro} is considered to be an ideal antiviral target for COVID-19 treatment (Li and Kang 2020). Results of *in silico* studies show that curcumin/curcuminoids can form strong bonds with the active site of SARS-CoV-2 M^{pro} (Ibrahim et al., 2020; Bahun et al., 2022). Due to high binding affinity and its binding with the interface region of M^{pro} may cause the protein conformational changes, indicating that curcumin/curcuminoids could be the potential ligands for COVID-19 therapy (Ibrahim et al., 2020; Li and Kang 2020; Kumar M. et al., 2021; Mahmud et al., 2021a; Babaeekhou et al., 2021; Teli et al., 2021; Halder et al., 2022). One more study demonstrated that demethoxycurcumin and bisdemethoxycurcumin had an optimum binding affinity with COVID-19 M^{pro} by molecular modeling and showed the stable state by molecular dynamic (MD) simulation assay, suggesting these could be one of the potential ligands for COVID-19 therapy (Mulu et al., 2021). Unfortunately, there are only two reports with *in vitro* validation of the inhibitory and selectivity effects of curcumin on SARS-CoV-2 infection to confirm its powerful potential on COVID-19 therapy (Kandeil et al., 2021; Bahun et al., 2022).

The nsp9 is single-strand RNA-binding protein of SARS-CoV (nsp9–SARS) which was found to be essential for viral replication (Frieman et al., 2012). nsp9 of SARS-CoV-2 can bind to the nidovirus RdRp-associated nucleotidyltransferase (NiRAN) domain relating the viral replication and transcription (Kumar M. et al., 2021), and it was also considered to be the therapy target for COVID-19 treatment (Zhang et al., 2020; Kumar et al., 2021b). There were several studies to screen the potential candidate compounds to bind or act on nsp9 by *in silico* tools (Barros et al., 2020; Chandra et al., 2021; Junior et al., 2021), and one *in vitro* cellular assay showed the inhibition on nsp9 can reduce the viral replication of SARS-CoV-2 (Littler et al., 2021). The molecular docking results showed that curcumin can bind to the ligand binding site of nsp9, and curcumin can form about 11 interaction sites with nsp9 (Kumar et al., 2021b).

The nsp15 of SARS-CoV-2 is a RNA uridylate-specific endoribonuclease (NendoU) and a conserved protein in coronavirus (Bai et al., 2021). nsp15 can degrade negative-strand viral RNA to protect virus from the host immune responses (Hackbart et al., 2020; Zhao et al., 2020; Gao et al., 2021), and the inhibition of nsp15 can also promote viral elimination by the host immune system (Kumar et al., 2021c). There were several *in silico*-based studies on nsp15 to find potential antiviral compounds or candidates (Mahmud et al., 2021b; Canal et al., 2021; Quimque et al., 2021; Zrieq et al., 2021), and only few studies have conducted further validation of the antiviral effects by *in vitro* or *in vivo* study (Canal et al., 2021; Kumar et al., 2021c). The molecular docking result showed curcumin can bind to nsp15, which might have the potential to inhibit SARS-CoV-2 replication (Kumar et al., 2021d).

Gaining deep insights into all those viral protein *in silico* studies of SARS-CoV-2, we infer that curcumin can bind and interact with several target viral proteins that assist in viral attachment (S protein), replication (nsp12, nsp9), posttranslational protein cleavage/modification (M^{pro}), and host immunity evasion (nsp15), suggesting that curcumin/curminoids provide a promising hit against COVID-19 even without any additional demonstration (*in vitro* and *in vivo* validation). To meet the urgent demand for managing the COVID-19 pandemic, curcumin use will be the first priority with high biosafety because the toxicity of curcumin is very low even in high amounts up to 12–18 g/day (Fu et al., 2021).

In Silico Study of Curcumin on Host Proteins Associated With COVID-19 Infection

As SARS-CoV-2 is a single-strand RNA virus, its mutation may quickly and easily evade the host immune system or targeting drugs; therefore, targeting on the inhibition of host proteins that participate in the viral infections will have the stable therapeutic advantages for COVID-19 treatment or prevention. The viral infection of SARS-CoV-2 is initiated by its S protein binding to ACE2 of the host cellular surface (Alcocer-Díaz-Barreiro et al., 2020; Jahanafrooz et al., 2022). Several *in silico* studies showed curcumin can bind to the S protein and ACE2 to restrict viral entry (Maurya et al., 2020; Nag et al., 2021). The molecular docking results showed that curcumin not only exhibits high interaction with ACE2 but also has the most potent binding with S^m and S^m–ACE2 complex (Nag et al., 2021), indicating that curcumin can be applied in treatments for different SARS-CoV-2 mutated strand infections. The human serine protease serine 2 (TMPRSS2) is a transmembrane protease of host cells; it cleaves and activates the S protein of SARS-CoV-2 to bind with ACE2 on the initiated stage of the viral infection (Singh et al., 2020) and reveals that TMPRSS2 is a suitable target for COVID-19 therapy. TMPRSS2 genes may be co-expressed with SARS-CoV-2 cell receptor genes ACE2 and basigin (BSG), but only TMPRSS2 is demonstrated to have tissue-specific expression in alveolar cells (Brooke and Prischi 2020). Acetaminophen (paracetamol) and curcumin can downregulate the expression of TMPRSS2 in human cells (Zarubin et al., 2020). In addition to the major mode of viral infection by S protein binding to ACE2, SARS-CoV-2 can infect host cells by endocytosis and follow the proteolytic activation by cathepsin L (Jackson et al., 2021; Takeda 2021). Based on the MD simulations, curcumin was found to be the inhibitor of TMPRSS2 (Umadevi et al., 2020; Jackson et al., 2021), and *in vitro* results showed curcumin treatment can also impede the activity of cathepsin L (Goc et al., 2021; Oso et al., 2022). Another key host cell membrane protein in SARS-CoV-2 infection is the glucose-regulating protein 78 (GRP78) receptor. GRP78, also termed as a HSP5A or binding immunoglobulin protein (Bip), is one member of the

TABLE 1 | *In silico* studies of curcumin/curcuminoids on the viral proteins and host proteins with SARS-CoV-2 infection.

	Protein	Description/functions	*In silico* binding sites	References
Viral proteins	nsp 12	RNA-dependent RNA polymerase, virus RNA replication	Curcumin: Lys545, Arg553, Ser759, Ser682, Arg555, Ala688, Val557dDiacetylcurcumin: Thr680, Asn691, Thr687, Lys545, Arg555, Asp623, Val557, Asp761	PDB ID: 7BV2 (Singh et al., 2021)
			Curcumin: Asn691, Asp623, Arg624	PDB ID: 6M71 (Kumar et al., 2021d)
	S protein	Receptor-binding domain (RBD) of spike protein	O-Demethyl demethoxycurcumin: Cys336, Asp364, Leu335, Phe338, Asp364, Val367, Leu368, Phe374, Phe374, Trp436	PDB ID: 6VSB (Umashankar et al., 2021)
			Curcumin: Arg328, Pro527, Lys529, Asn542, Ser98, Asn121, Arg190, Ser730, eu861, Asp867, Phe970, Asp994, The998	PDB ID: 6VSB (Babaeekhou et al., 2021)
			Curcumin: Leu452, Glu484, Phe490, Ser494, Tyr495 for S; Arg403, Try449, Leu452, Phe453, Lys484, Asp494 for Sm	PDB ID: 6M0J (Nag et al., 2021)
			Curcumin: Leu546, Gly548, Phe541, Asn856, Leu997, Ser967, Asp571, Ala570, Val976, Thr572, Asp979, Thr547, Arg1000, Ser975, Thr573, Asn978, Cys743, Thr573, Asn978, Cys743, Leu966	PDB ID: 6VSB (Jena et al., 2021)
			Curcumin: Tyr505, Ala387, Asp38, Gln493, Glu 35, His34, Glu 37, Arg393	PDB ID: 6VW1 (Kumar Verma et al., 2021)
	Mpro	Main protease, 3-chymotrypsin-like cysteine protease (3CLpro), there are 11 proteolytic cleavage sites of Mpro on the posttranslation of viral gene expressions	Curcumin: His41, Leu141, Asn142, Glu166, Gln189	PDB ID: 6M03 (Babaeekhou et al., 2021)
			Curcumin: Thr190, Pro168, Met165, Glu166, Cys145	PDB ID: 6LU7 (Kumar Verma et al., 2021)
			Curcumin: His163, Cys145, Gly143, Ser144, Leu141	PDB ID: 6LU7 (Ibrahim et al., 2020)
			Curcumin: Asn142, Gln192; demethoxycurcumin: Leu272, Thr199, Lys137; and bisdemethoxycurcumin: Phe294, Gln110, Glu240	PDB ID: 7BUY (Mulu et al., 2021)
			Curcumin: Gly143, Gln189, Thr190, Pro168, Leu141, Glu166, Cys145, Met165, Pro168	PDB ID: 6LU7 (Mahmud et al., 2021a)
			Curcumin: Thr26, Gly143, Cys145	PDB ID: 6LU7 (Teli et al., 2021)
			Curcumin: Gly143, Ser144	PDB ID: 6LU7 (Halder et al., 2022)
			Curcumin: Thr26, His41, Gln89	PDB ID: 6LU7 (Kandeil et al., 2021)
			Curcumin: Met49, Met165, Glu166, Arg188, Gln189, Gln192	PDB ID: 6M2N (Bahun et al., 2022)
			Curcumin: Leu141, Gly143, Ser144, Cys145, His163, Met165, Thr190	PDB ID: 6M2N (Adhikari et al., 2022)
			Curcumin: Thr190, Pro168, Met165, Glu166 and Cys145	PDB ID: 6LU7 (Umadevi et al., 2020)
	nsp 9	Nucleic acid-binding protein	Curcumin: Met16, Gly41, Gly42, Arg43, Val45, Phe60, Pro61, Lys62, Ser63, Ile69, Thr71	PDB ID: 6W4B (Kumar et al., 2021a)
	nsp 15	RNA uridylate-specific endoribonuclease (NendoU) activity, degrades viral RNA	n.a.	PDB ID: 6VWW (Kumar et al., 2021d)
Host proteins	ACE2	Angiotensin-converting enzyme-2, serve as viral spine protein receptor	Curcumin: Leu591, Lys94, Asn210, Glu564, Glu280, Tyr207, Asp206, Gly205, Tyr196, Ala99, Lys562, Ala396, Gln102, Trp566, Gln98, Val209, Pro565, Val212, Leu95	PDB ID:LR42 (Jena et al., 2021)
			Curcumin: Ser44, Ala46, Ser47, Gly66, Trp69, Ser70, Lys74, Ser77, Glu110, Met62, Leu73	PDB ID: LR42 (Kumar Verma et al., 2021)
			Curcumin: Ala348, His378, Asn394, Tyr385, His401, Glu402	PDB ID: 1R42 (Maurya et al., 2020)
			Curcumin: Ans210, Lys94, Leu91, Ala396, Lys562, Ala99, Try196, Gly205, Try207, Glu208, Glu564, Asp206, Gln102, Trp566, Gln98, Val209, Pro565, Val212, Leu95	PDB ID: n.a. (Kumar et al., 2021f)
	GRP78	Glucose-regulating protein 78 (GRP78) receptor, ER molecular chaperone, and cell surface GRP78 help viral infection	Curcumin: Thr39, Ile61, Glu201, Asp224, Phe258, Glu228	PDB ID: 5E84 (Allam et al., 2020)
			Curcumin: Ile426, Thr428, Thr434, Phe451	PDB ID: 5E84 (Sudeep et al., 2020)
	Cathepsin B/K/L	Host cysteine protease serves as viral spine protein activator	Curcumin: Gly143, Ser144 Cys145, His172	PDB ID: 3KW9 (Oso et al., 2022)
	TMPRSS2	Transmembrane serine protease 2, cleaving and activating S protein of SARS-CoV-2	Curcumin: Gly148, Asp147, Ser195	PDB ID: 2OQ5 (Umadevi et al., 2020)

n.a. non-available.

TABLE 2 | Clinical trial of curcumin on COVID-19 treatment of PubMed and ClinicalTrial.gov.

Formulation/design	Regimen	Administration	Patients (n)	Masking	Age (y)	Placebo (n)	Country	Authors
Nano-micellar gel	40 mg, four times/day	Oral	80	Double	19–69	40	Iran	Valizadeh et al. (2020)
Nano-micellar gel	40 mg, four times/day	Oral	40	Double	18–75	20	Iran	Hassaniazad et al., 2020*
Nano-micellar gel	80 mg, twice/day	Oral	60	Triple	18–65	30	Iran	Ahmadi et al. (2021)
Nano-micellar gel	40 mg, four times/day	Oral	40	Triple	18–75	20	Iran	Hassaniazad et al. (2021)
Nano-micellar gel	80 mg, twice/day	Oral	41	None	18–75	20	Iran	Saber-Moghaddam et al. (2021)
Nano-micellar gel	80 mg, thrice/day	Oral	60	None	18–75	30	Iran	Asadirad et al. (2022)
Nano-micellar gel	40 mg, four times/day	Oral	48	Double	30–65	24	Iran	Honarkar Shafie et al. (2022)
CurcuRougeTM	90 mg, twice/day	Oral	60	Double	65–75	30	Japan	Kishimoto et al. (2021)
Curcumin add 5 mg piperine	500 mg, twice/day	Oral	100	Double	20–75	50	Iran	Miryan et al., 2020*
Curcumin add 5 mg piperine	500 mg, thrice/day	Oral	100 in ICU)	Double	20–75	50	Iran	Askari et al., 2021*
Curcumin add 2.5 mg piperine	525 mg, twice/day	Oral	140	Double	>18	70	India	Pawar et al. (2021)
Artemisinin, boswellia, curcumin, vitamin C, and nanoparticle	Artemisinin 12/8.4 mg, curcumin 40/28 mg, boswellia 30/21 mg, and Vitamin C 120/84 mg, twice/day	Spray	50, 240, 252	Double	>18	16, 80, 84	Israel	NCT04382040*, NCT05037162*, NCT04802382*
Curcumin, quercetin, and vitamin D	Curcumin 42 mg, quercetin 65 mg, and vitamin D 90 units, four times/day	Oral	100	None	>18	50	Pakistan, Belgium	NCT05008003*
Curcumin, quercetin, and vitamin D	Curcumin 168 mg, quercetin 260 mg, and vitamin D 360 units, twice/day	Oral	50	None	>18	25	Belgium	NCT04844658*

* as the clinical trial application; ICU: intensive care unit.

70-kDa heat shock protein (HSP70) family and functions as the endoplasmic reticulum (ER)-resident molecular chaperone for the clearance of misfolded proteins (Dessie and Malik 2021). An intracellular ER stress increase may upregulate the GRP78 expression and induce GRP78 re-localization to the cell membrane as cell surface GRP78 (Elfiky et al., 2021). The cellular surface GRP78 can associate with the major histocompatibility complex class I (MHC-I) that may aid SARS-CoV-2 to get into the host cells for starting an infection or help viral release from infected host cells (Ha et al., 2020; Gonzalez-Gronow et al., 2021). *In silico* data showed curcumin could interact with the S protein binding site and ATPase domain of GRP78 (Allam et al., 2020; Sudeep et al., 2020), suggesting that curcumin can assist in preventing COVID-19 viral attachment and enter host cells by binding to and inhibiting GRP78 of host cell surface.

In silico studies of curcumin/curcuminoids on the viral proteins and host proteins with SARS-CoV-2 infection are given in **Table 1**. It was deems the same as the viral site; all *in silico* results of the host proteins on SARS-CoV-2 infection show that curcumin can bind and interact with several targeting host proteins that play roles in viral binding (ACE2) and S protein activation (TMPRSS2, cathepsin L), and aid viral entry host cells (GRP78). Multiple targeting effects of curcumin on the site of virus and host cells during SARS-CoV-2 infective stages show its high potential to be applied for COVID-19 prevention or therapy, but until now, validations by *in vitro* or/and *in vivo* studies are still few or nil, and strikingly, it is only applicable as a co-supplement or adjuvant for therapy; nowadays, filing for approval by the FDA to become a real anti-COVID-19 medicine is most challenging.

Clinical Trials of Curcumin on COVID-19 Treatment

Remarkably, there are numerous reports of *in silico*, *in vitro*, and *in vivo* studies demonstrating that curcumin has the therapeutic potential against the COVID-19 based on its anti-inflammation, antioxidant, and antiviral effects. In this section, we have reviewed many clinical trial applications and published reports regarding the antiviral potential of curcumin/curcuminoids on COVID-19 therapy from PubMed and clinicaltrials.gov. In all these clinical trials, curcumin/curcuminoids formulations are used as a therapeutic co-supplement in the treatment of COVID-19 patients (**Table 2**). Because of the drawback of poor bioavailability of curcumin, several strategies are applied to improve the oral bioavailability of curcumin in clinical trials containing nano-delivery systems (Hatamipour et al., 2019), with addition of adjuvants (Tabanelli et al., 2021) and new formulation (Flory et al., 2021). Inferiorly, the metabolic rate of curcumin is high in the intestine and liver, which causes its poor bioavailability. Extraordinarily, piperine is an inhibitor of glucuronidation in the liver and intestine (Kaur et al., 2018). Therefore, the combination of curcumin and 1% piperine is a

potential option for the management of COVID-19 based on several properties including antiviral, anti-inflammatory, immunomodulatory, anti-fibrotic, and antioxidant effects (Miryan et al., 2021). In other clinical trials, results of non-COVID-19 treatment revealed that co-supplementation of curcumin/curcuminoids with 1% piperine could significantly improve the bioavailability (Panahi et al., 2015a; Shoba et al., 1998), and this formulation provides more antioxidant (Panahi et al., 2016a,b) and anti-inflammatory effects (Panahi et al., 2015b; Rahimnia et al., 2015). Different ayurvedic therapeutic agents (*C. Longa* L, green tea, and *Piper nigrum*) suppress the entry of virus into host cells, transmission of pathogens, and concurrently promote the immunity. Curcumin and piperine (1-piperoylpiperidine) interact with each other and form a π–π intermolecular complex which enhances the bioavailability of curcumin by inhibiting glucuronidation of curcumin in the liver (Kumar G. et al., 2021). The dose of curcumin from two clinical trial applications and one report for COVID-19 treatment is 1,000 mg/day curcumin plus 0.5–1% piperine (Askari et al., 2021; Pawar et al., 2021). When compared with the control group, the clinical results indicated that patients with the manifestation of mild, moderate, and severe symptoms orally receiving curcumin with piperine formulations as the adjuvant symptomatic therapy for COVID-19 infection have better clinical outcomes, and it can also reduce morbidity and mortality (Miryan et al., 2020).

Furthermore, curcumin incorporated with a nano-delivery system can enhance water solubility, decrease degradation, control slow release, and increase absorptive efficiency (Chen et al., 2020; Li et al., 2020; Quispe et al., 2021), thereby increasing circulation time, pharmacokinetics, and biodistribution. Cumulative evidence unveiled that encapsulated curcumin with a nano-delivery system has more significant effects than native curcumin in the clinical trials for the treatment in metabolic syndrome to decrease triglyceride levels in serum (Bateni et al., 2021); in coronary elective angioplasty treatment to improve total cholesterol, triglycerides, and antioxidative capacity (Helli et al., 2021); and in knee osteoarthritis treatment to improve the severity of symptoms (Hashemzadeh et al., 2020). Curcumin is re-proposed as a potential antiviral key for the treatment of SARS-CoV-2 based on its relation to the infection pathways. Moreover, the use of curcumin-loaded nanocarriers for increasing its bioavailability and therapeutic efficiency was highlighted in several clinical trials (Dourado et al., 2021; Hassaniazad et al., 2021; Tahmasebi et al., 2021a; Tahmasebi et al., 2021b; Valizadeh et al., 2020; Hassaniazad et al., 2020; Ahmadi et al., 2021; Saber-Moghaddam et al., 2021; Asadirad et al., 2022; Honarkar Shafie, et al., 2022). Additionally, the potential of the nanostructured systems and their synergistic action with curcumin on the molecular targets for viral infections have been explored (Hassaniazad et al., 2020). One report illustrates that the administration of nano-curcumin can accelerate recovery from the acute inflammatory phase of COVID-19 by mediating inflammatory immune responses (decrease in serum IFN-γ and IL-17, TBX21 mRNA; increase in serum IL-4 and TGF-β, FOXP3 mRNA) (Ahmadi et al., 2021). COVID-19 patients receiving nano-curcumin supplements could significantly increase the O_2 saturation and decrease the scores of the Wisconsin Upper Respiratory System Survey (WURSS-24) in the third domain, fourth domain, and total score, indicating nano-curcumin supplementation could help decrease hypoxia and moderate the symptoms induced by SARS-CoV-2 infection (Honarkar Shafie, et al., 2022). After nano-curcumin treatment, a significant reduction in the frequency of Th17 cells, downregulation of Th17 cell-related factors, and decreased levels of Th17 cell-related cytokines were found in mild and severe COVID-19 patients, implying that curcumin could be a potential modulatory compound in improving the patient's inflammatory condition (Hassaniazad et al., 2020; Saber-Moghaddam et al., 2021). In all these clinical trials of COVID-19 treatment, nano-micelle curcumin was applied with the dose of 160 or 240 mg/day, and the dosage was lower than that of native curcumin treatment. Positively, hospitalized COVID-19 patients with oral administration of nano-curcumin formulation can modulate the inflammatory cytokine expression and secretion, such as IFN-γ, IL-1β, IL-6, and TNF-α (Kishimoto et al., 2021; Asadirad et al., 2022); regulate inflammatory immune responses to accelerate the recovery of the acute inflammatory phase; and shorten total recovery time (Ahmadi et al., 2021; Saber-Moghaddam et al., 2021). Additionally, oral spray with nano-curcumin formulation was developed and has entered phase III clinical trial for COVID-19 treatment in Israel (NCT04382040, NCT05037162, NCT04802382). Moreover, a new modified starch–curcumin formulation could increase curcumin absorption and distribution in blood circulation over 150 times compared to oral administration of native curcumin. This result showed this can significantly improve the anti-inflammatory effects and reduce the neutrophil/lymphocyte ratio with the administration of lower dose (Kishimoto et al., 2021).

Clinical trials of curcumin for COVID-19 treatment from PubMed and ClinicalTrial.gov are listed in **Table 2**. There are some points worth noting from all those clinical trials with curcumin formulations as the co-supplementation in COVID-19 treatment. As the sample size of those clinical trials was too small, their results cannot be representative and promising. Within 2 years from 2019, the mutants of SARS-CoV-2 (S^m) varied and increased, and the outbreak of the mutated strands was faster; consequently, patients diagnosed with COVID-19 were infected by different viral variants with different clinical symptoms, severity, and mortality. These several viral variants of COVID-19 spread simultaneously around the world, but most clinical trials with curcumin applications in COVID-19 treatment did not easily establish the sample criteria for the patient's viral mutants. There some pieces of evidence show that curcumin has a significant inhibitory effect on cytochrome P450 (CYP450) enzymes (Mashayekhi-Sardoo et al., 2021; Sharma et al., 2022), which play important roles in the phase 1 metabolism of several clinical drugs, such as steroids (Zhao N. et al., 2021). Generally, steroids are widely used for anti-inflammation in the clinical treatment of COVID-19 (Lin et al., 2021). Therefore, the safety of curcumin or curcuminoids was approved by the U.S. FDA, but the inhibitory effect of curcumin

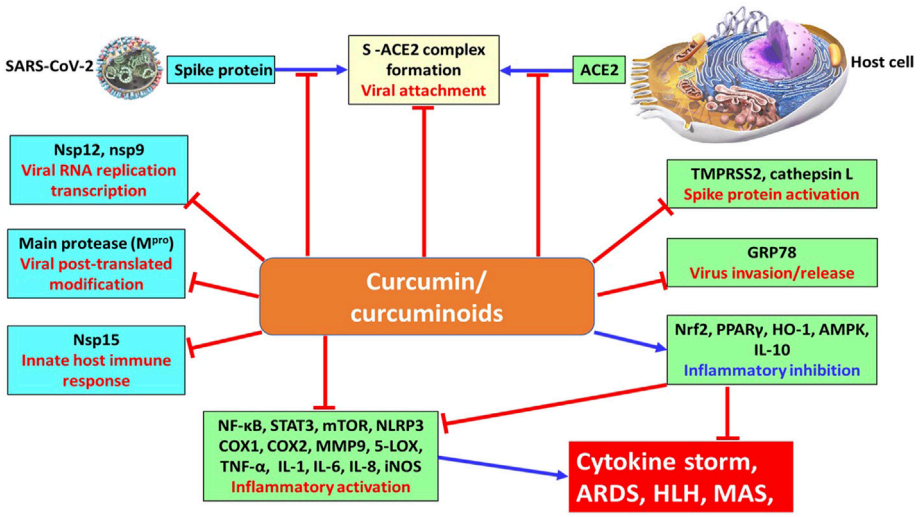

FIGURE 2 | Underlying mechanism of curcumin and curcuminoids on the viral protein and host cell proteins for combating the COVID-19 pandemic. stimulation; inhibition.

on CYP450 isoenzymes still needs more studies to investigate the interaction between curcumin and clinical drugs for treating COVID-19.

Promises and Perils of Curcuminoids on the Remedy of COVID-19

Curcumin attenuates oxidative stress and inflammation and regulates inflammatory and pro-inflammatory pathways associated with most chronic diseases (Quiñonez-Flores et al., 2016), and it presents future perspectives regarding the usage of curcumin as an immunomodulatory drug. Paradoxically, curcumin (along with many other dietary polyphenols), as abovementioned, can target multiple organs or cell lineages without a known receptor or a defined target. This, along with other chemical features of curcumin, has generated enormous difficulties in the detailed dissection of mechanistic pathways underlying its biomedical functions. Indeed, these features have been causing some confusion among drug developers and have been heavily debated recently (Jin et al., 2018; Heger 2017; Nelson et al., 2017). Taken together, the pharmaceutical potential of curcumin and curcuminoids in the remedy of COVID-19 is depicted in **Figure 2**. The underlying mechanisms of curcumin and curcuminoids on the viral protein and host cell proteins in combating COVID-19 infection are also illustrated, including proposed signaling pathways of stimulation and inhibition from both viral and host cellular sites. Recently, with the awareness of the immunomodulatory properties of curcumin, curcumin was successfully added to different food matrices to formulate functional foods that can improve human body immunity to abate the outbreak of SARS-CoV-2 (reviewed in Tripathy et al., 2021). Further investigation is needed to unequivocally determine whether curcumin might provide a therapeutic benefit in various diseases, particularly COVID-19. The utilization of purified active compounds like curcumin at higher than normal doses warrants additional scrutiny, including an accurate determination of the appropriate dose, dosing regimen, duration of treatment, and further clarification of the mechanism(s) of action as it pertains to viral infection. With regard to the treatment of COVID-19 infection or any other viral infection disease in which curcumin is administered *via* the oral route, another important issue concerns the increase in the bioavailability of curcumin. Despite its efficacy and safety, the therapeutic potential of curcumin is indeed still debated due to relatively poor bioavailability in humans including its poor solubility in water, chemical instability, and a low pharmacokinetic profile (Anand et al., 2007). Importantly, the oral bioavailability of curcumin is low due to a relatively low absorption by the small intestine coupled with an extensive reductive and conjugative metabolism in the liver and elimination through the gall bladder. Moreover, although curcumin is orally administered at a high dose, only a small quantity is detected in the blood plasma that is rapidly metabolized and excreted via faces and urine (Niu et al., 2012). Aforementioned limitations have restricted curcumin's therapeutic effectiveness in treating human diseases. Numerous studies have been conducted under heat and pressure with various formulations (amorphous) (Tran et al., 2019; Sunagawa et al., 2021) to increase the aqueous solubility and bioavailability of curcumin, which demonstrated that curcumin supplemented with nanoparticles of active substances or nano-formulations had enhanced bioavailability, controlled release, and increased stability. Additionally, lipid-based delivery systems and the encapsulation are mainly used for the enrichment of food products with health-promoting compounds. Notably, the delivery systems of curcumin (such as particles, micelles,

emulsions, and liposomes) have a demonstrably positive effect on augment bioavailability (Chebl et al., 2017; Guerrero et al., 2018; Tan et al., 2018).

CONCLUSION

Curcumin is a bioactive phytochemical that can be utilized as a nutraceutical or pharmaceutical in functional foods, supplements, and medicines. There are several limitations of curcumin use like low solubility and fast metabolism which restrict its absorption in the gastrointestinal tract and lead to poor oral bioavailability. Curcumin has multiple effects on the antioxidative stress, anti-inflammation, antiviral infections, but it has some inhibitory effects on the drug metabolism which need to be further clarified. To overcome these limitations, various curcumin formulations such as encapsulation in edible nanoparticles or microparticles to enhance its water dispersibility, chemical stability, and bioavailability are applied. Clinical trials of curcumin indicate safety, tolerability, and non-toxicity. However, the efficacy is questionable because of the small number of patients enrolled in each study. The challenges concerning research on curcumin's health benefits are given as follows: clarifying the relationship between curcumin's health benefits and the immunomodulation particularly in treatment of COVID-19 and conducting further human trials in which multiple research groups use the same samples and conditions. For the application of curcumin in COVID-19 treatment, further studies are still needed to optimize or improve its bioavailability following oral administration and to explore the degree of influence or interference of curcumin on clinical drugs metabolism. Moreover, this study after re-evaluation can afford a favorable application or approach of curcumin with evidence based on the drug discovery and development, pharmacology, functional foods, and nutraceuticals for effectively fighting COVID-19. Overall, curcumin is a promising ingredient of novel functional foods with protective efficacy in preventing or reducing the manifestation or complications of COVID-19 infection.

AUTHOR CONTRIBUTIONS

W-YH and JW conceived the articles collections and data analysis. M-JT, LH, and NK participated and designed the flow of article. Y-SF and C-FW prepared the manuscript draft and critically reviewed the manuscript. All authors have reviewed and approved the submitting version of manuscript.

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Inactivation and Recovery of High Quality RNA From Positive SARS-CoV-2 Rapid Antigen Tests Suitable for Whole Virus Genome Sequencing

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The diagnostic protocol currently used globally to identify Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection is RT-qPCR. The spread of these infections and the epidemiological imperative to describe variation across the virus genome have highlighted the importance of sequencing. SARS-CoV-2 rapid antigen diagnostic tests (RADTs) are designed to detect viral nucleocapsid protein with positive results suggestive of the presence of replicating virus and potential infectivity. In this study, we developed a protocol for recovering SARS-CoV-2 RNA from "spent" RADT devices of sufficient quality that can be used directly for whole virus genome sequencing. The experimental protocol included the spiking of RADTs at different concentrations with viable SARS-CoV-2 variant Alpha (lineage B.1.1.7), lysis for direct use or storage. The lysed suspensions were used for RNA extraction and RT-qPCR. In parallel, we also tested the stability of the viral RNA in the RADTs and the RNA extracted from the RADTs was used as a template for tiling-PCR and whole virus genome sequencing. RNA recovered from RADTs spiked with SARS-CoV-2 was detected through RT-qPCR with C_t values suitable for sequencing and the recovery from RADTs was confirmed after 7 days of storage at both 4 and 20°C. The genomic sequences obtained at each time-point aligned to the strain used for the spiking, demonstrating that sufficient SARS-CoV-2 viral genome can be readily recovered from positive-RADT devices in which the virus has been safely inactivated and genomically conserved. This protocol was applied to obtain whole virus genome sequence from RADTs ran in the field where the omicron variant was detected. The study demonstrated that viral particles of SARS-CoV-2 suitable for whole virus genome sequencing can be recovered from positive spent RADTs, extending their diagnostic utility, as a risk management tool and for epidemiology studies. In large deployment of the RADTs, positive devices could be safely stored and used as a template for sequencing allowing the rapid identification of circulating variants and to trace the source and spread of outbreaks within communities and guaranteeing public health.

Keywords: antigen testing, RT-qPCR, whole virus genome sequencing, lateral flow device, rapid antigen diagnostic test

INTRODUCTION

The diagnostic protocol currently used globally to identify Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection is RT-qPCR (1). RNA extracted from nasopharyngeal swabs is amplified to detect several viral structural and accessory genetic elements as suitable targets for this method (2). Although RT-qPCR has excellent sensitivity, the rapid spread of these infections and the epidemiological imperative to describe variation across the virus genome highlights the importance of sequencing (3). This in turn can enable refinement of detection methods (4) to facilitate the tracking of transmission pathways in nosocomial outbreaks (5) whilst highlighting superinfections and intra-host mutations resulting in the emergence of variants of concern (VOC) (6). SARS-CoV-2 rapid antigen detection tests (RADT) are designed to detect viral nucleocapsid protein with positive results suggestive of the presence of replicating virus and potential infectivity. RADT do not detect viral particle numbers as low as those detected by PCR, but are effective in detecting levels of virus likely to transmit infection (7). The frequent use of RADT testing in particular settings, such as meat processing plants (MPPs), can support risk-mitigation, in identifying and excluding highly infectious individuals from the workplace (8). The ability to recover viral RNA from spent positive RADT devices for subsequent whole virus genome sequencing (WvGS) would enable both the identification of virus lineage and definition of nucleotide polymorphisms, thus facilitating molecular epidemiological mapping of viral spread within these communities, as well as detecting the emergence of any new SARS-CoV-2 VOCs. This study provides proof of concept of using spent positive RADT kits to generate viral sequence data of sufficient quality to identify circulating variants and to trace the source and spread of outbreaks within communities.

METHODS

Recovery of SARS CoV-2 RNA From "Spent" RADT Test Devices

In this study, we used the Abbott Panbio™ COVID-19 Ag Rapid Test Device kit (Nasal) (Abbott Laboratories Ltd., USA) as RADT spiked with viable SARS-CoV-2 variant Alpha, lineage B.1.1.7 (Human nCoV19 isolate/England/MIG457/2020) grown in Vero E6 cells with a titer of 1.8×10^4 plaque forming units (PFU)/mL (9). For studying the correlation between recovery of the RNA from RADTs and concentration of SARS-CoV-2, the RADTs were inoculated in a 90° angle to the specimen well with 120 μL 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, and 1:16,000 dilutions of SARS-CoV-2 in duplicate. The buffer provided in the RADTs was used for preparing the dilutions. After inoculation, the RADTs were maintained on a flat surface for 15 min at room temperature, in accordance with the manufacturers' instructions. The appearance of control and test lines showed that the test was valid and capable of detecting cultured virus. The spent RADTs were then slowly filled with 700 μL viral lysis buffer (AVL) (QIAamp® Viral RNA Mini kit, Qiagen Ltd, UK) and then incubated for 10 min at room temperature. Each device was then transferred into a sterile 30 mL sample tube, vortexed for 5 s and centrifuged at 5,000 × *g* for 1 min. The lysed suspension (∼700 μL) was then used as a template for RNA extraction using a programmable QIAcube Platform (Qiagen Ltd., UK) according to the manufacturer's instructions.

The same protocol was used for the preparation of RADTs inoculated with neat and 1:16,000 dilution of SARS-CoV-2 with the aim of testing the stability of the RNA in these devices following incubation for maximum 7 days at 4 and 20°C after addition of buffer AVL.

In order to validate virus inactivation, eluate recovered from RADTs spiked with 120 μL of neat SARS-CoV-2 and 700 μL Buffer AVL was added to Vero E6 cells. Before addition to Vero E6 cells, cytotoxic components of the AVL buffer were eliminated from eluate using detergent removal spin columns (ThermoFisher, UK), which were shown to recover 100% of virus (10). The protocol demonstrated that viable virus could no longer be detected in the eluate from positive RADT test devices to which AVL buffer was added, but viable virus was detected when 700 μL of PBS was added. RNA suitable for WvGS was recoverable.

Protocol for Safe Virus Inactivation and Use for RT-qPCR and WvGS

The protocol described above was modified to facilitate the safe handling of real-field positive RADTs. SARS-CoV-2 positive RADTs were inoculated on-site with 700 μL AVL provided in ready to use aliquots in 1.5 mL tubes and transferred gently drop-by-drop using a single-use polyethylene Pasteur pipette (Fisher Scientific Ireland). After an incubation of 10 min, the RADT were transferred into a 50 mL sample tube, sealed and maintained at 4 or 20°C, then delivered to the laboratory (**Figure 1**). The next steps were performed in containment biosafety laboratory category 2 (BSL-2) facilities using standard BSL-2 work practices. The tubes were centrifuged for 5,000 × g for 1 min, and the discharged liquid collected in the bottom of the tubes (about 700 μL) was retrieved for RNA extraction and RT-qPCR using the method as described above. This protocol was used for the extraction of 30 RADTs from a number of MPPs in Ireland returning positive results. The samples were randomly chosen from a larger dataset of positive RADTs, including Abbott Panbio™ (Abbott Laboratories Ltd., USA) and Clinitest® Rapid COVID-19 Antigen Test (Siemens Healthineers, Germany) where voluntary participants from a number of MPPs provided their informed consent. Workers were invited to participate and provided with an information leaflet and consent form for signature. Anonymised data from the survey was provided to the research team, with ethical approval from UCD Human Research Ethics Committee (No.: LS-E-20-196-Mulcahy).

RT-qPCR Detection of SARS-CoV-2 and Whole Viral Genome Sequencing

The presence of SARS-CoV-2 RNA in purified samples, either from the experimental protocols or positive RADTs from MPPs, was confirmed by RT-qPCR targeting the nucleocapsid genes 1 (N1) and 2 (N2) and the human RNase P (RP). Three single

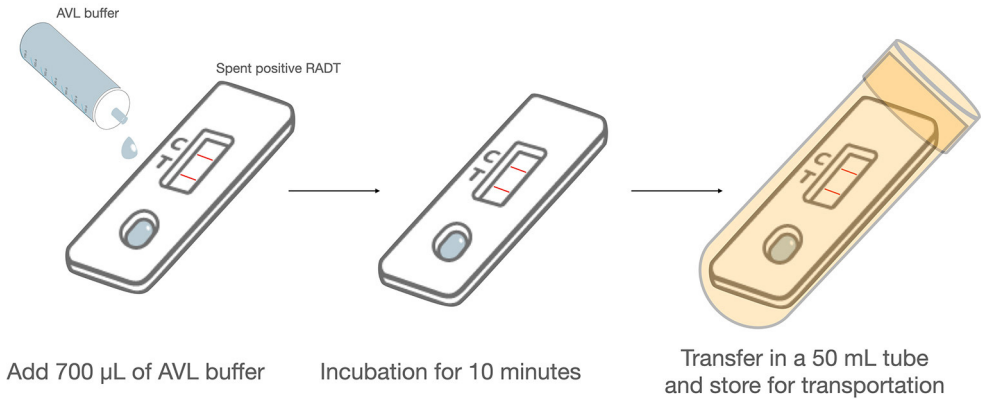

FIGURE 1 | Protocol for safe virus inactivation of RADT on-site. The experimental protocol was modified to facilitate the safe handling of real-field positive RADTs. SARS-CoV-2 positive RADTs are inoculated on-site with 700 µL AVL provided in ready to use aliquots and transferred gently drop-by-drop. After an incubation of 10 min, the RADT is transferred into a 50 mL sample tube, sealed and maintained at 4 or 20°C, then delivered to the laboratory.

TABLE 1 | Panel of primer and probes used for the RT-qPCR used in this study.

Label name	Description	Oligonucleotide sequence (5′ > 3′)	Label	Final conc.
2019- nCoV_N1-F	2019-nCoV_N1 Forward Primer	GAC CCC AAA ATC AGC GAA AT	None	500 nM
2019- nCoV_N1-R	2019-nCoV_N1 Reverse Primer	TCT GGT TAC TGC CAG TTG AAT CTG	None	500 nM
2019- nCoV_N1-P	2019-nCoV_N1 Probe	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	FAM, BHQ-1	125 nM
2019- nCoV_N2-F	2019-nCoV_N2 Forward Primer	TTA CAA ACA TTG GCC GCA AA	None	500 nM
2019- nCoV_N2-R	2019-nCoV_N2 Reverse Primer	GCG CGA CAT TCC GAA GAA	None	500 nM
2019- nCoV_N2-P	2019-nCoV_N2 Probe	FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1	FAM, BHQ-1	125 nM
RP-F	RNase P Forward Primer	AGA TTT GGA CCT GCG AGC G	None	500 nM
RP-R	RNase P Reverse Primer	GAG CGG CTG TCT CCA CAA GT	None	500 nM
RP-P	RNase P Probe	FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1	FAM, BHQ-1	125 nM

reactions were prepared using combined Primer/Probe Mix (1.5 µL) (IDT, USA) for each target (**Table 1**) adding DNase & RNase free water (6.5 µL) (Fisher Scientific Ireland), qScript XLT One-Step RT-qPCR ToughMix (2X) Low ROX (10 µL) (Quantabio, USA) and 2 µL of the template RNA, obtaining 20 µL of total reaction. A standard curve with 1:10 serial dilution of single stranded RNA (ssRNA) fragments of SARS-CoV-2 reference material (EU-JRC, Italy) was included on each RT-qPCR run along with negative extraction control. The cycling protocol of the complete reaction mix was incubated in a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) as follows: cDNA Synthesis (50°C for 10 min), initial denaturation (95°C, for 1 min) PCR cycling (40 cycles) included denaturation (95°C for 10 s) extension and data collection step (60°C for 1 min).

The extracted samples were also used as a template for multiplex PCR (tiling-PCR) according to the ARTIC panel (version 3) (11) and amplicons were prepared for WvGS, following the protocol for Illumina MiSeq sequencing (5). Raw data were quality assessed using FastQC (version 0.11.7) and pre-processed with fastp (version 0.20.1) (12). Consensus sequences were generated using the computational package iVar (version 1.0) (13). For phylogenetic analysis, sequences were aligned using a pipeline used previously (5, 6) which included the analysis with Nextclade (14) to identify differences between sequences and report sequence quality, while the pangolin tool was used for the assignment of epidemiological lineages (15). The sequences obtained were aligned using MAFFT (version 7) and for outlining the phylogenetic relationship among the sequences, a tree was generated with the Neighbor-Joining method (16) and visualized using FigTree (version 1.4.4) (https://github.com/rambaut/figtree).

RESULTS

RT-qPCR Detection of SARS-CoV-2 in Positive Spent RADT Test Device and Genomic Comparison Experimental Study

All RADT spiked with SARS-CoV-2 produced a test and control line within 15 min of inoculation. The RNA recovered from RADTs spiked with SARS-CoV-2 was detected through RT-qPCR and the C_t values ranged between 27.49 and 31.80 for the gene N1 and 28.19 and 31.91 for the gene N2 (**Figure 2**) with ∼1-C_t difference between each 1:2 dilution. There was no significant change in RNA detected by RT-qPCR overtime when RADTs were spiked with a high titer of SARS-CoV-2 (1×10^3 PFU/mL) following storage of RADTs at 4 or 20°C (**Figure 3A**) using neat concentration of cultured SARS-CoV-2, while the stability study demonstrated reduced detection

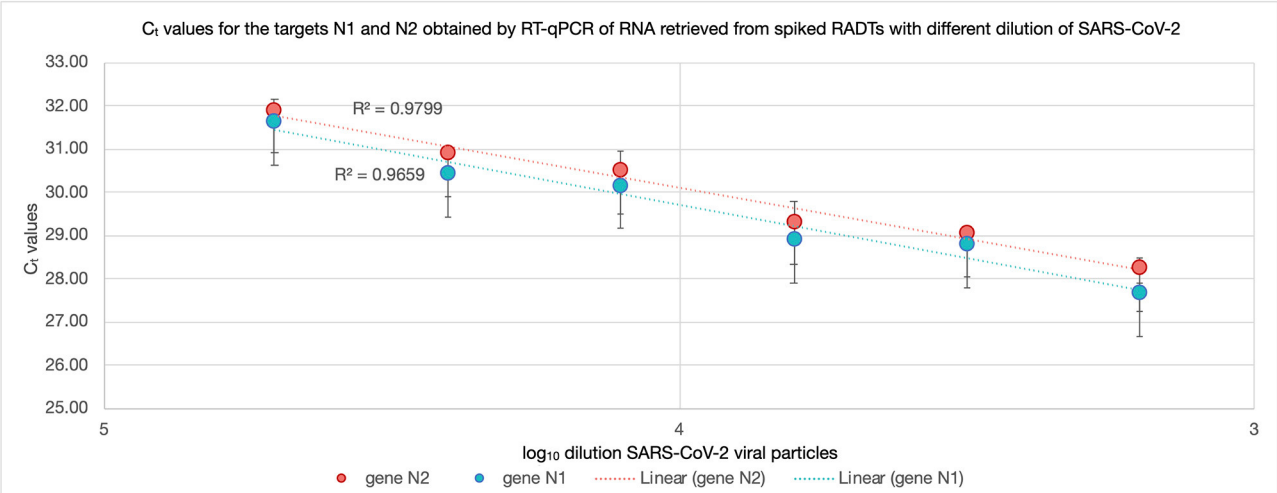

FIGURE 2 | C_t values for N1 and N2 of RNA recovered from RADTs spiked with different concentrations of SARS-CoV-2 viral particles. The RNA recovered from RADTs spiked with SARS-CoV-2 was detected through RT-qPCR and the C_t values ranged between 27.49 and 31.80 for the gene N1 (blue) and 28.19 and 31.91 for the gene N2 (red) reported in the y-axes. The $\log_{10}$ of the SARS-CoV-2 viral particles dilutions is presented in the x-axes. The R-squared (R^2) values are displayed in the dotted trend lines and the vertical bars on the points represent the standard deviation considering the average of the two C_t values recorded for each sample.

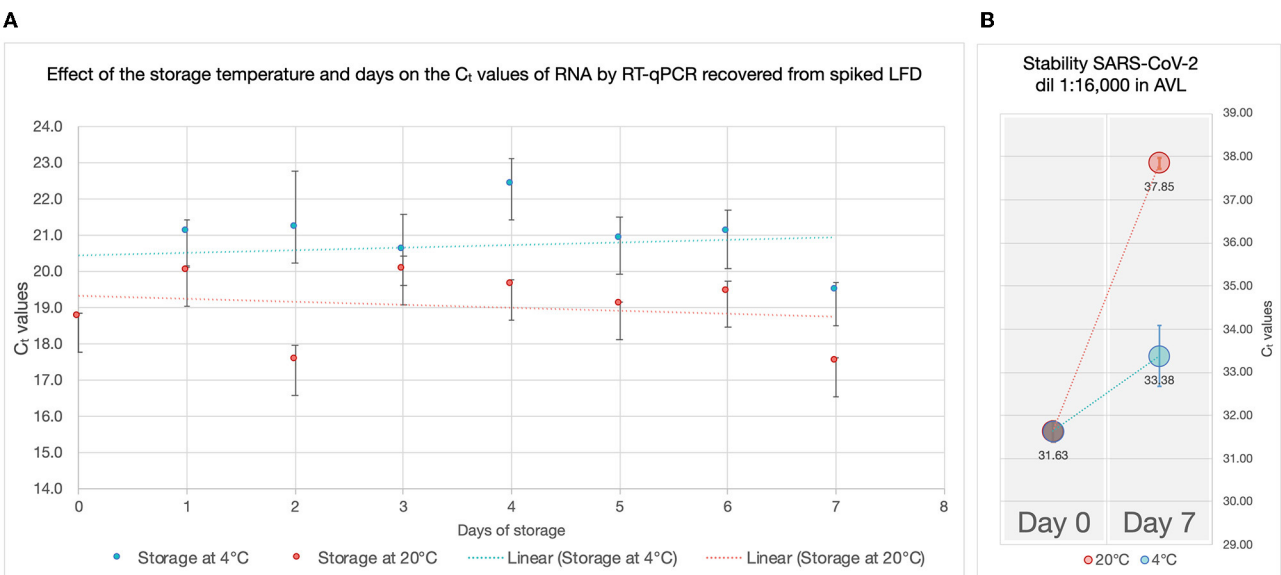

FIGURE 3 | Effect of the storage temperature and time on the C_t values of RNA by RT-qPCR recovered from spiked LFD. **(A)** C_t values (y-axes) of the RNA recovered from RADTs spiked with a high titer of SARS-CoV-2 (1×10^3 PFU/mL) following storage of RADTs at 4°C (blue) or 20°C (red) for 7 days. The vertical bars on the points represent the standard deviation considering the average of the two C_t values recorded for each sample and the trendlines (dotted lines) are shown. **(B)** Stability test. A diluted concentration (1:16,000) of the virus was used for spiking RADTs and stored at 4 and 20°C. The C_t values (y-axes) obtained on day 0 and 7 are shown in the plot along with the vertical bars on the points representing the standard deviation considering the average of the two C_t values recorded for the measurements.

of viral RNA by RT-qPCR when RADTs were inoculated with the 1:16,000 dilution (C_t = 31.63) at day 7. Low amounts of RNA in RADTs appear to be more stable when incubated at 4°C (C_t = 33.38) compared to 20°C (C_t = 37.85) (**Figure 3B**). A total of four RNA samples extracted from RADTs from the stability test were sequenced, including two RADTs stored at 4°C and two at 20°C after addition of buffer AVL and extracted after 2 and 7 days. The alignment of the sequences showed the perfect alignment with the sequence of the Human SARS-CoV-2 variant Alpha (lineage B.1.1.7, isolate England/MIG457/2020) (17, 18) used for spiking the RADTs (**Figure 4**).

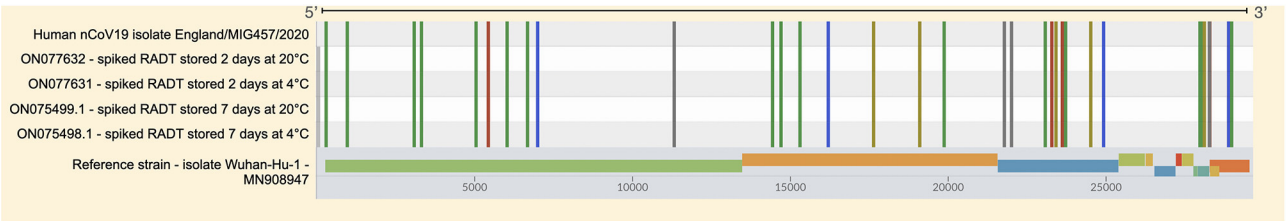

FIGURE 4 | Sequence alignment of WvG sequences obtained from RNA during the stability test. The spiked RADTs were stored at 4°C and 20°C after the addition of buffer AVL and the extraction protocol was performed after 2 and 7 days. The GenBank id of the four samples are included in the left side of the figure, the Human nCoV19 isolate/England/MIG457/2020 used for the spiking is included. The colored lines represent the mutations and polymorphisms common to the four WvGs compared to the reference WvG (MN908947 - Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1).

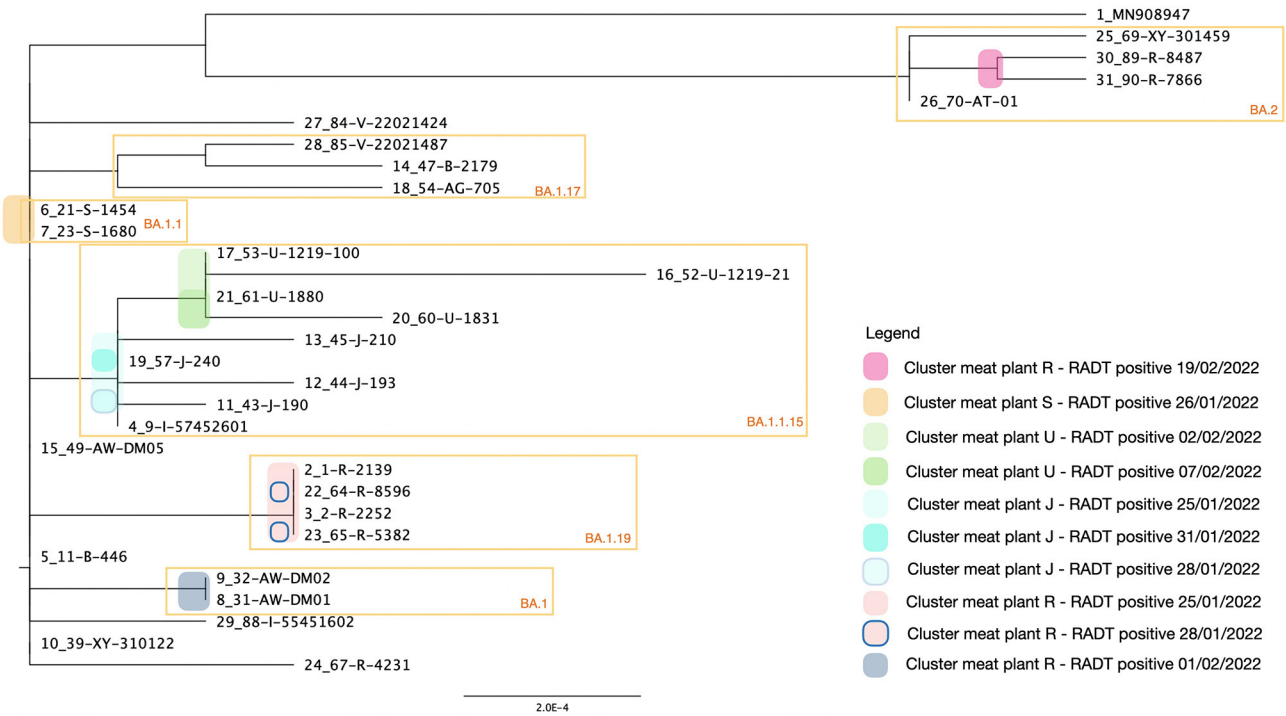

FIGURE 5 | Maximum likelihood phylogenetic tree of the 30 field-samples. The sequences were obtained from the positive RADTs provided by operators in the MPPs and treated with the developed protocol described in this study. The six yellow squares highlight the clusters and the colored spots on the branches of the tree, group the related samples with the same date of isolation. The clusters are annotated based on the average distance among the sequences and the tree is rooted with the reference MN908947 (Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1).

RT-qPCR Detection of SARS-CoV-2 in Positive Spent RADT Test Device and Genomic Comparison in Field Samples

The RNA extracted from positive RADTs from meat plants were tested for the presence of RNA of SARS-CoV-2 with the RT-qPCR. All the samples resulted positive with a Ct value included between 18.39 and 34.67 (**Supplementary Table 1**). All the samples were sequenced and the clade 21K (Omicron) was identified for 26 samples and four resulted 21L (Omicron). According to Nextclade Pango nomenclature, were identified seven different lineages: 9 BA.1.1.15 (30.0 %), 5 BA.1 (16.7 %), 4 BA.1.19 (13.3 %), 4 BA.1.1 (13.3 %), 4 BA (13.3 %), 3 BA.1.17 (10.0 %), and 1 BA.1.10 (3.3%). In addition the genome coverage ranged between 67.4 and 98.8% (**Supplementary Table 1**) while other parameters and details on the genomics sequence are presented in the **Supplementary Table 2**. The phylogenetic tree (**Figure 5**) highlighted the relationship among the samples clustered according to the MPPs and dates of positivity. In total were identified six clusters and four of them (BA.2, BA.1.1, BA.1.19, and BA.1) grouped samples originated from the same MPP. The cluster grouping the lineage BA.1.1.15 included samples from three different MPPs (U, J, and I), while samples

from MP R generated two distinct clusters with two lineages (BA.2 and BA.1.19) of the variant Omicron (clade 21L and 21 K respectively).

DISCUSSION

WvGS can be used to identify VOCs in the population at large (18) and can also be used at higher resolution to support epidemiological investigation of outbreaks (1, 5, 6, 17–19). As more of the Irish population is vaccinated, the application of WvGS becomes increasingly important to quickly identify and control new and emerging variants that could escape vaccinal protection particularly in elderly and vulnerable individuals (6). In this context, the source and spread of future virus outbreaks should be more aggressively tracked and traced to expedite its elimination from the Irish population.

RADTs in which virus has been inactivated have been used for years for example when transferring foot-and-mouth disease virus test samples from remote field locations to reference laboratories for characterization (20). The present study provides proof of the concept that sufficient SARS-CoV-2 viral genome can be readily recovered from positive RADT devices in which the virus has been safely inactivated to allow for high resolution sequencing. This is a useful extended finding which should be viewed in the context outlined above, as providing an additional source of material for WvGS.

Detection of lineages of the Omicron VOC from field samples and one lineage of the Alpha VOC experimentally, means there is no guarantee that other VOCs could be detected by the method described here. Only two different RADTs were used in this study and either the lysis buffers provided or the makeup of the lateral flow devices provided with other RADT kits could prevent the isolation of viral RNA for sequencing by the method described here. Further application of this method to recover RNA from positive RADTs and detect circulating variants will determine if this method can be utilized to detect other VOCs and be used with other RADT kits. Interestingly, the phylogenetic analysis highlighted the relationship among samples, and different clusters were identified, grouping in some cases, samples from the same MPPs. The limited number of samples for this trial and the relatively short period of time of the survey couldn't support more speculations on the direction of the infections however, the significant additional benefit derived from this study was proof of the concept that viral genome sequences could be obtained from spent positive RADTs. As the pandemic has evolved, tracking the spread of VOCs has become a priority for public health authorities. This study demonstrates the possibility of rapidly sequencing viruses associated with infections in workplace environments, such as MPPs, both to monitor the viral variants and lineages in circulation, and in future, with the validation of other available RADTs and the availability of WvGS obtained using this protocol, could be potentially applied to identify sources of infection, and the direction of person-to-person spread within workplaces.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, ON077632, ON077631, ON075499.1, ON075498.1.

AUTHOR CONTRIBUTIONS

SF, DS, and GMu designed the project. GMa, TR, GB, PW, and SF designed the experiments and wrote the manuscript. GMa and TR performed the experiments. GMa performed the genomic analysis. TR and GB cultivated SARS-CoV-2 and performed the experiment in CL3. LK and SM contributed on the extraction and library preparation for sequencing for the field-study. All authors read and approved the submitted 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/fpubh.2022.863862/full#supplementary-material

Supplementary Table 1 | Dataset 30 samples of RADTs resulted positive from a survey in MPPs in Ireland. The table presents the qualitative results of the sequencing and report of the results of the Nextstrain SARS-CoV-2 tool.

Supplementary Table 2 | Dataset 30 samples of RADTs resulted positive from a survey in MPPs in Ireland. The table presents metadata and results of the RT-qPCR and qualitative results of the sequencing, including Ct values, Nextclade Pango, total Nucleotide Missing and % completion genome.

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ORIGINAL RESEARCH
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Circulating Polyunsaturated Fatty Acids and COVID-19: A Prospective Cohort Study and Mendelian Randomization Analysis

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Higher circulating polyunsaturated fatty acids (PUFAs), especially omega-3 fatty acids, have been linked to a better prognosis in patients of coronavirus disease 2019 (COVID-19). However, the effects and causality of pre-infection PUFA levels remain unclear. This study aimed to investigate the observational and causal associations of circulating PUFAs with COVID-19 susceptibility and severity. We first performed a prospective cohort study in UK Biobank, with 20,626 controls who were tested negative and 4,101 COVID-19 patients, including 970 hospitalized ones. Plasma PUFAs at baseline (blood samples collected from 2007 to 2010) were measured by nuclear magnetic resonance, including total PUFAs, omega-3 PUFAs, omega-6 PUFAs, docosahexaenoic acid (DHA), linoleic acid (LA), and the omega-6/omega-3 ratio. Moreover, going beyond UK Biobank, we leveraged summary statistics from existing genome-wide association studies to perform bidirectional two-sample Mendelian randomization (MR) analyses to examine the causal associations of eight individual PUFAs, measured in either plasma or red blood cells, with COVID-19 susceptibility and severity. In the observational association analysis of each PUFA measure separately, total, omega-3, and omega-6 PUFAs, DHA, and LA were associated with a lower risk of severe COVID-19. Omega-3 PUFAs and DHA were also associated with a lower risk of testing positive for COVID-19. The omega-6/omega-3 ratio was positively associated with risks of both susceptibility and severity. When omega-6, omega-3, and their ratio are jointly analyzed, only omega-3 PUFAs remained significantly and inversely associated with both susceptibility and severity. The forward MR analysis indicated that docosapentaenoic acid (DPA-n3) and arachidonic acid (AA) might be causally associated with a lower risk of severe COVID-19, with OR (95% CI) per one SD increase in the plasma level as 0.89 (0.81, 0.99) and 0.96 (0.94, 0.99), respectively. The reverse MR analysis did not support any causal effect of COVID-19 on PUFAs. Our observational analysis supported that higher circulating omega-3 PUFAs, especially DHA, may lower the susceptibility to and alleviate the severity of COVID-19. Our MR analysis further supported causal associations of DPA-n3 and AA with a lower risk of severe COVID-19.

Keywords: COVID-19, polyunsaturated fatty acid, Mendelian randomization, prospective cohort, docosapentaenoic acid, arachidonic acid

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in over five million deaths in less than 2 years (1, 2). Understanding the role of nutrition in moderating susceptibility to and progression of COVID-19 is critical for the development of evidence-based dietary recommendations to prevent infection and to manage disease progression (3, 4). Omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) are of special interest because of their potent immunomodulatory effects, not only in mounting immune responses against viral infection but also in promoting inflammation resolution to avoid tissue damage (5–7). COVID-19 is an infectious disease characterized by cytokine storm and hyperinflammation in severe cases (8), presenting multiple possible points of action for PUFAs.

Recent observational studies have noted significant changes in the circulating levels of various PUFAs when comparing COVID-19 patients to healthy controls and across severity subgroups of patients. In general, total PUFAs, omega-6 PUFAs, linoleic acid (LA), and the omega-3 index measured as the percentage of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in red blood cell (RBC) fatty acids, are lower in COVID-19 patients and even lower in severe cases (9–12). A higher omega-3 index in patients was further associated with lower risks of requiring mechanical ventilation and death (9, 10). But conflicting patterns were also reported across cohorts and studies (11, 12), such as elevated levels of LA and arachidonic acid (AA) in COVID-19 patients (12). Moreover, the circulating levels of PUFAs in patients are likely confounded by immune responses to the viral infection and do not represent the effects of pre-infection circulating levels. There is a prospective cohort study that compared hospitalized COVID-19 patients to non-cases and found that almost all PUFA measures, including total PUFAs, omega-6 PUFAs, omega-3 PUFAs, LA, and DHA, are associated with a lower risk of severe COVID-19. The only exception is the omega-6/omega-3 ratio, which exhibits a positive association (13). However, the study did not distinguish the effects on susceptibility and severity, and the usage of non-cases without COVID-19 status as the control did not correct for selection bias in those receiving tests. Altogether, while these observational studies provide valuable insights, they are susceptible to residual confounding and reverse causation. The causal effects of circulating PUFAs on COVID-19 susceptibility and severity remain unclear.

Mendelian randomization (MR) is an analytic tool for inferring the causal effects of an exposure on an outcome of interest (14). MR uses randomly allocated genetic variants related to the exposure as instrumental variables, which are inborn and minimally affected by confounders and reverse causation (15). This method has been widely utilized in recent studies to evaluate the causal roles of specific risk factors in COVID-19, such as body mass index (BMI), white blood cells, some circulating proteins, and smoking (16–19). On the other hand, MR studies have also provided support for the causal clinical effects of circulating PUFAs (**Supplementary Table 1**). The genetically predicted circulating levels of various PUFAs have been associated with clinical biomarkers, such as blood lipids, white blood cell counts, and blood pressure (20–22). They were also directly associated with risks of specific diseases, such as cardiovascular diseases, diabetes, and cancers (23–27). Therefore, MR is a valuable and cost-effective tool to evaluate the causal roles of circulating PUFAs in COVID-19 susceptibility and severity.

In this study, we first performed an observational analysis in a prospective cohort, UK Biobank, with 4,101 COVID-19 patients, including 970 hospitalized ones, and 20,626 controls that were tested negative. We performed multiple comparisons across different case and control groups to evaluate the effects of six baseline plasma PUFA measures on COVID-19 susceptibility and severity. Furthermore, we applied bidirectional two-sample MR analyses to examine the causal associations between eight individual PUFAs and COVID-19. Genetic instruments for circulating PUFAs were obtained from previous genome-wide association studies (GWAS) of corresponding PUFAs measured in either plasma or RBC (28–30). Genetic associations with COVID-19 susceptibility and severity were obtained from GWAS meta-analyses conducted by the COVID-19 Host Genetics Initiative (HGI) (31). Our study, integrating observational and genetics-instrumented MR analyses, unraveled the effects of total and individual circulating PUFAs on the risks of COVID-19 susceptibility and severity.

MATERIALS AND METHODS

Ethical Considerations

The usage of individual-level data for this study was approved by the University of Georgia Institutional Review Board and UK Biobank (application no. 48818). All participants of UK Biobank and the Framingham Heart Study (FHS) provided written informed consent before joining these studies. Informed consent was not required for publicly available summary statistics. Our study follows the guidelines for strengthening the reporting of observational studies in epidemiology (STROBE, **Supplementary Table 2**) and strengthening the reporting of Mendelian randomization studies (STROBE-MR, **Supplementary Table 3**) (32).

Participants and Study Design

We performed an observational cohort study based on UK Biobank and then a bidirectional two-sample MR study with summary statistics from GWAS of PUFAs and COVID-19. UK Biobank is a population-based prospective study, including >500,000 participants aged 37–73 years at recruitment from 2006 to 2010 in the United Kingdom (33). The observational analysis was performed to examine the associations between six plasma PUFA measures and COVID-19 status in UK Biobank. The six plasma PUFA measures include total PUFAs, omega-3 PUFAs, omega-6 PUFAs, DHA, LA, and the calculated omega-6/omega-3 ratio. The MR study investigated the causal effects of eight individual PUFAs on COVID-19 susceptibility and severity. Genetic instruments for plasma PUFAs were obtained directly from published GWAS (28, 29). Genetic instruments for RBC PUFAs were determined based on a published GWAS,

but their summary statistics, not reported in the original study, were calculated by ourselves with the same statistical model and individual-level data from 2,462 FHS participants (30). Six PUFAs have genetic instruments for their circulating levels in both plasma and RBC, including α-linolenic acid (ALA), docosapentaenoic acid (DPA-n3), LA, γ-linolenic acid (GLA), dihomo-γ-linolenic acid (DGLA), and AA. Docosatetraenoic acid (DTA) only has genetic instruments for its RBC level, while DHA only for its plasma level.

Observational Analysis

Figure 1 displays the flow of participants throughout the observational study. To minimize the possibility of bias, we removed participants who had mismatched self-reported sex and genetic sex, sex chromosome aneuploidy, ten or more third-degree or closer relatives, or had withdrawn from UK Biobank. Our exposure variables were six PUFAs, as measured by nuclear magnetic resonance (NMR) in a random subset of plasma samples collected between 2007 and 2010 (13, 33, 34). We used the COVID-19 testing result and inpatient status as our outcome (data accessed on June 21, 2021). The specimen collection dates were March 16, 2020 to June 14, 2021 for those in England; February 11, 2020 to March 18, 2021 in Scotland; and January 13, 2020 to June 7, 2021 in Wales. Hospitalized COVID-19 patients were identified as those with positive PCR-based diagnosis and explicit evidence of being inpatients. Of note, being an inpatient does not necessarily indicate hospitalization for COVID-19 because patients in hospitals for any reason may be prioritized for COVID-19 testing (35). Inpatient status was not available for assessment centers in Scotland and Wales. To test the association with COVID-19 severity, we performed two separate analyses with different controls: (1) non-hospitalized COVID-19 patients, and (2) individuals who tested negative. To examine the association with COVID-19 susceptibility, we focused on all COVID-19 cases, which were tested positive for SARS-CoV-2. Individuals with negative tests were used as the control. This analysis of susceptibility was performed in two datasets: (1) participants from England, and (2) participants from England, Scotland, and Wales. For the 24,727 participants with both plasma PUFA measures and COVID-19 status, we applied logistic regression models on various case and control groups to estimate the associations of PUFAs with COVID-19 susceptibility and severity. Covariates included continuous variables, including age, BMI, and Townsend deprivation index, and categorical variables, including sex, ethnicity, and assessment center. Individuals with missing information in PUFA measures, COVID-19 status, or

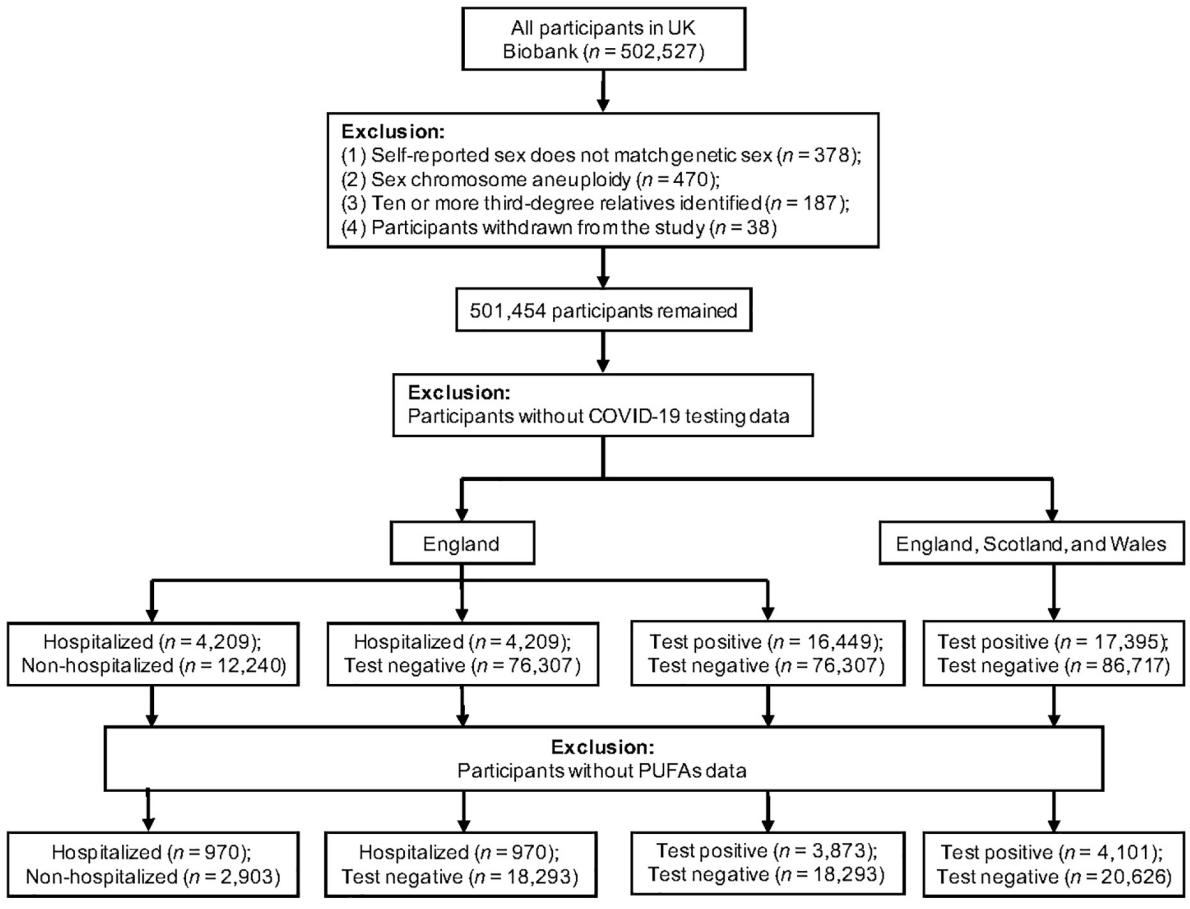

FIGURE 1 | Flowchart of the UK Biobank participants from recruitment to inclusion in the observational analysis.

covariates were excluded. All plasma PUFA measures were standardized to z scores and their comparable effect sizes were expressed per one standard deviation (SD) increase in the corresponding PUFAs. All analyses in the observational study were conducted using R version 4.0.0, and nominal significance was set at p-value < 0.05. Bonferroni correction for multiple testing [corrected P significance cutoff: 0.05/2 (outcomes)/6 (exposures) = 0.0042] was used to avoid the type I error (36).

Genetic Associations With Polyunsaturated Fatty Acids

Two types of circulating PUFAs were evaluated in our MR analyses, plasma and RBC PUFAs. For plasma PUFAs, single nucleotide polymorphisms (SNPs) were obtained from published GWAS of omega-3 PUFAs (n = 8,866) and omega-6 PUFAs (n = 8,631) in participants of European ancestry (28, 29). We selected SNPs for each plasma omega-3 and omega-6 PUFA, which reached genome-wide significance level ($P < 5 \times 10^{-8}$) and were restricted by linkage disequilibrium (LD) clumping to ensure independence ($r^2 < 0.001$ within a 10 Mb window). To ensure robustness and reduce false positives, we also used less stringent LD cutoffs ($r^2 < 0.01$, 0.1, and 0.3) to select SNPs associated with plasma omega-3 PUFAs. The same LD-related sensitivity analysis was not possible for plasma omega-6 PUFAs because their genome-wide summary statistics were not available. To examine the effects of RBC PUFAs, we obtained genetic associations at a genome-wide significance level ($P < 5 \times 10^{-8}$) identified by Tintle et al. (30). We used the individual-level data from the FHS to confirm the significance of these SNPs and calculate their effect sizes and standard errors. In the same linear mixed model, covariates included age, sex, and matrix of kinship coefficients in the FHS. We respectively selected independent ($r^2 < 0.001$, 0.01, 0.1, and 0.3 within a 10 Mb window) SNPs predicting RBC PUFAs at genome-wide significance ($P < 5 \times 10^{-8}$). We calculated F-statistics to test instrument strength (F-statistics > 10 for all plasma and RBC PUFAs) (37). Summary statistics for the genetic instruments for plasma and RBC PUFAs are openly available for public access (**Supplementary Tables 4, 5**).

Genetic Associations With COVID-19

To assess genetic associations with COVID-19 severity, we used three GWAS meta-analyses of only European participants which were conducted by the HGI (release 5, released on January 18, 2021) (31). First, we used the GWAS of severe COVID-19, labeled as study A2, that compared patients confirmed with very severe respiratory symptoms (n = 5,101) to the control group of general population samples (n = 1,383,241). Second, another HGI GWAS, labeled as study B2, compared hospitalized COVID-19 patients (n = 9,986) to general population samples (n = 1,877,672). The third severe COVID-19 GWAS utilized in our study, labeled as B1, compared hospitalized COVID-19 patients (n = 4,829) to non-hospitalized COVID-19 patients (n = 11,816). To assess genetic associations with COVID-19 susceptibility, we used one GWAS by HGI, labeled as study C2, that compared any COVID-19 case (n = 38,984) to population controls (n = 1,644,784). In addition to these four COVID-19 GWAS used in our primary analysis, we repeated MR analyses using the study A2, B1, B2, and C2 from HGI release 4 (released on October 20, 2020), to examine the consistency of our findings across different data releases. Detailed information about these GWAS is available at the COVID-19 HGI website.[1]

To assess reverse causality, we obtained strong ($P < 5 \times 10^{-8}$) and independent ($r^2 < 0.001$ within a 10 Mb clumping window) SNPs associated with COVID-19 phenotypes as genetic instruments. We also used a less stringent selection criterion ($P < 5 \times 10^{-6}$) to determine the robustness of our results.

Mendelian Randomization Analyses

Mendelian randomization was used to infer causality between PUFAs and COVID-19 by leveraging genetic data as instrumental variables. We scaled the odds ratio (OR) estimates per SD increment of plasma and RBC PUFAs (% of total fatty acids). We obtained the SNP-specific Wald estimate (ratio of the SNP-outcome effect divided by the SNP-exposure effect) when only one SNP was available. The inverse variance-weighted (IVW) method with a multiplicative random-effects model ($\geq$2 SNPs) was used as the primary analysis (38–40). We used the MR-Egger intercept test to evaluate the extent of unbalanced horizontal pleiotropy, which can lead to a biased causal effect estimate (39). In sensitivity analyses, we applied the MR-Egger and weighted median (WM) methods to account for pleiotropy (39–41). The MR-Egger method provides an unbiased causal estimate even when all SNPs are invalid instruments as long as that the horizontal pleiotropic effects are balanced across SNPs (39). However, MR-Egger can be imprecise and suffer from low statistical power, particularly when based on a small number of SNPs (e.g., <10) (39). The WM method gives robust causal estimates even when up to 50% of SNPs are invalid genetic instruments (41). To test the presence of heterogeneity among genetic instruments, we calculated Cochran's Q statistic for the IVW method and an extended version of Cochran's Q statistic (Rücker's Q') for the MR-Egger method (42, 43). We utilized Bonferroni correction [corrected P significance cutoff: 0.05/2 (outcomes)/7 (exposures) = 0.0036] for multiple testing. Additionally, we required a relationship to be nominally significant ($P < 0.05$) with both measures of the same PUFA (plasma and RBC) and in the case of COVID-19 severity, with different outcome GWAS (study A2, B2, and B1). All MR analyses were performed in R version 4.0.0 with the TwoSampleMR package version 3.6.9 (44).

RESULTS

Baseline Characteristics

The flow of UK Biobank participants throughout the observational study is described in **Figure 1**, while their baseline characteristics are summarized in **Table 1**. Across all

[1]https://www.covid19hg.org/results/

TABLE 1 | Characteristics of the UK Biobank participants at baseline.*

Characteristics	England				England, Scotland, and Wales	
	Hospitalized COVID-19	Non-hospitalized COVID-19	Test positive	Test negative	Test positive	Test negative
Participants, *n*	4,209	12,240	16,449	76,307	17,395	86,717
Participants with plasma PUFA measures, *n*	970	2,903	3,873	18,293	4,101	20,626
Age, y	59 [40–70]	51 [40–70]	52 [40–70]	59 [40–70]	52 [40–70]	59 [40–70]
Females, *n* (%)	445 (46)	1,559 (54)	2,004 (52)	9,771 (53)	2,123 (52)	11,145 (54)
Body mass index, kg/m^2 (SD)	29.55 (5.61)	27.96 (4.94)	28.36 (5.16)	27.69 (4.88)	28.36 (5.14)	27.71 (4.89)
PUFAs, mmol/l (SD)	4.82 (0.81)	4.92 (0.78)	4.89 (0.79)	4.97 (0.80)	4.89 (0.78)	4.96 (0.80)
Omega-3 PUFAs, mmol/l (SD)	0.48 (0.20)	0.49 (0.21)	0.49 (0.20)	0.53 (0.22)	0.49 (0.20)	0.53 (0.22)
DHA, mmol/l (SD)	0.21 (0.074)	0.22 (0.075)	0.22 (0.075)	0.24 (0.084)	0.22 (0.075)	0.23 (0.084)
Omega-6 PUFAs, mmol/l (SD)	4.34 (0.70)	4.42 (0.66)	4.40 (0.67)	4.44 (0.68)	4.40 (0.67)	4.44 (0.68)
LA, mmol/l (SD)	3.29 (0.70)	3.39 (0.65)	3.37 (0.67)	3.39 (0.69)	3.37 (0.66)	3.39 (0.68)

**Values are numbers (%) for categorical variables, mean (SD) or medians [range] for continuous variables. PUFAs, polyunsaturated fatty acids; DHA, docosahexaenoic acid; LA, linoleic acid.*

assessment centers in England, Scotland, and Wales, there were 104,112 participants with COVID-19 status. Among them, 17,395 were tested positive for COVID-19. Inpatient status was only reported by assessment centers in England. Of the 92,756 participants with COVID-19 status in England, 16,449 were tested positive, and 4,209 had confirmed inpatient status. Across England, Scotland, and Wales, COVID-19 patients were more likely to be male (*t*-test, $P = 0.008$), with higher BMI ($P = 9.34 \times 10^{-14}$), but younger than participants with negative testing results ($P < 2.2 \times 10^{-16}$). Across assessment centers in England, hospitalized COVID-19 patients were older ($P < 2.2 \times 10^{-16}$), were more likely to be male ($P = 2.44 \times 10^{-5}$), and had higher BMI ($P = 1.13 \times 10^{-14}$), when compared to non-hospitalized COVID-19 patients. The three known risk factors of severe COVID-19, age, sex, and BMI, were included as covariates in our observational association analysis.

Observational Association Analysis

Table 2 shows the observational associations between baseline plasma PUFAs and COVID-19 susceptibility and severity. Among participants from England who also had plasma data, there were 18,293 with negative testing results and 3,873 with positive tests. Among the COVID-19 patients, 970 were hospitalized and the other 2,903 were non-hospitalized. Comparing hospitalized patients to those tested negative, we observed a lower risk of COVID-19 severity per SD increase in total PUFAs (OR: 0.88; 95% confidence interval (CI): 0.82, 0.95; $P = 0.00051$), omega-3 PUFAs (OR: 0.82; 95% CI: 0.76, 0.89; $P = 8.07 \times 10^{-7}$), omega-6 PUFAs (OR: 0.91; 95% CI: 0.85, 0.98; $P = 0.012$), DHA (OR: 0.78; 95% CI: 0.72, 0.85; $P = 4.56 \times 10^{-9}$), and LA (OR: 0.92; 95% CI: 0.86, 0.99; $P = 0.023$). Using 2,903 non-hospitalized COVID-19 patients as the control group, there were consistently inverse associations of COVID-19 severity with total PUFAs ($P = 0.0012$), omega-3 PUFAs ($P = 0.0013$), omega-6 PUFAs ($P = 0.0047$), DHA ($P = 8.92 \times 10^{-5}$), and LA ($P = 0.0079$).

We further evaluated the effects of baseline plasma PUFAs on COVID-19 susceptibility by comparing COVID-19 patients to those tested negative. Among 24,727 participants in England, Scotland, and Wales, we found a lower risk of getting COVID-19 per SD increase in omega-3 PUFAs (OR: 0.92; 95% CI: 0.89, 0.96; $P = 2.27 \times 10^{-5}$) and DHA (OR: 0.91; 95% CI: 0.87, 0.94; $P = 1.41 \times 10^{-6}$). Among 22,166 individuals in England only, we also observed consistently significant associations for omega-3 PUFAs (OR: 0.92; 95% CI: 0.88, 0.96; $P = 4.29 \times 10^{-5}$) and DHA (OR: 0.91; 95% CI: 0.87, 0.94; $P = 3.00 \times 10^{-6}$).

The omega-6/omega-3 ratio was significantly associated with an increased risk of severe COVID-19, either by comparing hospitalized patients to participants who tested negative (OR: 1.13; 95% CI: 1.07, 1.20; $P = 1.48 \times 10^{-5}$) or to non-hospitalized patients (OR: 1.12; 95% CI: 1.03, 1.22; $P = 0.0061$). The ratio was also positively associated with COVID-19 susceptibility when comparing COVID-19 patients to those tested negative in England, Scotland, and Wales (OR: 1.06; 95% CI: 1.03, 1.10; $P = 0.00054$) or in England only (OR: 1.05; 95% CI: 1.02, 1.09; $P = 0.0030$). Notably, these PUFA measures are correlated with each other. For example, in the biggest sample from three regions ($n = 24{,}727$), there is a medium correlation between omega-6 and omega-3 PUFAs (Spearman's $\rho = 0.46$, $P < 2.2 \times 10^{-16}$). To evaluate if their COVID-19 associations are independent of each other, we jointly evaluate their effects in the same model (**Table 3**). Only the effects of omega-3 PUFAs persist after controlling for omega-6 PUFAs, the omega-6/omega-3 ratio, or both. In a model including all three PUFA measures, omega-3 PUFAs are associated with a lower risk of hospitalized COVID-19 when compared to those tested negative (OR: 0.86; 95% CI: 0.75, 0.98; $P = 0.029$), and a lower risk of testing positive in the England-only sample (OR: 0.89; 95% CI: 0.82, 0.96; $P = 9.93 \times 10^{-4}$) and in the sample from three regions (OR: 0.90; 95% CI: 0.84, 0.96; $P = 2.66 \times 10^{-3}$). Overall, our observational analysis showed that individuals with lower baseline levels of all five examined PUFAs were associated with a higher risk of hospitalized COVID-19, and those with lower levels of omega-3 PUFAs and DHA were also at a higher risk of COVID-19 susceptibility. On the other hand, the omega-6/omega-3 ratio was positively associated with the risks of both COVID-19

TABLE 2 | Associations of single polyunsaturated fatty acids (PUFAs) with COVID-19 susceptibility and severity.*

	COVID-19 severity						COVID-19 susceptibility					
	Hospitalized vs. non-hospitalized (*n* = 3,873)			Hospitalized vs. test negative (*n* = 19,263)			Test positive vs. test negative (*n* = 22,166)†			Test positive vs. test negative (*n* = 24,727)‡		
Plasma PUFAs	**β**	**SE**	***P***	**β**	**SE**	***P***	**β**	**SE**	***P***	**β**	**SE**	***P***
PUFAs	−0.14	0.043	0.0012	−0.13	0.037	0.00051	−0.029	0.019	0.13	−0.027	0.018	0.13
Omega-3 PUFAs	−0.14	0.044	0.0013	−0.20	0.040	8.07×10^{-7}	−0.083	0.020	4.29×10^{-5}	−0.082	0.019	2.27×10^{-5}
DHA	−0.18	0.045	8.92×10^{-5}	−0.25	0.042	4.56×10^{-9}	−0.098	0.021	3.00×10^{-6}	−0.097	0.020	1.41×10^{-6}
Omega-6 PUFAs	−0.12	0.043	0.0047	−0.090	0.036	0.012	−0.010	0.019	0.62	−0.0078	0.018	0.67
LA	−0.11	0.043	0.0079	−0.082	0.036	0.023	−0.0066	0.019	0.73	−0.0063	0.018	0.73
Omega-6/omega-3	0.11	0.042	0.0061	0.12	0.029	1.48×10^{-5}	0.053	0.018	0.0030	0.058	0.017	0.00054

**Only one PUFA measure was included in each logistic regression analysis. Effect sizes (β) per SD increase in the exposure, SEs, and P-values were obtained from the logistic regression analysis of COVID-19 susceptibility and severity. All models were adjusted for age, sex, ethnicity, BMI, Townsend deprivation index, and assessment center. PUFAs, polyunsaturated fatty acids; DHA, docosahexaenoic acid; LA, linoleic acid.*

†Data from England only.

‡Data from England, Scotland, and Wales.

TABLE 3 | Associations of multiple polyunsaturated fatty acids (PUFAs) with COVID-19 susceptibility and severity.*

Plasma PUFAs	**β**	**SE**	***P***	**Plasma PUFAs**	**β**	**SE**	***P***	**Plasma PUFAs**	**β**	**SE**	***P***
COVID-19 severity											
Hospitalized vs. non-hospitalized (*n* = 3,873)											
Omega3	−0.11	0.049	0.031	Omega6	−0.071	0.048	0.14				
Omega3	−0.12	0.066	0.069	Omega6/Omega3	0.026	0.064	0.69				
Omega6	−0.11	0.043	0.014	Omega6/Omega3	0.099	0.042	0.018				
Omega3	−0.043	0.078	0.58	Omega6	−0.091	0.052	0.080	Omega6/Omega3	0.069	0.068	0.31
Hospitalized vs. test negative (*n* = 19,263)											
Omega3	−0.19	0.044	1.61×10^{-5}	Omega6	−0.015	0.040	0.70				
Omega3	−0.17	0.058	3.08×10^{-3}	Omega6/Omega3	0.027	0.047	0.56				
Omega6	−0.078	0.036	0.030	Omega6/Omega3	0.12	0.029	3.94×10^{-5}				
Omega3	−0.15	0.068	0.029	Omega6	−0.028	0.043	0.52	Omega6/Omega3	0.039	0.049	0.43
COVID-19 susceptibility											
Test positive vs. test negative (*n* = 22,166), data from England only											
Omega3	−0.098	0.023	1.61×10^{-5}	Omega6	0.031	0.021	0.14				
Omega3	−0.089	0.031	3.97×10^{-3}	Omega6/Omega3	−0.0072	0.028	0.80				
Omega6	−0.0033	0.019	0.86	Omega6/Omega3	0.052	0.018	3.41×10^{-3}				
Omega3	−0.12	0.038	9.93×10^{-4}	Omega6	0.039	0.023	0.088	Omega6/Omega3	–0.027	0.031	0.38
Test positive vs. test negative (*n* = 24,727), data from England, Scotland, and Wales											
Omega3	−0.098	0.022	6.42×10^{-6}	Omega6	0.033	0.020	0.099				
Omega3	−0.075	0.029	9.46×10^{-3}	Omega6/Omega3	0.0093	0.026	0.72				
Omega6	−0.00060	0.018	0.97	Omega6/Omega3	0.058	0.017	5.99×10^{-4}				
Omega3	−0.11	0.035	2.66×10^{-3}	Omega6	0.035	0.022	0.10	Omega6/Omega3	–0.0071	0.029	0.80

**Two or three PUFA measures, shown on the same row, were included in each logistic regression analysis. Effect sizes (β) per SD increase in exposures, SEs, and P-values were reported. All models were adjusted for age, sex, ethnicity, BMI, townsend deprivation index, and assessment center. PUFAs, polyunsaturated fatty acids.*

susceptibility and severity. A joint analysis further support that these effects were mainly driven by omega-3 PUFAs.

Bidirectional Mendelian Randomization Analyses

We performed bidirectional MR analyses to examine the causal relationships between individual PUFAs and COVID-19. First, we performed a forward MR analysis to investigate the effects of PUFAs on COVID-19 susceptibility and severity. Second, we conducted a reverse MR analysis to evaluate the causal effects of genetically instrumented COVID-19 on PUFAs. All genetic instruments for PUFAs (F-statistics > 31.43) and COVID-19 (F-statistics > 30.81) were strong instruments. Six individual PUFAs have existing GWAS for their levels in plasma and RBC, and there are three GWAS on severe COVID-19 (i.e., HGI study A2, B2, B1). Only results that were consistent across these different GWAS were reported here.

In the forward MR study of plasma PUFAs, genetically instrumented one-SD increase in AA (OR: 0.96; 95% CI: 0.94, 0.99; P = 0.007) and DPA-n3 (OR: 0.89; 95% CI: 0.81, 0.99;

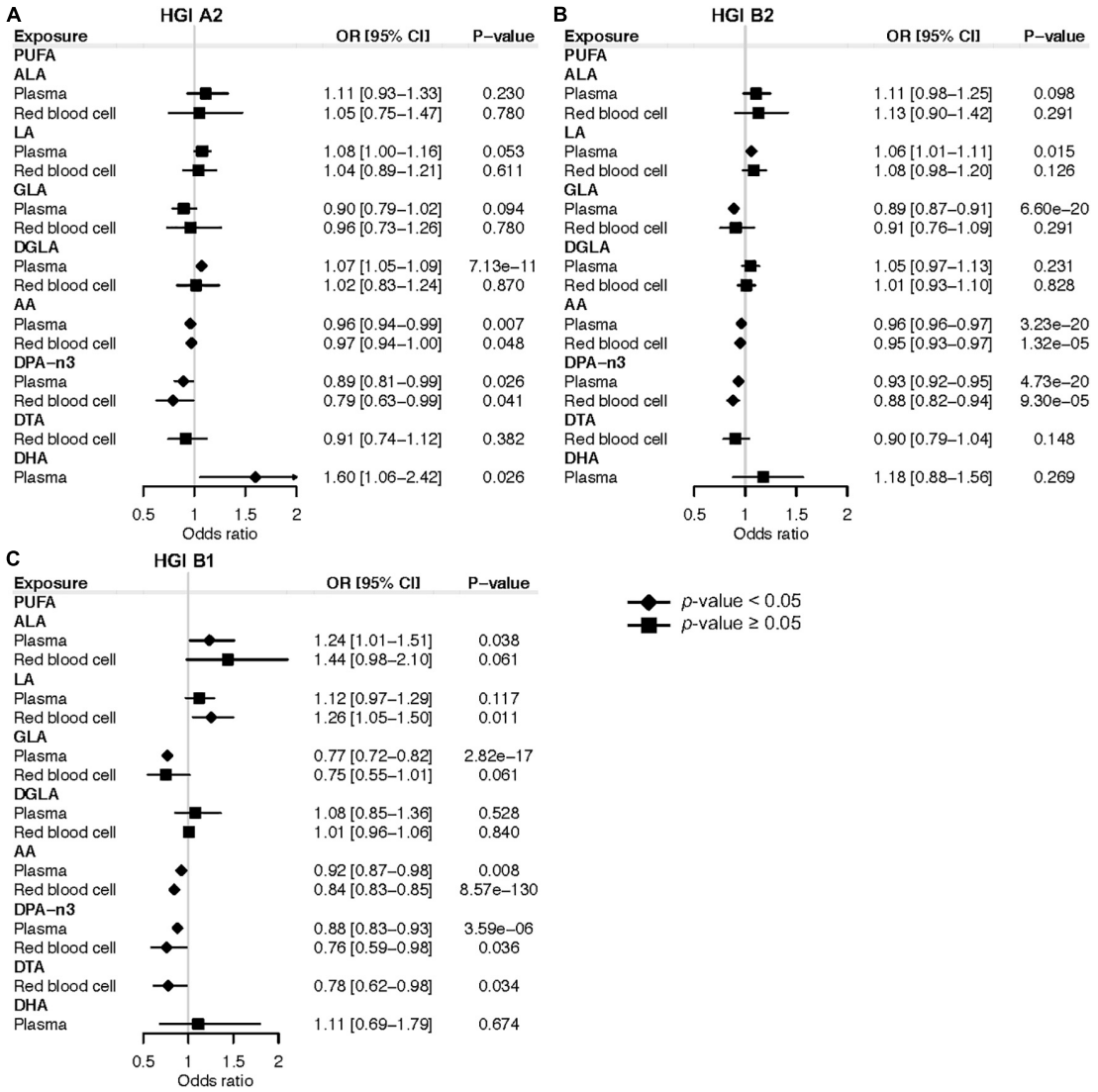

FIGURE 2 | Mendelian randomization estimates of the effects of polyunsaturated fatty acids on COVID-19 severity risk. **(A)** Mendelian randomization analysis based on the release 5 HGI A2. **(B)** Mendelian randomization analysis based on the release 5 HGI B2. **(C)** Mendelian randomization analysis based on the release 5 HGI B1. Odds ratios are scaled to a genetically predicted SD increase in polyunsaturated fatty acids. Associations with p-value < 0.05 were indicated with diamonds, while others with squares. Detailed summary statistics are available in **Supplementary Tables 6–8**. PUFA, polyunsaturated fatty acid; ALA, α-linolenic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; DPA-n3, docosapentaenoic acid; DTA, docosatetraenoic acid; DHA, docosahexaenoic acid; OR, odds ratio.

P = 0.026) were associated with a lower risk of very severe respiratory symptoms of COVID-19 based on HGI study A2 (**Figure 2A**). Consistently, genetically instrumented AA (OR: 0.96; 95% CI: 0.96, 0.97; $P = 3.23 \times 10^{-20}$) and DPA-n3 (OR: 0.93; 95% CI: 0.92, 0.95; $P = 4.73 \times 10^{-20}$) were associated with a lower risk of hospitalized COVID-19 based on HGI study B2, which used general population samples as the control (**Figure 2B**). Similar results were observed with HGI study B1, which used non-hospitalized COVID-19 patients as the control (**Figure 2C**). Besides plasma PUFAs, MR analyses with RBC PUFAs consistently support the protective effects of AA against severe COVID-19 based on HGI A2 (OR: 0.97; 95% CI: 0.94, 1.00; P = 0.048), B2 (OR: 0.95; 95% CI: 0.93, 0.97; $P = 1.32 \times 10^{-5}$), and B1 (OR: 0.84; 95% CI: 0.83, 0.85; $P = 8.57 \times 10^{-130}$) studies (**Figure 2**). For DPA-n3, its genetically instrumented RBC level was consistently associated with a lower risk of COVID-19 severity in our forward MR analysis with study A2 (OR: 0.79; 95% CI: 0.63, 0.99; P = 0.041), B2 (OR: 0.88; 95% CI: 0.82, 0.94; $P = 9.30 \times 10^{-5}$), and B1 (OR: 0.76; 95% CI: 0.59, 0.98; P = 0.036) (**Figure 2**). To ensure the robustness of findings, we selected genetic instruments based on various LD categories ($r^2 < 0.001$, 0.01, 0.1, and 0.3). The causal estimates of AA and DPA-n3 were consistent and at least nominally significant throughout all MR analyses (**Supplementary Tables 6–8**). Causal

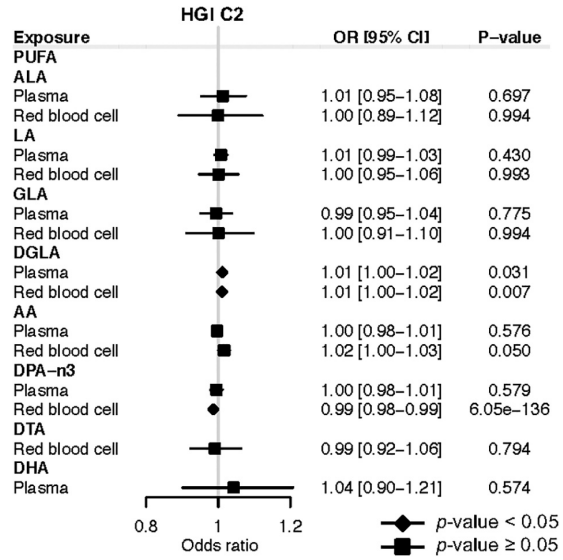

FIGURE 3 | Mendelian randomization estimates of the effects of polyunsaturated fatty acids on COVID-19 susceptibility risk based on the release 5 HGI C2. Odds ratios are scaled to a genetically predicted SD increase in polyunsaturated fatty acids. Associations with p-value < 0.05 were indicated with diamonds, while others with squares. Detailed summary statistics are available in **Supplementary Table 9**. PUFA, polyunsaturated fatty acid; ALA, α-Linolenic acid; LA, linoleic acid; GLA, γ-linoleic acid; DGLA, dihomo-γ-linoleic acid; AA, arachidonic acid; DPA-n3, docosapentaenoic acid; DTA, docosatetraenoic acid; DHA, docosahexaenoic acid; OR, odds ratio.

estimates for AA and DPA-n3 maintained the same effect directions in MR-Egger and WM methods, and sensitivity tests identified no evidence of horizontal pleiotropy or heterogeneity of effects (**Supplementary Tables 6–8**). Of note, while there were nominally significant associations between plasma DHA and very severe COVID-19 with HGI A2 and between RBC DTA and hospitalized COVID-19 with HGI B1, these two relationships were not replicated in analyses with the other two GWAS of severe COVID-19 (**Figure 2**).

In terms of COVID-19 susceptibility, we found that genetically instrumented one-SD increase of plasma DGLA (OR: 1.01; 95% CI: 1.00, 1.02; P = 0.031) was associated with an increased risk of any SARS-CoV-2 infection (**Figure 3**). MR analysis with RBC DGLA showed a similar pattern (OR: 1.01; 95% CI: 1.00, 1.02; P = 0.007). However, the association of genetically instrumented DGLA with the risk of testing positive for COVID-19 was not statistically significant using any other LD criteria for genetic instruments (**Supplementary Table 9**). Notably, our forward MR findings were confirmed using additional COVID-19 GWAS from HGI release 4 (**Supplementary Tables 10–13**). In summary, our forward MR analyses suggest that higher circulating levels of AA and DPA-n3 are associated with a lower risk of developing severe forms of COVID-19.

We further applied reverse MR analyses to investigate the causal effects of COVID-19 on each PUFA. Although several reverse MR analyses showed that genetically instrumented COVID-19 susceptibility or severity was associated with ALA, DHA, GLA, or DGLA, there was no consistent evidence for an effect of COVID-19 on these PUFAs using the conventional genome-wide significance threshold ($P < 5 \times 10^{-8}$) and the more lenient threshold ($P < 5 \times 10^{-6}$) for COVID-19 SNPs from HGI release 5 (**Supplementary Tables 14–21**). In addition, we used SNPs associated with COVID-19 from HGI release 4 and did not observe any causal effect of COVID-19 on PUFAs (**Supplementary Tables 22–29**). Importantly, the reverse MR results showed no significant association of genetically predicted COVID-19 severity with AA and DPA-n3, suggesting that the significant forward MR results are unlikely to be confounded by reverse causation. Lastly, we performed a supplemental and confirmatory MR analysis utilizing summary statistics of GWAS for four NMR-based plasma PUFA measures, omega-3 PUFAs, omega-6 PUFAs, DHA, and LA (45). The forward MR indicated that higher genetically predicted omega-3 PUFAs were associated with reduced risk of severe COVID-19 based on HGI release 5 study A2 (OR: 0.85; 95% CI: 0.72, 0.99; P = 0.034), B1 (OR: 0.76; 95% CI: 0.67, 0.86; $P = 8.81 \times 10^{-6}$), and B2 (OR: 0.85; 95% CI: 0.76, 0.95; P = 0.004) (**Supplementary Table 30**). No significant association was found for omega-3 PUFAs and COVID-19 susceptibility, nor for any other PUFA measures.

DISCUSSION

Our observational analysis in a prospective cohort showed that total PUFAs, omega-3 PUFAs, omega-6 PUFAs, DHA, and LA in baseline plasma samples were inversely associated with the risk of severe COVID-19. There were also inverse associations of omega-3 PUFAs and DHA with COVID-19 susceptibility. In contrast, the omega-6/omega-3 ratio was positively associated with both COVID-19 susceptibility and severity. A joint analysis of omega-6 PUFAs, omega-3 PUFAs, and their ratio further revealed that these effects were mainly driven by omega-3 PUFAs. In our bidirectional two-sample MR analyses, we provided evidence for the potential causal roles of higher circulating AA and DPA-n3 in a lower risk of COVID-19 severity.

Our observational findings are broadly consistent with previous observational studies and a pilot clinical trial. Julkunen et al. (13) also examined the UK Biobank cohort, although with smaller sample sizes and different controls. They showed that for total PUFAs, omega-3 PUFAs, omega-6 PUFAs, DHA, and LA, their absolute levels and relative percentages in total fatty acids were both inversely associated with the risk of severe COVID-19 when comparing patients to non-cases with unknown COVID-19 status. Our study corrected for potential selection bias by restricting the analysis to individuals with COVID-19 testing status and used those with negative tests or non-hospitalized patients as the controls. We confirmed the same inverse association patterns for severe COVID-19. We further showed that omega-3 PUFAs and DHA were inversely associated with COVID-19 susceptibility. Importantly, our joint analysis of omega-6 PUFAs, omega-3 PUFAs, and their ratio revealed that these effects were mainly driven by omega-3 PUFAs. Another study investigated the metabolic fingerprint of

COVID-19 severity in 581 samples from three cohorts, revealing inverse associations with severity for total PUFAs, omega-6 PUFAs, and LA. But inconsistent associations of omega-3 PUFAs, DHA, and the omega-6/omega-3 ratio were also observed across cohorts (11). Comparing the lipid profile of 42 severe COVID-19 patients to 22 healthy subjects, a study by Perez-Torres et al. (12) found that plasma GLA, DGLA, and EPA were decreased in COVID-19 patients, but LA and AA were elevated. Two small studies found that the omega-3 index was significantly lower in COVID-19 patients and was inversely associated with risks of requiring mechanical ventilation and death (9, 10). The differences in these observational studies are likely results of uncontrolled confounding factors or the usage of patients at different disease stages. In support of the associated protective effect of omega-3 fatty acids, the first randomized clinical trial of supplementing 1,000 mg omega-3 fatty acids in 128 critically ill COVID-19 patients showed that the intervention group had a significantly higher 1-month survival rate and improved respiratory and renal function (46). Altogether with the existing literature, our study supports the protective effects of omega-3 fatty acids against the development of severe COVID-19 and likely also against viral infection.

In our MR study, we examined whether specific individual PUFAs play causal roles in COVID-19 susceptibility and severity. We found that genetically instrumented circulating levels of AA and DPA-n3 are associated with a lower risk of severe COVID-19. AA is an omega-6 fatty acid, while DPA-n3 is an omega-3 fatty acid. Although these two specific PUFAs were not available in our observational analysis, their potentially causal protective effects are consistent with the inverse associations of both omega-6 PUFAs and omega-3 PUFAs with severe COVID-19. The potential protective roles of AA and DPA-n3 in severe COVID-19 have mechanistic support. It is usually generalized that omega-6 PUFAs are precursors to pro-inflammatory signaling molecules, such as the AA-derived prostaglandins (PGs) and leukotrienes, while omega-3 PUFAs, mainly EPA, DPA-n3, and DHA, give rise to anti-inflammatory signaling molecules, such as resolvins, protectins, and maresins. However, the underlying biochemistry and signaling pathways are complex, depending on specific mediating molecules and timing of actions (7, 47). First, both AA and DPA-n3 may modulate the inflammatory process and prevent the development of cytokine storm in COVID-19 patients. Both of them are well-known to serve as precursors of specialized pro-resolving mediators. In addition to resolvins, protectins, and maresins derived from DPA-n3, lipoxins derived from AA play essential roles in promoting the resolution of inflammatory responses and tissue repair (5, 7, 48). Notably, it has been highlighted that the roles of AA in initiating timely inflammatory responses through its derived PGs, such as PGE_2, may be as important as its roles in inflammatory resolution through lipoxins (6, 47). Second, both AA and DPA-n3 may inhibit virus entry into host cells. LA has been shown to directly and tightly bind the SARS-CoV-2 spike glycoprotein, reducing its interaction with the human ACE2 receptor (49). Similar inhibitory effects were observed for ALA, EPA, and DHA in a ligand screening study (50), which did not include AA and DPA-n3. Third, AA may suppress virus replication in host cells. In a pre-pandemic lipidomics study aiming to comprehensively characterize the host cell lipid response upon coronavirus infection, Huh-7 cells, a hepatocyte-derived carcinoma cell line, when infected with human coronavirus 229E (HCoV-229E), exhibit significantly elevated levels of LA and AA (51), a pattern that is also observed in a recent study of severe COVID-19 patients (12). Interestingly, exogenous supplementation of LA and AA in HCoV-229E-infected cells significantly decreased the virus genome copies in both cell lysates and supernatants, suggesting that LA and AA suppressed HCoV-229E virus replication. Similar suppressive effects were observed for the highly pathogenic Middle East respiratory syndrome coronavirus (MERS-CoV) (51), suggesting a general mechanism of LA and AA on coronavirus. Consistently, it has been known that unsaturated fatty acids, especially AA, can inactivate enveloped viruses, such as influenza and HIV (47). Our MR findings call for future studies into the mechanistic roles of AA and DPA-n3 in the development of severe COVID-19.

Our study has a number of strengths and novel features. Most notably, our study integrates two complementary research approaches, an observational analysis in a prospective cohort and a MR analysis. The observational analyses used, to our knowledge, the largest sample size to date. We also applied multiple comparisons and controls to significantly increase the credibility of the results. The two research approaches revealed consistent patterns. While the observational analyses highlighted omega-3 PUFAs to be negatively associated with both COVID-19 severity and susceptibility, our MR analyses confirmed that total omega-3 PUFAs and DPA-n3 may play causal roles in reducing the risk of severe COVID-19. There are multiple strengths associated with our MR analyses. To our knowledge, this is the first MR study examining the causal effects of PUFAs on COVID-19. It is also the first MR study of PUFAs that used genetic variants for RBC PUFAs, in addition to plasma PUFAs. RBC and plasma PUFAs are two lipid pools that reflect dietary input at varying time frames ranging from months to weeks, with RBC PUFAs reflecting longer-term dietary input and plasma PUFA more impacted by recent dietary intakes. There are medium to high correlations between PUFAs measured in the two sources (52–54). The inclusion of both RBC and plasma PUFAs has at least two benefits. It expanded the list of exposures to include those that only have genetic instruments in one source, including DTA in RBC and DHA in plasma. For PUFAs having genetic instruments in both sources, we only reported consistently significant results to reduce false positives. To obtain robust evidence and to ensure reproducibility across data releases, we confirmed the results with analyses based on four COVID-19 GWAS (A2, B2, B1, and C2) from HGI releases 5 and 4. Bonferroni correction was used to overcome the issue of multiple testing. We also applied sensitivity analysis with various LD cutoffs. Another strength is the application of bidirectional two-sample MR analyses to evaluate the direction of the causality and to rule out the impacts of reverse causation. Additionally, comparing our MR results between severe COVID-19 and any SARS-CoV-2 infection, we found that AA and DPA-n3 might mainly impact the severity of disease progression but not susceptibility to viral infection.

Our study has several limitations. First, we could not completely rule out the possibility that some genetic variants might be pleiotropic, although we applied multiple sensitivity analyses, including the heterogeneity test, MR-Egger, and WM method. We also applied the PhenoScanner to examine the pleiotropic effects of genetic instruments for AA and DPA-n3, which might provide alternative explanations for our MR observations (**Supplementary Table 31**) (55, 56). However, it is still difficult to distinguish if they represent horizontal or vertical pleiotropic effects. Second, another limitation in the MR analysis is that the population controls have no information on COVID-19 status in three COVID-19 GWAS used in our primary analysis, including the HGI A2, B2, and C2 studies. To mitigate this issue, we also utilized the HGI B1 study, which is another GWAS of COVID-19 using non-hospitalized patients as the control group. Third, dietary intakes of specific PUFAs, which influences their circulating levels, were not available in UK Biobank. So, our observational analysis did not investigate the direct or indirect effects of dietary PUFAs on COVID-19 risk. However, our MR study leveraging genetic instruments yields novel insights into their possible roles. Fourth, UK Biobank recruited healthier individuals and thus may not be representative of the general population. Fifth, the NMR-based measures of plasma PUFAs were collected over 10 years before the COVID-19 pandemic, and the time lag probably attenuates the magnitude of association. Sixth, the NMR-based method only measured two individual PUFAs (DHA and LA), while many other individual PUFAs (e.g., AA, ALA and EPA) were not available for the observational analyses. Notably, our MR study alleviates this limitation by using eight individual PUFAs (i.e., ALA, DPA-n3, DHA, LA, GLA, DGLA, AA, and DTA) from both RBC and plasma. Seventh, our study did not examine saturated or monounsaturated fatty acids. A previous study in UK Biobank found that the percentages of these two groups are both positively associated with the risk of severe COVID-19 (13). Eighth, our observational study could be affected by ascertainment bias in differential healthcare seeking and testing. Being an inpatient does not necessarily indicate hospitalization for COVID-19 because patients in hospitals for any reason may be prioritized for COVID-19 testing. Hospitalized patients and the observed effects of PUFAs might be driven by other diseases instead of COVID-19. One possible mitigation analysis is to use hospitalized non-COVID-19 patients as the control, which was not analyzed in this study. Ninth, our findings might not be extrapolated to other ethnicities because the study mainly focused on participants of European descent. Future studies in large non-European samples are needed to test the generalizability of our observations. Tenth, our study cannot thoroughly explain the mechanisms. Further mechanistic research is necessary to investigate the biological pathways underpinning the roles of PUFAs in severe COVID-19.

CONCLUSION

Our observational analysis in a prospective cohort shows that total PUFAs, omega-3 PUFAs, omega-6 PUFAs, DHA, and LA are inversely associated with the risk of severe COVID-19. Omega-3 and DHA may also be protective against SARS-CoV-2. A higher omega-6/omega-3 ratio has adverse effects on both COVID-19 susceptibility and severity. These associations are mainly driven by omega-3 PUFAs. Our MR study further suggests a possible causal role of AA and DPA-n3 in reducing the risk of severe COVID-19. Our findings call for further studies into the mechanistic roles of PUFAs in COVID-19. They also support the possible usage of circulating PUFA levels as biomarkers for identifying high-risk individuals and as therapeutic targets for managing COVID-19 patients.

DATA AVAILABILITY STATEMENT

The datasets (GWAS summary statistics) of COVID-19 analyzed for this study can be found in the COVID-19 Host Genetics Initiative (https://www.covid19hg.org/). The code for the analyses is available at https://github.com/yitangsun/COVID19_PUFA_MR.

AUTHOR CONTRIBUTIONS

YS and KY designed the study, provided statistical advice, and interpreted the data. YS performed data analysis, prepared visualizations, and wrote the manuscript. YS, RC, and AR provided material support during the study. KY critically revised the manuscript. All authors read and approved the final manuscript and took responsibility for the integrity of the work as a whole.

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SUPPLEMENTARY MATERIAL

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Epidemiology and Economic Burden of Continuing Challenge of Infectious Diseases in India: Analysis of Socio-Demographic Differentials

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Unlike other low- and middle-income countries, infectious diseases are still predominant, and non-communicable diseases (NCDs) are emerging without replacing the burden of infectious diseases in India, where it is imposing a double burden of diseases on households in the country. This study aimed to analyse the socio-economic and demographic differentials in the magnitude of economic burden and coping strategies associated with health expenditure on infectious diseases in India. National Sample Survey Organization (NSSO) data on "Key Indicators of Social Consumption in India: Health, (2017–18)" have been employed in this study. The findings of the study revealed that more than 33% of the individuals are still suffering from infectious diseases out of the total ailing population in India. Based on the various socio-economic and demographic covariates, infectious diseases are highly prevalent among individuals with marginalized characteristics, such as individuals residing in rural areas, females, 0–14 age groups, Muslims, illiterates, scheduled tribes (STs), and scheduled castes (SCs), large family households, and economically poor people in the country. The per capita out-of-pocket (OOP) expenditure on infectious diseases is INR 7.28 and INR 29.38 in inpatient and outpatient care, respectively. Whereas, monthly per patient OOP expenditure on infectious diseases by infection-affected populations is INR 881.56 and INR 1,156.34 in inpatient and outpatient care in India. The study found that people residing in rural areas, SCs followed by other backward classes (OBCs), illiterates, poor, and very poor are more dependent on borrowings, sale of assets, and other distressed sources of financing. However, under National Health Policy 2017, many initiatives, such as "Ayushman Bharat," PM-JAY, and National Digital Health Mission (NDHM) in 2021, have been launched by the government of India in the recent years. These initiatives are holistically launched for ensuring better health facilities, but it is early to make any prediction regarding its outcomes; hopefully, the time will define it over the passing of a few more years. Finally, the study proposed the need for proper implementations of policy initiatives, awareness against unhygienic conditions and contamination of illnesses, immunisations/vaccination campaigns, subsidized medical facilities, and the country's expansion of quality primary health-care facilities.

Keywords: infectious diseases, out-of-pocket expenditure, source of finance, coping, low- and middle-income countries

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INTRODUCTION

The narratives of public health are facing a significant challenge in demographic and epidemiological transitions, particularly in low- and middle-income countries (LMICs) (1–3). This transition has changed the pattern and distribution of morbidity and mortality among inhabitants and exaggerated the burden on these countries' pre-existing inadequate public health systems (1). Although several life-threatening diseases have been cured through various preventive, curative, and policy measures, infectious diseases are still one of the leading causes of death in LMICs (4, 5). These are the diseases caused by pathogenic microorganisms, such as viruses, bacteria, protozoa, parasites, and fungi. It spreads through direct or indirect interaction with unhygienic conditions (6, 7). Adverse living conditions are expected in LMICs, further augmenting the number and severity of infectious diseases in these countries (7–11).

World Health Organization (WHO) has ranked infections, such as lower respiratory infections, diarrheal diseases, and tuberculosis, in the top 10 causes of mortality worldwide in 2016. Most of the burden of these diseases has been observed in LMICs (12, 13). HIV/AIDS is still a significant cause of death in LMICs but left out from the list of top ten causes of death from 2000 to 2016 worldwide (13). Similarly, malaria prevalence has been reduced due to massive investment and policy initiatives in the past years. However, eliminating malaria is still a big challenge in most LMICs (14). New infectious diseases, such as avian flu, swine flu, and coronavirus, are also emerging at a higher rate and spreading more rapidly than ever in the communities (15, 16).

India is not an exception in such a transition. However, unlike other LMICs, infectious diseases are still predominant, and non-communicable diseases (NCDs) are emerging without replacing the country's burden of contagious diseases (17, 18). This double burden of diseases is a serious public health concern where NCDs are continuously increasing with prevalent contagious diseases, a significant cause of premature mortality among people in the country (17–21). Various long-standing infectious diseases, such as smallpox, guinea worm, polio, leprosy, cholera, and plague, have been controlled and eradicated from the community. However, diseases, such as dengue, malaria, typhoid, and tuberculosis, are still the common causes of febrile illness among people in India (17, 22, 23). In tuberculosis, India is the top country globally, with a 26% share of this disease in the global burden of diseases (13). After South Africa, India is the leading country suffering from HIV/AIDS worldwide, where 4.6 million people are infected with this life-threatening disease (13, 24, 25). Even among neonatal, the frequency of infectious diseases, such as typhoid, diarrhea, measles, tuberculosis, and jaundice, remains the primary cause of infant morbidity and mortality in India. The findings from various studies show that the burden of NCDs has increased from 37.9 to 61.8%, whereas the burden of communicable and infectious diseases is still at 27.5% for three decades in the country (21, 26).

Similarly, in a study, Banerjee and Dwivedi (27) observed that the prevalence of infectious diseases had slightly reduced between 2004 and 2014. But it is still stagnant and has become a significant challenge to public health. However, Paul and Singh (28) observed that the prevalence of infectious diseases in outpatient care increased nearly three times, from 8 to 26 per 1,000 in two decades (1995 to 2014). Also, typhoid fever incidence has been reported at 27.3 at the age under 5 years, 11.7 at 5 to 19 years, and 1.1 between 19 and 40 years per 1,000 person among residents of a low-income urban area of Delhi, India (29).

It is clear from the above discussion that infectious diseases are still contributing to both the physical and the economic burden of diseases in India (30). However, the Indian economy is one of the fastest-growing economies globally with a 6–7% annual average gross domestic product (GDP) growth rate but spends only 1%of its GDP on health (31, 32). Insufficient health insurance coverage also plays a crucial role in contributing to the country's increasing economic burden of diseases (33). This meager amount of public health spending and under-coverage of health insurance increases the household's dependence on out-of-pocket expenditure. The households' inability to cope with the economic burden of diseases pushes them into poverty (34–36).

Differences and vulnerabilities based on the socio-economic characteristics are common phenomena among the Indian population. Due to these vulnerabilities, the emergence and re-emergence of infectious diseases have been consistent among inhabitants for centuries. However, many contagious diseases have not even been controlled but also have been eliminated from society. Despite it, many of them are still more susceptible to the population in the country. Also, these diseases do not harm only individuals' health status but also impose an economic burden. A few studies, such as Visaria (21), Banerjee and Dwivedi (27), and Paul and Singh (28), have conducted an analysis of infectious diseases using nationally representative sample survey data but explored only the prevalence and its association with background characteristics. These studies lack to analyse the economic burden, especially out-of-pocket expenditure and finance's primary source, to cope with spending on infectious diseases. Considering the importance of the associated socio-economic covariates and economic dimensions compels us to analyse the problem accordingly. Hence, the study aims to analyse the prevalence and financial burden of out-of-pocket expenditure and source of finance on infectious diseases among various socio-economic and demographic covariates in India. Also, it is expected that the results of this study would provide a deep insight into the problem, which will work as a roadmap for the policymakers to control the same through appropriate policy initiatives.

METHODOLOGY

Data

The analysis is based on cross-sectional data from the National Sample Survey Organization (NSSO), 75th Round (2017–2018) on Key Indicators of Social Consumption in India:

Abbreviations: OOP, out-of-pocket; INR, Indian National Rupee; LMICs, low- and middle-income countries; SCs, scheduled castes; STs, scheduled tribes; OBCs, other backward classes; TCE, total consumption expenditure; NSSO, National Sample Survey Organization; HIV/AIDS, human immune virus/acquired immune deficiency syndrome; GDP, gross domestic product; NHP, national health policy.

Health (37). The survey consists of a sample of 113,823 households comprising 555,115 individuals. In inpatient care, 19,443 individuals, out of 58,214 ailments affected sample persons, reported suffering from at least any infectious diseases during the recall period of 365 days. While on outpatient care, 10,960 individuals, out of 39,778 ailments affected sample persons, reported suffering from at least any infectious conditions during 15-day recall period. Furthermore, all contagious diseases have been analyzed collectively as infectious diseases both in inpatient and outpatient care in India.

Methods

In this study, the prevalence (P_i) of infectious diseases has been calculated as follows: $Pi = \frac{1}{N}\sum_{i=1}^{n} I$, where "$N$" is the population size and "I" is the number of individuals affected by infectious diseases.

The economic burden of infectious diseases among various socio-economic covariates has been measured in terms of out-of-pocket expenditure on infectious diseases as a percentage share of total consumption expenditure (TCE) is given by: $OOP_{TCE} = \sum_{i=1}^{n} OOP_i / \sum_{i=1}^{n} MPCE_i \times 100$, where "$OOP_{(TCE)}$" stands for out-of-pocket expenditure as a percentage share of total consumption expenditure, "OOP_i" is the out-of-pocket expenditure on infectious diseases, and "$MPCE_i$" is the monthly per capita consumption expenditure of the ith individual.

While out-of-pocket expenditure on infectious diseases as a percentage share of total consumption expenditure (TCE) of a country's infection affected population at different threshold levels (reporting level, 5, 10, and 15%) is given by $OOP_{TCE_{iap}} = \sum_{i=1}^{n} OOP_i / \sum_{i=1}^{n} MPCE_{iap} \times 100$, where "$OOP_{TCE_{iap}}$" is the out-of-pocket expenditure on infectious diseases and "$MPCE_{iap}$" is the monthly per capita consumption expenditure of the country's infection-affected population.

Also, the average per capita OOP health expenditure on the infection has been measured as; $Per\ Capita\ OOP = \sum_{i=1}^{n} OOP_i/N$, where "$OOP_i$" is the out-of-pocket expenditure on infectious diseases and "N" denotes the total population. Furthermore, average per capita OOP expenditure on infection by the individuals particularly suffering from infectious diseases has been measured as; $Per\ Patient\ OOP = \sum_{i=1}^{n} OOP_i / \sum_{i=1}^{n} Q$, where "$OOP_i$" is the out-of-pocket expenditure on infectious diseases and 'Q' is the number of people affected with infectious diseases.

Finally, the source of finance to cope with OOP expenditure on infectious diseases has also been measured. Savings/income, borrowings, contributions from friends and relatives, sale of physical assets, and other resources have been taken as the individuals' key strategies or sources of finance. In the case of inpatient care, information on coping strategies has been given as the first and second primary sources of finance. While on outpatient care, the only first significant source of finance has been shown in the data source. Therefore, to ensure the similarities in inpatient and outpatient care, only the first important source of finance has been taken in the analysis. Besides the critical coping strategies, such as savings/income and borrowings, the remaining basis of finance has been taken collectively due to their less significant share and simplification of the analysis. The percentage of different sources of finance used to cope with the OOP health expenditure on the infection has been calculated as $Y = \sum_{i=1}^{n} U/V * 100$.

where "Y" is the percentage share of a source of finance, "U" is the source of finance, and "V" is the sum of all sources of finance.

Variables of the Study

Dependent Variable

The study's dependent variable is morbidity due to infectious diseases among inpatient and outpatient care individuals.

Independent Variable

In this analysis, the independent variables are the place of residence (rural/urban), sex (male, female, and transgender), age (0–14, 15–29, 30–59, and >60), education (illiterate, up to primary, secondary and graduation, and above), religious groups (Hindu, Muslims, and others), social groups (scheduled tribes, scheduled castes, other backward classes, and others), economic status (wealth quintiles, such as very poor, poor, average, rich, and very rich), households size (less than average and more than average), and region (north, north-east, east, central, west, and south). In Indian society, religion is one of the critical variables broadly divided into Hindus, Muslims, Sikhs, Buddhism, Christianity, Jainism, Zoroastrianism, and many smaller religious groups. But in this analysis, we have classified religion into three major categories, *viz*. Hindus, Muslims, and the remaining have been included in other religious groups because of their small size in the total population. The monthly per capita consumption expenditure (MPCE) has been taken as a proxy for income to measure the economic status of the individuals. It has been ranked from very poor to a very rich one. The analysis categorizes households' sizes into less than average and more than average. Furthermore, all states and union territories have been characterized into six regions based on their geographical locations: north, north-east, east, central, west, and south.

RESULTS OF THE STUDY

Summary Statistics

The details of sample persons and the respective estimated population in different demographic and socio-economic categories, i.e., place of residence, sex, age, religion, social classification, economic status, etc., have been given in **Table 1**. A total of 555,115 sample persons have been surveyed, representing the 1,140,187,554 total population of the country. Individuals suffering from various ailments have been shown in two categories, i.e., inpatient and outpatient. Where 58,214 individuals reported suffering from multiple diseases and were hospitalized during the last 365 days before the day of the survey, 19,443 individuals stated that they were affected by infectious ailments during this time. While on 15-day recall

period, a sample of 39,778 persons reported suffering from various diseases, out of which only 10,960 were affected with infectious diseases during this period.

Prevalence Rate and the Proportion of Population Spending on Infectious Diseases

Table 2 shows that out of 1,000 persons, 8.26 persons reported infectious diseases in inpatient care, which is 33.90% of the country's total ailing population. Furthermore, the percentage share of population spending and their income regarding their total consumption expenditure (TCE) on infectious diseases has been measured at different threshold levels. The finding shows that nearly 0.66, 0.54, and 0.45% of the population suffering from infections are spending more than 5, 10, and 15% of their TCE on infectious diseases in India. The analysis shows a significant variation among demographic and socio-economic covariates in the prevalence of infectious diseases. Therefore, the association of several demographic and socio-economic variables with infectious diseases has been analyzed in the study. The prevalence is highest among individuals residing in the urban area (10.05 individuals), among transgender people (24.92 individuals), 60+ age group (12.21 individuals), other religious groups (10.78 individuals), other social groups (9.12 individuals), illiterate people (10.19 individuals), and people coming from the southern part of the country (12.19 individuals) than their respective correspondents in the study.

Similarly, in outpatient care, the prevalence has been reported at 25.41 persons out of 1,000 persons in India and is 33.97% of the country's total ailing population. Furthermore, the findings reveal that instead of their absolute numbers, nearly 2.13, 1.84, and 1.63% population suffering from infections are spending more than 5, 10, and 15% of their TCE on infectious diseases in India. Whereas, based on various socio-economic and demographic variables, the prevalence and percentage of people suffering from infectious diseases is highest among people residing in rural areas (25.58 individuals), among females (26.53 individuals), among 0–14 age groups (40.04 individuals), among other religious groups (29.83 individuals), more than average family size (26.97), among SCs (26.60 individuals), among the central region of the country (31.93 individuals) than their corresponding variables in the analysis. Also, the proportion of people suffering from infectious diseases out of the total ailing population is highest among people residing in the rural area, male, 0–14 age group, Muslims, scheduled tribes (STs), education up to secondary level, very poor economic status, large family size, and northern regions of the country. However, a similar trend has been found in the percentage and prevalence of infection-affected populations among various socio-economic and demographic variables. Still, it differs in the case of absolute numbers of people suffering from infections and the population spending OOP expenditure equal to their TCE at various threshold levels in the study. The analysis shows that the dependent age groups, such as 60+ and 0–14, are more suffering from infectious diseases. While education emerges as a critical preventive factor, an increase in the number of years spent in an educational institution positively impacts the dominance of infectious diseases in communities. Further, the prevalence of infectious diseases is growing, with an increase in India's economic level. In comparison to the emergence of the ailments, it is describing the ability to report and access healthcare facilities by the economically sound section of the society in India. Indeed, these figures confirm the notion that people belonging to low-income families may not be able to hospitalize their family members during severe life-threatening ailments due to their insufficient financial resources or low economic status in India.

Level of Out-of-Pocket Expenditure on Infectious Diseases

Table 3 shows that in the case of inpatient care, the overall average per capita OOP expenditure on infectious diseases is INR 7.28 in the country. Whereas, for various socio-economic and demographic variables, it is highest among urban areas (INR 10.42), among transgender people (INR 8.57), among 60+ age groups (INR 11.64), among other religious groups (INR 9.35), among different social groups (INR 10.34), among educated up to secondary level (INR 8.98), among economically rich people (INR 15.38), among less than average households (INR 7.98), and people populated in the southern region (INR 9.72) of India. Furthermore, we have also calculated the average monthly per capita OOP expenditure of infection-affected population. Results show that, on average, INR 881.56 has been spent per month on inpatient care in India. The distinction between various socio-economic and demographic covariates has been found in the analysis. The average monthly per capita OOP expenditure on infectious diseases is relatively low among people residing in rural areas, transgender people, Muslims, STs, people educated up to primary level and illiterates, economically poor, and people from the north-east region of the country.

The OOP health expenditure on infectious diseases as a share of total consumption expenditure has been calculated as 0.34% in the study. On various demographic and socio-economic variables, it is relatively highest among individuals residing in rural areas (0.36%), females (0.34%), above 60+ (0.47%), and Hindus (0.35%). Among different social groups, it is 0.36% in other social groups. Among different education groups, illiterates (0.49%), among various economic groups, very poor wealth quintile (0.41%), among other households' size, more than average (0.37%), and among different geographical regions, eastern region (0.53%) spends a higher share of TCE on infectious diseases as compared to their respective counterparts in the country. On the other hand, OOP health expenditure on infectious diseases out of total consumption expenditure of infection-affected population has also been measured at different threshold levels in the study. The analysis illustrates that 36.04% of total consumption expenditure is spent on infectious diseases by the country's infection-affected population at the reporting level. It has been observed at different threshold levels: 31.59, 28.06, and 25.23%, with their corresponding levels as 5, 10, and 15%, respectively. The analysis shows that it declines with an increase in the infection's consumption expenditure from reporting to above threshold levels.

Similarly, India's overall average per capita OOP health expenditure on infectious diseases in outpatient care is INR 29.38. Furthermore, based on numerous socio-economic and

TABLE 1 | Summary statistics of the sample population.

Variables	Estimated and sample population	Inpatient		Outpatient	
		Total ailing population	Population ailing from infectious diseases	Total ailing population	Population ailing from infectious diseases
All India	1,140,187,554	27,783,232	9,419,082	85,269,522	28,969,799
	(555,115)	(58,214)	(19,443)	(39,778)	(10,960)
Place of residence					
Rural	804,273,325	17,989,881	6,041,486	54,781,294	20,571,709
	(325,883)	(32,441)	(11,048)	(20,802)	(6,506)
Urban	335,914,229	9,793,351	3,377,596	30,488,228	8,398,090
	(229,232)	(25,773)	(8,395)	(18,976)	(4,454)
Sex					
Male	589,257,319	14,075,161	4,833,223	39,474,970	14,352,788
	(283,200)	(30,033)	(10,076)	(18,948)	(5,372)
Female	550,864,001	13,704,591	4,584,209	45,793,674	14,617,011
	(271,878)	(28,177)	(9,365)	(20,829)	(5,588)
Transgender	66,234	3,480	1,650	878	0
	(37)	(4)	(2)	(1)	0
Age					
0–14	301,230,284	4,676,254	2,827,072	17,561,555	12,063,353
	(155,647)	(10,208)	(6,322)	(7,946)	(5,162)
15–29	318,486,584	5,198,084	1,996,661	10,555,762	5,921,053
	(152,909)	(10,750)	(4,215)	(4,316)	(2,044)
30–59	441,153,086	12,628,168	3,626,929	35,162,767	8,485,415
	(203,797)	(26,185)	(7,032)	(15,746)	(2,792)
60+	79,317,600	5,280,726	968,420	21,989,438	2,499,978
	(42,762)	(11,071)	(1,874)	(11,770)	(962)
Religion					
Hindu	925,019,675	22,107,923	7,399,669	66,622,950	22,844,158
	(412,512)	(44,026)	(14,485)	(29,273)	(8,089)
Muslim	161,096,251	3,939,529	1,436,344	13,039,977	4,512,422
	(83,001)	(7,805)	(2,534)	(6,750)	(1,901)
Others*	54,071,628	1,735,780	583,069	5,606,595	1,613,219
	(59,602)	(6,383)	(2,424)	(3,755)	(970)
Social groups					
STs	103,490,979	1,710,964	658,676	5,221,281	2,421,788
	(75,256)	(6,885)	(2,998)	(2,783)	(1,079)
SCs	223,840,510	5,296,687	1,824,955	15,555,824	5,955,188
	(94,062)	(9,621)	(3,228)	(6,502)	(bib2,046)
OBCs	512,112,220	12,169,154	4,191,417	36,144,771	13,170,645
	(222,766)	(23,369)	(7,713)	(15,943)	(4,648)
Others	300,743,845	8,606,427	2,744,034	28,347,646	7,422,178
	(163,031)	(18,339)	(5,504)	(14,550)	(3,187)
General education					
Illiterate	297,266,691	8,996,019	3,028,885	30,587,274	10,580,025
	(147,275)	(17,117)	(5,694)	(14,156)	(4,503)
Up to primary	498,126,420	11,108,187	3,989,067	34,039,172	12,678,283
	(226,320)	(23,076)	(8,429)	(15,038)	(4,246)

(Continued)

TABLE 1 | Continued

Variables	Estimated and sample population	Inpatient		Outpatient	
		Total ailing population	Population ailing from infectious diseases	Total ailing population	Population ailing from infectious diseases
Secondary	250,785,824	5,703,816	1,819,081	14,596,197	4,314,580
	(126,888)	(12,880)	(3,946)	(7,260)	(1,646)
Graduation and above	94,008,619	1,975,210	582,049	6,046,879	1,396,911
	(54,632)	(5,141)	(1,374)	(3,324)	(565)
Wealth quintile					
Very poor	534,658,598	9,524,508	3,358,934	30,175,229	14,049,087
	(218,202)	(18,152)	(6,414)	(10,970)	(4,309)
Poor	268,007,766	6,975,140	2,318,274	19,937,428	6,601,036
	(134,525)	(13,823)	(4,616)	(9,342)	(2,709)
Average	164,381,344	5,179,085	1,764,899	15,379,224	4,368,359
	(97,343)	(11,484)	(3,869)	(8,123)	(1,966)
Rich	107,479,414	3,857,699	1,301,897	12,010,674	2,613,975
	(67,558)	(8,777)	(2,797)	(6,785)	(1,312)
Very rich	65,660,432	2,246,800	675,078	7,766,967	1,337,342
	(37,487)	(5,978)	(1,747)	(4,558)	(664)
Households size**					
Less than average	687,672,512	18,922,107	6,515,377	57,874,634	18,547,100
	(289,829)	(39,646)	(13,530)	(23,870)	(6,173)
More than average	452,515,042	8,861,125	2,903,705	27,394,888	10,422,699
	(265,286)	(18,568)	(5,913)	(15,908)	(4,787)
Regions***					
North	160,836,817	3,768,445	1,174,847	10,385,958	3,809,508
	(105,232)	(10,349)	(3,030)	(7,187)	(2,009)
Northeast	44,044,744	658,172	259,272	1,025,085	490,600
	(72,324)	(7,129)	(2,926)	(1,436)	(581)
Central	284,261,422	5,362,634	1,923,078	18,086,039	9,075,577
	(106,814)	(9,520)	(3,156)	(6,416)	(2,497)
East	250,025,083	5,526,488	1,645,190	19,914,913	6,697,091
	(94,334)	(9,632)	(2,811)	(7,649)	(2,269)
West	159,468,994	4,116,808	1,471,742	12,813,455	4,201,608
	(68,771)	(7,457)	(2,631)	(5,313)	(1,374)
South	241,550,494	8,350,685	2,944,953	23,044,072	4,695,415
	(107,640)	(14,127)	(4,889)	(11,777)	(2,230)

Figures are based on the author's calculations from the NSSO 75th rounds.

Values in parentheses are sample sizes.

[*] Include remaining religions such as Christianity, Sikhism, Jainism, Buddhism, Zoroastrianism, and others.

[**] Households size is categorized into two categories viz. less than average and more than average.

[***] North: J&K, Himachal Pradesh, Punjab, Chandigarh, Uttarakhand, Haryana, Delhi, Rajasthan; Northeast: Sikkim, Arunachal Pradesh, Nagaland, Manipur, Mizoram, Tripura, Meghalaya, Assam; East: Bihar, West Bengal, Jharkhand, Odisha; Central: Utter Pradesh, Chhattisgarh, Madhya Pradesh; West: Gujrat, Daman, and Diu, Dadar and Nagar Haveli, Maharashtra, Goa; South: Andhra Pradesh, Karnataka, Lakshadweep, Kerala, Tamil Nadu, Pondicherry, Andaman and Nicobar, Telangana.

demographic covariates, it is highest among urban areas (INR 32.65), among women (INR 30.05), among 0–14 age groups (INR 41.46), among other religious groups (INR 35.12), among different social groups (INR 31.09), among illiterate people (INR 38.63), among economically wealthy people (INR 38.95), among less than average households (INR 30.94), and people populated in the eastern region (INR 38.52) of India. At the same time, we calculated the average monthly per capita OOP expenditure of infection-affected population. Results show that INR 1,156.34 has been spent per month on outpatient care in India. Unlike inpatient care, it is highest in urban areas, rich wealth quintiles, and relatively weaker sections (socio-economically), such as female, 0–14 age groups, and Muslims, OBCs, illiterates' individuals than their corresponding counterparts in India. Furthermore, OOP health expenditure on infection as a percentage share of total consumption expenditure

TABLE 2 | Socio-economic and demographic covariates in the prevalence rate (out of 1,000), and the population spending OOP as a percentage of total consumption expenditure (TCE) on infectious diseases at various thresholds levels in India (2017–18).

Variables	Inpatient					Outpatient				
	Prevalence of infectious diseases affected population (out of 1,000)	Percentage of individuals suffering from infectious diseases out of total ailing population	Population spending more than 5% of TCE on infectious diseases[#]	Population spending more than 10% of TCE on infectious diseases[#]	Population spending more than 15% of TCE on infectious diseases[#]	Prevalence of infectious diseases affected population (out of 1,000)	Percentage of individuals suffering from infectious diseases out of total ailing population	Population spending more than 5% of TCE on infectious diseases[#]	Population spending more than 10% of TCE on infectious diseases[#]	Population spending more than 15% of TCE on infectious diseases[#]
All India	8.26	33.90	7,566,479 (0.66)	6,206,458 (0.54)	5,118,207 (0.45)	25.41	33.97	24,313,160 (2.13)	21,028,176 (1.84)	18,607,805 (1.63)
Place of residence										
Rural	7.51	33.58	4,908,874 (0.61)	4,109,357 (0.51)	3,458,224 (0.43)	25.58	37.55	17,686,381 (2.20)	15,389,695 (1.91)	13,795,506 (1.72)
Urban	10.05	34.49	2,657,605 (0.79)	2,097,101 (0.62)	1,659,982 (0.49)	25.00	27.55	6,626,779 (1.97)	5,638,481 (1.68)	4,812,299 (1.43)
Sex										
Male	8.20	34.34	3,928,688 (0.67)	3,195,816 (0.54)	2,647,006 (0.45)	24.36	36.36	11,958,945 (2.03)	10,497,358 (1.78)	9,473,398 (1.61)
Female	8.32	33.45	3,636,635 (0.66)	3,009,487 (0.55)	2,470,045 (0.45)	26.53	31.92	12,354,215 (2.24)	10,530,818 (1.91)	9,134,407 (1.66)
Transgender	24.92	47.42	1,156 (1.74)	1,156 (1.74)	1,156 (1.74)	0	0	0	0	0
Age										
0–14	9.39	60.46	2,355,396 (0.78)	1,935,175 (0.64)	1,577,076 (0.52)	40.05	68.69	10,651,752 (3.54)	9,361,453 (3.11)	8,424,760 (2.80)
15–29	6.27	38.41	1,593,614 (0.50)	1,297,719 (0.41)	1,097,511 (0.34)	18.59	56.09	4,834,563 (1.52)	4,152,473 (1.30)	3,648,294 (1.15)
30–59	8.22	28.72	2,848,611 (0.65)	2,320,435 (0.53)	1,910,599 (0.43)	19.23	24.13	6,762,962 (1.53)	5,781,272 (1.31)	4,988,549 (1.13)
60+	12.21	18.34	768,858 (0.97)	653,128 (0.82)	533,020 (0.67)	31.52	11.37	2,063,884 (2.60)	1,732,978 (2.18)	1,546,202 (1.95)
Religion										
Hindu	8.00	33.47	5,988,086 (0.65)	4,943,440 (0.53)	4,066,503 (0.44)	24.70	34.29	19,296,804 (2.09)	16,804,927 (1.82)	14,905,312 (1.61)

(Continued)

TABLE 2 | Continued

Variables	Inpatient					Outpatient				
	Prevalence of infectious diseases affected population (out of 1,000)	Percentage of individuals suffering from infectious diseases out of total ailing population	Population spending more than 5% of TCE on infectious diseases[#]	Population spending more than 10% of TCE on infectious diseases[#]	Population spending more than 15% of TCE on infectious diseases[#]	Prevalence of infectious diseases affected population (out of 1,000)	Percentage of individuals suffering from infectious diseases out of total ailing population	Population spending more than 5% of TCE on infectious diseases[#]	Population spending more than 10% of TCE on infectious diseases[#]	Population spending more than 15% of TCE on infectious diseases[#]
Muslim	8.92	36.46	1,116,550 (0.69)	902,428 (0.56)	759,880 (0.47)	28.01	34.60	3,723,305 (2.31)	3,156,296 (1.96)	2,792,440 (1.73)
Others*	10.78	33.59	461,843 (0.85)	360,589 (0.67)	291,823 (0.54)	29.83	28.77	1,293,052 (2.39)	1,066,953 (1.97)	910,053 (1.68)
Social groups										
STs	6.36	38.50	498,904 (0.48)	392,379 (0.38)	316,222 (0.31)	23.40	46.38	1,914,754 (1.85)	1,667,578 (1.61)	1,485,687 (1.44)
SCs	8.15	34.45	1,403,728 (0.63)	1,056,581 (0.47)	892,188 (0.40)	26.60	38.28	4,841,682 (2.16)	4,171,069 (1.86)	3,606,080 (1.61)
OBCs	8.18	34.44	3,430,279 (0.67)	2,893,105 (0.56)	2,397,502 (0.47)	25.72	36.44	11,261,062 (2.20)	9,731,786 (1.90)	8,657,122 (1.69)
Others	9.12	31.88	2,233,568 (0.74)	1,864,393 (0.62)	1,512,295 (0.50)	24.68	26.18	6,295,662 (2.09)	5,457,743 (1.81)	4,858,916 (1.62)
General education										
Illiterate	10.19	33.67	2,449,347 (0.82)	2,038,858 (0.69)	1,699,493 (0.57)	35.59	34.59	9,037,526 (3.04)	7,928,451 (2.67)	6,948,430 (2.34)
Up to primary	8.01	35.91	3,158,874 (0.63)	2,508,953 (0.50)	2,048,849 (0.41)	25.45	37.25	10,695,820 (2.15)	9,245,178 (1.86)	8,195,519 (1.65)
Secondary	7.25	31.89	1,482,428 (0.59)	1,260,815 (0.50)	1,031,526 (0.41)	17.20	29.56	3,496,711 (1.39)	2,928,393 (1.17)	2,644,182 (1.05)
Graduation and above	6.19	29.47	475,829 (0.51)	397,832 (0.42)	338,338 (0.36)	14.86	23.10	1,083,103 (1.15)	926,154 (0.99)	819,673 (0.87)

(Continued)

TABLE 2 | Continued

Variables			Inpatient					Outpatient		
	Prevalence of infectious diseases affected population (out of 1,000)	Percentage of individuals suffering from infectious diseases out of total ailing population	Population spending more than 5% of TCE on infectious diseases[#]	Population spending more than 10% of TCE on infectious diseases[#]	Population spending more than 15% of TCE on infectious diseases[#]	Prevalence of infectious diseases affected population (out of 1,000)	Percentage of individuals suffering from infectious diseases out of total ailing population	Population spending more than 5% of TCE on infectious diseases[#]	Population spending more than 10% of TCE on infectious diseases[#]	Population spending more than 15% of TCE on infectious diseases[#]
Wealth quintile										
Very poor	6.28	35.27	2,868,802	2,404,820	2,074,478	26.28	46.56	12,116,112	10,500,884	9,350,584
			(0.54)	(0.45)	(0.39)			(2.27)	(1.96)	(1.75)
Poor	8.65	33.24	1,784,914	1,488,153	1,268,611	24.63	33.11	5,587,556	5,043,970	4,444,407
			(0.67)	(0.56)	(0.47)			(2.08)	(1.88)	(1.66)
Average	10.74	34.08	1,372,420	1,084,720	864,461	26.57	28.40	3,621,808	3,063,676	2,688,672
			(0.83)	(0.66)	(0.53)			(2.20)	(1.86)	(1.64)
Rich	12.11	33.75	1,044,103	855,777	630,876	24.32	21.76	2,007,087	1,655,098	1,447,385
			(0.97)	(0.80)	(0.59)			(1.87)	(1.54)	(1.35)
Very rich	10.28	30.05	496,240	372,989	279,780	20.37	17.22	980,597	764,548	676,756
			(0.76)	(0.57)	(0.43)			(1.49)	(1.16)	(1.03)
Household size**										
Less than average	9.47	34.43	5,088,301	4,092,923	3,272,155	26.97	32.05	14,638,243	12,229,554	10,479,367
			(0.74)	(0.60)	(0.48)			(2.13)	(1.78)	(1.52)
More than average	6.42	32.77	2,478,178	2,113,536	1,846,052	23.03	38.05	9,674,918	8,798,622	8,128,438
			(0.55)	(0.47)	(0.41)			(2.14)	(1.94)	(1.80)
Regions***										
North	7.30	31.18	946,173	725,453	616,859	23.69	36.68	3,231,769	2,764,717	2,491,067
			(0.59)	(0.45)	(0.38)			(2.01)	(1.720	(1.55)
Northeast	5.89	39.39	228,147	162,731	115,253	11.14	47.86	367,352	334,918	312,119
			(0.52)	(0.37)	(0.26)			(0.83)	(0.76)	(0.71)

(Continued)

TABLE 2 | Continued

Variables	Inpatient					Outpatient				
	Prevalence of infectious diseases affected population (out of 1,000)	**Percentage of individuals suffering from infectious diseases out of total ailing population**	**Population spending more than 5% of TCE on infectious diseases#**	**Population spending more than 10% of TCE on infectious diseases#**	**Population spending more than 15% of TCE on infectious diseases#**	**Prevalence of infectious diseases affected population (out of 1,000)**	**Percentage of individuals suffering from infectious diseases out of total ailing population**	**Population spending more than 5% of TCE on infectious diseases#**	**Population spending more than 10% of TCE on infectious diseases#**	**Population spending more than 15% of TCE on infectious diseases#**
East	6.77	35.86	1,650,676 (0.58)	1,447,197 (0.51)	1,242,895 (0.44)	31.93	50.18	8,022,211 (2.82)	6,829,588 (2.40)	6,044,282 (2.13)
Central	6.58	29.77	1,252,856 (0.50)	982,828 (0.39)	774,223 (0.31)	26.79	33.63	5,645,658 (2.26)	5,088,658 (2.04)	4,658,459 (1.86)
West	9.23	35.75	1,215,744 (0.76)	1,050,639 (0.66)	891,142 (0.56)	26.35	32.79	3,361,856 (2.11)	2,945,451 (1.85)	2,565,950 (1.61)
South	12.19	35.27	2,272,883 (0.94)	1,837,610 (0.76)	1,477,834 (0.61)	19.44	20.38	3,684,314 (1.53)	3,064,844 (1.27)	2,535,928 (1.05)

Figures are based on the author's calculations from the NSSO 75th rounds.

#Values in parentheses show the percentage share of the total population.

**Include remaining religions such as Christianity, Sikhism, Jainism, Buddhism, Zoroastrianism, and others.*

***Households size is categorized into two categories viz. less than average and more than average.*

****North: JandK, Himachal Pradesh, Punjab, Chandigarh, Uttarakhand, Haryana, Delhi, Rajasthan; Northeast: Sikkim, Arunachal Pradesh, Nagaland, Manipur, Mizoram, Tripura, Meghalaya, Assam; East: Bihar, West Bengal, Jharkhand, Odisha; Central: Utter Pradesh, Chhattisgarh, Madhya Pradesh; West: Gujrat, Daman, and Diu, Dadar and Nagar Haveli, Maharashtra, Goa; South: Andhra Pradesh, Karnataka, Lakshadweep, Kerala, Tamil Nadu, Pondicherry, Andaman and Nicobar, Telangana.*

TABLE 3 | Socio-economic and demographic covariates in the level of out-of-pocket (OOP) expenditure as a share of total consumption expenditure (TCE) on infectious diseases in India (2017–18).

Variables	Inpatient							Outpatient						
	Average per capita OOP expenditure on infectious diseases (INR)	**OOP expenditure on infectious diseases as a percentage of TCE (%)**	**Per capita OOP expenditure of the individuals suffering from infectious diseases (INR)**	**OOP expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(% at reporting level)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(5%)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(10%)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(15%)***	**Average per capita OOP expenditure on infectious diseases (INR)**	**OOP expenditure on infectious diseases as a percentage of TCE (%)**	**Per capita OOP expenditure of the individuals suffering from infectious diseases (INR)**	**OOP expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(% at reporting level)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(5%)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(10%)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(15%)***
All India	7.28	0.34	881.56	36.04	31.59	28.06	25.23	29.38	1.06	1156.34	35.82	31.74	28.70	26.26
Place of residence														
Rural	5.97	0.36	794.98	42.50	37.99	34.39	31.41	28.01	1.29	1095.23	44.16	39.93	36.59	33.83
Urban	10.42	0.31	1036.43	29.83	25.42	21.96	19.27	32.65	0.77	1306.03	25.81	21.92	19.22	17.18
Sex														
Male	7.22	0.33	880.25	35.94	31.47	27.92	25.06	28.75	1.03	1180.53	34.86	30.88	27.98	25.66
Female	7.35	0.34	883.13	36.17	31.72	28.22	25.41	30.05	1.09	1132.59	36.86	32.69	29.47	26.91
Transgender	8.57	0.30	344.07	10.43	7.67	5.40	3.12	0	0	0	0	0	0	0
Age														
0–14	6.93	0.37	737.93	32.49	27.93	24.21	21.25	41.46	1.82	1035.20	41.49	37.03	33.38	30.37
15–29	5.31	0.24	847.16	33.67	29.22	25.79	23.01	21.63	0.75	1163.30	34.14	30.22	27.35	25.00
30–59	8.17	0.36	993.24	39.71	35.33	31.87	29.09	25.33	0.87	1317.06	32.48	28.66	26.05	24.06
60+	11.64	0.47	953.51	36.65	32.15	28.68	25.84	37.16	1.10	1178.92	33.37	29.23	26.11	23.74
Religion														
Hindu	7.52	0.35	939.49	38.56	34.09	30.51	27.63	28.88	1.03	1169.49	35.51	31.43	28.37	25.92
Muslim	5.25	0.26	589.02	26.85	22.49	19.10	16.39	30.32	1.22	1082.32	39.06	34.98	31.92	29.46
Others*	9.35	0.32	867.01	27.22	22.78	19.46	16.90	35.12	1.13	1177.24	32.88	28.80	25.98	23.79
Social group														
STs	2.73	0.17	428.34	25.17	20.92	17.77	15.33	18.79	0.81	803.15	27.68	23.85	21.17	19.01
SCs	6.04	0.34	740.79	36.94	32.59	29.42	26.92	29.68	1.14	1115.62	34.38	30.52	27.77	25.64
OBCs	6.95	0.34	849.19	36.28	31.80	28.21	25.29	30.38	1.12	1181.39	38.42	34.26	31.04	28.45
Others	10.34	0.36	1133.42	36.82	32.33	28.66	25.73	31.09	0.99	1259.80	35.07	30.89	27.78	25.30
General education														
Illiterate	8.34	0.49	818.58	41.18	36.70	33.14	30.24	38.63	1.53	1085.49	36.13	31.97	28.79	26.19
Up to primary	5.67	0.29	707.85	30.83	26.39	22.93	20.22	27.46	1.07	1078.73	37.14	33.00	29.79	27.24

(Continued)

TABLE 3 | Continued

Variables	Inpatient							Outpatient						
	Average per capita OOP expenditure on infectious diseases (INR)	**OOP expenditure on infectious diseases as a percentage of TCE (%)**	**Per capita OOP expenditure of the individuals suffering from infectious diseases (INR)**	**OOP expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(% at reporting level)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(5%)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(10%)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(15%)***	**Average per capita OOP expenditure on infectious diseases (INR)**	**OOP expenditure on infectious diseases as a percentage of TCE (%)**	**Per capita OOP expenditure of the individuals suffering from infectious diseases (INR)**	**OOP expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(% at reporting level)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(5%)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(10%)***	**OOP health expenditure as a percentage of TCE of the individuals suffering from infectious diseases *(15%)***
Secondary	8.98	0.36	1237.33	42.76	38.34	34.74	31.78	25.81	0.84	1500.09	36.85	32.97	30.29	28.20
Graduation and above	7.97	0.21	1287.96	28.91	24.38	20.81	17.96	19.85	0.52	1335.55	25.41	21.55	18.98	17.01
Wealth quintile														
Very poor	4.83	0.41	768.83	62.86	58.21	54.32	51.06	27.96	1.38	1063.93	46.77	42.59	39.24	36.48
Poor	6.18	0.32	714.71	36.77	32.32	28.82	25.85	24.97	0.97	1013.90	34.45	30.17	26.87	24.19
Average	8.75	0.33	814.81	30.47	26.06	22.59	19.86	37.63	1.14	1416.05	36.62	32.37	29.10	26.51
Rich	15.38	0.40	1269.47	33.05	28.58	24.96	22.06	28.99	0.68	1192.02	21.08	17.39	15.03	13.27
Very rich	14.82	0.21	1441.86	20.70	16.38	13.17	10.82	38.95	0.67	1912.17	24.45	20.80	18.54	16.86
Households size**														
Less than Average	7.98	0.32	842.32	31.43	27.01	23.60	20.91	30.94	0.87	1147.30	27.87	23.94	21.14	18.99
More than average	6.22	0.37	969.60	50.50	45.92	42.02	38.74	27.00	1.71	1172.43	71.14	66.43	62.27	58.60
Regions***														
North	6.62	0.25	906.54	32.01	27.58	24.02	21.21	30.82	0.99	1301.05	30.28	26.34	23.59	21.36
Northeast	2.24	0.12	380.27	18.50	13.80	10.31	7.98	12.57	0.51	1128.54	40.24	36.43	33.34	30.55
East	8.77	0.53	1296.07	64.88	60.29	56.33	52.92	38.52	1.77	1206.64	45.19	40.93	37.71	35.11
Central	3.34	0.20	506.92	28.49	24.19	21.08	18.65	28.16	1.27	1051.23	41.60	37.64	34.53	31.83
West	9.19	0.35	996.02	36.38	31.83	28.04	24.91	32.17	1.04	1221.09	32.28	28.36	25.47	23.33
South	9.72	0.36	797.15	28.02	23.62	20.27	17.67	20.15	0.55	1036.58	26.00	21.78	18.61	16.23

Figures are based on the author's calculations from the NSSO 75th rounds.

**Include remaining religions such as Christianity, Sikhism, Jainism, Buddhism, Zoroastrianism, and others.*

***Households size is categorized into two categories viz. less than average and more than average.*

****North: JandK, Himachal Pradesh, Punjab, Chandigarh, Uttarakhand, Haryana, Delhi, Rajasthan; Northeast: Sikkim, Arunachal Pradesh, Nagaland, Manipur, Mizoram, Tripura, Meghalaya, Assam; East: Bihar, West Bengal, Jharkhand, Odisha; Central: Utter Pradesh, Chhattisgarh, Madhya Pradesh; West: Gujrat, Daman, and Diu, Dadar and Nagar Haveli, Maharashtra, Goa; South: Andhra Pradesh, Karnataka, Lakshadweep, Kerala, Tamil Nadu, Pondicherry, Andaman and Nicobar, Telangana.*

is 1.06% on outpatient care in the study. Whereas, at various socio-economic and demographic variables, it is highest among rural inhabitants (1.29%), females (1.09%), 60+ age groups (1.82%), Muslims (1.22%), scheduled castes (1.14%), illiterates (1.53%), very poor wealth quintiles (1.38%), more than average households' size (1.71%), and coming from eastern region (1.77%) than their respective counterparts in the analysis.

Furthermore, the percentage share of OOP health expenditure on infectious diseases as a share of total consumption expenditure of infection-affected populations has also been measured at different threshold levels in the study. The findings show that it is 35.82% of the total consumption expenditure of the country's infection-affected population at the reporting level. At the same time, it reduces proportionately with the increase in the threshold levels as 5, 10, and 15% in the analysis, respectively. Moreover, a similar trend of deterioration with an increase in threshold has been observed among various socio-economic and demographic variables. But the proportion has been found highest among patients inhabiting rural areas, females, 0–14 age groups, Muslims, OBCs, education up to primary levels, economically very poor people, accompanying large family size, and an inhabitant of the eastern region of India, respectively.

Source of Finance to Cope With Out-of-Pocket Expenditure on Infectious Diseases

Table 4 shows that in the case of inpatient care, 86.4% of the infected population are using savings/income as a first source to finance the infection-derived expenditure in India. Simultaneously, the share of borrowings and other remaining coping strategies are only 8.4 and 5.0% in the country. Not using any coping strategies to finance health expenditure on infectious diseases has been reported in the study, which constituted only a 0.3% share of the infection-affected population in the country. Furthermore, concerning the individuals' residence and sex, the percentage of savings/income is highest among people residing in the urban area (89.1%) and female (87.5%) patients. In comparison, borrowings are significantly prevalent among rural (10.1%) and male (9.0%) patients in India. In the patients' age, both savings/income (87.2%) and borrowings (9.2%) are highly employed as a source of finance by the 0–14 age group in the study. While concerning religion, the share of both sources of finance for coping is relatively less among Muslim people, but other residual coping strategies are highest in the study.

Among social categories, savings/income are employed by STs (88.1%), and borrowings are used mainly by SCs (10.2%) in India. Furthermore, savings/income (90.6%) are highly used by educated people suffering from infectious diseases in the analysis. Still, the dependency on borrowing (10.1%) is relatively highest among illiterate people in the country. Among different economic statuses, the share of savings/income is highest among wealthy people, but the poor are more dependent on borrowings in the study. The analysis shows that savings/income and borrowings are the leading sources of finance for coping among individuals related to large family size. Finally, savings/income with a 95.1% share is the top strategy for dealing in the north-east region. In contrast, individuals from the southern part have a larger share of borrowing to finance their inpatient care expenditure in India.

In outpatient care, savings/income with a 92.9% share is the leading strategy to finance health-care expenditure in India. However, it has been seen that the percentage of borrowings is not very much significant in the study. But as a source of finance, borrowings and other coping strategies contribute only 1.5 and 2.8% to the total share of expenditure on infectious diseases in the country. The findings also illustrate that nearly 3.0% of people suffering from infection did not report any source of finance in the analysis. Based on various socio-economic variables, savings/income is primely used by urban (94.2%) inhabitants and male (93.2%) patients in India. It is highest among different age groups among 0–14 with a 94.1% share. Although the overall percentage of borrowings is less significant among all coping strategies, dependence has been higher among the aged, Muslims, educated, and very poor. Again, it has been seen that 93.9% OBCs are dependent on savings/income while not reporting any source of finance for coping and other remaining strategies are highest among STs in India. The share of savings/income is highest among educated people in the country on educational background. Finally, the percentage of savings/income is highest in the northern region. At the same time, the share of people who did not report any copying strategies is highest in the north-eastern part of India.

DISCUSSION AND CONCLUSION

Overall, the results indicate a significant existence of infectious diseases that are still a big threat to India's public health. Although several horizontal and vertical policy initiatives to cure, control and eradicate infectious diseases have been taken into account by the governments, but could not provide any landmark changes in this regard. It has been perceived that these infectious diseases are not easily controllable until the worse surroundings, such as the lack of cleanliness, open defecation, and many other associated factors, are addressed. In inpatient and outpatient cases, infectious diseases are significantly prevalent among rural areas, females, transgender, children (0–14 age group), aged persons (60+ age group), SCs, non-Hindu communities, and illiterate people in India. In the analysis, the relatively lower percentage of poor people suffering from infectious diseases does not illustrate their better health conditions. Still, it reflects their un-reportability to health-care facilities than economically prosperous people. This results from their insufficient financial resources and fulfills this notion that accessibilities of health-care facilities are still far from the reach of these economically marginalized sections of Indian societies.

Furthermore, the analysis shows that OOP expenditure on infectious diseases is comparatively high in outpatient care. Per patient OOP expenditure on contagious diseases has been found lower among most socio-economically vulnerable groups, such as rural inhabitants, transgender people, Muslims, scheduled tribes, illiterates, educated up to primary level, and poor wealth quintile in India. Also, a declining trend of average per capita OOP expenditure with an increase in the thresholds level has been seen. The result further elaborates that people rely more on savings/income to cope with infectious diseases. Still,

TABLE 4 | Socio-economic and demographic covariates in the source of finance to cope with out-of-pocket (OOP) expenditure on infectious diseases in India (2017–18).

Variables	Inpatient				Outpatient			
	Savings/ Income	Borrowings	Others[#]	Not reported any source of finance	Savings/ Income	Borrowings	Others[#]	Not reported any source of finance
All India	86.4	8.4	5.0	0.3	92.9	1.5	2.8	3.0
	(85.9–87.9)	(7.9–9.4)	(4.4–5.8)	(0.1–0.4)	(91.9–93.9)	(1.1–2.0)	(2.1–3.5)	(2.4–3.6)
Place of residence								
Rural	85.6	10.1	5.1	0.3	92.4	1.8	2.8	3.4
	(84.3–86.9)	(9.1–11.2)	(4.2–6.0)	(0.03–0.5)	(91.2–93.6)	(1.1–2.4)	(2.0–3.5)	(2.6–4.1)
Urban	89.1	5.9	5.0	0.3	94.2	1.0	2.9	2.1
	(87.8–90.6)	(5.1–6.9)	(3.9–6.1)	(0.2–0.5)	(92.6–95.9)	(0.4–1.6)	(1.6–4.3)	(1.3–3.0)
Sex								
Male	86.1	9.0	5.0	0.3	93.2	1.5	2.9	2.7
	(84.8–87.8)	(7.9–10.3)	(4.0–6.0)	(0.1–0.6)	(91.8–94.6)	(0.8–2.2)	(1.8–3.6)	(1.9–3.5)
Female	87.5	8.2	5.2	0.2	92.7	1.6	2.7	3.3
	(86.2–88.7)	(7.3–9.1)	(4.3–6.1)	(0.1–0.3)	(91.3–94.1)	(0.9–2.3)	(1.9–3.6)	(2.4–4.2)
Transgender	1.0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
Age								
0–14	87.2	9.2	4.5	0.6	94.1	1.3	2.2	2.4
	(85.6–88.7)	(8.0–10.5)	(2.9–4.5)	(0.1–1.2)	(92.8–94.1)	(0.7–1.9)	(1.3–3.1)	(1.7–3.2)
15–29	85.5	9.0	6.0	0.1	91.4	2.2	4.1	2.4
	(83.4–88.2)	(7.3–10.7)	(4.3–7.8)	(0.05–0.2)	(88.8–94.1)	(0.8–3.5)	(1.9–6.2)	(1.4–3.4)
30–59	87.1	8.4	4.7	0.1	93.7	1.1	2.2	3.7
	(85.5–89.0)	(7.2–9.9)	(3.6–6.1)	(0.01–0.2)	(91.9–95.4)	(0.3–1.9)	(1.3–3.2)	(2.4–5.1)
60+	87.0	6.3	8.0	0.1	88.3	2.7	4.6	4.8
	(84.6–89.4)	(4.8–8.2)	(6.0–10.0)	(0.02–0.2)	(84.0–92.7)	(0.5–4.9)	(1.5–7.8)	(2.3–7.3)
Religion								
Hindu	87	8.6	4.7	0.2	93.1	1.4	2.7	3.0
	(86.0–88.2)	(8.0–9.6)	(4.1–5.7)	(0.1–0.3)	(92.0–94.3)	(0.9–1.9)	(1.9–3.4)	(2.4–3.7)
Muslims	85.6	7.9	6.2	0.6	91.8	2.8	3.6	2.2
	(83.0–88.6)	(5.8–10.2)	(4.6–8.1)	(−0.2–1.5)	(89.1–94.5)	(0.9–4.6)	(1.8–5.3)	(1.1–3.4)
Others*	87.2	8.7	4.3	0.2	93.4	0.6	2.7	4.6
	(83.8–90.6)	(5.7–11.9)	(2.8–5.9)	(0.1–0.3)	(90.2–96.5)	(−0.03–1.2)	(0.5–4.8)	(1.8–7.4)
Households size**								
Less than average	86.5	8.5	5.4	0.2	91.8	0.2	3.3	3.5
	(85.4–87.8)	(7.7–9.4)	(4.6–6.5)	(0.1–0.3)	(90.4–93.1)	(1.1–2.4)	(2.4–4.3)	(2.7–4.4)
More than average	87.4	8.7	4.0	0.3	95.0	1.1	1.9	2.1
	(85.8–89.2)	(7.4–10.4)	(3.1–4.9)	(−0.001–0.8)	(93.7–96.3)	(0.4–1.8)	(1.0–2.8)	(1.5–2.8)
Social groups								
STs	88.1	6.2	5.5	0.4	88.2	0.4	4.2	7.3
	(85.8–90.5)	(4.6–7.8)	(4.0–7.4)	(0.2–0.7)	(84.5–91.8)	(0.05–0.7)	(1.8–6.6)	(4.4–10.1)
SCs	86.3	10.2	4.0	0.2	92.7	1.4	1.9	4.3
	(84.4–88.4)	(8.5–12.1)	(3.1–5.0)	(0.01–0.4)	(90.4–94.9)	(0.4–2.3)	(0.8–2.9)	(2.5–6.1)
OBCs	86.1	9.7	4.5	0.2	93.9	1.4	2.7	2.2

(Continued)

TABLE 4 | Continued

Variables	Inpatient				Outpatient			
	Savings/ Income	Borrowings	Others[#]	Not reported any source of finance	Savings/ Income	Borrowings	Others[#]	Not reported any source of finance
	(84.6–87.8)	(8.7–11.1)	(3.7–6.1)	(0.03–0.3)	(92.5–95.3)	(0.8–2.0)	(1.7–3.8)	(1.4–2.9)
Others	88	6.1	5.7	0.5	93.0	2.3	3.2	2.1
	(86.1–89.8)	(5.0–7.5)	(4.6–7.3)	(0.03–1.0)	(90.9–95.1)	(1.0–3.6)	(1.7–4.8)	(1.3–2.9)
General education								
Illiterate	84.5	10.1	5.5	0.5	92.2	1.3	3.0	3.7
	(82.6–86.8)	(8.6–11.8)	(4.2–7.1)	(0.04–0.9)	(90.5–94.0)	(0.7–1.9)	(1.8–4.2)	(2.5–4.8)
Up to primary	88	8.1	4.2	0.2	93.6	1.7	2.3	2.8
	(86.8–89.2)	(7.3–9.2)	(3.7–5.2)	(0.09–0.3)	(92.2–95.0)	(0.9–2.6)	(1.5–3.1)	(1.9–3.6)
Secondary	86.6	8.4	5.2	0.2	93.3	1.3	3.8	2
	(84.6–89.0)	(6.8–10.2)	(3.8–6.8)	(−0.1–0.4)	(90.3–96.2)	(−0.04–2.6)	(1.2–6.4)	(1.1–2.9)
Graduation and above	90.6	3.8	5.6	0.1	91.2	2.5	3.3	3.4
	(86.3–95.1)	(2.2–5.7)	(1.6–9.9)	(−0.05–0.3)	(86.4–95.9)	(−0.7–5.8)	(0.7–5.8)	(0.7–6.1)
Wealth quintile								
Very poor	85.6	9.3	5.3	0.3	91.5	1.8	3.2	3.7
	(83.9–87.5)	(7.9–10.8)	(4.4–6.6)	(−0.1–0.7)	(89.8–93.1)	(1.0–2.5)	(2.0–4.3)	(2.7–4.7)
Poor	84.2	11	5.4	0.2	94.8	1.5	2	1.8
	(81.9–86.7)	(9.4–12.8)	(3.6–7.6)	(0.05–0.4)	(93.2–96.4)	(0.5–2.5)	(1.0–2.9)	(1.0–2.7)
Average	88.1	8.2	3.7	0.5	94.3	1.4	2.8	2.1
	(86.2–90.1)	(6.7–10.0)	(2.8–5.0)	(0.1–0.9)	(92.4–96.3)	(0.3–2.4)	(1.4–4.3)	(1.2–3.1)
Rich	90.5	5.7	4.0	0.2	95.7	1.3	1.5	2.5
	(88.8–92.5)	(4.5–7.4)	(2.8–5.3)	(0.03–0.3)	(93.6–97.8)	(−0.4–2.9)	(5.8–2.4)	(1.0–4.1)
Very rich	91.1	2.5	6.1	0.2	89.2	0.4	5.7	5.5
	(89.0–93.3)	(1.6–3.8)	(4.4–8.2)	(−0.01–0.3)	(82.4–96.0)	(−0.2–1.0)	(0.05–11.3)	(1.1–9.9)
Regions***								
North	89.6	7.6	2.6	0.4	95.4	1.3	0.9	3.3
	(87.6–91.8)	(6.0–9.5)	(1.6–3.8)	(0.01–0.7)	(93.3–97.5)	(0.2–2.4)	(0.2–1.7)	(1.4–5.2)
Northeast	95.1	2.6	2.0	0.3	89.6	1.2	1.2	8.1
	(93.5–96.7)	(1.3–3.9)	(1.0–3.1)	(0.1–0.4)	(82.5–96.6)	(−0.4–2.8)	(−0.3–2.8)	(1.3–14.8)
Central	87.1	8.1	5.0	0.3	93.6	1.6	3.4	1.4
	(84.7–89.6)	(6.1–10.4)	(4.0–6.2)	(−0.004–0.6)	(91.6–95.6)	(0.7–2.6)	(1.8–5.0)	(0.6–2,2)
East	88.2	6.0	5.6	0.6	92.3	0.7	4.0	3.0
	(86.5–90.3)	(4.8–7.2)	(4.4–7.1)	(−0.1–1.4)	(90.4–94.3)	(0.4–1.1)	(2.4–5.5)	(1.8–4.3)
West	92.4	5.2	2.3	0.1	92.7	2.7	1.6	3.0
	(90.7–94.5)	(3.5–7.0)	(1.6–3.1)	(0.02–0.2)	(89.8–95.6)	(0.7–4.7)	(0.03–3.2)	(1.5–4.5)
South	81.0	13.0	7.2	0.1	91.2	1.7	2.7	5.2
	(79.0–83.2)	−11.6	(5.5–9.1)	(0.04–0.2)	(88.9–93.5)	(0.7–2.8)	(1.7–3.7)	(3.4–7.1)

Figures are based on the author's calculations from the NSSO 75th rounds.

Values in parentheses are 95% confidence interval.

[#]*Includes the remaining source of finance such as the sale of physical assets, contributions from friends and relatives, and other sources.*

[*]*Include remaining religions such as Christianity, Sikhism, Jainism, Buddhism, Zoroastrianism, and others.*

[**]*Household size is categorized into two categories viz. less than average and more than average.*

[***]*North: JandK, Himachal Pradesh, Punjab, Chandigarh, Uttarakhand, Haryana, Delhi, Rajasthan; Northeast: Sikkim, Arunachal Pradesh, Nagaland, Manipur, Mizoram, Tripura, Meghalaya, Assam; East: Bihar, West Bengal, Jharkhand, Odisha; Central: Utter Pradesh, Chhattisgarh, Madhya Pradesh; West: Gujrat, Daman, and Diu, Dadar and Nagar Haveli, Maharashtra, Goa; South: Andhra Pradesh, Karnataka, Lakshadweep, Kerala, Tamil Nadu, Pondicherry, Andaman and Nicobar, Telangana.*

borrowings are employed significantly to manage inpatient care than outpatient care in India. Also, socio-economically deprived people, such as rural people, SCs, illiterate, and very poor, are more dependent on borrowing than others. This fact exaggerates the belief that these people are still reliant on borrowings to attain basic needs, including health, due to their improper inclusion in the country's main streams. Furthermore, an interesting fact regarding education status and different sources of finance for coping has been observed in the study. The analysis states a positive impact on the use of savings/income with an increase in the individuals' education level.

This study concludes that infectious diseases are still a significant threat to public health in India. Various life-threatening, long-lasting contagious diseases such as smallpox, cholera, plague, dengue, and flu pandemic, have been controlled, cured, and eradicated through numerous vertical and horizontal disease control programmes. But several re-emerging infectious diseases are increasing the challenges of health care, treatment behavior, health-care costs, and source of finance for coping in the country. The global emergence of COVID-19 has challenged humanity's survival in India and worldwide (38). Since its emergence, the continuously rising number of active cases has warned the world of its death toll. With the government's imposition of social distancing through lockdown, the only way to prevent such pandemic has radically broken the entire social structure, cultural values, and economic systems (15, 16). This evidence hypothesizes that socially and economically vulnerable sections of societies become worse when they suffer from such contagious diseases. It increases their burden of health care through OOP health expenditure and reduces their productive efficiency during the spell of ailments. For instance, in the study, SCs depend more on borrowings to finance infectious diseases than other social groups in India. The idea of dependency on borrowings to fund health-care expenditure communicates that these deprived sections are still at the mercy of their masters and very far from the mainstream of the country.

Additionally, to curtail the share of OOP expenditure on health care, an increase in the percentage of GDP on health care and universalisation of insurance coverage is the need of the hour (36). According to the budget estimates for the fiscal year 2018, around 1.3% of India's GDP has been spent on public health, whereas it is a minimum of 6–7% of GDP in most European countries. Only 12% of the urban and 13% of the rural population are under the protection coverage through Rashtriya Swasthya Bima Yojana (RSBY) in the country (39). Though various health insurance schemes have been launched by the country's union and state governments, people without insurance coverage are still considerable due to the improper implementations. As a result, nearly 86% of the rural population and 82% of the urban population were not covered under any public or private scheme of health expenditure support in India (39). To fulfill the objectives of maximum population coverage, another plan, "Ayushman Bharat," PM-JAY the world's largest government-funded health-care scheme, was launched by the Government of India in 2018 under National Health Policy 2017. The scheme provides a health cover of Rs. 5 lakhs per family per year, specifically targeted 10.74 crore poor and vulnerable families (∼50 crore beneficiaries) (40). The plan ensures financial protection against catastrophic health expenditure and access to affordable and quality health care for all country's citizens (41).

In addition, for ensuring the quality of health-care services, the Government of India had initiated the "National Digital Health Mission (NDHM)" in 2021. The mission will create a national digital health ecosystem to support universal health coverage in an efficient, accessible, inclusive, affordable, timely, and safe manner. The system will provide a wide range of data, information, and infrastructure services to the people (41, 42). However, these initiatives have been holistically launched for ensuring better health facilities, but it is early to make any prediction regarding its outcomes; hopefully, the time will define it over the passing of a few more years.

Also, spreading awareness among people about cleanliness, sanitation, and free from open defecation has positively impacted the country's individuals' health. The findings tabled the fact that Swachh Bharat Mission-Gramin (SBM-G) has reduced the number of diarrheal cases. They avoided more than 14 million Disability-Adjusted Life Years (DALYs) between 2014 and October 2019 (43, 44). Recently, to prevent the community transmission of the coronavirus disease, propagation of awareness through multiple media platforms changed the public attitudes and behavior toward susceptible people to this contagious disease (15, 45). The awareness campaigns not only reduced the chances of contact with coronavirus but also encouraged the asymptomatic individuals to conduct health protocols, such as self-isolation and social distancing, in the country (45). Therefore, the result deliberates the need for proper implementations of policy initiatives against ailments, outcome-oriented implementation of health-care schemes among targeted population, ensuring quality of public health-care system and its expansion nearer to the people's doorsteps, immunisations/vaccinations drives, subsidized medical facilities to vulnerable sections, and awareness programmes against unhygienic conditions in the country.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: www.mospi.gov.in.

AUTHOR CONTRIBUTIONS

BR conceived the idea and prepared the initial draft of the manuscript. BR and RT performed the statistical analysis. RT revised the manuscript. Both authors read and approved the final manuscript.

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Determining SARS-CoV-2 non-infectivity state–A brief overview

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From the beginning of the COVID-19 pandemic, it has claimed over 6 million lives, and globally the pandemic rages with detrimental consequences, with the emergence of new more infectious and possibly virulent variants. A clinical obstacle in this battle has been to determine when an infected individual has reached a non-infectious state. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) can be transmitted under diverse circumstances, and various rules and regulations, along with different testing methods, have been applied in an attempt to confine the transmission. However, that has proven to be a difficult task. In this review, we take together recently published data on infectivity and transmission of SARS-CoV-2 and have combined it with the clinical experience that physicians in Iceland have accumulated from the pandemic. In addition, we suggest guidelines for determining when patients with COVID-19 reach a non-infectious state based on a combination of clinical experience, scientific data, and proficient use of available tests. This review has addressed some of the questions regarding contagiousness and immunity against SARS-CoV-2.

KEYWORDS

COVID-19, SARS-CoV-2, transmission of SARS-CoV-2, non-infectious state, viral testing

Initial infectious state

The average incubation period for SARS-CoV-2 is 2–7 days, with over 98% of symptomatic patients falling ill within 12 days (1–4). The different variants of SARS-CoV-2 have different mean incubation times, the omicron variant (B.1.1.529) has a mean incubation period of 3.2 days as compared to 4.4 days for the delta variant (B.1.617.2) (5). The delta variant also has a shorter mean incubation period as compared to previous variants (6). The most common way to diagnose COVID-19 infection is by using reverse transcription polymerase chain reaction (RT-PCR) on samples collected from the upper respiratory tract (nasopharyngeal and/or oropharyngeal swabs). SARS-CoV-2 virus in infected individuals can be detected by RT-PCR for an average of 17.0 days in samples taken from the upper respiratory tract, with the highest levels in the first week (7).

The mean duration of viral shedding is variable depending on what type of sample is being measured. Viral shedding can be detected by RT-PCR in samples from the lower respiratory tract for an average of 14.6 days while samples from stool are positive for an average of 17.2 days (7).

In some cases, patients can remain SARS-CoV-2 positive by RT-PCR for a prolonged time, up to 230 days in an immunocompromised patient (8) but also in previously healthy individuals for 60–110 days (9–11). However, it is unclear, in these cases, whether the cause of prolonged viral shedding is the retention of the virus in the body or re-infection. It has been reported that patients with COVID-19 can be tested negative after disease followed by a positive test (re-detectable positive) both with the same and different variants or even co-infected by multiple variants of the virus (12, 13). Many individuals test positive for COVID-19 without showing symptoms, after diagnosis, many patients develop symptoms, but some remain asymptomatic. The reported percentage of individuals that remain asymptomatic throughout the disease varies widely. Based on testing in the general population and in defined groups for COVID-19, the percentage of asymptomatic COVID-19 positive individuals that do not develop symptoms ranges from 12.2 to 62.5% (14–18). In addition, asymptomatic infections of COVID-19 have been shown to be more common when the SARS-CoV-2 virus has a specific mutation, the 11083G>T mutation (19). Symptomatic, pre-symptomatic, asymptomatic individuals, and patients with COVID-19 can transmit COVID-19 to others (20–22).

There are similar viral loads at the start of infection among asymptomatic and symptomatic patients (7, 16) and viral load does not seem to correlate with disease severity as there is no statistical difference in viral load between asymptomatic and hospitalized patients with COVID-19 (23). Higher viral load in nasopharyngeal samples is more common in patients with an unfavorable outcome; however, a high viral load is not an independent risk factor for intensive care unit (ICU) admission or death (24).

The viable SARS-CoV-2 virus, cultured in Vero cells, can be isolated from the samples of patients with COVID-19 up to 24 days after symptom onset with more success in the earlier days (25–29). Viable SARS-CoV-2 virus cannot be isolated from all RT-PCR confirmed patients, as it is highly dependent on a high viral load (27). The likelihood of isolating a viable virus is significantly higher in the first week after the onset of symptoms than in the second and after 10 days, the probability drops to 6.0% (30). The virus is most commonly isolated from nasopharyngeal swabs and sputum but has also been isolated from saliva (25), endotracheal samples (28), stool (31), and urine (32). The viable virus has been cultured in samples from symptomatic, pre-symptomatic, asymptomatic, and re-detectable positive patients with COVID-19 (33–35).

The infectivity of SARS-CoV-2 that varies among patients with COVID-19 depends on multiple factors, such as their vaccination status. However, the highest viral load and infectivity are noted during the first 5 days of the symptomatic state.

SARS-CoV-2 antibody response

Seroconversion following SARS-CoV-2 exposure has been under intense investigation

Seroconversion (when antibodies against SARS-CoV-2 can be detected) has been reported to be at around day 12 after the onset of symptoms with some individual variability that is not associated with disease severity (36). The clinical significance of the individual isotype responses has been evaluated. Interestingly, higher immunoglobulin M (IgM), immunoglobulin M (IgG), and immunoglobulin M (IgA) SARS-CoV-2 specific antibodies have been associated with worse clinical outcomes (37–41). Patients with high levels of IgG and IgA anti-receptor-binding domain (RBD) antibodies were more likely to require hospital admission, mechanical ventilation, and fatal outcomes when compared to patients with lower levels. Non-hospitalized patients also had lower neutralizing antibodies (42). Patients with COVID-19 in the ICU had higher levels of IgA for RBD, S1, and N protein when compared to non-hospitalized patients (41).

It has become increasingly common to test for the presence of SARS-CoV-2-specific antibodies in the serum of individuals. It is performed for numerous reasons 1) to determine if an individual has been infected with the SARS-CoV-2 virus, 2) to determine the level of protection against re-infection, 3) to determine the level of protection in vaccinated individuals, and 4) to determine if an individual is contagious or not. However, it remains to be completely resolved if serological status against SARS-CoV-2 can be used to determine the non-infectious state. Excluding RT-PCR and measuring only antibodies for SARS-CoV-2 are not sufficient to indicate whether an individual has been infected in the first week of disease but after 21–35 days, the sensitivity of pooled IgG/IgM measurements rises to 96.0% (43).

The sensitivity and specificity of the various commercially available SARS-CoV-2-specific serologic assay kits vary, based on testing method and manufacturer. Currently, there are at least 222 commercialized SARS-CoV-2 antibody immunoassays that have received CE-*in vitro* diagnostic (IVD) certification (44).

Immunological memory after COVID-19 and neutralizing antibodies

Immunological memory is formed after infection, but how long the memory lasts is highly dependent on the type of

infection. Neutralizing antibodies are of special importance as they prevent the binding of the pathogen to the host cells. Studies have shown that antibodies formed after COVID-19 last at least 6 months or more in most patients, but the level of antibodies decreases with time (18, 45–47). Levels of IgG specific for the spike protein of SARS-CoV-2 are stable for over 6 months and the number of spike-specific memory B cells is higher 6 months after infection when compared to 1 month (46, 48).

The half-life of anti-spike protein IgG antibodies has been shown to be 184 days, with a shorter half-life for men (49). The spike protein consists of the S1 and S2 subunits, with the RBD situated within S1. The half-life of antibodies against different parts of the spike protein differs, with antibodies against S1 having the shortest half-life at 115 days, 125 days for antibodies against the RBD, and 344 days for antibodies against the S2 part (50).

The main neutralizing antibodies for SARS-CoV-2 have been found to be directed against the spike protein of the virus and the RBD domain, presumably, as they prevent respiratory epithelial cellular entry by the virus (51). Although the majority of today's known neutralizing antibodies disrupts angiotensin-converting enzyme 2 (ACE2) binding to the RBD, others have been found to recognize epitopes outside this site (52). The half-life of neutralizing antibodies against SARS-CoV-2 has been documented to be from 90 to 114 days (46, 53). Only 20.2% of the mean serum levels of SARS-CoV-2-specific antibodies in convalescent and vaccinated individuals is needed to confer a 50% protection against severe infection (53). Only time will tell how the antibodies for SARS-CoV-2 are maintained in the long term. However, it has been estimated that antibodies will maintain a 50% protection against COVID-19 infection for 1.5–2 years, while 50% protection against serious infection would last several years (49, 53). This can clearly represent a weakness in the efficacy of the vaccines, as the spike protein is prone to mutate, making the SARS-CoV-2 virus more infectious (54).

Vaccine targets

After the SARS outbreak in 2003, studies reported neutralizing antibodies against the SARS-CoV spike protein (55). Since both SARS-CoV and SARS-CoV-2 utilize the attachment of spike protein to the human ACE2 receptor to invade host cells, it is crucial to develop neutralizing antibodies against the spike protein to induce protection against SARS-CoV-2 infection (56). The spike protein thus became the main target of vaccine development (52). Presently, Sputnik V, ChAdOx1, Spikevax, BNT162B2, Vidprevtyn, VLA2001, COVID-19 Vaccine Janssen, Nuvaxovid, and COVID-19 Vaccine from Sinovac all target the spike protein. This can clearly represent a weakness in the efficacy of the vaccines, as the spike protein is prone to mutate (57–59).

Recent results from a retrospective study based on the U.S. registry have shown that transfusion of plasma with high anti-SARS-CoV-2 antibody levels was associated with a lower risk of death when compared to plasma with lower antibody levels (60). In Iceland, Ronapreve^{TM} was used successfully in treating patients against the first variants of concern. However, with the emergence of the Omicron variant, the usefulness of many of the monoclonal antibody biologicals is dwindling. Sotrovimab^{TM} was effective against the BA.1 Omicron subvariant, but the effectiveness against the BA.2 Omicron subvariant is negligible.

Examining the neutralizing antibodies in seropositive and seronegative individuals after receiving the BNT162b2 mRNA (Pfizer) vaccine, it was seen that individuals who had been infected with SARS-CoV-2 produced antibodies with a higher neutralization potency and were less susceptible to escape variants of the virus. Suggesting that booster doses of the vaccine that induced a higher frequency of memory B cells are able to produce a broader range of neutralizing antibodies and target the escape variants (61). Moreover, neutralizing IgG and IgA antibodies have been detected in the breast milk of lactating women, reaching stable levels 14 days after the second dose (62). The third dose of BNT162b2 has also been shown to have a 93% effectiveness in preventing COVID-19-related hospital admission, 92% effectiveness in preventing against severe disease, and 81% effectiveness in preventing death, when compared to two doses administered at least 5 months before (63).

The declining efficacy of BNT162b2 in protecting against SARS-CoV-2 infections of the BNT162b2 vaccine has been reported 6 months after being fully vaccinated (two doses), the reason probably being due to waning immunity, rather than new variants, such as Delta. Importantly, however, the effectiveness of the vaccine in protecting against severe disease and hospital admissions did not wane (64). A fourth dose, administered 4 months after the third, has been shown to be efficacious against symptomatic disease. It did not show any substantial differences in humoral responses when compared to the third, suggesting that the third dose induced the maximal immunogenicity of the vaccine, whereas the fourth dose was able to restore the antibody levels (65).

The omicron variant harbors over 30 mutations in the coding region for the spike protein (66). Models, validated by experimental results, have suggested that the omicron variant is 2.8 times more contagious than the delta variant (67). This is of concern regarding the efficacy of the current vaccines targeting the spike protein of the SARS-CoV-2 virus. It has been shown that the efficacy of the BNT162b2 vaccine is still maintained, although at a lower level (68). One month (66, 69) after the second dose of the mRNA-1273 vaccine, the neutralization titers were 35 times lower against the omicron variant than the delta variant. While a booster dose of the vaccine increased the neutralization

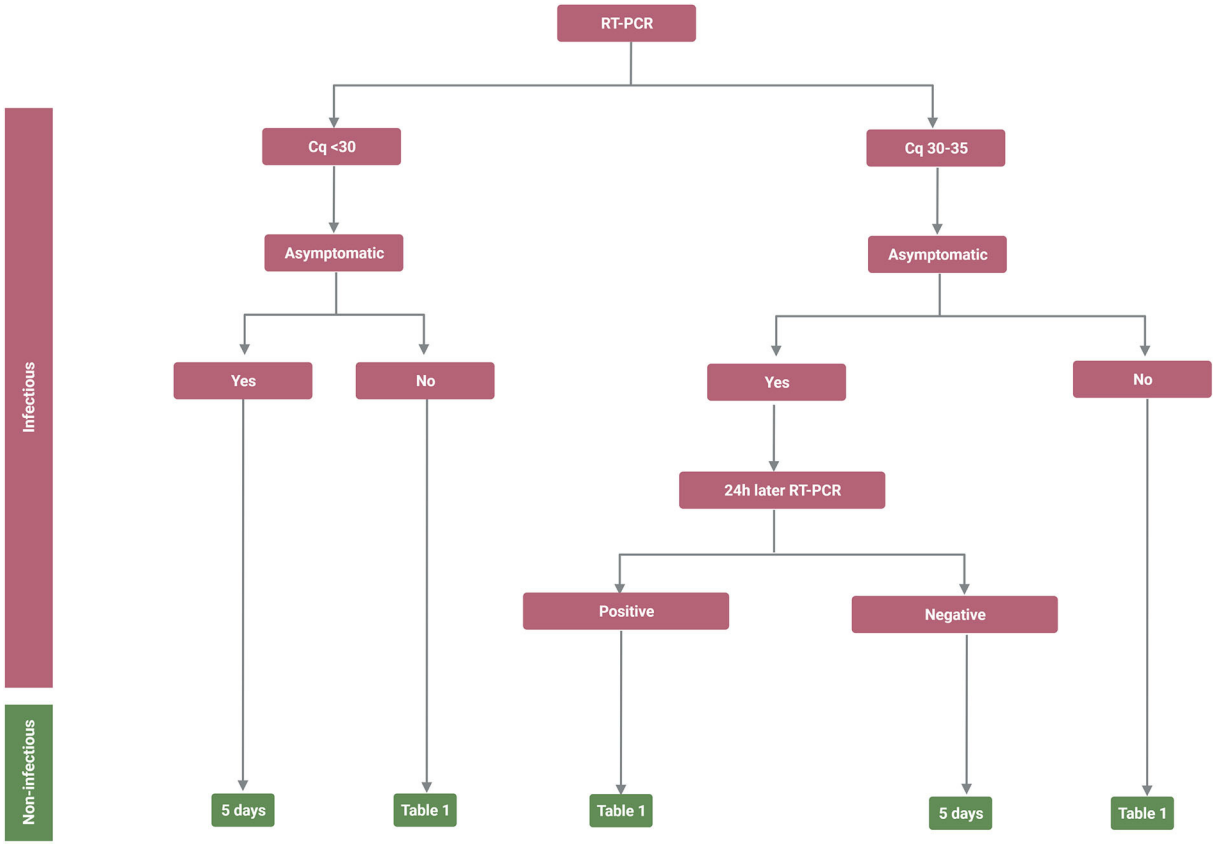

FIGURE 1
Infectivity and non-infectivity of fully vaccinated, immune competent, patients infected with Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Flow chart depicting the suggested strategy on how to isolate SARS-CoV-2 infected individuals to minimize the likelihood of releasing infectious individuals back into society. The flow chart takes into account that it is the last symptom or reverse transcription polymerase chain reaction (RT-PCR) measurement (Cq value) that defines in what category the individual will place since patients will be in different stages of the disease when they are sampled. *It should be noted that the positive predicted value of the antigen test can vary and it may therefore be important to confirm the diagnosis with RT-PCR. Created with BioRender.com.

titer against the omicron variant 20-fold when compared to 1 month after the second dose of the vaccine (70). Three doses of mRNA COVID-19 vaccine have also been associated with protection against both the omicron and delta variants (65, 71), with the fourth dose able to restore the antibody levels comparable to the third dose, but not showing any difference in the levels of omicron-specific neutralizing antibodies (65).

Thus, it is clear that vaccines protect against infections with SARS-CoV-2 and even though the virus mutates, they still offer some protection against the new variants. However, vigilance is needed offering the science community a challenge to develop new vaccines tailored against new virus variants. Even though initial vaccines offer less protection against newer variants but they are still helpful as demonstrated by their ability to protect against severe disease, hospitalization, and death (72).

Is it possible to determine non-infectivity?

A clear defining criterion when patients with COVID-19 cease to be infectious remains to be determined. This has a wide range of implications, such as public health recommendations, the safety of healthcare personnel, and international traveling to name a few.

Scientifically validated guidelines on this matter are required. Unfortunately, major differences exist between the current guidelines, and they are everchanging. The most common clinical and biological markers used to determine non-infectivity are viral RNA copies, viable viral isolation, symptom score, serology, and days from initial symptoms. Viral viability studies are the gold standard but are not practical for widespread use. Case studies where patients with COVID-19 have been followed have shown that infectivity can

TABLE 1 Determination of non-infectivity in relation to vaccination status and symptoms.

Vaccination status	Symptoms	Days of isolation until non-infectivy
Three doses[a]	No or mild symtpoms	5 days, no need for second PCR or antibody measurements
Two doses[b]	No symptoms or low symtpoms	7 days, no need for second PCR or antibody measurements
Partially and unvaccinated[c]		10 days, no need for second PCR or antibody measurements.
Serious COVID-19 disease, indipendent of vaccionation status	Serious COVID-19 symptoms needing dexamethasone, tocilizumab, ICU admission, respirator.	14 days and patient has N-protein specific antibodies and/or 1 negative PCR test OR 21 days after diagonsis

[a]Three doses of vaccine or two doses of vaccine and recovered from COVID-19.
[b]Two doses of vaccine or one dose of vaccine and recovered from COVID-19.
[c]No vaccination or only one dose.

be maintained for a longer time than the 20-day transmission-based precautions recommended by the Centers for Disease Control and Prevention (CDC) (26, 73) or the 14–20 days of isolation as recommended by the European Center for Disease Prevention and Control (ECDC) for individuals with severe symptoms (74).

The quantification cycle (Cq, also known as threshold cycle (Ct)) value from the RT-PCR has also been used as a surrogate marker for infectivity, where Cq < 35 is regarded as positive. Thus, a value of <20 has been shown to correlate with high viral load, whereas values of >35 might reflect a low contagious state with no detectable viable virus (27, 75). However, detection power is significantly affected by various factors (76), such as sampling location, swab technique, days from exposure, duration, and severity of symptoms among others. It is also important to note that one sample only gives a point estimate in time and the Cq values are most likely low (high viral load) before symptom onset and in the post-infectious state and as previously mentioned, re-infections with SARS-CoV-2 are common. However, a meta-analysis shows that a pooled estimate of how many people are re-detectable positive, in a cohort of recovered COVID-19 patients post discharge, is 14.8% and that the time from onset of symptoms to being re-detectable positive is 35.4 days (77). COVID-19 could therefore have a longer disease duration than previously thought.

Rapid antigen tests have significantly lower sensitivity and specificity than RT-PCR assays. These have been suggested as an alternative quick way to differentiate between contagious and non-contagious individuals (78, 79). In addition, in a comparative study of 122 CE-marked SARS-CoV-2 antigen rapid diagnostic tests (Ag RDTs), 78.6% met the authors' 75% sensitivity criteria in samples with relatively high viral load (Cq ≤ 25). Finally, only 20.8% met that criterion in samples with moderate viral load (Cq >25 to <30) and the majority were negative in samples with low viral load (Cq > 30). A Cochrane review on rapid antigen tests shows that these tests have a higher sensitivity for symptomatic patients with COVID-19 than asymptomatic (72.0 vs. 58.1%, respectively) and in patients that have Cq ≤25 than in those with higher Cq (94.5 vs. 40.7%, respectively). In addition, in the first week of symptoms, the average sensitivity of the rapid antigen tests was higher than in the second week (78.3 vs. 51.0%, respectively) (78). Another recent study found that the rate of false negative rapid antigen testing was noted in 87 of the 807 tests. Furthermore, the negative predictive value correlated strongly with the time of symptoms with a negative predictive value of rapid antigen testing being 80–100% for symptoms lasting < 5 days, whereas, the negative predictive value for the longer duration was only 50% (80). Thus, since very high viral load most often coincides with a symptomatic state, it is clear that rapid antigen tests are, at best, only useful in identifying potentially infected individuals with symptoms highly suggestive of COVID-19 that should be corroborated with PCR testing (81). It has been shown that if viral load fell below 10^6 copies/ml in patients with COVID-19, it was not possible to culture viable virus despite positive RT-PCR up to day 28 (27). In addition, in an earlier report, it was suggested that patients with Cq above 33–34 by RT-PCR technology might not be contagious and might be used to determine if they could be safely discharged or relieved from strict confinement. Similar findings have been observed by others suggesting that Cq values above 30–33 might be used to define the viability of replicating the SARS-CoV-2 virus (1). In addition, comparing Cq values between laboratories can be problematic, as large variation has been found with a quantitative comparison between samples (82). Where the method of sampling and sampling location are of importance, with the nasopharyngeal swabs still being the gold standard while throat swabs are not recommended due to the low sensitivity and positive predictive value (83). Thus, based upon these and similar findings, this has been further stratified into the following groups of viral load: high (Cq 17–25), moderate (Cq 25–30), and low (Cq 30–36).

Based on the data discussed above and the clinical experience of the physicians managing the pandemic in Iceland, a flow chart (Figure 1, Table 1) was created, proposing a strategy on how to determine a low risk for viral transmission (non-infectivity) in fully vaccinated individuals based upon their symptomatic state. A similar strategy has already been proven to be highly successful at Landspitali University Hospital in Reykjavik, Iceland.

Concluding remarks

Numerous attempts have been made to provide evidence-based protocols to establish non-infectivity, particularly for determining when to stop quarantine of infected individuals, when healthcare workers can return to work, and more importantly for infected immunocompromised individuals.

While rapid antigen tests do not have the sensitivity or specificity of RT-PCR tests, they can contribute to the removal of asymptomatically infected or pre-symptomatic SARS-CoV-2 spreading individuals from the general population. In addition, in selected cases in asymptomatic individuals, in-depth SARS-CoV-2-specific IgM/IgG/IgA levels might provide a better overview of the individuals' timeline of infectivity.

Thus, the suggested flow chart will hopefully provide some insight into how to minimize the likelihood of releasing an infected and contagious individual from all restrictions either within the hospital or within general public settings.

Author contributions

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

Conflict of interest

The 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.

Publisher's note

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Dihydropyridine-derived calcium channel blocker as a promising anti-hantavirus entry inhibitor

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Hantaviruses, the causative agent for two types of hemorrhagic fevers, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), are distributed from Eurasia to America. HFRS and HPS have mortality rates of up to 15% or 45%, respectively. Currently, no certified therapeutic has been licensed to treat hantavirus infection. In this study, we discovered that benidipine hydrochloride, a calcium channel blocker, inhibits the entry of hantaviruses *in vitro*. Moreover, an array of calcium channel inhibitors, such as cilnidipine, felodipine, amlodipine, manidipine, nicardipine, and nisoldipine, exhibit similar antiviral properties. Using pseudotyped vesicular stomatitis viruses harboring the different hantavirus glycoproteins, we demonstrate that benidipine hydrochloride inhibits the infection by both HFRS- and HPS-causing hantaviruses. The results of our study indicate the possibility of repurposing FDA-approved calcium channel blockers for the treatment of hantavirus infection, and they also indicate the need for further research *in vivo*.

KEYWORDS

Hantaan virus, hantavirus, bunyavirales, hemorrhagic fever with renal syndrome, hantavirus pulmonary syndrome, antivirals, benidipine hydrochloride, calcium channel blocker

Introduction

Hantaviruses, the causative agents for hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) worldwide (Jiang et al., 2017), belong to the genus *Orthohantavirus*, family *Hantaviridae*, within the order *Bunyavirales*. Hantaviruses are enveloped viruses containing tripartite negative-stranded RNA and a large (L) genome, including medium (M) and a small (S) segment, which encode RNA-dependent RNA polymerase (RdRp or LP), glycoprotein

precursor (GPC), and nucleocapsid protein (NP), respectively (Vaheri et al., 2013). During virus maturation, GPC is cleaved into Gn and Gc by the host enzyme, and this cleavage facilitates virus attachment to cellular receptors and subsequent membrane fusion (Cifuentes-Munoz et al., 2014; Mittler et al., 2019; Serris et al., 2020).

The distribution of HFRS and HPS was defined by the geographical distribution of the natural host of these causative viruses (Kabwe et al., 2020). HFRS is mainly endemic in Eurasia, primarily caused by the Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus (DOBV), and Puumala virus (PUUV). HPS is an epidemic in the Americas, with SNV and ANDV as the predominant pathogens. HTNV and SEOV are the two primary pathogens of HFRS in China, and HTNV is responsible for severe cases (Jonsson et al., 2010). There are currently no certified pharmaceutical agents approved by the U.S. Food and Drug Administration (FDA) to treat hantavirus infection, considering the mortality of up to 15% for HFRS and 45% for HPS, respectively. It underlines the urgent need to develop new therapeutic antivirals (Brocato and Hooper, 2019; Ye et al., 2019; Munir et al., 2020). The repurposed use of FDA-approved drugs is an effective strategy to identify potential antivirals, considering clinical safety, and was adopted during the SARS-CoV-2 pandemic (Riva et al., 2020; Santos et al., 2020; Zhou et al., 2020). In addition to SARS-CoV-2, this strategy has been applied to various emerging and re-emerging viruses, such as the Ebola virus, Zika virus, and severe fever with thrombocytopenia syndrome virus (SFTSV) (Johansen et al., 2015; Barrows et al., 2016; Li et al., 2019).

Hantavirus, an enveloped virus, binds to cellular receptors through viral glycoprotein and enters cells *via* endocytosis (Mittler et al., 2019; Noack et al., 2020; Guardado-Calvo and Rey, 2021). The interfering entry process is, therefore, an attractive strategy for combating hantavirus infection. Vesicular stomatitis virus (VSV) is widely used to screen viral entry inhibitors due to its multiple advantages including the ability to be handled under lower biosafety conditions and the ability to pseudotype various viral envelope proteins (Mayor et al., 2021). Based on recombinant VSV-based antiviral screening, we found that benidipine hydrochloride, a calcium channel inhibitor, is a potential beneficial countermeasure against hantavirus. By blocking membrane-bound calcium channels, calcium channel inhibitors reduce the intracellular calcium level, which may have protective effects on vascular endothelial cells (Yao et al., 2006). Furthermore, benidipine HCl exhibits pan-anti-hantaviral activity when applied to pseudotyped viruses carrying different hantaviral glycoproteins. As a result of our study, we believe that benidipine HCl and other calcium channel inhibitors hold promise as potential therapeutic agents for treating hantavirus infection. We believe further *in vivo* studies are warranted.

Methods

Cells, viruses, and reagents

African green monkey kidney Vero-E6 (ATCC, CCL-81), human non-small-cell lung carcinoma (A549) (ATCC; CCL-185), Syrian golden hamster kidney BHK-21 (ATCC; CCL-10), human embryonic kidney HEK-293T, and human hepatoma Huh7 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) in 5% CO_2 at 37°C, as described previously (Ye et al., 2020). DMEM without Ca^{2+} was obtained from Yuchun Bio (Shanghai, China).

HTNV (strain 76-118) was propagated and titrated in Vero-E6 cells, as previously indicated (Ye et al., 2015; Ye et al., 2019).

The primary mouse monoclonal antibodies against HTNV NP (1A8) were produced in a lab, as mentioned earlier (Cheng et al., 2016). An antibody against GFP was purchased from Abbkine (Wuhan, China), and tubulin/GAPDH was purchased from Sangon Biotech (Shanghai, China). Horseradish peroxidase (HRP) or infrared dye-conjugated secondary antibodies were obtained from Sangon Biotech and Li-Cor Biosciences (Lincoln, NE, United States), respectively.

Benidipine hydrochloride (HCl) was purchased from MedChemExpress (NJ, United States) and cilnidipine, felodipine, nicardipine HCl, nifedipine, nisoldipine, and nitrendipine were purchased from TargetMol (Shanghai, China). Manidipine was purchased from APExBIO Technology (Houston, TA, United States). The calcium chelator BAPTA-AM was purchased from GlpBio (Montclair, CA, United States).

Cytotoxicity assay

Cell viability was calculated as previously mentioned (Ye et al., 2020). Briefly, Vero-E6 and A549 cells were incubated with serial dilutions of each drug for 24–48 h. Cell viability was assayed using Cell Counting Kit-8 (CCK8) (TargetMol), with absorbance (A) at 450 nm being measured using a BioTek HT synergy instrument.

Rescue of recombinant vesicular stomatitis virus bearing the Hantaan virus glycoprotein precursor

The plasmid-bearing VSV antigenome lacking VSV-G ORF but with an additional GFP ORF was synthesized at GenScript (Nanjing, China), containing unique restriction sites for foreign gene expression: 5′ flanked by the T7 bacteriophage promoter, 3′ flanked by hepatitis delta virus ribozyme (HDVRz), and the T7 terminator sequence. The codon-optimized GPC genes of

HTNV (NC_005219) were PCR amplified and inserted into the VSV antigenome plasmid, resulting in the rVSV-HTNV-G vector.

BHK-21 cells were seeded into a six-well plate overnight and infected with vaccinia virus bearing T7-pol (kindly provided by the Wuhan Institute of Virology, CAS) for 2 h. Then, the cells were transfected with helper plasmids encoding VSV-N, VSV-P, VSV-L, and VSV-G (kindly provided by the Wuhan Institute of Virology, CAS). The transfection ratio for each plasmid was 5:3:5:1:8, with a total of 11 μg per well. Transfections were performed using the Hieff Trans Liposomal Transfection Reagent (Yeasen, Shanghai, China). Cytarabine (TargetMol) was added after transfection at a concentration of 100 μg/ml, and the culture supernatant was collected 72 h post-transfection and used to blind infect VeroE6 cells. After several passages, the cytopathic effect (CPE) in the cell monolayer was noticeable and indicated a successful rescue. The rescued virus was referred to as rVSV-HTNV-G and verified by HTNV GPC-specific antibodies. Viruses were propagated and tittered on Vero E6 cells, and the titer was determined using plaque assays.

Preparation of the hantavirus glycoprotein precursor pseudotyped vesicular stomatitis virus

GPC genes of HTNV (NC_005219), SEOV (AB027521), PUUV (U14136), DOBV (L33685), ANDV (AF291703), and SNV (L25783) were codon-optimized and synthesized at GenScript (Nanjing, China). All target genes were cloned into the pCAGGS vector, as previously indicated (Yao et al., 2020). Pseudotyped VSV (pVSVΔG-GFP) was stored in our laboratory, where the coding region of the G protein was replaced with an enhanced green fluorescent protein, and the VSV-G protein was expressed in the trans form. BHK-21 cells were transfected with pCAGGS-GPC of each hantavirus, 24 h later, and then pVSVΔG-GFP-bearing VSV-G was added at a multiplicity of infection (MOI) of 1 for 1 h at 37°C. The monolayer was then washed with Dulbecco's Phosphate-Buffered Saline (DPBS, Cellgro) three times, and the fresh medium was replenished. After 36 h of incubation, the culture supernatant was clarified by low-speed centrifugation, aliquoted, and stored at −80°C.

Western blot analysis

As indicated in different experiments, cells in six-well plates were treated with benidipine HCl and infected with viruses. Cells were washed twice with DPBS and lysed with RIPA buffer (Beyotime, P0013C, or P0013D). Samples were quantified using a BCA kit (Thermo Fisher Scientific), and 20 μg or 40 μg aliquots of each cell lysate were boiled for 10 min and subjected to 12% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) and blotted with indicated primary antibodies, followed by secondary antibodies conjugated to infrared dyes or HRP and visualized using an Odyssey Infrared Imaging System (Li-Cor Biosciences) or Tanon 5200SF Imaging System (Shanghai, China).

Immunofluorescence assay

Cells in 24-well plates were treated with drugs and infected with rVSV, rVSV-HTNV-G, or different pVSV and were imaged with an IX71 fluorescence microscope (Olympus, Tokyo, Japan) 24 h post-infection. For the immunofluorescence assay, cells were seeded onto coverslips in 24-well plates at a confluence of 60%–70%. Then, cells were treated with benidipine HCl and infected with HTNV, subjected to IFA at the indicated time points, p. i., following an established protocol (Ye et al., 2019). Cells were imaged with a BX60 fluorescence microscope (Olympus).

Quantitative reverse transcription PCR

Total RNA of HTNV or rVSV-HTNV-G infected cells was extracted and reverse transcripted using the Hifair® 1st Strand cDNA Synthesis SuperMix (Yeasen), according to the instructions provided by the manufacturer. qRT-PCR was performed using the Hieff® qPCR SYBR Green Master Mix (Yeasen) on a CFX96 Real-Time system (Bio-Rad). The mRNA expression level of each target gene was normalized to the corresponding GAPDH expression level. The primers used for gene amplification were as follows: GFP (forward: 5′-CTG GACGGCGACGTAAACG -3'; reverse: 5′-CCAGGGCACGGG CAGCTTGC -3′), HTNV S segment (forward: 5′-GAGCCT GGAGACCATCTG -3'; reverse: 5′-CGGGACGACAAAGGA TGT -3′), and GAPDH (forward: 5′- ACCCACTCCTCCACC TTTG -3'; reverse: 5′- ATCTTGTGCTCTTGCTGGG -3′).

Statistical analysis

Statistical analysis was performed using a two-tailed unpaired t-test in GraphPad Prism software (La Jolla, CA, United States). Data are presented as means ± standard deviations (SDs) (n = 3 or otherwise indicated). All experiments were repeated at least three times.

Results

Benidipine hydrochloride inhibits Hantaan virus infection in different cell lines

The repurposing of FDA-approved drugs is an effective method of screening potential antiviral agents since the safety

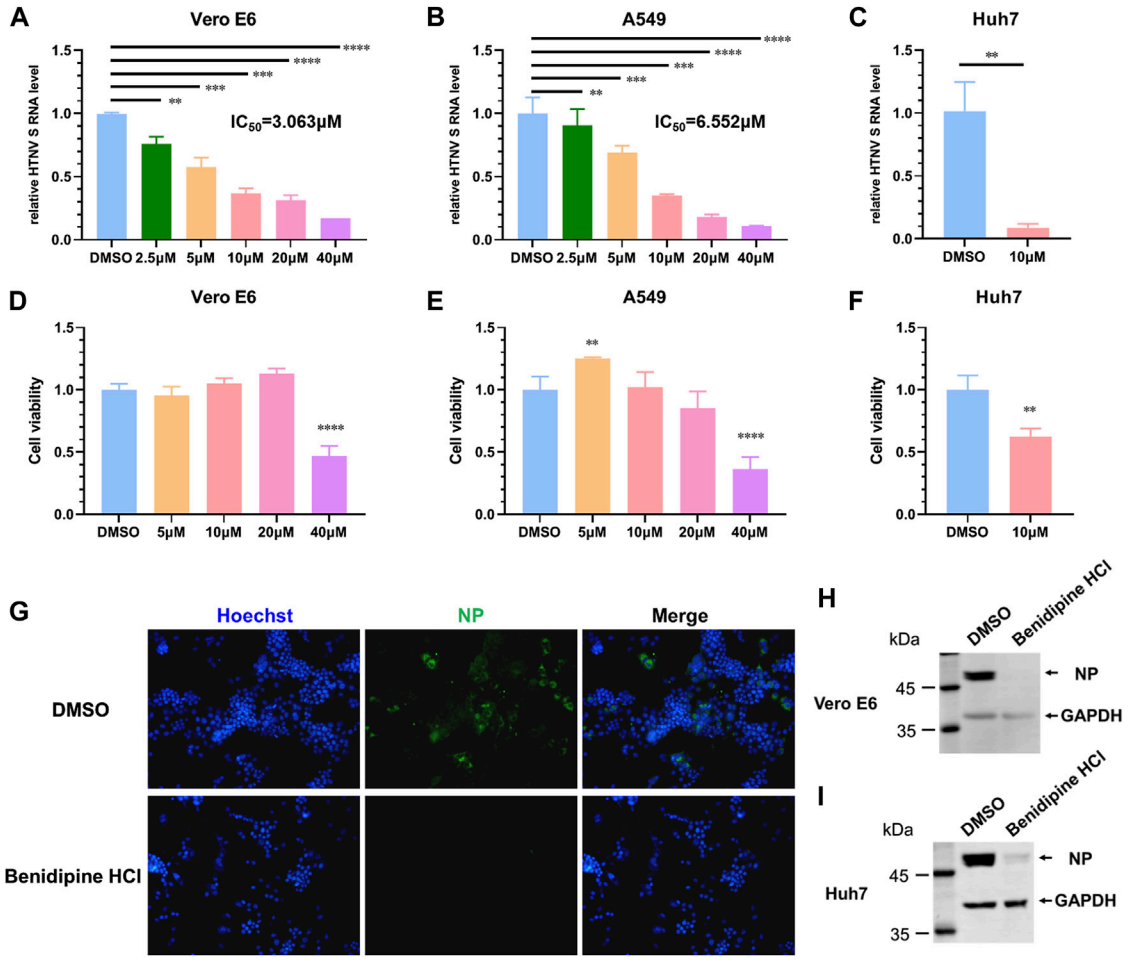

FIGURE 1
Benidipine hydrochloride inhibits HTNV infection. **(A–C)** Benidipine hydrochloride inhibited HTNV in Vero, A549, and Huh7 cells with dose-dependent effects. Benidipine hydrochloride was applied to cell monolayers at the indicated concentrations for 1 hour, and then, HTNV was inoculated at MOI 1 with the same concentrations of benidipine hydrochloride. After adsorption, the inoculum was discarded, and benidipine hydrochloride was added to the fresh medium. Total RNA was extracted 24 hpi, and the HTNV S segment RNA level was measured by qRT-PCR and normalized to GAPDH. IC_{50} was indicated in the panel (IC_{50} refers to 50% inhibitory concentration). Vero cells **(D)**, A549 cells **(E)**, and Huh7 cells **(F)** were analyzed for viability using the CCK8 assay after 24 h of benidipine hydrochloride treatment. The inhibition of the expression of HTNV structural proteins by benidipine hydrochloride. **(G–I)** Vero cells were treated with 10 μM benidipine hydrochloride and infected with HTNV; after 24 h of infection, coverslips were stained with the NP-specific antibody 1A8 **(G)**. After being exposed to 10 μM benidipine hydrochloride, Vero cells **(H)** or Huh7 cells **(I)** were infected with HTNV. After 24 h of infection, cells were lysed and immunoblotted with the antibody 1A8. Student's t-test was used to compare mean values between the benidipine hydrochloride-treated group and the vehicle control group (DMSO). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

of these drugs has been demonstrated in clinical trials. By leveraging this strategy, we found that benidipine hydrochloride (HCl) significantly inhibited HTNV replication. Benidipine HCl was added to Vero cells 1 hour prior to HTNV infection; the cells were inoculated with HTNV at a multiplicity of infection (MOI) of 1, and benidipine HCl was added during and after virus adsorption. The relative intracellular RNA levels of the HTNV S segment were determined by quantitative real-time PCR (qRT-PCR) at 24 h after infection. A dose-dependent reduction of the HTNV S segment was observed in cell monolayers treated with benidipine in comparison with the DMSO vehicle, with an IC_{50} of 3.063 μM (Figure 1A). However, CCK8 assays did not reveal any effect on cell viability (Figure 1D). Inhibition of HTNV infection by benidipine HCl was also observed on A459 cells with an IC_{50} of 6.552 μM (Figures 1B,E) and Huh7 cells (Figures 1C,F), both permissive cell lines for replication of HTNV. Furthermore, in HTNV-infected Vero E6 cells, treated with benidipine HCl, the number of NP-positive cells was remarkably reduced (Figure 1G). Additionally, benidipine HCl treatment significantly reduced the relative protein levels of NP (Figures 1H,I).

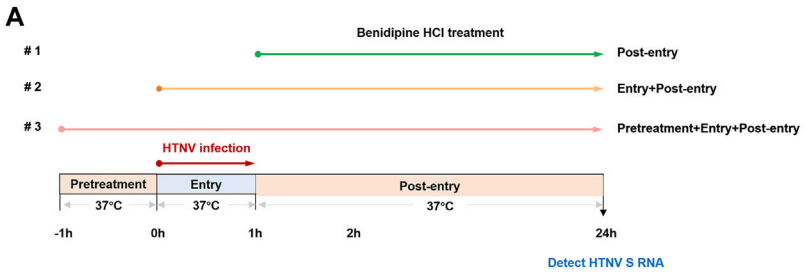

FIGURE 2
In vitro anti-HTNV mechanism of benidipine hydrochloride. **(A)** Benidipine hydrochloride was administered to Vero E6 cells, as described in the schematic diagram. After HTNV infection, cells were treated with benidipine hydrochloride (#1), or added with HTNV prior to adsorption (#2), or 1 h before HTNV and added throughout the infection procedure (#3). **(B)**The effect of benidipine hydrochloride on the entry of HTNV. As depicted in **(A)**, the relative intracellular S segment RNA level of HTNV was determined at 24 h post-infection using a variety of methods. *$p < 0.05$; **$p < 0.01$.

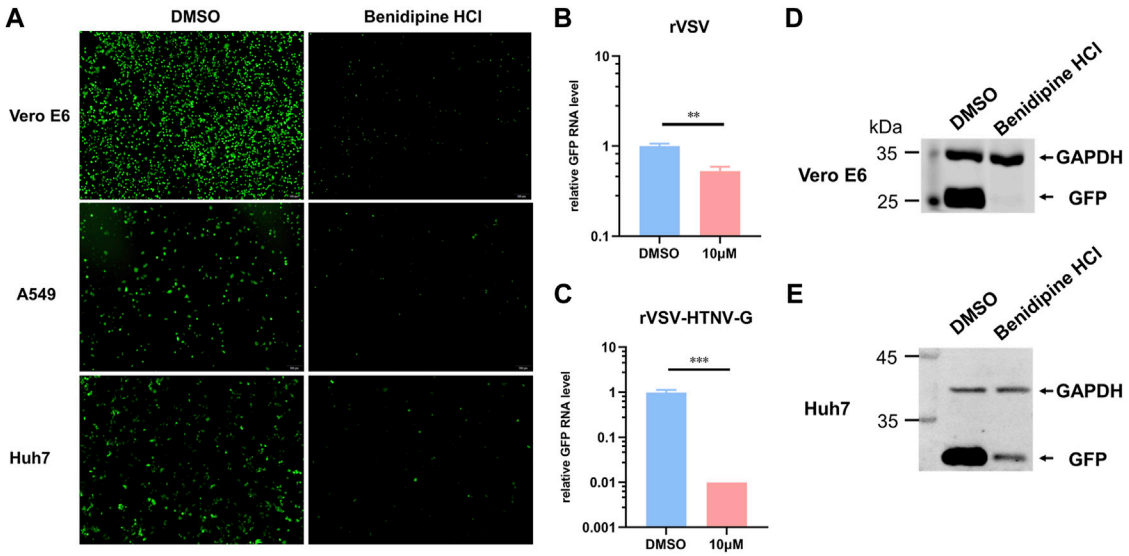

FIGURE 3
Effect of benidipine hydrochloride on the entry stages of HTNV. **(A)** Vero E6, A549, and Huh7 cells were treated with benidipine hydrochloride or the DMSO vehicle and then inoculated with rVSV-HTNV-G at MOI 1, and GFP-positive cells were photographed 24 h after infection. **(B,C)** Vero E6 cells were treated with benidipine hydrochloride or the DMSO vehicle and then infected with rVSV **(B)** or rVSV-HTNV-G **(C)**. After 24 h post-infection, total RNA was extracted, and GFP RNA levels were determined by qRT-PCR and normalized to GAPDH. **(D, E)** Vero **(D)** and Huh7 **(E)** cells were treated with 10 μM benidipine hydrochloride and infected with rVSV-HTNV-G; the cells were lysed 24 h after infection and blotted with antibodies against GFP and GAPDH. **$p < 0.01$; ***$p < 0.001$.

Benidipine hydrochloride inhibits Hantaan virus infection by blocking virus entry interfering with virus internalization

Following this, we examined the mechanisms by which benidipine HCl inhibits HTNV infection. Upon treating Vero E6 cells with 10 μM benidipine HCl before virus adsorption, or during virus adsorption and afterward, or after virus adsorption, intracellular levels of the viral S segment RNA were detected 24 h post-infection (Figure 2A). A gradual decrease in the HTNV RNA level was observed over the course of benidipine HCl treatment, during and after the treatment, and after adsorption, suggesting that benidipine HCl inhibited HTNV entry into the cell (Figure 2B).

For further validation of benidipine HCl's entry inhibitory effects on HTNV, we tested its effect on a recombinant VSV expressing GFP with glycoprotein substituted for HTNV GPC

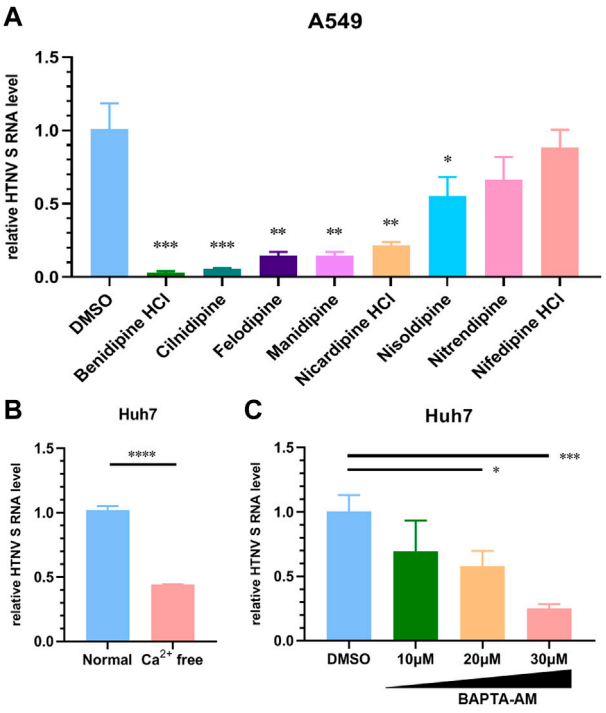

FIGURE 4
Different dihydropyridine-derived calcium channel blockers inhibit HTNV infection. **(A)** A549 cells were treated with the indicated CCBs or the DMSO vehicle and infected with HTNV at MOI 1, and the relative level of S segment RNA was determined by normalizing to GAPDH. **(B)** Huh7 cells were cultured in Ca^{2+}-free or normal medium and then infected with HTNV (MOI = 1), 24 hpi; the relative intracellular HTNV RNA level was measured by qRT-PCR. **(C)** Huh7 cells were infected with HTNV upon treatment with BAPTA-AM, 24 hpi. The relative intracellular HTNV RNA level was measured by qRT-PCR. *p < 0.05; **p < 0.01; ***p < 0.001.

(rVSV-HTNV-G). In Figure 3A, the number of GFP-positive cells was significantly lower in benidipine HCl-treated cells than in DMSO-treated cells, indicating that benidipine HCl inhibited rVSV-HTNV-G infection in different cell lines. Additionally, the level of GFP RNA within cells was detected 24 h following infection with rVSV and rVSV-HTNV-G, indicating that benidipine HCl is more effective at inhibiting rVSV-HTNV-G than rVSV (Figures 3B,C). In addition, Vero E6 and Huh7 cells treated with benidipine HCl had significantly lower levels of GFP protein than Vero E6 and Huh7 cells treated with DMSO (Figures 3D,E).

Calcium channel blockers inhibit Hantaan virus infection through reducing cellular Ca^{2+} uptake

As a calcium channel blocker derived from dihydropyridine (DHP), benidipine HCl is commonly used in the treatment of hypertension. In order to determine whether other members of the DHP process exhibit similar HTNV inhibition functions, we tested the anti-HTNV activity of an array of DHPs. In Figure 4A, cilnidipine, felodipine, amlodipine, manidipine, nicardipine, and nisoldipine inhibit HTNV replication on A549 cells at 10 μM, suggesting that multiple DHP-derived calcium channel blockers have a common anti-HTNV effect. The HTNV RNA level was also significantly reduced when Huh7 cells were treated with Ca^{2+}-free medium as compared to the normal medium (Figure 4B). Likewise, the HTNV RNA level was also decreased in a dose-dependent manner when Huh7 cells were treated with BAPTA-AM, a calcium chelator (Figure 4C). Thus, these results suggest that calcium channel blockers inhibit HTNV infection by reducing the Ca^{2+} influx.

Benidipine hydrochloride exhibits the broad-spectrum anti-hantaviral entry activity

In order to examine whether benidipine HCl inhibits other hantaviruses, we rescued various VSV pseudoviruses enveloped with pathogenic hantaviral glycoproteins, including SEOV, PUUV, DOBV, SNV, and ANDV. As expected, benidipine HCl significantly decreased the infection rate of those pVSVs (Figure 5), demonstrating that it has a broad-spectrum anti-hantavirus activity.

Discussion

The development of a drug is a complex process that involves a great deal of uncertainty and takes a long time. The repurposing of licensed drugs is an alternative strategy for the development of antivirals (Johansen et al., 2015; Barrows et al., 2016; Li et al., 2019; Riva et al., 2020; Santos et al., 2020; Zhou et al., 2020). Dihydropyridine-derived calcium channel blockers have been used for a long time to treat hypertension and angina pectoris (Yamamoto et al., 1990). As a new long-acting drug, benidipine HCl is approved for its ability to bind to the voltage-gated calcium channel's DHP-binding site (Yao et al., 2006). In addition to its anti-hypertensive and cardioprotective properties, benidipine HCl also exhibits renoprotective and endothelial protective properties (Karasawa and Kubo, 1990; Yao et al., 2000; Matsubara and Hasegawa, 2005). In addition, because benidipine HCl only has mild side effects and has a high level of clinical safety, it is attractive for its potential application against other diseases.

As an acute disease with high mortality, HFRS is transmitted primarily through the inhalation or consumption of rodent-contaminated air or food (Jiang et al., 2017; Kabwe et al., 2020). Endothelial permeability increases and renal damage

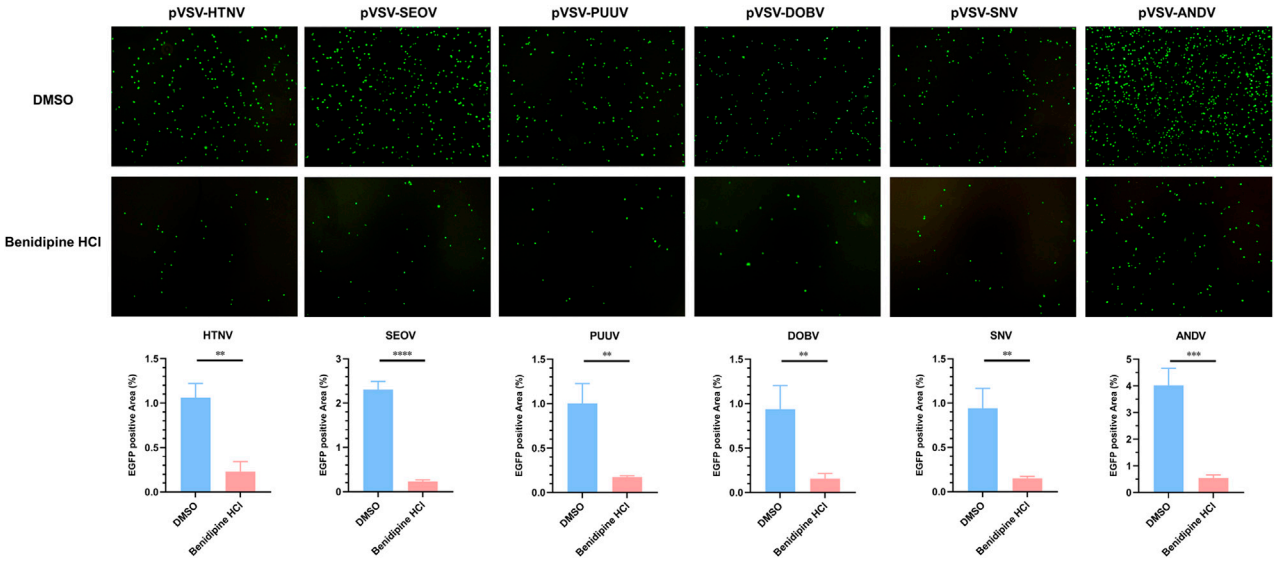

FIGURE 5
Benidipine hydrochloride inhibits multiple hantaviruses' glycoprotein pseudotyped VSV infection. Various pseudotyped VSV bearing different hantaviral glycoproteins were incubated with 10 μM benidipine HCl or the vehicle DMSO, after which the mixture was used to infect A549 cells, and the GFP-positive cells were photographed 24 h post-infection. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

are the main symptoms of HFRS caused by HTNV or SEOV (Jiang et al., 2017). Currently, there are no licensed antivirals against these viruses, and the first-line treatment option is supportive therapy. Since the responsible pathogens are biosecurity-related, treatment measures are needed for these viruses.

The present study demonstrated that benidipine HCl has a powerful antiviral effect on HTNV in a variety of cells with an IC_{50} at a low micromolar level. Furthermore, benidipine HCl significantly inhibited the entry of rVSV-bearing HTNV GPC into cells, and the inhibition is more potent than that of VSV itself. Pretreatment of cells with benidipine HCl during virus infection and after virus adsorption results in the most effective inhibition of HTNV compared to other methods. The results indicate that benidipine HCl inhibits HTNV mainly at the entry stage.

Additionally, both the calcium-free medium and BAPTA-AM, a cellular calcium chelator, inhibit HTNV replication, along with numerous calcium channel blockers derived from DHP. These results suggest that the level of intracellular Ca^{2+} correlates with the replication level of HTNV and other hantaviruses. In fact, West Nile virus (WNV) is one example of a virus that induces Ca^{2+} influx, which is crucial for efficient viral replication (Scherbik and Brinton, 2010). Aside from this, Ca^{2+} can regulate multiple pathways through signal transduction (Bagur and Hajnóczky, 2017) and could serve as an active center for some special proteins, such as the annexin family, which has been shown to be involved in the regulation of multiple virus infections. Furthermore, DHP-derived calcium channel blockers target different calcium channel subtypes and differ in half-life times, which may result in different inhibitory efficacies against HTNV. Nevertheless, further research is necessary to identify the specific mechanism.

Viral hemorrhagic fever (VHF) poses a serious health threat to humans, particularly in less-developed countries. There are several types of viruses that can cause VHF, such as filoviruses, arenaviruses, bunyaviruses, and alphaviruses. Other CCBs, in addition to benidipine HCl, may inhibit VHF-induced virus infection by interfering with the virus entry stage (Lavanya et al., 2013; Sakurai et al., 2015; DeWald et al., 2018; Li et al., 2019). A number of CCBs were also evaluated to see if they were effective in stopping HTNV infection, and multiple drugs were found to be effective. In light of the wide-spectrum nature of CCBs against VHF viruses, we investigated the effect of benidipine HCl against hantavirus pseudotyped viruses and concluded that this agent was able to inhibit the main pathogenic hantaviruses responsible for both HFRS and HPS. This host-targeting treatment is unlikely to result in drug-resistant strains. Further research into how Ca^{2+} regulates the replication of HTNV may provide additional information regarding the hantavirus lifecycle and strengthen our understanding of its virology.

Overall, we found that benidipine HCl, as well as other CCBs, can inhibit hantavirus infection. This is similar to what has been observed for other viruses, which indicates that an *in vivo* study of benidipine HCl against hantavirus infection is warranted.

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

JP and WY performed most of the experiments; HZ and WY prepared recombinant and pseudotyped VSVs; HL, YW, LZ, LQ, and YY provided experimental technique support; LC, LZ, and YD provided experimental materials; BW, AQ, YL, and FZ provided administrative support; BW, ZX, FZ, and WY offered financial support; BW, WY conceived the study and wrote the manuscript; BW, JP, YL, FZ, and WY checked and finalized the manuscript. All authors have made substantial contributions to the manuscript.

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Conflict of interest

The 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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Factors associated with hemorrhagic fever with renal syndrome based maximum entropy model in Zhejiang Province, China

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Background: Hemorrhagic fever with renal syndrome (HFRS) is a serious public health problem in China. The geographic distribution has went throughout China, among which Zhejiang Province is an important epidemic area. Since 1963, more than 110,000 cases have been reported.

Methods: We collected the meteorological factors and socioeconomic indicators of Zhejiang Province, and constructed the HFRS ecological niche model of Zhejiang Province based on the algorithm of maximum entropy.

Results: Model AUC from 2009 to 2018, is 0.806–0.901. The high incidence of epidemics in Zhejiang Province is mainly concentrated in the eastern, western and central regions of Zhejiang Province. The contribution of digital elevation model ranged from 2009 to 2018 from 4.22 to 26.0%. The contribution of average temperature ranges from 6.26 to 19.65%, Gross Domestic Product contribution from 7.53 to 21.25%, and average land surface temperature contribution with the highest being 16.73% in 2011. In addition, the average contribution of DMSP/OLS, 20-8 precipitation and 8-20 precipitation were all in the range of 9%. All-day precipitation increases with the increase of rainfall, and the effect curve peaks at 1,250 mm, then decreases rapidly, and a small peak appears again at 1,500 mm. Average temperature response curve shows an inverted v-shape, where the incidence peaks at 17.8°C. The response curve of HFRS for GDP and DMSP/OLS shows a positive correlation.

Conclusion: The incidence of HFRS in Zhejiang Province peaked in areas where the average temperature was 17.8°C, which reminds that in the areas where temperature is suitable, personal protection should be taken when

going out as to avoid contact with rodents. The impact of GDP and DMSP/OLS on HFRS is positively correlated. Most cities have good medical conditions, but we should consider whether there are under-diagnosed cases in economically underdeveloped areas.

KEYWORDS

MaxEnt, HFRS, socio-economic factors, ecological data, meteorological data

Introduction

Hemorrhagic fever with renal syndrome (HFRS) is a rodent viral disease caused by hantaviruses (HVs), which are distributed in Eurasia and have a case fatality rate of 5–15%; HVs can also cause hantavirus cardiopulmonary syndrome (HCPS), which develop mainly in the Americas and have a case fatality rate of up to 40% for HCPS (1). Since there are no specific drugs, HFRS and HCPS can only be treated with symptomatic support. HFRS caused by Hantaan (HTNV) and Seoul (SEOV), HTNV and SEOV which belongs to genus in Orthohantavirus family Hantaviridae, order Bunyavirales (2). Whole virus-inactivated vaccines against HTNV or SEOV are currently licensed in Korea and China, but the protective efficacy of these vaccines is uncertain (1).

HFRS is a serious public health problem in China. The epidemic reached the peak in 1986, with a total of 115,804 cases reported. As government launched a series of disease control and prevention measures, the incidence began to decrease (3). HFRS was first observed in the Heilongjiang Province of China in the 1930s. It remained poorly understood until 1978 when Hantaan Virus and its reservoir *Apodemus agrarius* were discovered by Lee et al. (4). During 2006–2012, 77,558 human cases and 866 fatal cases of HFRS were reported, with an average annual incidence rate of 0.83 per 100,000 and a case fatality rate of 1.13%. About 84.16% of the total cases were concentrated in 9 provinces, with the highest incidence in spring and autumn/winter (5).

Zhejiang Province is an HFRS endemic region, and the first case was reported in 1963. From 1963 to 2020, the morbidity and mortality rates decreased significantly, however, the geographical distribution of endemic areas has been expanding to all of Zhejiang Province (6).

Studies had shown hantavirus infection dynamics: changes in climate (7–9), environmental condition affect the risk of zoonotic transmission via changes in reservoir dynamics (8); nephropathia epidemica is more likely to occur with intense vegetation activity, soil with low water content condition (10). Previous analyses in China suggest rainfall, mouse density and autumn crop yield are correlated with the incidence of HFRS (11). A study in Xi'an, China showed HFRS is correlated to rainfall, rodent density and lags of temperature (12). Another research observed HTNV stabilities and results show at 4 degrees wet conditions particularly, HTNV is detectable after 96 days, and sensitive to drying (13).

Materials and methods

Data collection and case definition

The data from 2009 to 2018 on HFRS cases were obtained from the Chinese Notifiable Disease Reporting System. Information of HFRS cases includes age, gender, residential address and date of illness onset. According to the health industry standard of the People's Republic of China for diagnostic criteria of HFRS, HFRS cases were classified as suspected cases, clinically diagnosed cases and confirmed cases (6). Zhejiang Province is located on the southeast coast of China, with 11 cities and 90 county (**Supplementary Figure 1**).

Meteorological data (DEM01-11) were obtained from China Meteorological Data Sharing Service System.[1] It included sunshine hours, average relative humidity, average land surface temperature, 20–8 precipitation, 8–20 precipitation, 20–20 precipitation, average air pressure, average air temperature, daily maximum temperature, average wind speed and maximum wind speed. Layers for yearly average meteorological data from 2009 to 2018 were generated using the kriging interpolation method with ArcGIS 10.2.

The socio-economic factors and ecological data used in this study were obtained from the Resource and Environmental Science Data Center of the Chinese Academy of Sciences.[2] They included normalized difference vegetation index (NDVI), enhanced vegetation index (EVI), Annual NDVI (aNDVI); clay, sand, silt; gross domestic product (Zjgdp), and digital elevation model. Digital elevation model (DEM) can be regarded as an image, a typical raster data obtained

1 http://data.cma.cn/

2 http://www.resdc.cn

by sampling the image plane coordinates and height. Defense Meteorological cSatellite Program Nighttime Lighting Index (DMSP/OLS), DMSP/OLS imageries were acquired by the Defense Meteorological Satellite, mainly used for urban expansion research. We used ArcGIS 10.2 to set the grid for layers of different variables to the same geographic boundaries and cell sizes to extract data for Zhejiang Province.

Statistical methods

The maximum entropy principle is to predict the unknown information of a target area by incomplete known information. Using known species distribution and ecological environment data, the non-random relationship between environmental characteristics and species distribution in the known species distribution area is studied to find the probability distribution with maximum entropy as the optimal distribution to predict the suitable habitat for a species (14).

The maximum entropy model (MaxEnt) is a valuable software for ecological niche models (ENM) study to estimate the habitat suitability of a species through occurrence data and a set of environmental variables (15). It is well documented that MaxEnt has great advantages in epidemiological studies of natural epidemics, detecting the main meteorological factors influencing the high incidence of infectious diseases (16–18) and predicting the impact of future climate change on local species (19, 20). MaxEnt is a machine learning algorithms based on the maximum information entropy to construct the model. The prediction structure is accurate, and its response curve depends on the data characteristics. It can obtain more accurate results with only a small number of sample data (21).

To select the best model, we consulted with epidemiologists on which factors might be associated with the occurrence of HFRS and performed cross-correlation analysis to effectively identify multicollinearity. We selected a validity variable from the variables of multicollinearity. Different regularization multipliers (RM) were adjusted in MaxEnt, then we selected the features that contributed most to the model, thus reducing model overfitting (20, 22).

In this study, ecological niche models of HFRS were constructed using Maxent 3.4.1. Spatial distribution of HFRS cases from 2009 to 2018 was dependent variable; meteorological factors, socio-economic factors and ecological data were independent variables. In our modeling, 75% of the data are randomly selected as the training set and the remaining 25% as the test set. The stability of the model is verified by the (cross-validation) method, and the result of the average of 10 modeling repetitions is used as the model result (21). In the parameter setting, Regularization multiplier was set as 1, Replicate type was set as bootstrap, Replicates was set as 10, Max number of background points was set as 10,000, Max iterations was set as 500. In general, AUC values < 0.7 are considered low accuracy, 0.7–0.9 are considered useful for applications, and >0.9 are considered high accuracy (23, 24).

Results

From 2009 to 2018, the cumulative number of HFRS reported cases in Zhejiang Province was 4240, The annual numbers of HFRS cases in each year during 2009–2018 were 438, 458, 541, 501, 526, 385, 362, 349, 353, and 327. Cases were reported in 80% of counties in Zhejiang province, and the number of counties reporting cases each year from 2009 to 2018 were 62, 56, 59, 61, 65, 63, 61, 62, 71, and 63.

Figure 1 is the receiver operating characteristic (ROC) curve for the again averaged over 10 replicate, and AUC for different years were given in ENMs. The AUCs for 12 models, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2009–2010 average of 2009–2018 were 0.901, 0.879, 0.867, 0.857, 0.848, 0.882, 0.869, 0.872, 0.869, 0.869, 0.808, and 0.759. (Part of 2009–2018*: We selected all variables that contributed more than 5% in the above 11 models to be included in this model).

Figure 2 shows the predicted potential risk map of HFRS from 2009 to 2018 in Zhejiang Province.

The legend on the right side of the picture shows the different risk levels in red, orange, green and blue in descending order, with red representing the highest risk and, and blue representing the lowest risk. It can be concluded from the above risk map that the high incidence of epidemics in Zhejiang Province is mainly concentrated in the eastern and western regions, as well as in the central region.

Table 1 shows the contribution of each variable to the final training MaxEnt model in different years used in this study, showing the mean and average of the 10 replicate runs. According to **Table 1**, it can be seen that zjdem, average temperature and GDP contribute the most to the MaxEnt model constructed in Zhejiang Province.

DEM that made the greatest contribution varied significantly by years, ranging from 4.22 to 26.0% from 2009 to 2018. Average temperature had the second highest contribution, ranging from 6.26 to 19.65% during 2009–2018. The third highest contribution is gross domestic product, contribution during 2009–2018 ranging from 7.53 to 21.25%. The contribution of average land surface temperature ranked fourth, and it fluctuates greatly from year to year, with the highest being 16.73% in 2011, but the lowest being only 1.67% in 2018. In addition, the average contribution of DMSP/OLS, 20-8 precipitation and 8-20 precipitation were all in the range

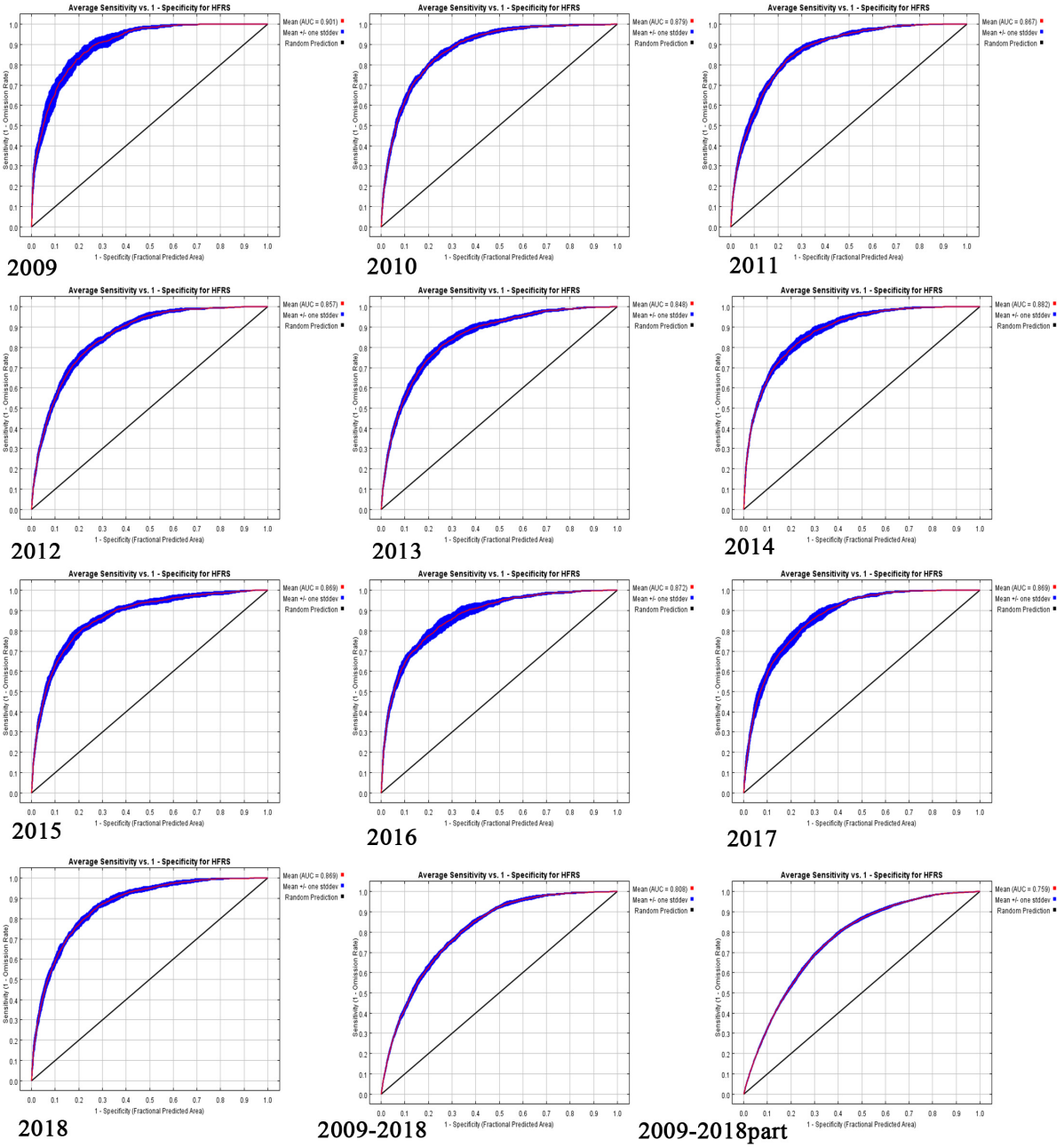

FIGURE 1
The receiver operating characteristic (ROC) curve for HFRS from 2009 to 2018.

of 9%, even though the contribution of DMSP/OLS was as high as 17.8% in 2010.

The following picture shows the results of the jackknife test of variable importance. In Figure 3, the dark blue bar is the separate contribution of the variable and the light blue bar is the contribution that does not include that variable.

Figure 4 shows the response curves for the 4 variables that contributed the most to this study after excluding the variables that contributed less than 5%.

Figure 4A shows the effect curve of All-day Precipitation, and it can be seen that the incidence of HFRS increases with the increase of rainfall, and the effect curve peaks at 1,250 mm, then decreases rapidly, and a small peak appears again at 1,500 mm; through Figure 4B, we can see that average temperature response curve shows an inverted v-shape, the incidence of HFRS increases with the increase of average temperature, and the incidence peaks at 17.8°C, followed by a rapid decrease; through Figure 4C, we can see the response curve of HFRS and

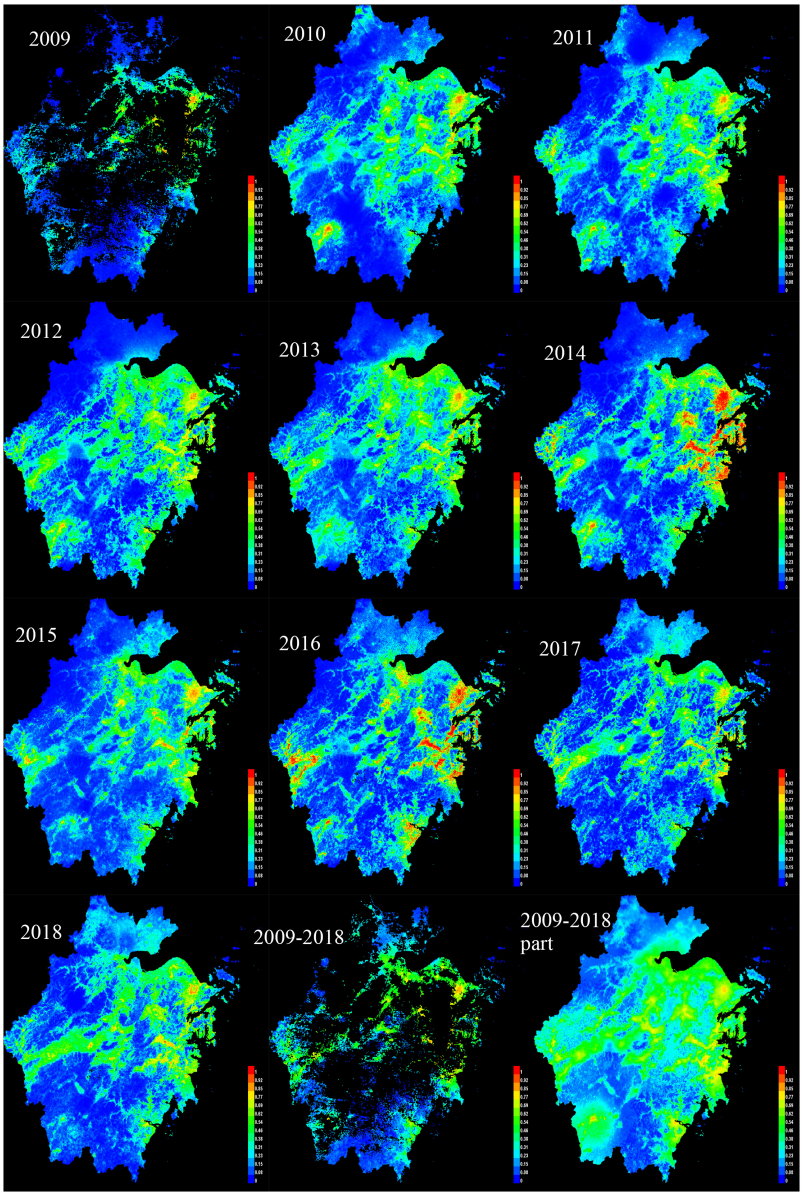

FIGURE 2
The predicted potential risk map of HFRS from 2009 to 2018 in Zhejiang Province.

GDP showing a positive correlation—the incidence increases with the increase of GDP; through **Figure 4D**, we can see the response curve of DMSP/OLS Nighttime Light Data (DMSP/OLS), also show positive correlation—the incidence rate increases with the increase of DMSP/OLS.

Discussion

The advantage of the maximum entropy model is that it is based on the prediction of positive points, avoiding the problem of missing negative data, and it predicts the probability magnitude of risk, which is more widely used in the field of infectious diseases. The prevention and control of any infectious disease cannot be separated from three major elements: infectious source, transmission route and susceptible population. HFRS is a natural epidemic disease, and the influence of meteorological factors on natural epidemic disease needs to be considered from various aspects.

Yuan et al. constructed a maximum entropy model with 16 environmental factors, and then they considered NDVI, rainfall variance and elevation as the main environmental factors affecting landslide hazard (25). Sun et al. constructed ENM of severe fever with thrombocytopenia syndrome (SFTS) using

TABLE 1 The contribution of each variable to the final training MaxEnt model.

Variables	2009 (%)	2010 (%)	2011 (%)	2012 (%)	2013 (%)	2014 (%)	2015 (%)	2016 (%)	2017 (%)	2018 (%)	2009–2018 (%)
zjdem	15.75	6.38	7.56	4.22	18.43	13.06	11.00	10.19	12.16	26.00	20.98
demo8	19.65	16.25	6.53	8.20	7.11	10.26	12.30	9.67	6.26	9.20	14.63
gdp	9.79	7.53	12.94	20.09	16.79	13.32	11.09	19.11	21.25	8.55	11.81
demo3	8.57	11.01	16.73	3.42	14.89	10.98	11.19	3.29	9.69	1.67	10.79
zjdmsp	6.02	17.80	13.96	13.05	8.61	11.51	14.31	11.26	2.92	13.61	9.05
demo4	0.90	1.16	3.11	19.22	11.08	0.88	2.93	1.35	4.51	0.76	9.00
demo5	6.22	11.12	0.69	6.90	0.79	13.83	2.80	2.30	5.90	3.04	5.95
demo7	1.75	1.73	1.42	2.33	3.80	1.49	3.03	4.53	2.04	2.58	3.20
ndvi	4.76	2.46	3.82	3.59	2.39	2.25	4.40	4.05	13.05	3.44	2.76
demo2	3.40	6.21	8.05	5.62	2.67	10.82	4.79	10.02	3.33	3.57	2.75
andvi	2.51	5.04	4.57	4.41	2.55	3.07	3.33	6.08	7.21	4.52	2.12
demo9	1.16	2.41	1.38	0.71	1.72	1.82	1.33	0.99	1.60	1.46	1.75
evi	1.82	1.33	2.32	1.57	2.79	2.32	6.55	2.01	2.42	1.97	1.59
demo1	3.09	3.64	2.08	1.19	2.49	0.89	0.69	1.81	1.49	13.58	1.43
demo6	9.23	1.06	12.09	2.07	0.92	0.98	4.39	4.84	1.38	0.99	0.37
demo10	1.34	1.82	0.64	0.64	0.47	0.94	1.99	0.96	0.58	0.54	0.56
demo11	0.94	1.66	0.54	0.57	0.47	0.39	1.85	5.57	1.71	2.48	0.55
sand	1.71	0.35	0.42	0.36	0.49	0.52	0.53	1.14	1.00	0.45	0.09
silt	0.60	0.80	0.32	1.22	1.12	0.48	1.16	0.41	0.84	0.70	0.40
clay	0.82	0.24	0.82	0.61	0.40	0.18	0.37	0.47	0.67	0.88	0.23

MaxEnt, and they found yearly average temperature, altitude, yearly average relative humidity and yearly accumulated precipitation accounted for 94.1% contribution for ENM (17). Fang et al. found that HFRS incidence in Shandong Province is mainly associated with the seasonal environmental variables: temperature, precipitation and humidity (26). Our study confirmed the contribution of mean temperature to the incidence of HFRS in Zhejiang Province ranged from 6.26 to 19.65% in 2009–2018.

Firstly, temperature have impact on people's willingness of travel, and the probability of HFRS infection will increase when people go out in favorable temperature and sunny weather (27); secondly, temperature will affect the reproduction and activities of host animals and vector organisms, and only when the density of host animals and vector organisms reaches a certain level, human can be infected when they go out (27).

We likewise found some fluctuations in the effect of all-day precipitation on the onset of HFRS, with the effect curve peaking at 1,250 mm and then declining rapidly, with another small peak at 1,500 mm. This is consistent with many studies in which there is a certain lag in the onset of HFRS by rainfall. Studies have confirmed that there is a lag effect of meteorological factors on the onset of HFRS, and the conclusions obtained from different study areas vary from several weeks to several months (27–29). Cao et al. found that the effect of temperature on HFRS varies widely among regions with different temperature zones, ranging from a lag of 1 month in temperate regions to 3 months in subtropical regions (29).

Our findings concern seasonal patterns of HFRS in Zhejiang Province of China that there are two peak incidences of HFRS, one from May to June and the other from November to January (6). The host of SEOV- *Rattus norvegicus* is more common in urban while the host of HTNV—*Apodemus agrarius* is more likely to inhabit rural areas (30). Zhejiang Province has shifted from a single peak incidence in winter in the early stage to two peak incidences in winter and spring at this stage, based on the reasons described below, which is also a renewal of previous concepts. The first reason is that the main host animal of the spring peak is the *Rattus norvegicus* (26), and the main host animal in the urban residential area is the *Rattus norvegicus*, which lives very closely with humans, resulting in an increase in the incidence of urban areas. Secondly, the medical conditions of urban residents are significantly better than those in rural areas, and their medical security and transportation conditions have played a key role (31).

Our results found that the incidence of HFRS shows a positive correlation with GDP and DMSP/OLS response curves, which is consistent with our earlier findings and could further explain why Ningbo city has the province's most number of cases, compared with other cities. GDP (32) and DMSP/OLS are socioeconomic indicators, and many scholars have used DMSP/OLS to study urban development and agreed that DMSP/OLS has strong potential for urbanization research (33, 34).

DMSP/OLS images can be used as a characterization of human activities and become a good data source for human

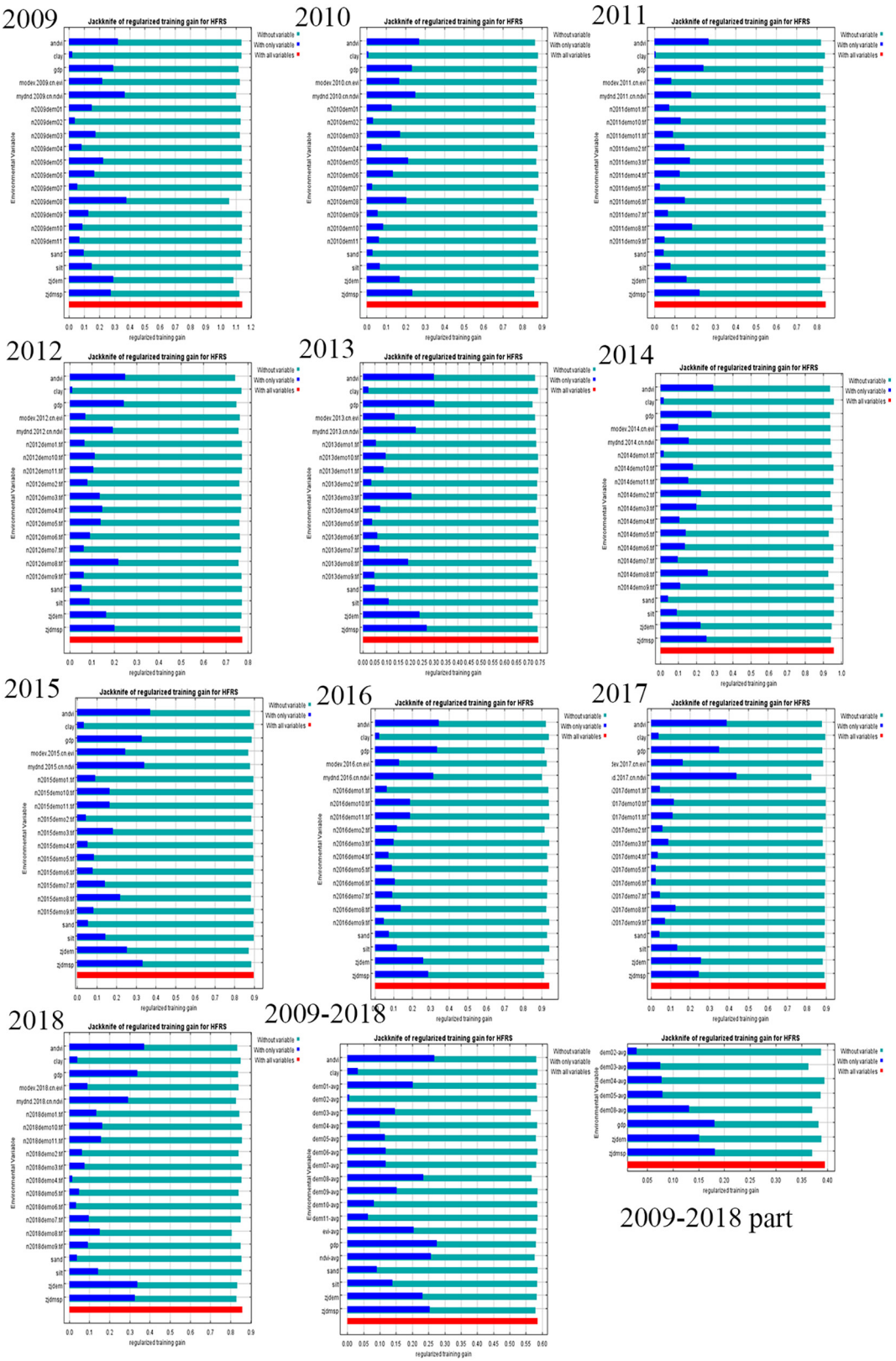

FIGURE 3
The results of the jackknife test of variable importance.

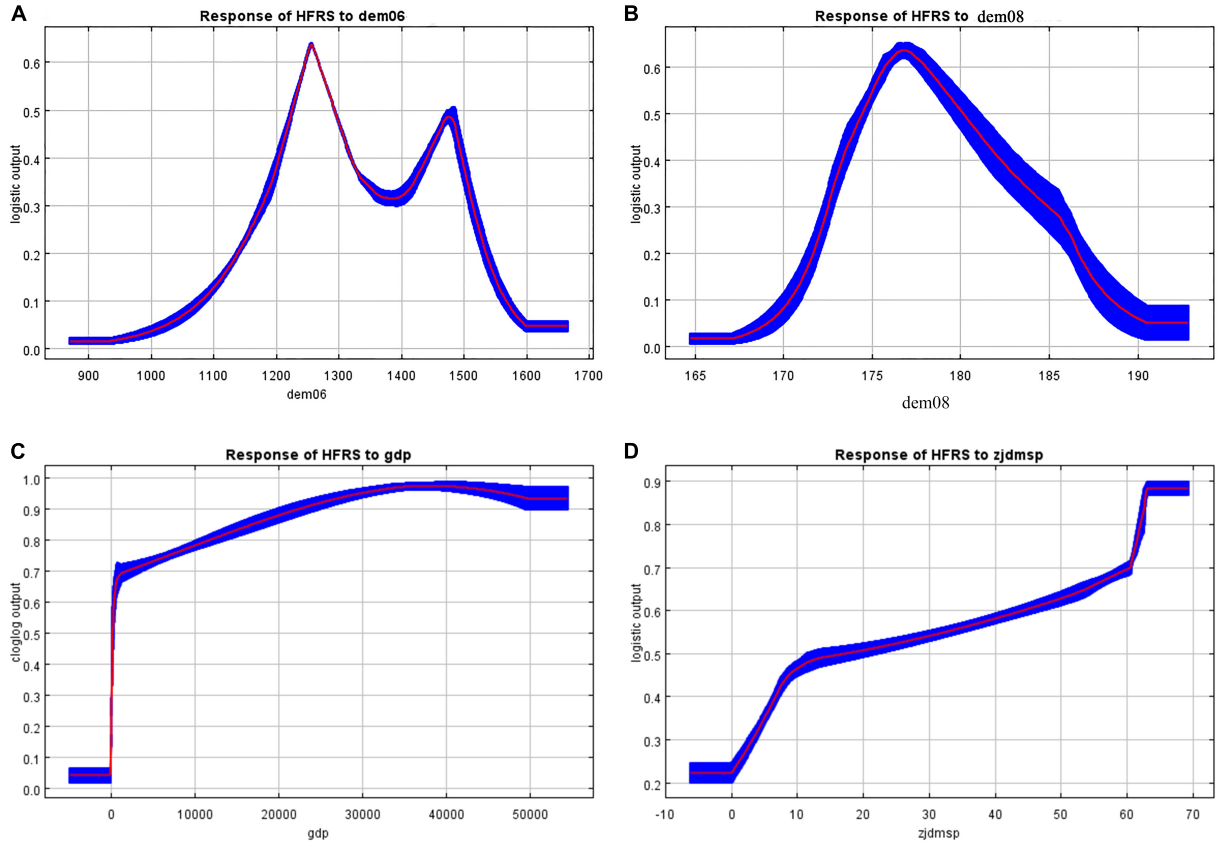

FIGURE 4
The response curves contributed to the MaxEnt model of HFRS.

activity monitoring studies. One of the main advantages of this data is that it does not depend on high spatial resolution, which is usually around 1 km, so it is easier to process DMSP/OLS data; another important feature is that it covers information closely related to the distribution of factors such as population and city layout, like traffic roads and residential places (33).

Zhejiang Province is mainly concentrated in the eastern and western regions, which is consistent with the results of our earlier study (6). The city with the highest incidence rate in Zhejiang Province is Ningbo, which is inseparable from the ability of local medical institutions to detect and diagnose diseases (35). Ningbo is a sub-provincial city with developed economy, convenient transportation and high level of medical services. Due to the rapid development of Ningbo, a large number of rural areas have been merged into the city, and the living environment of the urban-rural integration area is not perfect, which is also one of the reasons for the high incidence of local diseases.

The peak incidence in summer mainly is related to indoor infection caused by the breeding of domestic rodent, and the peak incidence in winter is related to exposure to wild rodent in the field (36). The HFRS monitoring in Zhejiang Province found that *Apodemus agrarius* accounted for 59.71% of the total number of wild rodent captured, and *Rattus norvegicus* accounted for 10.77% of the total number of domestic rodent captured (37). The clinical symptoms and outcomes of HTNV-caused HFRS are usually more serious than those of SEOV-caused (38).

Our study found that the incidence of HFRS varies greatly among regions in Zhejiang Province, but Zhoushan city always has the lowest incidence in the province. Another study found that the incidence of SFTS in Zhoushan city ranks among the top in the province, and we speculate that local rainfall and humidity have a certain relationship with this incidence (39). The MaxEnt model has great advantages in predicting the survival of endangered species. Scholars have used the optimized MaxEnt model to predict the distribution of Quasipaa boulengeri in different provinces in China is important environment variables (40).

The advantage of this study is that two environmental variables and two economic indicators associated with the incidence of HFRS in Zhejiang Province were found by incorporating meteorological factors and socioeconomic indicators. The ecological locus model with maximum

entropy explored the incidence risk areas and provided a reference basis for the rational allocation of medical resources in the province.

This study has the following disadvantage: the international algorithms of ecological niche models are complex and diverse, and we only used the most widely used and stable maximum entropy algorithm to analyze the HFRS epidemic in Zhejiang Province, thus we are unable to achieve an in-depth and comprehensive analysis. Since only some counties in Zhejiang Province are under surveillance, the lack of molecular epidemiological analysis of the impact is also a major shortcoming of the study.

Conclusion

Although many models have been used for the study of risk factors of infectious diseases, the present study confirmed that the maximum entropy model for the study of risk factors of HFRS in Zhejiang Province was in good agreement with the results of previous studies. In summary, the incidence of HFRS peaks in areas where the average temperature is 17.8 degrees Celsius, which reminded us that in areas where temperature is suitable, personal protection should be taken when going out to avoid contact with rodents. The incidence of HFRS by rainfall is more complicated and fluctuates to a certain extent, which is also consistent with the conclusions of most studies, and there is a certain lag in the impact on the disease. The impact of GDP and DMSP/OLS on HFRS is positively correlated. Most cities have good medical conditions, but it also reminds us whether there are under-diagnosed cases in economically underdeveloped areas.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author/s.

Ethics statement

This study was reviewed and approved by the Ethics Committee of the Zhejiang Provincial Center for Disease Control and Prevention (No. 2020-021). All data used in this study followed the Law of the Prevention and Treatment of Infectious Diseases in the People's Republic of China. As only surveillance information was analyzed, this study did not involve human research, informed consent was waived by the Ethical Institutional Review Board.

Author contributions

RZ: conceptual, methodology, writing—original draft preparation and funding acquisition. NZ: analysis and modeling. YL: software. TL: improve the language. JS: project administration and writing and editing. FL: writing—review and supervision. ZW: supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.967554/full#supplementary-material

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