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Inflammation and organic damage in COVID-19: What have we learned 2 years into the pandemic?

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Inflammation and organic damage in COVID-19: What have we learned 2 years into the pandemic?

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Editorial: Inflammation and organic damage in COVID-19: what have we learned 2 years into the pandemic?

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Editorial on the Research Topic
Inflammation and organic damage in COVID-19: what have we learned 2 years into the pandemic?

Acute coronavirus disease 2019 (COVID-19) presents a wide spectrum of clinical manifestations, from asymptomatic infection to severe pneumonia or multisystemic failure. In addition, nearly 3 years after the pandemic, now it is known that there are persistent forms of COVID-19, known as long-COVID, with long-term effects in different organs and systems. These complications related to SARS-CoV-2 infection, which significantly affect the quality of life of many convalescent patients, are not restricted to severe presentations of COVID-19; hence, many patients with persistent symptoms have never been hospitalized. The mechanisms explaining long COVID are not yet well delimited. Recent findings related to immunity alterations together with inflammation and endothelial damage induced by the virus, along with certain predisposing factors, would favor the development of these complications.

In this regard, the implication of the ABO blood group in the COVID-19 disease was formulated early at the beginning of the pandemic, and it has now been established that the A blood group is associated with more susceptibility and severe symptoms of COVID-19, while the O blood group shows protection against viral infection (1). Tamayo-Velasco et al. detail in a complete review how the presence of anti-antigen A and B antibodies in group 0 patients confers a protective effect against protein S of the virus, which could open new avenues for prognostic and therapeutic stratification. The presence of a high viral load in some individuals determines the status of persistent viraemia, which has also been shown to be an independent factor associated with bad prognosis in COVID-19 (2). In this sense, in a short prospective study, Roy-Vallejo et al. report how the presence of detectable viremia in some patients is associated with a greater inflammatory response characterized by an increase in IL-6 levels and poor evolution. Following this line, in an interesting prospective study, Melhorn et al. prove persistence of inflammatory and vascular mediators 5 months after hospitalization in a cohort

of COVID-19 patients compare with healthy and septic controls. In fact, IL-6 again, along with TNF, SAA, CRP, Tie2, Flt-1, and PIGF, was significantly increased in the post-COVID group.

The post-acute sequelae of COVID-19 (PACS) represent a heterogeneous group of symptoms characterized by cardiovascular, general, respiratory, and neuropsychiatric sequelae. PACS can be classified into two categories: PACS cardiovascular disease, characterized by a group of cardiovascular conditions that develop during the chronic phase of the disease, and PACS cardiovascular syndrome (PACS-CVS), which lacks clear evidence of cardiovascular disease (3). In this Research Topic, Aparisi et al. provide insights into the role of the cardiopulmonary exercise test (CPET) in evaluating PACS-CVS. However, it is important to note that there is a lack of evidence-based recommendations for managing this elusive condition. Nonetheless, CPET should be implemented due to its ability to assess the pathophysiology of exercise limitation.

In about 25% of patients with severe COVID-19 disease (WHO Severity Grade 3 and 4), a restrictive ventilatory defect has been revealed. This and other facts justify that a significant percentage of COVID-19 patients present respiratory failure not only during the acute illness of the disease but also chronically, months after overcoming it. SARS coronavirus induces the upregulation of type I collagen (4). At 1 year after ICU admission in a cohort of 105 critically ill patients from several Spanish hospitals, in an interesting prospective multicenter study, González et al. have found that 32.2% of these patients persisted with respiratory alterations, 10% still had moderate/severe lung diffusion (DLCO) involvement (<60%), and 53.7% had a fibrotic pattern on CT. Moreover, patients had a mean (SD) number of symptoms of 5.7 ± 4.6, and 61.3% met the criteria for post-COVID syndrome at 1 year. Thus, there is a compelling clinical need to identify circulating fibrosis markers in COVID-19 leading to pulmonary pro-fibrotic responses that can identify candidate patients suffering from long-term COVID with respiratory alterations. In this regard, Brusa et al. report another circulating biomarker, known as the Targeting Matrix Metalloproteases Pathway-1 (TIMP-1), which has been associated with disease severity and the systemic inflammatory index, suggesting a promising non-invasive prognostic biomarker for structural respiratory damage in COVID-19 patients.

Beyond the local and systemic inflammatory response, endothelial dysfunction (ED) or endotheliitis has been demonstrated to play a critical role in COVID-19 acute organ disfunction and may also be related to long-term systemic symptoms. ED favors both inflammatory activation and local coagulation, leading to hypercoagulability states (HS), microthrombosis, and hypoperfusion, more markedly in microcirculation (5). Due to this, cardiovascular pathologies such as myocardial damage and thromboembolic events (TE) have been frequently related to COVID-19 (6). In a comprehensive review, Izquierdo-Marquisá et al. detail how myocardial injury is present in around one-third of hospitalized COVID-19 patients, and this condition is associated with worse in-hospital outcomes, with over 50% mortality. Myocardial injury-related mechanisms are varied (myocarditis related to viral infection, ED, or HS), and quick identification is key to being able to treat it early. Beyond the classic diagnostic tests of myocardial injury (electrocardiogram and echocardiogram) and cardiac biomarkers (such as troponin and natriuretic peptides), the identification of new affordable and bedside biomarkers seems essential to identify this potentially fatal situation. Recent studies have evaluated the role of MR-proadrenomedullin (MR-proADM), a novel marker of ED in sepsis and pneumonia (7, 8). It is a pro-hormone with vasodilator properties synthesized by endothelial cells. High levels of MR-proADM achieved an excellent accuracy to predict mortality and poor outcome in patients with COVID-19 (9). In this sense, Spoto et al. demonstrate how this molecule complements troponin, a canonical biomarker of myocardial damage, improving its prognosis accuracy and risk stratification in a cohort of COVID-19 patients with myocardial injury. Despite the rationale that early antiplatelet therapy would lower the risk of cardiovascular events on the basis of their antithrombotic and anti-inflammatory properties, the effectiveness of this approach remains controversial (10). In this regard, Zong et al. perform a systematic review and meta-analysis, including early observational studies and recent randomized controlled trials (RCTs) assessing the effect of antiplatelet therapy in adult patients with COVID-19. Based on 23 observational studies, including 87,824 COVID-19 patients, antiplatelet treatment has been found to favor a lower risk of mortality (odds ratio: 0.72, 95% confidence interval: 0.61–0.85; p-value < 0.01). However, the narrative synthesis of RCTs showed conflicting evidence, which did not support adding antiplatelet therapy to the standard care. This discrepancy seems to suggest that there are subgroups of COVID-19 patients who could benefit from this therapy, while in others such a benefit would not exist. It is necessary to carry out new and larger RCTs that evaluate antiplatelets from an individualized or personalized point of view based on the endotype of the candidate patient. In this respect, biomarkers of TE can be useful. D-dimer has shown to be a robust predictor associated with bad outcomes in COVID-19 (11). Interestingly, in a multicenter study, Ronderos Botero et al. demonstrate how the D-dimer prognostic value has also not varied in successive pandemic waves. Thus, TE biomarkers can be useful, not only at the prognostic level but also to individualize treatments.

The association of COVID-19 with prevalent gastrointestinal distress, characterized by the fecal shedding of SARS CoV 2 RNA or persistent antigen presence in the gut, has been scarcely evaluated (12). In this Research Topic, Moon present a review addressing gastrointestinal symptoms and describing data on the gut–lung axis, viral transmission to the gut, and its influence on gut mucosa and the microbial community.

We hope that this Research Topic provides original information to the scientific community on the "hot" topic

Abbreviations: COVID-19, acute coronavirus disease 2019; ED, endothelial dysfunction; HS, hypercoagulability state; PACS, post-acute sequelae of severe acute respiratory syndrome coronavirus 2 infection; CPET, cardiopulmonary exercise test; RCT, randomized clinical trials; TE, thromboembolic events.

of long COVID and the medium- and long-term effects of SARS-CoV-2 infection.

Author contributions

DA drafted the manuscript. LG and DA critically revised the manuscript for important intellectual content. 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.

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MINI REVIEW
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ABO Blood System and COVID-19 Susceptibility: Anti-A and Anti-B Antibodies Are the Key Points

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The implication of the ABO blood group in COVID-19 disease was formulated early, at the beginning of the COVID-19 pandemic more than 2 years ago. It has now been established that the A blood group is associated with more susceptibility and severe symptoms of COVID-19, while the O blood group shows protection against viral infection. In this review, we summarize the underlying pathophysiology of ABO blood groups and COVID-19 to explain the molecular aspects behind the protective mechanism in the O blood group. A or B antigens are not associated with a different risk of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection than that of other antigens. In this case, the cornerstone is natural anti-A and anti-B antibodies from the ABO system. They are capable of interfering with the S protein (SARS-CoV-2) and angiotensin-converting enzyme 2 (ACE2; host cell receptor), thereby conferring protection to patients with sufficient antibodies (O blood group). Indeed, the titers of natural antibodies and the IgG isotype (specific to the O blood group) may be determinants of susceptibility and severity. Moreover, older adults are associated with a higher risk of bad outcomes due to the lack of antibodies and the upregulation of ACE2 expression during senescence. A better understanding of the role of the molecular mechanism of ABO blood groups in COVID-19 facilitates better prognostic stratification of the disease. Furthermore, it could represent an opportunity for new therapeutic strategies.

Keywords: ABO blood group, COVID-19, anti-A antibody, SARS-CoV-2 spike protein, ACE2 (angiotensin converting enzyme 2)

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INTRODUCTION

At 2 years since the beginning of the COVID-19 pandemic (1, 2), people worldwide continue to suffer deaths and important changes in their lifestyles (3). Although vaccines are being encouraged to hinder the spread of this pandemic (4, 5), the pathophysiology of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is not well understood. Many studies identified multiple risk factors during the first wave to identify and treat susceptible patients early (6–8). Old age (9), male (10), and comorbidities, such as hypertension (11), were related to severity. Some routine biomarkers (12) and specific cytokines (13, 14) have also been proposed.

Similarly, a possible implication of the ABO blood group was formulated (15, 16). Currently, as multiple studies have reported (17–22), it seems clear that the A blood group is associated with more susceptibility and severe symptoms in COVID-19, while the O blood group shows protection against viral infection. Despite many descriptive studies on this tendency, few studies have focused on the implicated molecular mechanisms. Several studies have focused on angiotensin-converting enzyme 2 (ACE2) as the host cell receptor (23), S protein of the virus (24), and antigens or antibodies of the ABO system (25). However, no studies have specifically and directly deepened our understanding of the implications of ABO blood groups and their possible implications in developing future therapeutic strategies.

Therefore, we summarized the underlying pathophysiology of ABO blood groups and COVID-19. We exhaustively analyzed the role of A, B, AB, and O antigens in the disease and its molecular aspects. The functions of natural anti-A and anti-B antibodies are the cornerstone. We examined the importance of immunoglobulin (Ig) isotypes and their plasma concentrations by focusing on the consequences of immunosuppressive status according to the ABO system in patients with COVID-19. We examined how the complex interrelations between antibodies, the virus, and the host cell receptor relate to the protective molecular mechanism.

RELATIONSHIP BETWEEN ABO BLOOD GROUP AND COVID-19 SEVERITY AND SUSCEPTIBILITY

The Role of ABO Antigens in COVID-19

In 1901, Nobel Prize winner Karl Landsteiner discovered the ABO system (26). Erythrocytes, endothelial and epithelial respiratory cells, and digestive endothelial cells synthesize ABH carbohydrate epitopes. The addition of N-acetylgalactosamine or galactose to the H antigen (precursor chain) allows the appearance of A and B antigens, respectively. Thus, the O blood group only expresses the H antigen, whereas the AB blood group expresses both the A and B antigens (27, 28). In the Caucasian population, the O and A groups were the most frequent (45 and 40%, respectively), followed by the B group (11%) and AB group (4%). In contrast, group B is overexpressed in black and Asian populations (20 and 27%, respectively) (28). These differences in the ABO system are associated with some peculiarities. Blood group A is linked to hypercoagulability, cardiovascular events, and a higher risk of colon and gastric cancer (29). Group B is more susceptible to infections by *Escherichia coli* (30). The O blood group showed reduced thrombotic risk due to lower plasma von Willebrand factor (VWF) and coagulation factor VIII levels (29, 31).

Studies on COVID-19 also found more comorbidities in patients with the A blood group than those with the other groups (16, 17, 29). This subgroup of patients has a higher Charlson comorbidity index (32) and more cardiovascular diseases, especially hypertension (20), when infected with SARS-CoV-2. Moreover, according to the ABO blood group, these innate differences are not confounders. Multiple studies have confirmed the increased susceptibility, severity, and death risk in the A blood group, an independent risk factor for COVID-19 (17, 18, 20–22, 33). The implication of the ABO system was also strongly evidenced in a genome-wide associated study (GWAS) that identified a 3p 21.31 gene cluster related to the ABO blood group and respiratory failure in COVID-19 (34). We can expect new findings from genome-wide association analyses to explain better the importance of the ABO system in the severity and mortality of patients with COVID-19.

Descriptive and genetic studies based on ABO phenotypes found clear evidence about the implication of the ABO system in susceptibility and disease severity. However, no direct molecular interrelation between ABO system antigens and the virus has elucidated the mechanism involved in the susceptibility of the ABO blood group.

Anti-A and Anti-B Antibodies Are the Cornerstones

Antigens of ABO blood group are present in the cell membrane. However, they do not directly modulate the SARS-CoV-2 infectious capacity. Natural anti-A or anti-B antibodies in patients with the A, B, or O blood groups are freely present in plasma, providing a decisive connection with the virus.

The Direct Connection Between Antibodies, S Protein of SARS-CoV-2, and ACE2 Receptors in the Host Cell

The infectious capacity of SARS-CoV-2 has been characterized previously. The virus binds to the cell surface *via* its S protein, cell receptor-binding domains (RBDs), and virus-cell membrane fusion domains (35). The S protein binds to the host cell receptor's ACE2 (36). ACE2 is present in virtually all organs, but lung alveolar epithelial cells and enterocytes of the small intestine (37) are important in this context. Moreover, the transmembrane protease serine subfamily member 2 (TMPRSS2), a cell surface protein expressed by endothelial cells in the respiratory and digestive tracts, is used by the virus for S protein priming (38). Enhanced entry correlated with optimal functions of both TMPRSS2 and ACE2. Similarly, in ACE2 expressing cells, dendritic-cell-specific ICAM3-grabbing nonintegrin (DC/L-SIGN) facilitates the infectious capacity; however, an adequate ACE2 correlation is required (39). These mechanisms promulgated in SARS-CoV-2 have also been confirmed in COVID-19 (40, 41). ACE2 is the main host cell receptor for the viral S protein (no other receptor has been discussed), and its function is probably improved by the proper interaction between TMPRSS2 and DC/L-SIGN (42).

Immunoglobulins can bind to or block different proteins. Anti-A and anti-B antibodies from the ABO system are natural Igs in serum. It has been reported that the presence of anti-A antibodies (and probably anti-B antibodies) prevents the interaction between the viral S protein of the virus and ACE2 on the cell surface (**Figure 1**). The molecular mechanism is not yet fully understood. However, several hypotheses have been proposed. It seems that carbohydrates or glycosylated epitopes are present in the cell membrane of both SARS-CoV-2 and ACE2 and it is known the strong binding between natural antibodies from the ABO system and carbohydrate molecules, such as A or

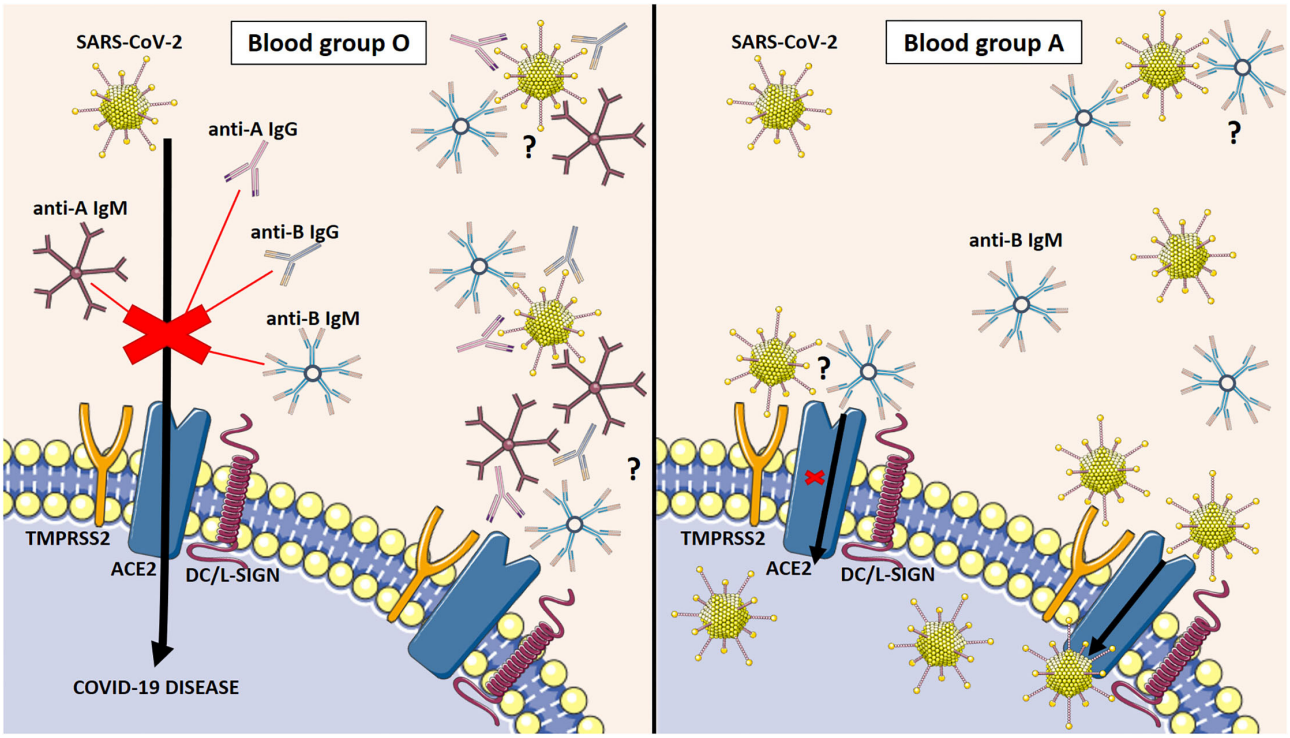

FIGURE 1 | Molecular mechanism that explains the susceptibility and severity of COVID-19 disease depending on the ABO blood group. The presence of anti-A antibodies and probably anti-B antibodies inhibit the interaction between the S protein of the virus and the ACE2 on the cell surface in the O blood group (Left side). The absence of antibodies in the A blood group facilitates the entrance of SARS-CoV-2 into the host cell and the consequent viral infection (Right side). ACE2: angiotensin-converting enzyme 2. TMPRSS2: transmembrane protease serine subfamily member DC/L-SIGN; dendritic-cell-specific ICAM3-grabbing nonintegrin.; Natural antibodies bind glycosylated or carbohydrate epitopes in the S protein of SARS-CoV-2 (on top) or ACE2 (below).

B antigens. On the one hand, the S protein could be decorated with A or B carbohydrate epitopes able to be recognized by the natural anti-A or -B antibodies from blood group O, B, and A individuals (24). On the other hand, natural anti-A or -B antibodies can directly bind to the ACE2 glycosylated region (43). In this case, possible competitive inhibition of ACE2 by both natural (anti-A/anti-B) antibodies and SARS-CoV-2 may induce early ACE2 downregulation in blood group O, increasing the production of multiple inflammatory cytokines (44) in the first step of the infection. Patients with the O blood group suffered a consequent cytokine drop during the hospital stay, while non-O patients maintained their cytokine levels associated with hyperinflammation. An early, effective, and moderate cytokine release functions in immunocompetent patients, while disease severity is linked to persistent immune dysregulation after infection, associated with high cytokine levels for days or weeks. These findings could explain the optimal activation of the immune response and the effective viral clearance of SARS-CoV-2 infection in the patients with the O blood group. In any case, the interaction between natural antibodies and ACE2 or S proteins should prevent viral infection *via* transfusion rules. Therefore, natural anti-A or -B antibodies protect patients with the O blood group against severe disease and mortality in COVID-19. Comparatively, antibodies operate *via* the same mechanism as future specific treatments against SARS-CoV-2 (45). The more anti-A or-B antibodies present in the plasma (O blood group), the reduced infectious capacity (19). In contrast, the absence of antibodies (AB blood group) or one of them (A or B blood groups) is associated with a higher risk for poor outcomes in COVID-19 (20). Descriptive and epidemiological studies have corroborated this tendency during the pandemic in all populations (15, 17, 18, 20–22, 32, 33, 46).

Immunoglobulin Isotype of Anti-A and Anti-B Antibodies

Natural anti-A or -B antibodies from the ABO system differ from most naturally occurring antibodies because of their exclusive expression in individuals lacking the corresponding antigen (A or B antigen)(47). They exhibit high polyspecificity and polyreactivity to multiple antigens, not only those included in the ABO system (48). The main isotype of natural Ig is M (IgM) in all ABO blood groups with specific natural antibodies (A, B, and O blood groups), reaching all groups with similar plasma concentrations of IgM antibodies. By cons, the presence of anti-A/B antibodies with the IgG isotype was restricted to the O blood group (**Figure 2**). Indeed, anti-A or anti-B IgG were found in almost 90% (34/38) of O blood group donors (predominance of IgG2). Meanwhile, only 14% of patients with the A blood group had anti-B IgG, and 4% with B blood group had anti-A IgG. None of the AB blood group samples contained anti-A

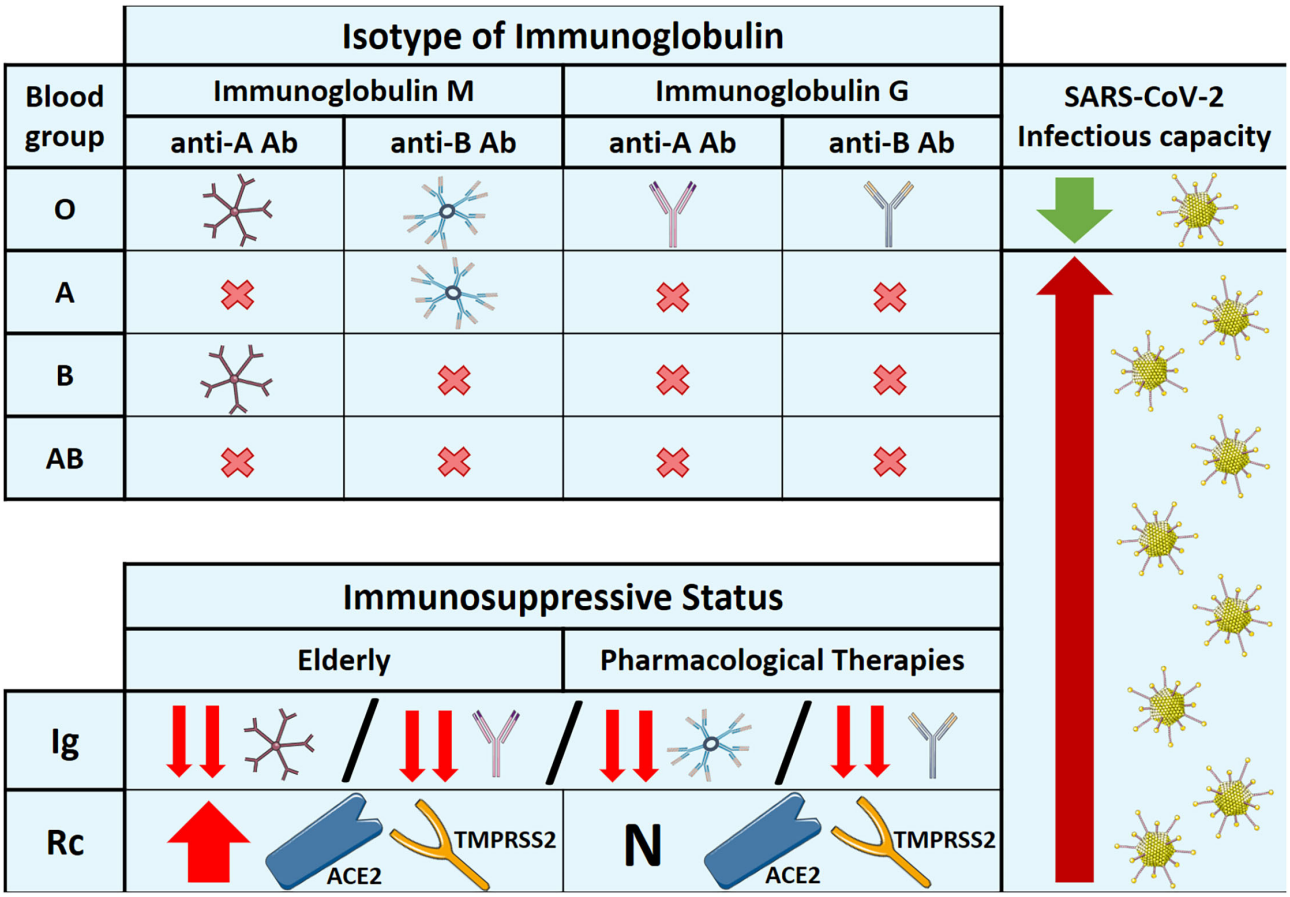

FIGURE 2 | Specific isotype of immunoglobulin in each ABO blood group, expression of natural antibodies and receptors in immunosuppressive situations, and their relationship with the infectious capacity of SARS-CoV-2. Ab: antibody. Rc: receptor. Ig: immunoglobulin. ACE2: angiotensin-converting enzyme 2. TMPRSS2: transmembrane protease serine subfamily member. N; normal expression.

or anti-B antibodies of any isotype (IgM or IgG) (25). Until now, there has been no explanation for this finding. It would be relevant, for example, in hemolytic disease of the newborn because only newborns of blood group O mothers develop the hemolytic disease after ABO-incompatible pregnancies (49). In COVID-19, studies have shown that patients with the O blood group are under-represented, whereas patients with groups A, B, and AB are over-represented (20). We previously explained that the higher the plasma concentration of natural anti-A or anti-B antibodies (O blood group), the higher the protective effect against SARS-CoV-2 infection. Nevertheless, it is not only the plasma level of natural antibodies but also the isotype of Ig. IgG (restricted to the O blood group) may strongly avoid the interaction between ACE2 and the S protein compared to the IgM isotype.

Immunosuppressive Status and Plasma Antibody Levels

A strong immune system is crucial for overcoming infections. It includes both an optimal innate and adaptive immune response, with adequate antibody production by B cells. Unfortunately, many situations can weaken the immune system, reducing cell-mediated immune function and humoral immune responses. This decline in immune capacity is associated with reduced antibody levels, making individuals more suitable for infections and disease severity. Older individuals are one of the most recognized cases (9). Aging reduces the production of B and T cells in the bone marrow and thymus and diminishes the function of mature lymphocytes in secondary lymphoid tissues (50). Similarly, immunosuppressive treatments (glucocorticoids, cytotoxic drugs, other immunomodulatory agents, or new immunosuppressive therapies) can also compromise the immune system (51). In addition, infections can have immunosuppressive effects in the local environment (52), increasing susceptibility and severity of infectious diseases and decreasing the efficacy of vaccination (53). Moreover, different studies have revealed, in severe cases of COVID-19, that the presence of immune downregulation with profound immunosuppression was the primary phenomenon. Immunological alterations vary and are classified into different subsets or phenotypes. One of these immunophenotypes is characterized by the coexisting alterations in T cells' numbers, subset composition, cycling, activation, and gene expression (54, 55).

It has been shown for SARS-CoV that the interference between natural anti-A antibodies in the O blood group was dose-dependent and still detected at a plasma dilution of up to 1/32. Indeed, patients with the O blood group with low anti-A antibodies were not inhibitory in the host cell adhesion assay (24). The lack of or drop in antibodies due to any immunosuppressive situation creates ABO discrepancies (56). Therefore, it is of value to determine whether the ABO group performed both forward (red blood cell antigen) and reverse (anti-A and anti-B antibodies in plasma). Once we know the importance of natural antibodies from the ABO system in COVID-19, we should evaluate only the reverse type in terms of protection against viral infection. Accordingly, patients with the O, A, or B blood groups (forward type) that associate lack of antibodies would behave as the AB blood group (reverse type). The current situation favors infections and worsens outcomes for a large number of people, especially older adults and patients who are immunosuppressed.

While aging decreases plasma levels of antibodies, studies have demonstrated increased ACE2 and TMPRSS2 expression in older adults (10, 57). The first study described a significant expression of ACE2 in older males in both mouse models and human organs (10). The second study demonstrated the overexpression of ACE2 and TMPRSS2 in the upper respiratory tract of aged ferrets compared to young animals (57). Moreover, a recent study found that ACE2 levels increase during aging in mouse and human lungs due to telomere shortening or dysfunction (58). It involves the transcriptional level, where ACE2 promoter activity is dependent on DNA damage response (58). Therefore, both the upregulation of ACE2 and the decrease in antibodies make the elderly more susceptible to severe infection by SARS-CoV-2 (**Figure 2**).

DISCUSSION

After this exhaustive assessment regarding the implications of the ABO blood system in COVID-19, we make the following key points: i) The presence or absence of any antigen of the ABO system is related to different susceptibilities, presenting more comorbidity in patients with antigen A (A blood group), while the absence of antigens (O blood group) is associated with lower thrombotic and cardiovascular risk. This is one of the reasons why the number of patients infected with SARS-CoV-2 who were hospitalized with worse outcomes belongs to the non-O blood group. However, there is no direct molecular relationship between ABO system antigens and the virus that can explain the true mechanism involved in the susceptibility of the ABO blood group. ii) Natural anti-A and -B antibodies from the ABO system are capable of interfering with the S protein (SARS-CoV-2) and ACE2 (host cell receptor). The presence of high plasma concentrations of antibodies in the O blood group confers greater protection to these patients. iii) The isotype of natural antibodies would be decisive because the A, B, and O blood groups present IgM, but only the O blood group presents anti-A and anti-B IgG antibodies in the plasma. iv) Immunosuppressive status, such as in older adults and patients with some diseases or undergoing pharmacological treatments, is associated with a lack of antibodies. This creates the ABO discrepancies. Patients with the O, A, or B blood groups would behave as patients with the AB blood group, making them more susceptible to infection.

Some questions might be interesting to consider and could open future investigations in this area. The first is related to the exact mechanism by which natural antibodies from the ABO blood system avoid the interaction between the S protein of the virus and ACE2 on the cell surface (20, 24, 43, 44). An experimental model is required to understand whether antibodies only block the S protein, perhaps together with SARS-CoV-2, to competitively inhibit ACE2 in host cells or whether both molecular mechanisms are possible. These findings would help us to underline the pathophysiology of the ABO blood groups in the same way that the lower plasma von Willebrand factor (VWF) and coagulation factor VIII levels in the O blood group are well described (29, 31). For example, suppose our natural anti-A or -B antibodies would constantly bind or block ACE2. In that case, O blood group individuals could present persistent ACE2 downregulation, resulting in increased production of multiple inflammatory cytokines (44). Furthermore, these antibodies might be associated with protection against cardiovascular diseases, similar to ACE inhibitors, conferring a lower risk in the O blood group. Therefore, it would be necessary for anti-A and anti-B antibodies to bind to the same proteins, or one of them must demonstrate more affinity or interfere with SARS-CoV-2 more efficiently.

Another important issue is the antibody isotype. As mentioned before, the IgM isotype is present in A, B, and O blood groups, while anti-A and anti-B with IgG isotypes are almost unique to the O blood group (25). The better outcome of the O blood group is confirmed in COVID-19, but this effect depends on the higher plasma level of natural antibodies compared to the rest of the blood groups (24), or perhaps IgG isotypes confer more protection or both. The IgG isotype interferes more strongly than IgM, explaining the protective status of the O blood group. However, elucidation would require complicated and specific laboratory assays, COVID-19 cases, healthy donors, and all blood groups and isotypes of Igs.

Finally, studying older patients might determine whether the upregulation of ACE2 or the decrease in antibodies with senescence is significant (10, 57). Moreover, the implications of ACE2 upregulation would lead to specific studies based on different symptoms in old and young patients. Patients with ACE2 overexpression in the gastrointestinal tract are associated with more diarrhea (59). In fact, there is evidence demonstrating a direct association between endothelitis and severe COVID-19 (60). Therefore, ACE2 may be a relevant factor in this phenomenon.

CONCLUSION

In conclusion, natural anti-A and B antibodies from the ABO system interfere with the S protein (SARS-CoV-2) and ACE2 (host cell receptor), conferring protection to patients with sufficient antibodies (O blood group). The titers of natural antibodies and IgG isotype (specific to the O blood group) are determinants of susceptibility and severity. Older adults are associated with a higher bad outcomes risk due to the lack of

antibodies and the upregulation of ACE2 expression. There is no doubt that more investigations would be beneficial to understand the role and molecular mechanism of ABO blood groups in COVID-19 fully and help develop novel therapeutic strategies.

AUTHOR CONTRIBUTIONS

ÁT-V, MP-P, and FÁ conceptualized the study. ÁT-V, IF, SP-G, and DA-O contributed to methodology and investigation. ÁT-V wrote the manuscript. ÁT-V and DA-O contributed to figures. MP-P, FÁ, and DA-O wrote and reviewed the article. ÁT-V and DA-O visualized the study. All authors critically revised the manuscript, reviewed the final version, and agreed with the content of the work.

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MINI REVIEW
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Myocardial Injury in COVID-19 and Its Implications in Short- and Long-Term Outcomes

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COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still a pandemic with high mortality and morbidity rates. Clinical manifestation is widely variable, including asymptomatic or mild respiratory tract illness to severe pneumonia and death. Myocardial injury is a significant pathogenic feature of COVID-19 and it is associated with worse in-hospital outcomes, mainly due to a higher number of hospital readmissions, with over 50% mortality. These findings suggest that myocardial injury would identify COVID-19 patients with higher risk during active infection and mid-term follow-up. Potential contributors responsible for myocardial damage are myocarditis, vasculitis, acute inflammation, type 1 and type 2 myocardial infarction. However, there are few data about cardiac sequelae and its long-term consequences. Thus, the optimal screening tool for residual cardiac sequelae, clinical follow-up, and the benefits of a specific cardiovascular therapy during the convalescent phase remains unknown. This mini-review explores the different mechanisms of myocardial injury related to COVID-19 and its short and long-term implications.

Keywords: SARS CoV-2, infection, COVID-19, inflammation, organ failure, biomarkers, prognosis

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INTRODUCTION

In December 2019, the first cases of pneumonia caused by a new virus called Severe Acute Respiratory Syndrome 2 (SARS-CoV-2) were noted in Wuhan, China. This new infection was named Coronavirus disease 2019 (COVID-19) (1) and it disseminated all over the world, being declared as a global pandemic on March, 2020 by the World Health Organization (WHO). It has overloaded many healthcare systems and has been considered the worst sanitary crisis since the pandemics of Influenza in 1918. Despite substantial progress in clinical research, new viral strains are still a challenge for the healthcare system. Therefore, understanding the potential contributors of hospital readmissions after COVID-19 might improve long-term outcomes (2).

THE SARS-CoV-2 VIRUS

SARS-CoV-2 Origin

Human epidemiological data suggest a zoonotic origin of SARS-CoV-2 from a Seafood Market in China. Early reports suggested that bats were the most likely initial hosts and its transmission to human involved an intermediate animal Once most of the animal trading markets in China were closed, infected human have become the main source of the infection (3–5).

SARS-CoV-2 Structure

SARS-CoV-2 is an enveloped ribonucleic acid (RNA) virus with a double-layered lipid envelope. Its name refers to its core shell with surface projections which features a solar corona (Latin: corona = crown). There are four coronaviruses subfamilies: alpha- and beta- subfamilies, originated from mammals (bats); and gamma- and delta- subfamilies, from pigs and birds. While alpha-coronaviruses cause asymptomatic or mildly symptomatic infection, beta-coronaviruses may cause severe disease (6).

SARS-CoV-2 belongs to the beta-coronaviruses, such as Middle East Respiratory Syndrome (MERS-CoV) and SARS-CoV. SARS-CoV-2 and SARS-CoV share around the 80% of their genome (7).

The most important envelope proteins in SARS-CoV-2 are: Spike (S) protein that mediates the viral entry into the host cell through ACE2 receptor; Membrane (M) and Envelope (E) protein which are responsible for the membrane structure. The nucleocapsid is mainly composed of the N protein (8).

SARS-CoV-2 Transmission Methods

SARS-CoV-2 predominant route of transmission from person-to-person is through respiratory droplets and contact (3). While its infectivity (R0) is around 2.2–2.7, the R0 for SARS-CoV was 3 and 2–5 for MERS-CoV (9).

Droplet transmission occurs when mucous membranes, such as mouth, nose and eyes, are exposed to infectious respiratory droplets of someone within 1 m who has respiratory symptoms. Indirect transmission can occur through fomites on surfaces in the environment around the infected person (e.g., Stethoscope) (10).

Airborne transmission may occur during procedures that generate aerosols: e.g., endotracheal intubation, nebulized treatments, bronchoscopy, tracheostomy, non-invasive positive-pressure ventilation or cardiopulmonary resuscitation (10, 11). Some evidence suggests a fecal-to-oral transmission, but to date it has not been proven (12).

Pathogenesis

Extrapolations from knowledge about other similar beta-coronaviruses, like SARS-CoV and MERS-CoV, are used to understand SARS-CoV-2 pathogenesis (8, 13–15).

The entrance of the virus into the host cells is mediated by the union between the Spike protein of SARS-CoV-2 and the angiotensin-converting enzyme 2 (ACE2) and protein priming by the serine protease TMPRSS2. TMPRSS2 transcription is regulated by androgenic hormones which can explain, partially (7) the higher mortality and incidence in men.

Previous studies about SARS-CoV showed that the effectiveness of the virus banding to ACE2 could be an important determinant of the virus transmissibility. Consequently, the increased transmissibility of SARS-CoV-2 may be due to its higher affinity of binding to the ACE2 receptor than SARS-CoV (16).

Viral genome replication and translation is held after the cell entry and RNA has been released into the cytoplasm. When this replication occurs in the epithelial cells of the respiratory tract it causes severe pneumonia or Acute respiratory distress syndrome (ARDS) (17).

Proposed mechanisms for the pathophysiology of multi-systemic injury secondary to SARS-CoV-2 infection are direct cytotoxicity, endothelial cell damage and thrombo-inflammation, dysregulation of the renin-angiotensin–aldosterone system (RAAS) and dysregulation of the immune response (18, 19). The role of each mechanism in the pathophysiology of COVID-19 is still not fully delimited. Some of these mechanisms are unique to COVID-19 (ACE2-mediated viral entry and dysregulation of the RAAS). However, the microcirculation dysfunction and the pathogenesis caused by the systemic release of cytokines are also present in sepsis (20) (**Figure 1**).

MYOCARDIAL INJURY IN SARS-CoV-2

The ACE2 receptors are highly expressed in cardiovascular cells and are involved in blood pressure regulation and myocardial function (21). Cardiovascular manifestations of COVID-19 are variable, including myocardial injury, thromboembolism, arrhythmia, acute coronary syndrome, heart failure or cerebrovascular accidents. These cardiovascular complications have been associated with worse short and long-term outcomes (22, 23). The mechanisms of cardiovascular damage are not clearly understood and hypotheses are based on SARS-CoV-2 resemblance to other coronaviruses.

Myocardial injury is diagnosed when serum levels of cardiac troponin (cTn) are above the 99^{th} percentile upper reference limit (24). Initial studies suggested that myocardial injury was present in around 20–30% of COVID-19 patients (23, 25–29). The incidence of myocardial injury increases with COVID-19 severity and has prognostic implications (30). The suggested mechanisms for SARS-CoV-2-related cardiac damage are: (1) cardiomyocytes injury; (2) endothelial cells injury and endothelialitis; (3) indirect injury from hypercoagulability state; (4) ischemic myocardial injury; and (5) indirect injury from cytokine storm (**Figure 2**).

Direct Cardiomyocytes Injury

Myocarditis related to viral infection is widely described (31). Few studies about fulminant myocarditis in COVID-19 patients have been published (32–36) and suggest that direct myocardial infection is produced through the ACE2 receptor. Cardiomyocyte apoptosis induced by SARS-CoV-2 has been proved *in vitro* (37). However, the pathophysiology of this injury is not clearly defined to date, only one study has displayed viral genome particles in

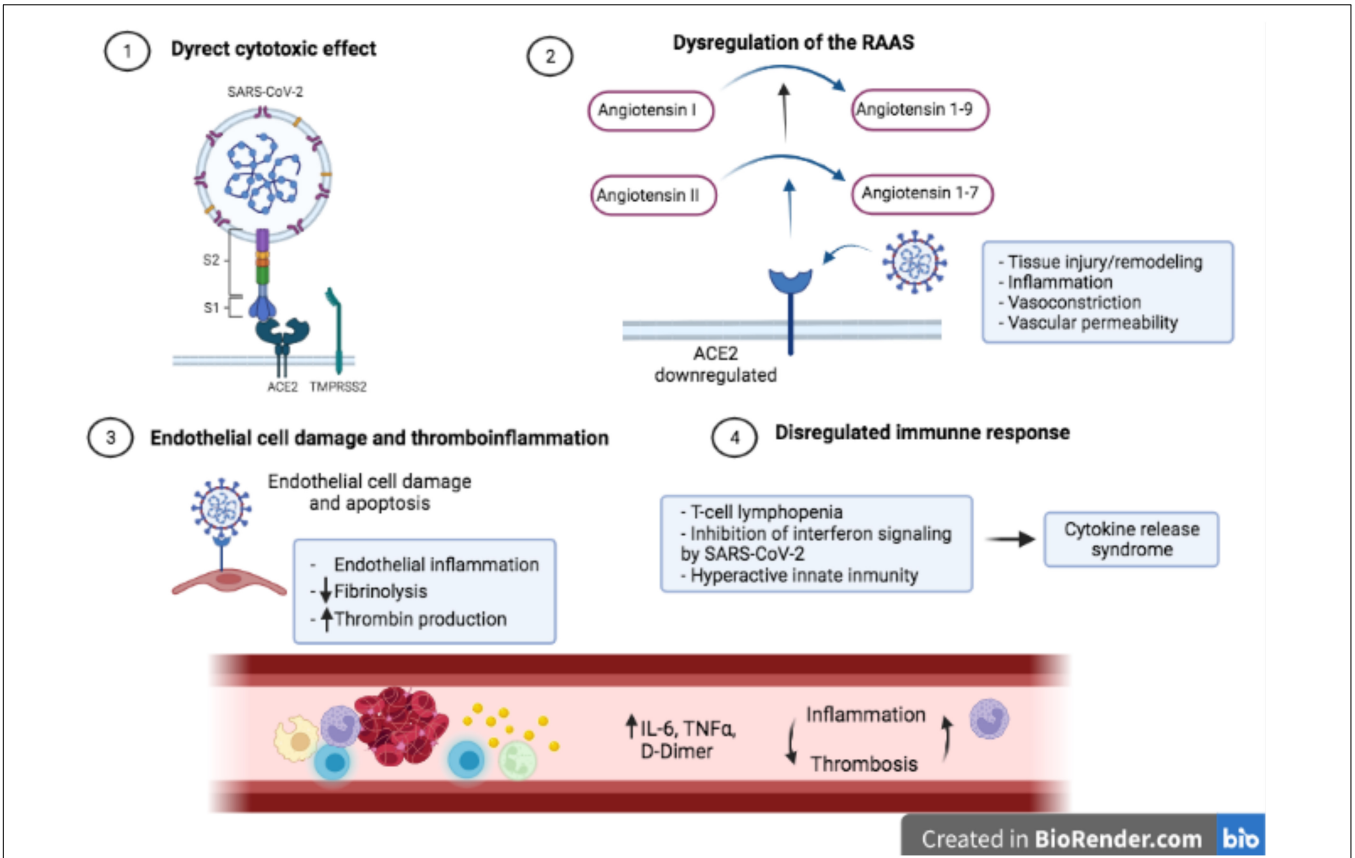

FIGURE 1 | Pathophysiology of COVID-19 [adapted from Gupta et al. (18)]: **(1)** Direct virus-mediated cell damage. SARS-CoV-2 enters into host cells through the union between the spike protein and ACE2 receptor in the presence of TMPRSS2 protease; **(2)** Downregulation of ACE2 leads to a dysregulation of the RAAS and consequently to an increase of angiotensin I and angiotensin II; **(3)** Virus entrance to endothelial cell damage induces apoptosis and endothelialitis; **(4)** T-cell lymphopenia, inhibition of interferon signaling and hyperactive innate immunity produces a dysregulation of the immune response and a cytokine storm syndrome.

the cardiomyocytes (38) while SARS-CoV-2 is principally found inside macrophages or interstitial cells (32, 39, 40).

Endothelial Cells Injury

Endothelial cells infection by SARS-CoV-2 ends up into cell injury of tissues supplied by the affected vasculature. Fibrin deposition and activation of the terminal portion of the complement cascade in the context of endothelial inflammation has been confirmed in autopsies of COVID-19 patients (41).

Hypercoagulability State

Thrombotic events such as pulmonary embolism, venous thromboembolism, vascular cerebral accident, and myocardial infarction have been related to COVID-19 disease (42, 43), as well as disseminated intravascular coagulopathy (DIC) in 71% of COVID-19 non-survivors (44). However, the precise mechanisms which activates the coagulation system are not fully understood and are partially attributed to the cytokine storm and the dysregulation of the immune response. In addition to the hypercoagulability and endothelial dysfunction, the immobility of critical patients and the associated venous stasis complete the 3 Virchow criteria for a high risk of venous thrombosis. Finally, COVID-19 treatments would have interactions with antiaggregant and anticoagulant therapies and increase the risk of thromboembolic events (17).

Myocardial Ischemia

The hypercoagulability and inflammatory stage may lead to myocardial ischemia because of a thrombotic event (type I myocardial infarction) or because of a mismatch between myocardial oxygen supply and demand (type II myocardial infarction). Patients with previous history of cardiovascular disease seem to have a higher risk of myocardial ischemia during viral infections than those without cardiovascular disease (45, 46).

Cytokine Storm Syndrome

SARS-CoV-2 infection has been related to a cytokine storm that may end up to a systemic inflammatory reaction, sepsis, and multiorgan failure (47). Few studies have suggested myocardial injury in the setting of systemic inflammation but without cardiomyocytes virus infiltration, implying that in this setting, myocardial injury could be related to the cytokine storm (48). Among all cytokines, interleukin-6 (IL-6) has an important

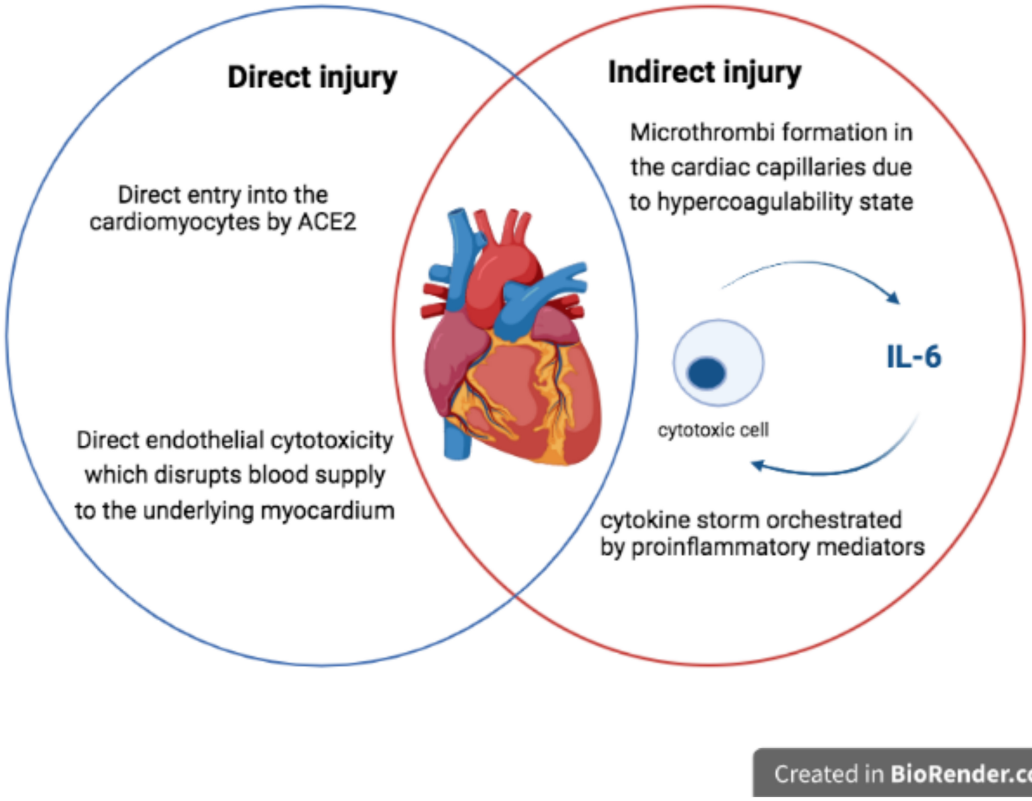

FIGURE 2 | Mechanisms for myocardial damage in COVID-19 [adapted from Siripanthong et al. (72)].

position in COVID-19, not only because of its stimulating effects in cytokine storm, but also because of its cardiovascular effects. Some studies have revealed that IL-6 produces cardiac dysfunction as a consequence of decreasing papillary muscles contractility. In addition, IL-6 has been associated with arrhythmic (49) events and higher levels of myocardial injury biomarkers, as a consequence of its role in atherosclerotic events (50–52), cardiac fibrosis (53), pulmonary hypertension (54) and higher cardiovascular risk (55).

PROGNOSTIC IMPLICATIONS OF MYOCARDIAL DAMAGE IN COVID-19 PATIENTS

Myocardial injury is present in around one-third of hospitalized COVID-19 patients (23, 25–29, 56, 57). Higher cTn levels predict worse outcomes in COVID-19 hospitalized patients, including a higher risk of death and mechanical ventilation (**Supplementary Table 1**). Consequently, the measurement of troponin levels could be a useful tool to guide patient management during their hospitalization (58, 59).

Myocardial injury in COVID-19 patients has been associated with cardiovascular risk factors such as high blood pressure or diabetes mellitus, with heart failure, ischemic cardiovascular disease and chronic renal disease (26, 29, 60). In terms of laboratory findings, it is associated with lower hemoglobin levels and higher inflammatory markers (26, 29, 56).

Cardiovascular inflammation, microvascular dysfunction, ischemia, and myocardial injury, usually found in COVID-19 patients, are known precursors of cardiac arrhythmias and prolonged QT intervals (61, 62). Sinus tachycardia is the most frequently arrhythmia present in COVID-19, probably related to many causes (hypoperfusion, hypoxia, fever. . .). New onset or preexisting atrial fibrillation is the second most frequent arrhythmia, being present in 10–14% of hospitalized patients and 22% of critical COVID-19 patients (63–65). Atrial fibrillation and sinus tachycardia are independent predictors of severity, myocardial injury, and worse outcomes of COVID-19 patients (65). Regarding ventricular arrhythmias, Guo et al. reported an incidence of malignant ventricular arrhythmias in 6% of hospitalized patients. These findings are similar to those found during influenza infection (66). Another report form Du et al. found that arrhythmias were registered in a 60% of patients but only two patients died because of a malignant arrhythmia (67). Since the beginning of the pandemic, early reports proposed hydroxychloroquine or azithromycin as effective drugs against SARS-CoV-2, further studies found that cardiac arrest was more frequent in patients who received these drugs (68).

To date, only few studies regarding the cardiovascular long-term consequences after recovery fromCOVID-19 have been published (**Supplementary Table 1**), suggesting worse long-term

outcomes (69–72). In our previous published study of a cohort with 172 patients who survived COVID-19 hospitalization, myocardial injury was associated with poor prognosis, mainly due to a higher number of readmissions (71). In the same direction, Kini et al. (70) found that the risk of death at 30 days was significantly increased in those patients who had myocardial injury during the acute phase. Finally, Xie et al. (69) showed that beyond 1 month after infection, COVID-19 patients have higher risk of a cardiovascular event; consequently, specific cardiovascular follow-up should be included in care pathways of COVID-19 survivors.

Myocarditis and myocardial injury related to SARS-CoV-2 infection can produce functional and morphologic sequelae on the heart, particularly in those with preexisting cardiac disease (73–75). Cardiovascular magnetic resonance (CMR) imaging has been used as a tool to assess cardiac involvement in patients who survived COVID-19. A multicenter study with 148 recovered COVID-19 patients (74) showed that myocardial injury was associated with CMR abnormalities in around 50% of the patients. Three different patterns of injury were observed: non-infarct myocarditis-pattern injury (27%), ischemic pathology (22%), and non-ischemic non-specific scar (5%). In a 6% of the patients, dual pathology (ischemic and non-ischemic patterns) were observed. No global functional ventricular consequences were found. In addition, a German study that included patients which were recently recovered from COVID-19, CMR revealed cardiac abnormalities in 78% of patients, such as decreased left ventricular ejection fraction and higher left ventricle volumes. Endomyocardial biopsy in patients with cardiac involvement found in CMR studies, showed active lymphocytic inflammation (75). CMR studies in recovered COVID-19 patients have found some disorders that could be responsible for future arrhythmias or heart failure. Further investigation of long-term cardiovascular consequences of COVID-19 is required.

CONCLUSION AND FUTURE PERSPECTIVES

The COVID-19 pandemic is still causing significant morbidity and mortality worldwide. Close monitoring of cardiovascular system in patients with COVID-19 may help to identify high- vs. low-risk patients. Patients with COVID-19 infection and previous cardiovascular disease present a poor prognosis and a higher risk of overall mortality. Further investigation regarding the mechanism, manifestations, and prognosis of myocardial injury in COVID-19 patients is required to improve therapies and prognosis.

AUTHOR CONTRIBUTIONS

AI-M, HC-G, ÁA, BV, and NR-B contributed to the literature review and manuscript drafting. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.901245/full#supplementary-material

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BRIEF RESEARCH REPORT
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SARS-CoV-2 Viremia Precedes an IL6 Response in Severe COVID-19 Patients: Results of a Longitudinal Prospective Cohort

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Background: Interleukin 6 (IL6) levels and SARS-CoV-2 viremia have been correlated with COVID-19 severity. The association over time between them has not been assessed in a prospective cohort. Our aim was to evaluate the relationship between SARS-CoV-2 viremia and time evolution of IL6 levels in a COVID-19 prospective cohort.

Methods: Secondary analysis from a prospective cohort including COVID-19 hospitalized patients from Hospital Universitario La Princesa between November 2020 and January 2021. Serial plasma samples were collected from admission until discharge. Viral load was quantified by Real-Time Polymerase Chain Reaction and IL6 levels with an enzyme immunoassay. To represent the evolution over time of both variables we used the graphic command *twoway* of Stata.

Results: A total of 57 patients were recruited, with median age of 63 years (IQR [53–81]), 61.4% male and 68.4% Caucasian. The peak of viremia appeared shortly after symptom onset in patients with persistent viremia (more than 1 sample with > 1.3 log10 copies/ml) and also in those with at least one IL6 > 30 pg/ml, followed by a progressive increase in IL6 around 10 days later. Persistent viremia in the first week of hospitalization was associated with higher levels of IL6. Both IL6 and SARS-CoV-2 viral load were higher in males, with a quicker increase with age.

Conclusion: In those patients with worse outcomes, an early peak of SARS-CoV-2 viral load precedes an increase in IL6 levels. Monitoring SARS-CoV-2 viral load during the first week after symptom onset may be helpful to predict disease severity in COVID-19 patients.

Keywords: SARS-CoV-2, viremia, interleukin 6 (IL-6), prognosis, COVID-19

INTRODUCTION

One of the most feared complications of the disease caused by the coronavirus SARS-CoV-2 (COVID-19), is the development of an Acute Respiratory Distress Syndrome (ARDS), which can affect 15.6–31% of patients (1). Siddiqui and Mehra (2) proposed that ARDS is part of the final stage of the disease, in which clinical features are mainly the consequence of the host hyperinflammatory response and a cytokine storm; whereas the stage I (early infection) is mainly caused by viral replication and the early immune response. However, to the best of our knowledge, this proposal has not been validated.

Since the outbreak of the COVID-19 pandemic, many efforts have been made to find early risk factors and biomarkers able to predict the evolution toward the cytokine storm. In this sense, older age, obesity and comorbidities such as hypertension, diabetes and coronary heart disease have been associated with higher risk of death (3, 4). On the other hand, increased levels of C reactive protein (CRP), lactate dehydrogenase (LDH), and D-dimer, among others, have been shown to be related to the development of ARDS and mortality (3, 5, 6).

In this context, Interleukin 6 (IL6) has been described as one of the most useful biomarkers (7). In a previous work, we showed that IL6 could be a severity biomarker but also a guide to select COVID-19 patients who could benefit from treatment with tocilizumab, an inhibitor of the IL6 receptor (8). Another important biomarker is the presence of SARS-CoV-2 RNA in peripheral blood (viremia), which has been associated with disease severity and a hyperinflammatory state (9, 10). Saji et al. (11) showed that the combination of SpO2/FiO2, IL6 and the presence of SARS-CoV-2 viremia at admission had the highest accuracy to predict fatal outcomes. Bermejo et al. (12) and Myhre et al. (13) found that the presence of SARS-CoV-2 viremia at admission correlated with increased levels of IL6, CRP, and ferritin. In addition, a proteomic analysis showed that the expression of viral response and interferon/monocytic pathway proteins such as IL6 and one of its regulators, the Nicotinamide phosporibosyl transferase (NAMPT), were upregulated in patients with quantifiable SARS-CoV-2 viremia at admission, compared to those with undetectable viremia (14).

In a previous study of our group, we found that viremia was associated with Intensive Care Unit (ICU) admission and in-hospital death, and it was a better biomarker than IL6 (10). In this regard, since SARS-CoV-2 infection is involved in triggering IL6 expression, viremia as an indicator of the systemic viral shedding, could be related with the IL6 response and be useful as an early biomarker (phase of viral response). Nevertheless, the factors determining an IL6 increase in COVID-19 patients have not been well established yet and the association over time between SARS-CoV-2 viremia and IL6, has not been assessed in a prospective cohort with serial samples.

Considering our previous results, the aim of this study was to evaluate the relationship between the presence of SARS-CoV-2 viremia and the time evolution and IL6 levels in a prospective cohort of COVID-19 hospitalized patients.

MATERIALS AND METHODS

Study Design, Population, and Data Collection

This work is a secondary analysis of samples from a prospective observational cohort assembled to validate the predictive value of SARS-CoV-2 viremia (ongoing manuscript). The study included patients hospitalized for COVID-19 in Hospital Universitario La Princesa (HUP) between November 1st 2020 and January 15th 2021.

The inclusion criteria were: (a) positive Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) for SARS-CoV-2 in nasopharyngeal and throat swabs at most 48 h prior to hospitalization; (b) acceptance to participate in the study and oral or written consent; (c) age older than 18 years. The exclusion criteria were: (a) patients without an initial viremia determination in the first 24–36 h after admission; (b) patients unlikely to be followed-up because they were candidates to be transferred to other health facilities.

Clinical, laboratory and therapeutic data were collected from electronic clinical records and included in an anonymized database. Baseline clinical and laboratory data are to those obtained at admission day.

The need for hospitalization was decided by the physicians at the emergency room based on clinical criteria, without the intervention of the research team. Patient's treatment and management was decided by each attending physician based on the hospital protocols and their clinical judgment. Attending physicians were blind to the viremia results.

Sample Size

The sample size was estimated in 49 patients to validate the primary objective of the study "SARS-CoV-2 viremia as a biomarker of disease severity" (ongoing manuscript) based on the results of our previous retrospective studies (10, 15); nevertheless, 57 patients were finally included.

Sample Collection

Serial plasma and serum samples were collected from admission until discharge. In the first week, samples were collected every 48 h, with the first sample in the first 24–36 h. Thereafter, samples were collected twice a week. All samples were frozen at –80°C and stored in the Microbiology Department facilities.

SARS-CoV-2 RNA Extraction and Detection

Firstly, a nucleic acid extraction of samples was performed by the automatic eMAG® Nucleic Acid Extraction System (Biomerieux, France). Detection of viremia was performed with rRT-PCR using TaqPath™ COVID-19 CE81 IVD RT-PCR Kit [Thermo Fisher Scientific, United States (TFS)], according to the manufacturer's instructions, by a QuantStudio™ 5 Real Time PCR System (Applied Biosystems, United States). Amplification curves were analyzed with QuantStudio™ Design and Analysis software version 2.4.3 (Applied Biosystems, United States) and interpreted by a clinical microbiologist. To increase sensitivity, two wells were used for each sample. Two positive controls (one of 20,000 copies and another of 200 copies) and two negative controls were added in each run in duplicate.

Quantification of Viral Load

A standard curve was established using a positive control with a known concentration (TaqPath Positive Control from TaqPath™ COVID-19 Control Kit) of 10,000 copies/μl of the SARS-CoV-2 genomic regions targeted by the TFS assay. Ten-fold serial dilutions of the positive control were made up to 1 copy. Nine wells of each of the concentrations and of the negative control were added to the run. The rRT-PCR was performed by QuantStudio™ 5 Real Time PCR System and a standard curve was obtained plotting DNA concentration against cycle threshold (Ct) values. The amplification curves were analyzed with QuantStudio™ Design and Analysis software version 2.4.3 (Applied Biosystems, United States). The results of the nine wells with 1 copy were omitted because they were widely dispersed.

Viral load was calculated from Ct values using the standard curve as reference and was expressed as copies/ml and the logarithm with base 10 (log10). Due to the variability and lack of accuracy obtained with the lower levels of SARS-CoV-2 viremia, only viremias > 1.3 log10 (20 copies/ml) were considered quantifiable.

Interleukin 6 Measurement

Serum samples collected in the same extraction as the plasma used for SARS-CoV-2 viremia determinations were used to assess IL6 levels. IL6 levels were retrospectively quantified in triplicate with the Human IL6 Duoset enzyme-immunoassay from R&D Systems Europe Ltd. (Abingdon, United Kingdom), following the manufacturer's instructions.

Variables

For analysis using viremia as a quantitative variable, all values were used. However, to define viremia as categorical, positive viremia was considered when values were higher than 1.3 log10 (namely 20 copies/ml, which was the threshold for quantifiable viremia) and negative when values were below this threshold. Persistent viremia was defined as more than one positive viremia in the first week of hospitalization.

Two different variables were used to evaluate IL6 levels: (a) a quantitative variable defined as IL6 concentration, expressed in pg/ml, (b) a dichotomic variable, which considered levels of IL6 as high when at least one IL6 determination was higher than 30 pg/ml or low if all determinations were below 30 pg/ml. This threshold was based on our previous study, where we showed that IL6 > 30 pg/ml was associated with poor respiratory outcomes (8). The average levels of IL6 and viral load were defined as the arithmetic mean of all their determinations in each patient.

Statistical Analysis

We used Stata 14.0 for Windows (Stata Corp. LP, College Station, TX, United States) for all the analysis described below. Quantitative variables were represented as median and Interquartile Range (IQR), and the Mann Whitney or Kruskall Wallis tests were used to assess significant differences, since all quantitative variables followed a non-normal distribution. Qualitative variables were described as counts and proportions and Chi square or Fisher's exact test was used for comparisons.

In order to comparatively show levels of IL6 and viral load through the two first weeks of follow-up, we used as time variable the number of days from the beginning of symptoms to collection of each sample. To represent the mean evolution over time of both variables we used the graphic command *twoway* from Stata with the option fractional polynomial prediction with 95% confidence interval (CI). Since it is well known that blockade of IL6 receptor with tocilizumab can result in an increase of IL6 serum levels (16), we decided to carry forward the last observation before tocilizumab treatment [last observation carried forward (LOCF) strategy] to replace IL6 values in the remaining visits of the first 2 weeks for those patients treated with tocilizumab in order to avoid the bias of excluding this important population (see comparative baseline characteristics in **Supplementary Table 1**). Furthermore, we also applied LOCT strategy for those patients who died or were discharged before the 5th visit (14th day after admission), in order to obtain a more homogeneous number of determinations all along the follow-up.

To determine which variables were associated with high levels of IL6, we performed a multivariable logistic regression analysis that was first modeled by adding all the variables with a *p*-value lower than 0.15 in the bivariable analysis. The final model was reached through backward stepwise removal of variables with *p*-value higher than 0.15.

Ethics

This study was approved by the Research Ethics Committee of Hospital Universitario La Princesa, Madrid (register number 4267; 22-10-2020), and it was carried out following the ethical principles established in the Declaration of Helsinki. As proposed by AEMPS (Agencia Española de Medicamentos y Productos Sanitarios, The Spanish Agency for Medicines and Medical Devices), only oral consent was required due to the COVID-19 emergency (17). However, a written information sheet was also

offered to all patients. After being informed about the study, all included patients (or their representatives) gave informed consent, which was registered in the electronic clinical chart.

This article was written following the STROBE initiative (Strengthening the Reporting of Observational studies in Epidemiology).

RESULTS

Study Population and Sample Characteristics

A total of 57 patients were recruited, with median age of 63 years (IQR 53–81), 61.4% were male, 68.4% Caucasian, and 75.4% had previous comorbidities. The median time from symptom onset to first sample was 8 days (IQR 4–10). Baseline clinical characteristics according to IL6 levels are shown in **Table 1**. During patients' hospitalization, 301 serum samples were collected, with a median number of 3 samples per patient (IQR 2-5).

Nine patients were treated with tocilizumab, who on average, showed data suggesting a more severe disease, although differences did not reach statistical significance (**Supplementary Table 1**).

Most patients who progressed to a severe disease started this evolution 7–14 days after the symptoms onset. In addition, patients with a more benign course were discharged at the end of the first week after admission. For clinical consistency, we decided to analyze only the samples corresponding to the first 2 weeks of hospitalization, a maximum of 5 samples per patient. Thus, IL6 levels were measured in 228 samples, with a median of 3.6 pg/ml (IQR 0–21 pg/ml). Baseline clinical characteristics of patients depending on IL6 status are shown in **Table 1**. SARS-CoV-2 viremia was determined in 234 samples, with the highest percentage of positive viremia (36.8%) at admission (visit 1).

Time Course of Interleukin 6 and SARS-CoV-2 Viremia

The average serum levels of IL6 and SARS-CoV-2 viral load were moderately but significantly correlated (r = 0.41, p = 0.0014; **Supplementary Figure 1**).

Figure 1A shows the evolution over time of IL6 and viremia analyzed using data from the whole population (including IL6 after tocilizumab treatment). The peak of viremia appeared early, at the first days after symptom onset (day 3–5), and quickly decreased. On the other hand, the highest levels of IL6 were found at day 20. The wide 95% CI suggested a high heterogeneity, especially at both extremes of the time course. **Figure 1B** shows the results when the LOCF strategy (see Statistical section for further information) was used to minimize the increase of IL6 induced by tocilizumab (see **Supplementary Figure 2** for raw data in cases treated or not with tocilizumab). With LOCF strategy, the peak of IL6 was smaller, but the time course of IL6 production was quite similar to that obtained from raw data. Hereinafter, the relationship between IL6 and SARS-CoV2 viral load shown corresponds to results obtained with the LOCF strategy.

Relationship Between Interleukin 6 and SARS-CoV-2 Viremia

A total of 19 patients had high IL6 (**Table 1**), of them 11 (57.9%) had persistent viremia compared to 5 patients (13.2%) in the low IL6 group (p = 0.001), with an odds ratio of 9.1 (95%CI 2.5–32.6) (**Figure 2E**). In the graphic representation of IL6 and viral load according to high/low IL6 (**Figures 2A,B**), an early and minor peak of IL6 was found in the low IL6 group, together with a small peak of viremia at day 4. On the other hand, patients with at least one IL6 above 30 pg/ml had an early high viremia around day 3 and a progressive increase of IL6 especially after day 12.

When the prediction of IL6 and viral load was calculated according to persistent viremia status, remarkable differences were obtained (**Figures 2C,D**). In the persistent viremia group, viral load showed a peak around day 4, whereas IL6 had a two-phase increase: one at the first days from symptom onset and then a subsequent progressive increase after day 5. Regarding non-persistent viremia the increase of IL6 was slow from symptom onset until day 20. The median of the average levels of IL6 were 3.6 pg/ml (IQR 1.0–9.2 pg/ml) in the non-persistent viremia group and 21.4 pg/ml (IQR 12.3–44.9) in patients with persistent viremia ($p < 0.001$).

Prediction of Interleukin 6 and SARS-CoV-2 Viremia According to Demographic Factors

The effect of demographic factors on IL6 levels and SARS-CoV-2 viremia was also assessed. The median of the average IL6 concentration was significantly higher in males (11.3 pg/ml [IQR 3.3–27.5] than in females (2.5 pg/ml [IQR 0.7–9.2 pg/ml]; p = 0.005). In the group of patients with high IL6, 84.2% were male compared to 50% in the group with low IL6 (p = 0.02). No differences were found in the average viral load (11.1 copies/ml [IQR 0–197.3 copies/ml] vs. 2.3 copies/ml [IQR 0–6.8 copies/ml]; p = 0.08) or in the percentage of patients with persistent viremia (34.3% vs. 18.2%; p = 0.24) between males and females, respectively. However, predicting curves for IL6 and viral load were substantially different depending on sex (**Figure 3A**). In males, curves had a fast increase in viral load with a peak around day 2 from symptom onset and a later rise in IL6 levels until day 20, while women showed a small increase in viral load and IL6 between day 2 and 7 approximately.

The effect of age on IL6 levels and viral load was also considered. The average levels of IL6 and viremia did not correlate with age (r = 0.21, p = 0.13; and r = 0.19, p = 0.16; respectively). Moreover, no differences were found when age was categorized as < 75 years and > 75 years (p = 0.57 and p = 0.88, for IL6 and viral load, respectively). In the group with high IL6, 31.6% of patients were older than 75 years, the same percentage as in the low IL6 group (p = 1). Regarding persistent viremia, the proportion of patients older than 75 years was 29.3% in the group

TABLE 1 | Baseline clinical characteristics of the study population according to IL6 levels.

	Study population (*n* = 57)	Low IL6 (*n* = 38)	High IL6 (*n* = 19)	*P*-value
Age; median (IQR)	63 (53–81)	60 (49–81)	72 (59–81)	0.21
Male sex; *n* (%)	35 (61.4)	19 (50)	16 (84.2)	0.02
Race/ethnicity; *n* (%)				0.009
-Caucasian	39 (68.4)	22 (57.9)	17 (89.4)	
-Latin-American	16 (28.1)	15 (39.5)	1 (5.3)	*0.01
-Asian	2 (3.5)	1 (2.6)	1 (5.3)	
Comorbidities; *n* (%)	43 (75.4)	30 (79)	13 (68.4)	0.5
Age-adjusted Charlson's Comorbidity Index; median (IQR)	3 (1–5)	2.5 (1–5)	4 (1–5)	0.37
Days from symptom onset to first sample; median (IQR)	8 (4–10)	8 (4–12)	6 (3–8)	0.12
Persistent viremia; *n* (%)	16 (28.1)	5 (13.2)	11 (57.9)	0.001
Clinical progression^; *n* (%)	12 (21.1)	3 (7.9)	9 (47.4)	0.001
Intensive Care Unit; *n* (%)	8 (14)	3 (7.9)	5 (26.3)	0.1
In-hospital mortality; *n* (%)	5 (8.8)	0	5 (26.3)	0.003

**Significant differences were only found between Caucasians and Latin-Americans. ^Clinical progression was defined as a worsening of at least one point on the WHO COVID Ordinal Outcomes Scale (33) during a 14-day follow-up after admission.*

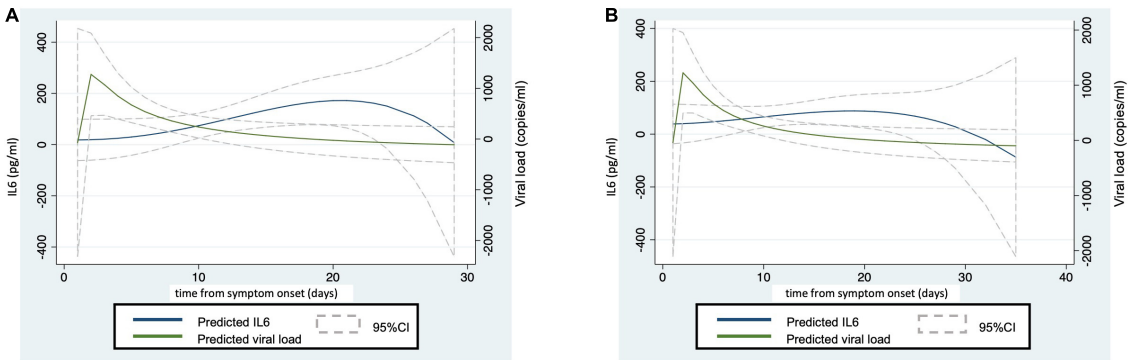

FIGURE 1 | The peak of viral load precedes the IL6 increase. Graphic representation of time-course of IL6 levels and SARS-CoV-2 viral load from symptom onset. **(A)** representation of raw data. **(B)** Representation of data after applying the LOCF strategy. The fractional polynomial prediction was performed using the *twoway* command of Stata.

with non-persistent viremia and 37.5% in those with persistent viremia ($p = 0.55$). In the prediction curves according to age and sex, all parameters increased with age except for viral load in males, which peaked between 40 and 50 years (**Figure 3B**).

Regarding ethnicity, there were only differences between Caucasians and Latin-Americans in the average IL6 levels (11.9 [IQR 2.9–35.2] vs. 2.9 [IQR 1.0–4.3]; $p = 0.005$), and the percentage of patients with high IL6 (89.5% vs. 5.3%, $p = 0.01$) (**Table 1**). No differences were found depending on ethnicity in the average viral load or the percentage of patients with persistent viremia.

Finally, a multivariable analysis showed that the presence of high IL6 was associated with persistent viremia (OR 10.0 [95%CI 2.0–49.5]; $p = 0.005$); conversely, female sex (OR 0.17 [0.03–0.9]; $p = 0.04$) and Latin-American origin (OR 0.06 [0.01–0.7]; $p = 0.02$) had a protective effect.

DISCUSSION

This study assessed the relationship between IL6 and SARS-CoV-2 viremia. The most relevant finding was the different time course of IL6 and viremia: the peak of viremia appeared shortly after symptom onset in patients with persistent viremia and also in those with at least one measure of IL6 > 30 pg/ml, in which it was followed days later by a progressive increase in IL6. Moreover, the presence of persistent viremia in the first week of hospitalization was associated with higher levels of IL6. Both IL6 and SARS-CoV-2 viral load were higher in males, with a progressive increase with age that occurred earlier in males.

Our findings are consistent with the COVID-19 phases first described by Siddiqui and Mehra (2). These authors described an early stage characterized by a viral response (SARS-CoV-2 viremia) and a final stage caused by a hyperinflammatory state characterized by increased levels of IL6. To date, only two studies have evaluated SARS-CoV-2 viremia and systemic cytokines in a longitudinal design, but none of them considered the different time course of the increase in viremia and IL6 (18, 19). Van Riel et al. (18) only included 20 patients and found that the levels of IL6 were associated with critical disease but not with the presence of viremia; while Brasen et al. (19) found and association between maximum viral load and IL6. Neither of them assessed the temporal course of both biomarkers. In our previous work (10),

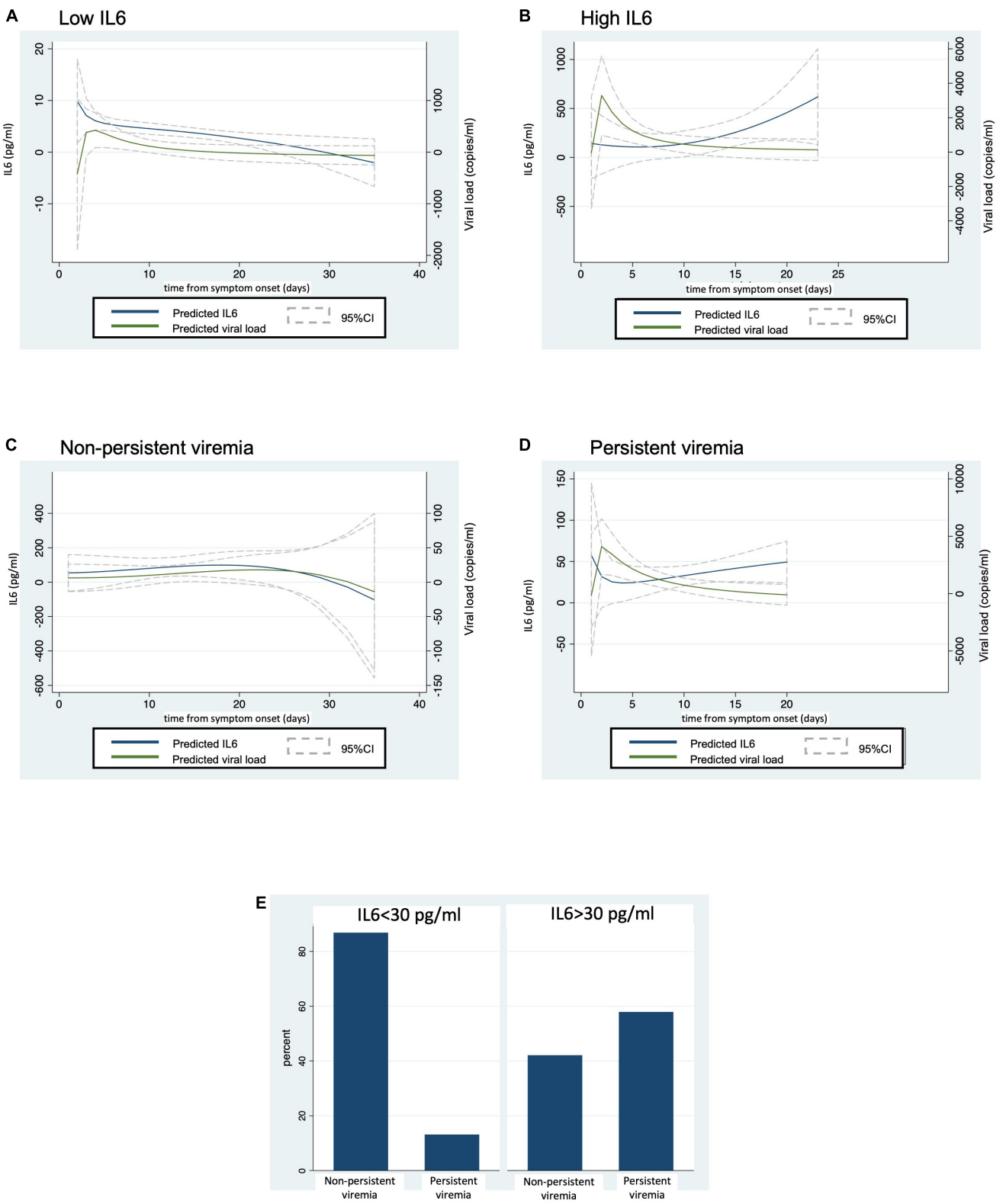

FIGURE 2 | Patients with worse outcomes have an early peak of SARS-CoV-2 viral load before a prominent increase in IL6 levels. Graphic representation of IL6 levels and SARS-CoV-2 viral load from symptom onset in: **(A)** patients with low IL6; **(B)** patients with at least one IL6 > 30 pg/ml (high IL6); **(C)** non-persistent viremia; and **(D)** persistent viremia. **(E)** Represents the percentage of patients with persistent viremia according to IL6 levels (low vs. high). **(A–D)** The fractional polynomial predictions were performed using the *twoway* command of Stata.

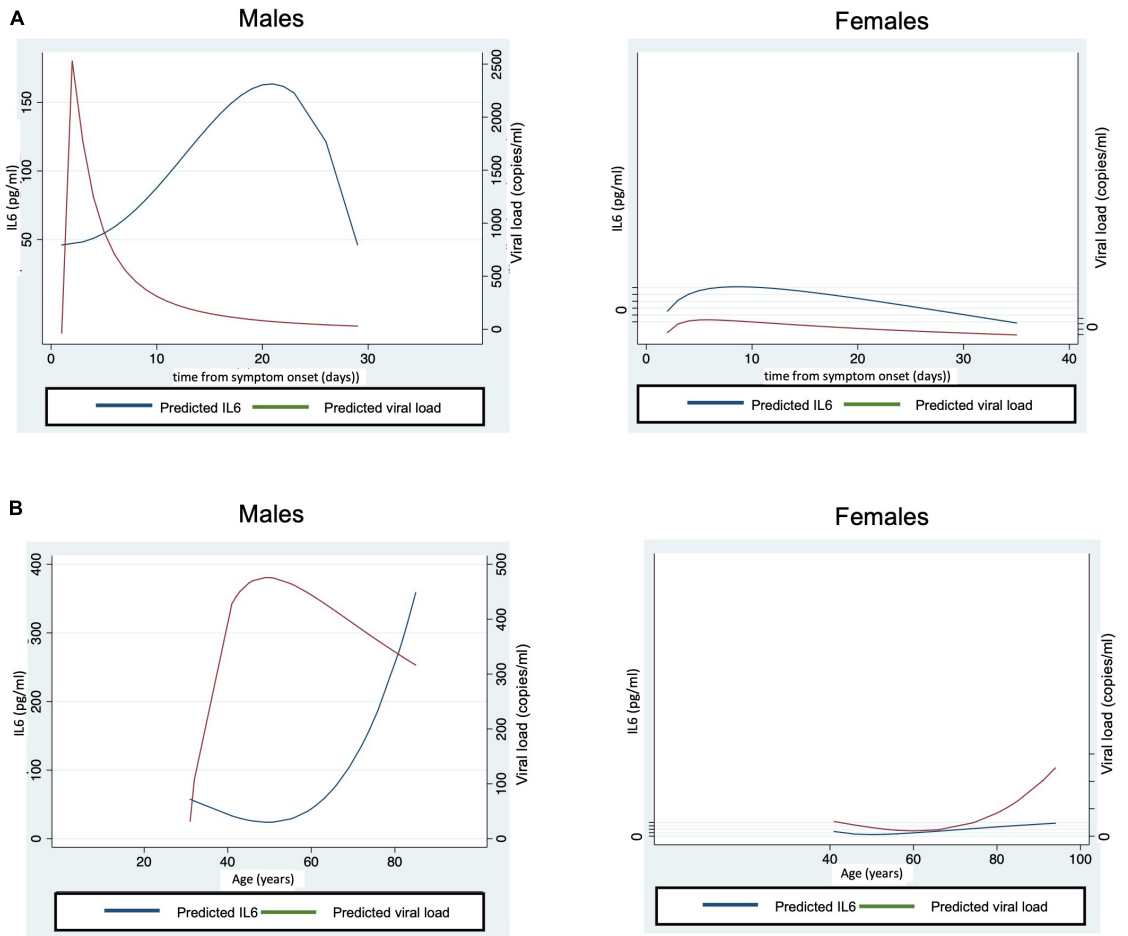

FIGURE 3 | Males had more relevant increases of IL6 and viral load. **(A)** Represents the levels of IL6 and viral load from symptom onset by sex (both panels using the same scale), while **(B)** shows levels of IL6 and viral load by age and sex (both panels using the same scale). The fractional polynomial predictions were performed using the *twoway* command of Stata.

we described that the presence of relevant SARS-CoV-2 viremia was associated with higher risk of death and ICU admission. Furthermore, viremia was the most useful biomarker for these outcomes, being superior to IL6, lymphopenia and LDH. In the present study, we show that the SARS-CoV-2 viremia appears early in the course of the disease, standing out as a relevant, simple and early biomarker.

Since the beginning of the pandemic, CRP, and IL6 levels have been used as prognostic biomarkers in COVID-19; in addition, IL6 activity has been targeted for treatment by the anti-IL-6 receptor antibody but also to guide treatment and predict response to tocilizumab (8, 20, 21). Moreover, a previous study of our group showed that high levels of IL6 (above 30 pg/ml) were associated with worse prognosis of COVID-19, and also with a better response to tocilizumab, thereby suggesting a role of IL6 levels in guiding treatment and predicting response to this therapeutic agent (8). However, Ong et al. (22) and Liu et al. (23) showed that IL6 in COVID-19 patients peaked after the worsening of respiratory function, suggesting that when proinflammatory biomarkers rise, lung damage might be already established. In our cohort, 68.8% of patients with persistent viremia had at least one IL6 $>$ 30 pg/ml in the later hyperinflammatory phase of the disease. Taking into account the high percentage of patients with persistent viremia who develop an hyperinflammatory response, these patients might be considered as candidates for intensive treatment and surveillance, or even for early treatment with IL6 blockade.

Nevertheless, these findings might not be extensive to all patients. A more severe course of COVID-19 and higher levels of IL6 have been previously described in older males (24). In this sense, genetic and hormonal factors have been proposed to be involved in age and sex related differences in COVID-19 (24, 25). One of the most studied SARS-CoV-2 related proteins is ACE2, the membrane receptor needed for the virus internalization, which is encoded by the gen of the same name located in the X chromosome (25, 26). ACE2 expression increases with age and in male sex in COVID-19 patients (27, 28). ACE2 expression also correlates with SARS-CoV-2 infectivity in cells of the respiratory tract (29) and with higher viral

loads in nasopharyngeal swabs (30). Whether endothelial and vascular ACE2 is related to viral load in peripheral blood has not been assessed yet, but it is plausible that higher levels of systemic ACE2 lead to increased viremia. Another proposed mechanism to explain sex differences is the effect of Toll Like Receptor (TLR) pathways, especially TLR7. This receptor, which recognizes viral single strain RNA and enhances IL6 production, is also located in X chromosome. TLR7 was one of the most important susceptibility genes found in an Italian cohort of COVID-19 patients, where 6.3% of young males with life-threatening disease presented missense variants of this gene (31).

Regarding ethnicity, patients with a Latin-American origin had lower levels of IL6 in our cohort. In this sense, other cytokines and chemokines such as MCP-1, IL-10, IL-15, CXCL10, and CCL2 have been associated with SARS-CoV-2 viremia (12, 18). It is possible that the immune response of COVID-19 patients is enhanced by molecular pathways different from IL6, which may play a relevant role in patients without an IL6 increase. However, this hypothesis needs to be further evaluated with studies with bigger sample size than our cohort.

This study has several limitations. First of all, the sample size of our cohort was small, although it was sufficient to find different patterns in the kinetics of IL6 and viral load. Secondly, all patients included were hospitalized and their first sample was obtained at a median of 7 days after symptom onset, therefore, data from the first days of the disease were limited. In addition, information about different variants of SARS-CoV-2 in our cohort could not be obtained because viral sequencing was not available in our facilities. However, at the time our study was performed, the most prevalent variant in Madrid was the original strain (32).

In conclusion, in those patients with worse outcomes, an early peak of SARS-CoV-2 viral load precedes around 5–10 days a prominent increase in IL6 levels. This finding was very clear in males older than 40 years. Therefore, monitoring SARS-CoV-2 viral load during the first week after symptom onset may be helpful to stratify the severity of patients and predict those who are at high risk of developing hyperinflammatory syndrome and ARDS.

DATA AVAILABILITY STATEMENT

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 the Research Ethics Committee of Hospital Universitario La Princesa, Madrid. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ER-V, LC, IG-A, and DR-S designed the study and wrote the first draft of the manuscript. MCL, EÁ, AB, JH, JO, SR-G, MCS, CM, EG, BG, RM, II, AV, AS-A, DA, JÁ, PP, MT, MU, JG-R, RG-V, JA, RC, and CS included patients in the study and collected data. BQ, CA, CM-C, and EF-R extracted and processed samples. ER-V, AT-M, NZ, AM-R, and LF performed laboratory determinations. ER-V, AS-G, and IG-A analyzed the data. All authors reviewed the final draft.

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One Year Overview and Follow-Up in a Post-COVID Consultation of Critically Ill Patients

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The long-term clinical management and evolution of a cohort of critical COVID-19 survivors have not been described in detail. We report a prospective observational study of COVID-19 patients admitted to the ICU between March and August 2020. The follow-up in a post-COVID consultation comprised symptoms, pulmonary function tests, the 6-minute walking test (6MWT), and chest computed tomography (CT). Additionally, questionnaires to evaluate the prevalence of post-COVID-19 syndrome were administered at 1 year. A total of 181 patients were admitted to the ICU during the study period. They were middle-aged (median [IQR] of 61 [52;67]) and male (66.9%), with a median ICU stay of 9 (5–24.2) days. 20% died in the hospital, and 39 were not able to be included. A cohort of 105 patients initiated the follow-up. At 1 year, 32.2% persisted with respiratory alterations and needed to continue the follow-up. Ten percent still had moderate/severe lung diffusion (DLCO) involvement (<60%), and 53.7% had a fibrotic pattern on CT. Moreover, patients had a mean (SD) number of symptoms of 5.7 ± 4.6, and 61.3% met the criteria for post-COVID syndrome at 1 year. During the follow-up, 46 patients were discharged, and 16 were transferred to other consultations. Other conditions, such as emphysema (21.6%), COPD (8.2%), severe neurocognitive disorders

(4.1%), and lung cancer (1%) were identified. A high use of health care resources is observed in the first year. In conclusion, one-third of critically ill COVID-19 patients need to continue follow-up beyond 1 year, due to abnormalities on DLCO, chest CT, or persistent symptoms.

Keywords: COVID-19, CT abnormalities, intensive care unit (ICU), lung function, SARS, SARS-CoV-2, post-COVID syndrome, sequelae

INTRODUCTION

Since the beginning of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in December 2019, more than 300 million COVID-19 cases have been confirmed globally, and more than 5.7 million people have died (1). A far from negligible proportion of hospitalized patients (20–67%) may develop a more severe disease resulting in acute respiratory distress syndrome (ARDS) (2, 3). This has generated a surge of patients who require respiratory support with invasive or non-invasive mechanical ventilation (IMV and NIMV) (3, 4), overburdening ICUs worldwide.

COVID-19 continues to be a public health emergency of international concern due to the enormous global disease burden. As a result of this situation, there is growing interest in the long-term sequelae after recovery from acute COVID-19. Previous reports indicate that at 6 months of follow-up, at least three-quarters of COVID-19 survivors discharged from the hospital still had persisting symptoms (5–7). Importantly, patients with more severe acute disease and those who were critically ill during their hospital stay had a higher risk of lung diffusion impairment (up to 56%) and radiological abnormalities (4, 6). To date, the literature on 1-year outcomes after hospital discharge is diverse (8, 9) and has not focused on critically ill COVID-19 survivors. Specifically, a study published recently (10) found that those who were critically ill during the hospital stay presented more pulmonary damage on chest CT (87%) and lung diffusing impairment (54%) at the 12-month follow-up.

In this respect, we aimed to describe what happens to the patients who needed ICU admission due to COVID-19 infection 1 year after their hospital discharge. We deeply describe the clinical follow-up, which includes an evaluation of symptoms, respiratory assessment (including lung volumes, DLCO, and 6-minute walking test) and a chest CT scan 3, 6, and 12 months after hospital discharge. Moreover, a questionnaire to evaluate persistent symptoms and post-COVID syndrome was performed at 1 year of follow-up in all patients.

MATERIALS AND METHODS

Study Design and Population

This was a prospective observational study performed in patients who had a critical care admission due to COVID-19 between March and August 2020 in Hospital Universitari Arnau de Vilanova and Hospital Universitari Santa Maria in Lleida (Spain). The study is a subset of the ongoing multicenter study CIBERESUCICOVID (NCT04457505) and follows the Strengthening the Reporting of Observational Studies (STROBE) statement.

The study was approved by the Medical Ethics Committee (CEIC/2273). Informed consent was acquired (written and/or verbal) from all patients.

The main objective of this study was to describe the following at 1 year after a critical COVID-19 infection: (1) a general perspective of these patients, (2) the follow-up of the survivors in the context of a clinical post-COVID unit, and (3) the prevalence of post-COVID syndrome in these patients.

Inclusion and Exclusion Criteria

All patients were positive for SARS-CoV-2, were older than 18 years and had been admitted to the ICU. Follow-up of patients who survived was based on the following exclusion criteria: (i) treatment with palliative care, (ii) follow-up in another center, and (iii) severe mental disability that made it impossible to assess pulmonary function.

Clinical Data Collection

Clinical Data During Hospital Stay

Patient sociodemographic and comorbidity data and clinical, vital, ventilator, and laboratory parameters were recorded at the hospital and ICU admission. We also collected data on the length of ICU and hospital stays, the duration of mechanical ventilation and the need for and duration of prone positioning, treatments received, complications during hospitalization and death.

Follow-Up Visit in the Post-COVID Unit

Patients were evaluated at 3, 6, and 12 months after hospital discharge. General and respiratory symptoms, as well as quality of life and anxiety and depression, were assessed as previously described (11). The protocols for the pulmonary function tests, 6-minute walking test and chest CT scan of the thorax were also previously described (9).

The post-COVID unit is a consultation based on the joint evaluation of a pulmonologist (JG), two nurses (MA, SS), and a physiotherapist (AM) with experience in the management of post-COVID and chronic respiratory patients. Patients were discharged when they had clinically recovered from pulmonary damage due to COVID-19. Nevertheless, many others were referred to other consultations due to previous existing pulmonary conditions (such as COPD or emphysema) or other comorbidities (neurological, cardiological, etc.).

Abbreviations: CT, computed tomography; COVID-19, coronavirus disease 2019; ICU, intensive care unit; ARDS, acute respiratory distress syndrome; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; 6MWT, 6-minute walking test.

Post-COVID Syndrome

We aimed to describe post-COVID syndrome prevalence after 12 months of hospital discharge in all critical COVID-19 survivors. There have been several definitions of this condition proposed in the last year (12). A recent study supported by the World Health Organization (WHO) (13) suggested post-acute COVID-19 as the presence of symptoms such as fatigue, shortness of breath, and cognitive dysfunction that impact daily quality of life after 3 months of probable or confirmed SARS-CoV-2 infection, which are not explained by other alternative diagnoses. Symptoms might be persistent or new onset within at least 2 months.

We evaluated these domains (fatigue, shortness of breath, and cognitive dysfunction) by using standardized and validated questionnaires. The Functional Assessment of Chronic Illness Therapy (FACIT) is a questionnaire that assesses self-conception of fatigue and its impact on health-related quality of life in the last 7 days. It contains 13 items from 0 (not very fatigued) to 4 (very fatigued), where a higher score indicates a better quality of life (14, 15). The British Columbia Cognitive Complaints Inventory (BC-CCI) is a 6-item scale that measures perceived cognitive impairments such as problems with concentration, memory, expressing thoughts, word finding, slow thinking, and difficulty solving problems in the past 7 days (16). A higher score reveals more severe cognitive complaints (17). Finally, we used the modified Medical Research Council (mMRC) scale to define the presence of dyspnea in routine clinical practice.

Statistical Analysis

Descriptive statistics of the mean (standard deviation) and median (25th percentile; 75th percentile) were estimated for quantitative variables with normal and non-normal distributions, respectively. The absolute and relative frequencies were calculated for qualitative variables. Relative frequencies were calculated excluding missing data. Categorical variables were compared using the chi-squared test or Fisher's exact test, whereas continuous variables were compared using the non-parametric Mann–Whitney *U* test or *t*-test, depending on whether the variable was normally distributed (Shapiro Wilk test). The *p*-value for the trend was computed from the Pearson test when the variable was normal and from the Spearman test when it was continuous non-normally distributed. For categorical variables, the p value for the trend was computed from the Mantel–Haenszel test. The p value threshold defining statistical significance in all analyses was set at 0.05. Data management and statistical analyses were performed using R (version 4.0.2; R Foundation for Statistical Computing) (18).

RESULTS

General Description of Hospital Stay

A total of 181 patients were admitted to the ICU due to COVID-19 between March and August 2020. Briefly, they were predominantly middle-aged (median [IQR] 61 [52–67] years old) males (66.9%) with obesity, hypertension and diabetes mellitus as the most frequent comorbidities. Of the total cohort, 37 (20.4%) patients did not survive hospital stay. As expected, the non-survivors showed higher comorbidity, were more severe at ICU admission and presented more frequently with acute renal failure than survivors (**Table 1**; **Supplementary Table 1**).

TABLE 1 | Baseline characteristics.

	ALL ***n*** **= 181** **Median [IQR], mean (sd) or n (%)**	**Survivors** ***n*** **= 144** **Median [IQR], mean (sd) or n (%)**	**Non-survivors** ***n*** **= 37** **Median [IQR], mean (sd) or n (%)**	***P*-value**	***n***
Sociodemographic data					
Age, *years*	61.0 [52.0;67.0]	60.0 [48.0;66.0]	67.0 [62.0;73.0]	**<0.001**	181
Sex, *female*	60 (33.1%)	51 (35.4%)	9 (24.3%)	0.279	181
Smoking history				0.038	181
Non-smoker	90 (49.7%)	74 (51.4%)	16 (43.2%)		
Former	57 (31.5%)	49 (34.0%)	8 (21.6%)		
Current	12 (6.63%)	7 (4.86%)	5 (13.5%)		
Unknown	22 (12.2%)	14 (9.72%)	8 (21.6%)		
Time from symptoms to hospital admission, *days*	7.00 [5.00;9.00]	7.00 [5.00;9.00]	6.00 [4.00;8.00]	0.336	180
Time from symptoms to ICU admission, *days*	8.00 [6.00;11.0]	8.00 [7.00;11.0]	8.00 [5.00;11.0]	0.678	180
Comorbidities					
Obesity	81 (45.5%)	60 (42.6%)	21 (56.8%)	0.174	178
Hypertension	78 (43.1%)	58 (40.3%)	20 (54.1%)	0.186	181
Diabetes mellitus (Type I/II)	42 (23.2%)	25 (17.4%)	17 (45.9%)	**0.001**	181
Chronic heart disease	22 (12.2%)	13 (9.03%)	9 (24.3%)	**0.021**	181
COPD/Bronchiectasis	14 (7.73%)	9 (6.25%)	5 (13.5%)	0.166	181
Chronic renal disease	11 (6.08%)	6 (4.17%)	5 (13.5%)	**0.049**	181
Asthma	10 (5.52%)	10 (6.94%)	0 (0.00%)	0.218	181
HIV	2 (1.10%)	1 (0.69%)	1 (2.70%)	0.368	181
Immunological disorders	1 (0.55%)	0 (0.00%)	1 (2.70%)	0.204	181

IQR, interquartile range [p25;p75]; sd, standard deviation; HIV, human immunodeficiency virus. Bold values are statistically significant p-values.

Focusing on the survivors, the median (IQR) ICU stay was 9 (5–24.2) days, and the overall hospitalization duration was 22 (13–37) days. During the ICU stay, 50.7% of patients required IMV with a median (IQR) duration of 17 (10–25) days. Prone positioning was needed in 47.2% of the patients. Patients were mostly treated with corticosteroids (79.2%), hydroxychloroquine (59.7%), lopinavir/ritonavir (56.9%), tocilizumab (49.3%), and remdesivir (25.0%). Moreover, 95.8% of patients received thromboprophylaxis therapy, and 96.5% received antibiotic therapy. The most frequent complications were septic shock (25.7%) and acute renal failure (16.7%) (**Table 1**; **Supplementary Table 1**).

Post-COVID Unit: Clinical Follow-Up

Figure 1 shows the flowchart of the study and the clinical management during the clinical consultation. After hospital discharge, of the 144 eligible patients, 36 were unreachable or decided not to participate in the follow-up, one was severely disabled, and two underwent follow-up in another center. This left 105 patients who started the clinical follow-up in the post-COVID unit at 3 months after hospital discharge. Patients who did not attend the follow-up visit showed similar sociodemographic and clinical characteristics (except smoking habit and hospital duration) to the patients who did attend the consultation (**Supplementary Table 2**).

Three-Month Follow-Up

Of the 105 patients, 97 and 93 were able to perform pulmonary function tests and 6MWTs, respectively (**Table 2**). At this point, the proportions of patients with abnormal TLC and DLCO were 38.6 and 82%, respectively. In general, the patients had exercise test results that were lower than expected values (19) with a mean (SD) percent predicted 6-minute walk distance (PP-6MWD) of 83.7% (26) and an average oxygen saturation of 95.3% (1.98). The CT scans showed a high proportion of lung affectation, most frequently with ground-glass opacities (56.6%), followed by mixed ground-glass opacities (29.3%) and consolidation (17.2%). Forty-three (43.4%) and 28 (28.3%) patients showed reticular and fibrotic patterns, respectively, and the mean (SD) of pulmonary lobes affected by ground-glass or consolidation was 3.0 (2.0) with a mean (SD) TSS of 5.8 (4.6) (**Table 2**).

After the clinical and functional evaluations, 15 patients were discharged and another 3 transferred for the following consultations: 2 for virtual pulmonary nodules consultation and 1 for psychiatry consultation (**Figure 1**). Consequently, 83% of patients required a second follow-up visit in the post-COVID unit (**Figure 2A**).

Six-Month Follow-Up

Before this point, one patient died, and another was unreachable and did not attend the follow-up, so 85 patients were evaluated (**Figure 1**). Of these followed patients, 79 had available pulmonary function tests, showing proportions of abnormal TLC and DLCO of 31.5 and 83.6%, respectively. The PP-6MWD mean (SD) was 91.4% (19.9). Chest CT showed a slight improvement in some parameters regarding density, type of lesions, and TSS (**Table 2**).

After the clinical assessment, the clinician decided to discharge 15 patients and to transfer another 13 for different consultations: ten to other pulmonary consultations (five to COPD/emphysema and the rest to asthma/vascular/ventilation/pulmonary nodules and lung cancer fast diagnostic track [FDT] consultations), and three to neurology, hematology, and cardiology (**Figure 1**).

This meant that two-thirds of the patients (67%) in this consultation needed to continue with follow-up (**Figure 2A**). Again, this was due to the high proportion of patients who did not recover lung diffusion capacity to within the normal range because of COVID-19 damage (**Figure 2B**).

Twelve-Month Follow-Up

Two patients died before the upcoming visit, and five were unreachable and did not attend the follow-up (**Figure 1**). This left 50 patients evaluated in the consultation, of which 38 required a pulmonary function test, and 41 also received a chest CT.

Of these patients, 40.9 and 70.2% had abnormal TLC and DLCO values, respectively (**Table 2**). Forty-three, eight and 23 percent of patients did not recover normal values of DLCO, TLC and distance in the 6MWT, respectively (**Figures 2B–D**). Of these, nine patients (10% of the initial 105 patients) had moderate/severe affectation of DLCO with values below 60%. The mean (SD) PP-6MWD was 95.3% (21.3). The chest CT of these more affected patients showed a high proportion of abnormalities, with the most frequent finding being interlobular septal thickening (100%) and bronchiectasis (90.2%), with all of this in the context of the presence of reticular and fibrotic patterns in 53.7 and 36.6% of patients, respectively. The number of lobes affected by ground-glass or consolidation remained high (mean [SD] of 3.5 [1.4]) (**Table 2**). Fifty-three percent of patients had abnormal TSS values at this point (**Figure 2E**).

The pulmonary function, 6MWT and chest CT scan of these 50 patients at 3, 6, and 12 months are depicted in **Supplementary Table 3**.

After a careful evaluation, the clinician decided to discharge 16 patients. This decision meant that 32.2% of patients, based on the clinical point of view, needed to continue to be monitored beyond 12 months after hospital discharge due to pulmonary sequelae of critical COVID-19.

Symptoms Related to Post-COVID Syndrome at 12 Months of Follow-Up

To assess the prevalence of post-COVID syndrome 1 year after hospital discharge, a telephone survey was conducted of all 105 initial patients. Three patients had died, and five patients did not respond, so we finally contacted 97 patients.

Thirty-seven percent of patients suffered from mild/moderate/severe cognitive complaints based on the BC-CCI scale, and 33 and 45% had abnormal values in the fatigue and dyspnea scales, respectively. This results in 61.3% of patients showing at least one altered domain. Additionally, the patients had a mean (SD) number of symptoms of 5.7 (4.6), with the most frequent being reduced fitness (700.1%), concentration and/or memory problems (50.5%), muscle weakness (46.4%), tingling and/or pain in the extremities (43.3%), and erectile dysfunction (38.8%), among many others (**Table 3**).

TABLE 2 | Description of pulmonary function, 6MWT, and chest CT findings of patients followed at 3, 6, and 12 months.

	Three months Mean (sd) or n (%)	Six months Mean (sd) or n (%)	Twelve months Mean (sd) or n (%)	*p* for trend
Post-COVID consultation discharge	*n* = 104	*n* = 105	*n* = 105	**<0.001**
Exitus	0 (0.00%)	1 (0.95%)	3 (2.86%)	
None	87 (83.7%)	57 (54.3%)	32 (30.5%)	
Loss to follow-up	0 (0.00%)	1 (0.95%)	6 (5.71%)	
Yes	17 (16.3%)	46 (43.8%)	64 (61.0%)	
Pulmonary function				
FVC, %	*n* = 97	*n* = 78	*n* = 38	
	78.1 (15.5)	79.8 (14.7)	86.5 (16.8)	**0.009**
FEV1, %	*n* = 96	*n* = 78	*n* = 38	
	86.0 (17.4)	87.1 (16.5)	91.2 (17.7)	0.138
FEV1 to FVC ratio (categorical)	*n* = 95	*n* = 77	*n* = 37	0.556
≥70%	92 (96.8%)	74 (96.1%)	35 (94.6%)	
<70%	3 (3.16%)	3 (3.90%)	2 (5.41%)	
TLC, %	*n* = 96		*n* = 22	
	82.9 (18.6)	86.3 (18.5)	84.5 (15.6)	0.404
TLC, % (categorical)	*n* = 96	*n* = 70	*n* = 22	0.679
≥80%	59 (61.5%)	48 (68.6%)	13 (59.1%)	
≤50–80%	33 (34.4%)	20 (28.6%)	9 (40.9%)	
<50%	4 (4.17%)	2 (2.86%)	0 (0.00%)	
RV, %	*n* = 96	*n* = 69	*n* = 22	
	90.2 (42.1)	88.3 (34.5)	88.8 (29.5)	0.793
DLCO, *mL/min/mmHg*	*n* = 94	*n* = 79	*n* = 37	
	67.6 (14.7)	65.6 (13.3)	70.6 (13.9)	0.508
DLCO, *mL/min/mmHg* (categorical)	*n* = 94	*n* = 79	*n* = 37	0.553
≥80%	17 (18.1%)	13 (16.5%)	11 (29.7%)	
≤60–80%	51 (54.3%)	36 (45.6%)	17 (45.9%)	
<60%	26 (27.7%)	30 (38.0%)	9 (24.3%)	
Six-minute walking test				
PP-6MWD*, %	*n* = 93	*n* = 77	*n* = 37	
	83.7 (26.0)	91.4 (19.9)	95.3 (21.4)	**0.005**
Oxygen saturation, %	*n* = 95	*n* = 77	*n* = 38	
Initial	96.5 (1.26)	96.6 (1.32)	96.7 (1.10)	0.414
Final	95.1 (2.57)	95.1 (2.87)	95.1 (1.62)	0.941
Minimal	94.1 (2.71)	94.3 (2.89)	94.3 (2.15)	0.516
Average	95.3 (1.98)	95.6 (1.87)	95.5 (1.37)	0.374
Chest CT scan findings				
Density	*n* = 99	*n* = 81	*n* = 41	
Ground-glass	56 (56.6%)	32 (39.5%)	20 (48.8%)	0.171
Mixed ground-glass	29 (29.3%)	33 (40.7%)	27 (65.9%)	**<0.001**
Consolidation	17 (17.2%)	12 (14.8%)	3 (7.32%)	0.155
Internal structures	*n* = 99	*n* = 81	*n* = 41	
Interlobular septal thickening	81 (81.8%)	62 (76.5%)	41 (100%)	**0.047**
Bronchiectasis	76 (76.8%)	65 (80.2%)	37 (90.2%)	0.082
Atelectasis	22 (22.2%)	17 (21.0%)	11 (26.8%)	0.651
Solid nodule	31 (31.3%)	32 (39.5%)	18 (43.9%)	0.126
Non-solid nodule	2 (2.02%)	6 (7.41%)	0 (0.00%)	0.962
Lesions	*n* = 99	*n* = 81	*n* = 41	0.989
Fibrotic	28 (28.3%)	25 (30.9%)	15 (36.6%)	
None	28 (28.3%)	22 (27.2%)	4 (9.76%)	
Reticular	43 (43.4%)	34 (42.0%)	22 (53.7%)	
No. of lobes affected by ground-glass	*n* = 98	*n* = 81	*n* = 41	
or consolidative opacities	3.06 (2.02)	2.62 (1.95)	3.56 (1.43)	0.443
Total severity score	*n* = 99	*n* = 81	*n* = 41	
	5.88 (4.60)	4.48 (3.68)	4.63 (2.26)	**0.033**

*sd, standard deviation; FVC, forced vital capacity; FEV, forced expiratory volume; DLCO, diffusion capacity of the lungs for carbon monoxide; TLC, total lung capacity; RV, residual volume; PP-6MWD, percent predicted 6-minute walk distance. *The PP-6MWD was calculated from standardized prediction equations using the formula PP-6MWD = 6MWD/Predicted 6MWD × 100. Bold values are statistically significant p-values.*

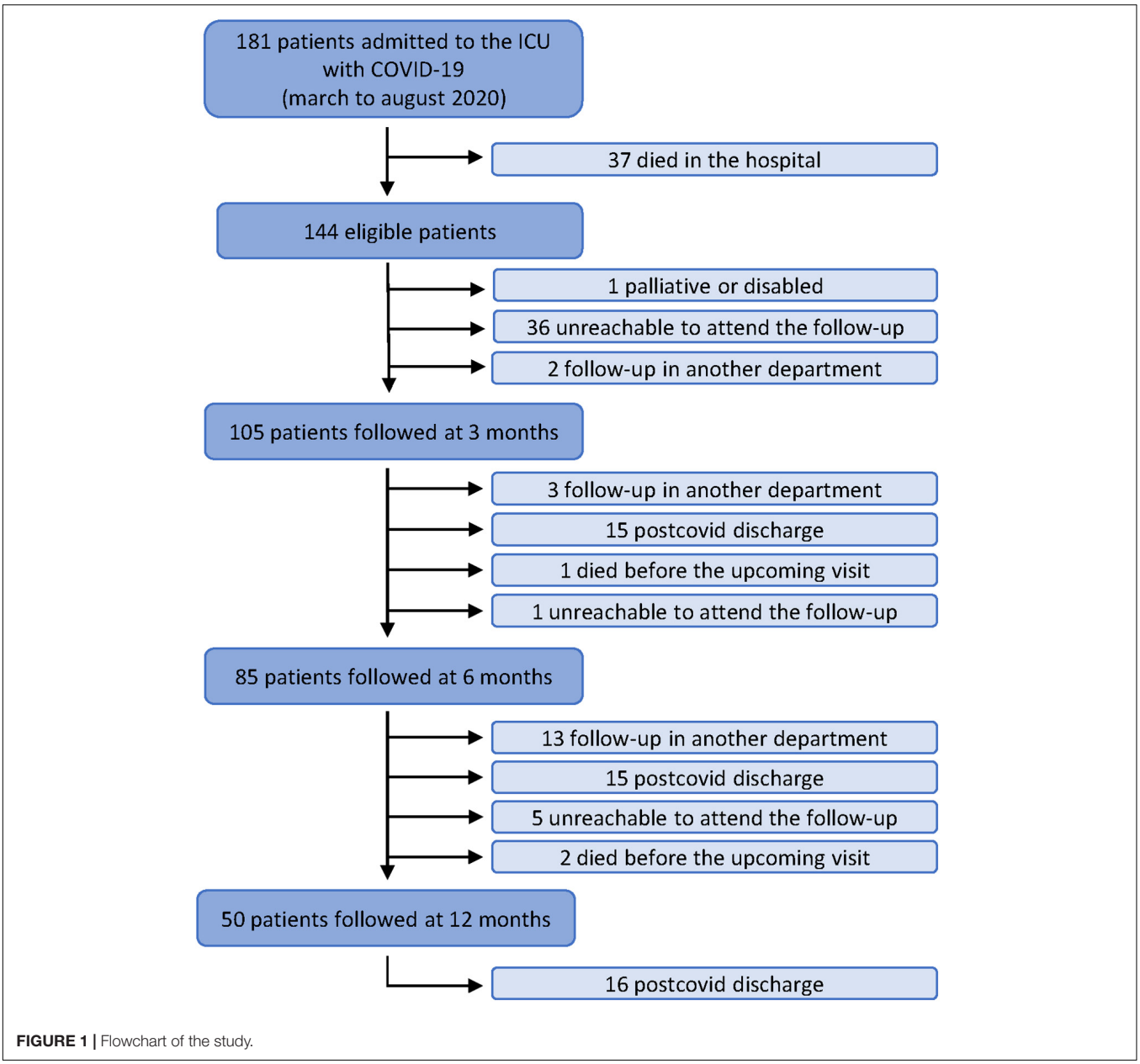

FIGURE 1 | Flowchart of the study.

There were no differences in symptoms, including FACIT, BC-CCI, and mMRC scores, between patients who needed to complete the follow-up in the post-COVID unit vs. discharged patients (**Supplementary Table 4**). Additionally, no significant correlation was observed between objective respiratory measurements and symptoms. Only the mMRC scale showed a significant correlation with DLCO ($r = -0.3$; $p = 0.027$) and the FACIT score with the 6MWT ($r = 0.3$; $p = 0.04$) and TSS ($r = 0.3$; $p = 0.04$) (**Supplementary Figure 1**).

Additional Diagnosis and Health Care Use During the 1-Year Follow-Up

During the follow-up, three patients died (**Supplementary Table 5**). In the clinical context of this post-COVID unit, many other conditions were diagnosed and treated (**Supplementary Table 6**). Those other conditions included neurological/cognitive problems, coagulation disorders, cardiological problems, diaphragm elevation, and morbid obesity. More importantly, in one patient, a new diagnosis of pulmonary adenocarcinoma was made, and three had a high level of suspicion of either a new diagnosis or a recurrence of lung cancer. Twenty-one (20%) and eight (7.6%) patients were recently diagnosed with emphysema and spirometric COPD, respectively. After careful clinical evaluation, two patients were recruited and accepted into a randomized clinical trial of antifibrotics in post-COVID-19 patients in another hospital.

The use of the national health system was high (**Supplementary Table 7**). The mean (SD) number of outpatient

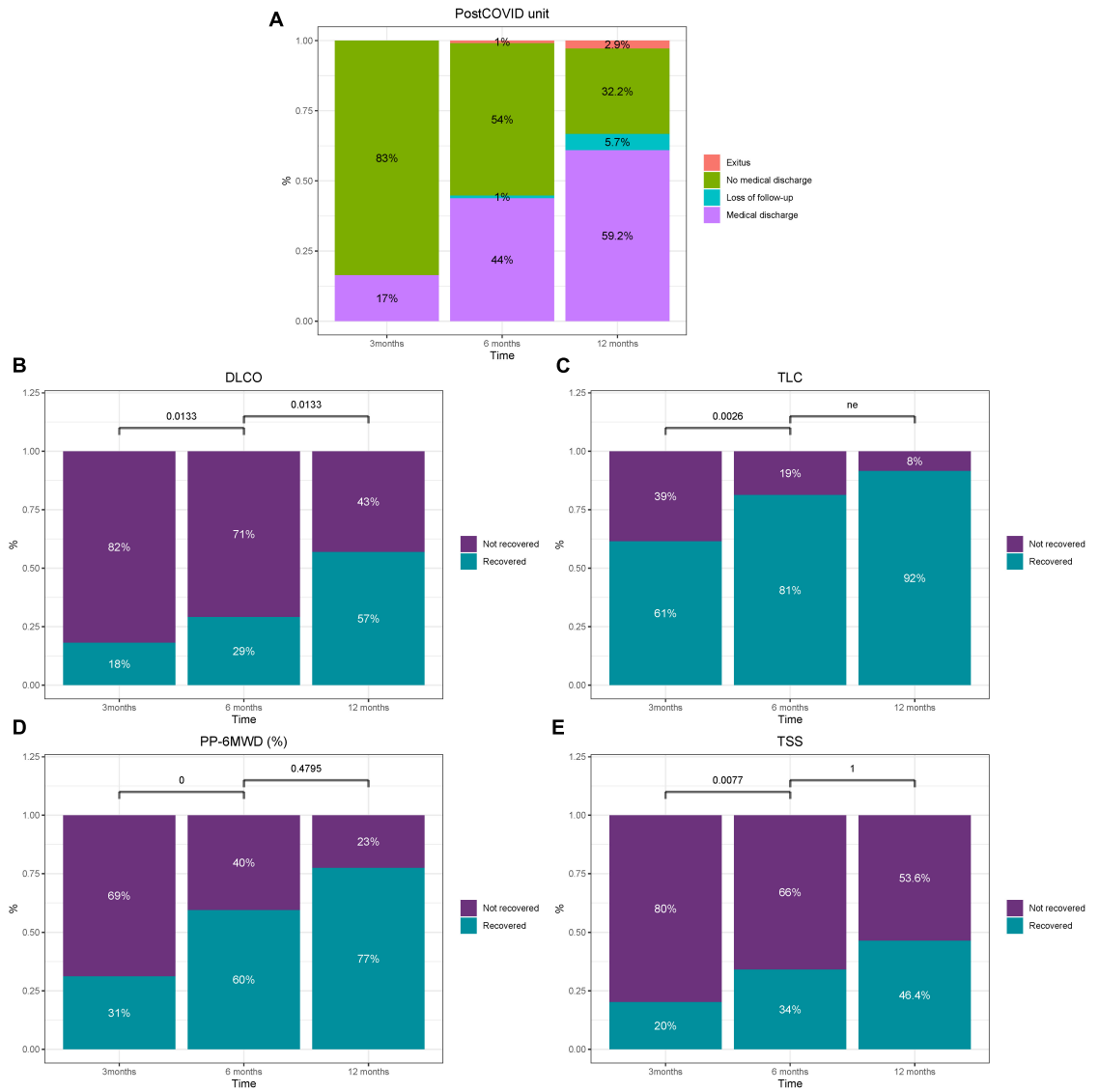

FIGURE 2 | Overview of clinical decisions and percentage of recovered patients regarding post-COVID unit **(A)**, DLCO **(B)**, TLC **(C)**, 6MWT **(D)**, and TSS **(E)** over time. *P*-values were computed using McNemar's test. ne, not estimable.

clinic visits were 12.4 (9.25), with a mean of 5.8 (4.5) and 2.3 (2.7) phone and emergency department visits, respectively. Thirteen patients (13.4%) needed hospitalization, and one was admitted to the ICU. Thirty-six patients (37.1%) attended a pulmonary rehabilitation program.

DISCUSSION

Our report describes an overview of critically ill patients due to COVID-19 between March and August 2020 and the clinical follow-up of survivors in a single center post-COVID critical care unit for 1 year. The most relevant findings of this study are: first, 32% of patients needed to continue the follow-up in a post-COVID unit beyond 1 year. A total of 10% of these patients had moderate/severe affectation of DLCO (values below 60%), and chest CT showed a high proportion of fibrotic (53.7%) and reticular (36.5%) patterns. Second, during the follow-up period, other conditions and comorbidities (related or not to COVID-19), such as emphysema, COPD, neurocognitive disorders, and lung cancer, were identified. Third, at the 12-month follow-up, a highly variable number of symptoms and post-COVID syndrome were very common (even in discharged patients). Fourth, a high use of health care resources is observed in the first year.

There are numerous studies regarding pulmonary sequelae after COVID-19 at 12 months (8, 10, 20). These prospective cohorts of patients already point to a high prevalence of pulmonary involvement represented by an abnormal DLCO and many chest CT findings. This is especially important in those with the most severe disease in the acute phase, where 54% of patients

TABLE 3 | Prevalence of persistent symptoms and post-COVID syndrome at the 1-year follow-up.

	Twelve-month follow-up *n* = 97 Mean (sd) or n (%)	*n*
Post-COVID syndrome		
BC-CCI		96
None or minimal cognitive complaints	60 (62.5%)	
Mild cognitive complaints	19 (19.8%)	
Moderate cognitive complaints	13 (13.5%)	
Severe cognitive complaints	4 (4.17%)	
Total score	3.89 (4.76)	96
FACIT score	36.8 (12.3)	96
Score < 30	32 (33.3%)	
Dyspnea		94
0	51 (54.3%)	
1	31 (33.0%)	
2	9 (9.57%)	
3	3 (3.19%)	
Post-COVID syndrome*	57 (61.3%)	93
Sequelae symptoms		
Number of symptoms	5.77 (4.66)	97
Reduced fitness	68 (70.1%)	97
Concentration and/or memory problems	49 (50.5%)	97
Muscle weakness	45 (46.4%)	97
Tingling and/or pain in extremities	42 (43.3%)	97
Erectile dysfunction	26 (38.8%)	67
Sleeping problems	36 (37.1%)	97
Joint complaints	32 (33.0%)	97
Reduced vision	31 (32.0%)	97
Hoarseness	27 (28.1%)	96
Hair loss	26 (26.8%)	97
Smell or taste disorder	26 (26.8%)	97
Changes in menstruation	8 (26.7%)	30
Reduced hearing	24 (24.7%)	97
Headache	21 (21.6%)	97
Dizziness	20 (20.6%)	97
Palpitations	20 (20.6%)	97
Skin rash	17 (17.5%)	97
Sore throat or difficulty swallowing	14 (14.4%)	97
Chest pain	14 (14.4%)	97
Loss of appetite	8 (8.25%)	97
Diarrhea or vomiting	6 (6.19%)	97
Patient Global Impression of Severity (PGI-S)		**97**
None	48 (49.5%)	
Mild	14 (14.4%)	
Moderate	22 (22.7%)	
Severe	12 (12.4%)	
Very severe	1 (1.03%)	
Vaccination		
COVID-19 vaccination	79 (82.3%)	96
COVID-19 brand names		78
Pfizer	36 (46.2%)	
Moderna	11 (14.1%)	
AstraZeneca	27 (34.6%)	
Janssen	4 (5.13%)	
Administered doses	1.34 (0.48)	79
Time to first vaccination, *days*	317 (95.7)	79
SF-12		95
Physical score	45.7 (11.1)	
Mental score	48.1 (13.3)	

(Continued)

TABLE 3 | (Continued)

**Post-COVID syndrome is defined as alterations in fatigue, cognitive disorders, and/or dyspnea. sd, standard deviation; BC-CCI, British Columbia Cognitive Plain Inventory; FACIT, Functional Assessment of Chronic Illness Therapy; SF-12, 12-Item Short Form Survey.*

have abnormal DLCO values and 87% have at least one abnormal chest CT pattern at 1 year of follow-up (10). However, to date, the literature focusing on the long follow-up of critically ill survivors of COVID-19 is scarce (21). Gamberini et al. (21) described 51.5% of patients with abnormal DLCO, with 70.3% of patients having fibrotic changes on chest CT and 40.5% having ground-glass opacities or consolidation at 1 year. These data are even more worrisome than ours, probably because this group focused on invasively ventilated patients. Further studies are needed to create or validate scores to identify patients at high risk of pulmonary sequelae on chest CT (22).

Although all of these studies assessed pulmonary sequelae after COVID-19 at 12 months (8, 10, 20), none of them provided information about the clinical management and follow-up in a real post-COVID consultation. Our work demonstrates that during follow-up, many comorbidities (related to COVID-19 or not) could be diagnosed and should be managed, such as COPD, emphysema, lung cancer, or other non-respiratory conditions. Moreover, the clinical nature of this consultation allowed us to discriminate COVID-19 respiratory sequelae from previous existing pulmonary conditions (and those not previously diagnosed), such as COPD and emphysema.

Another important issue is persistent symptoms and post-COVID syndrome in critically ill COVID-19 survivors. The literature shows that a wide variety of symptoms and impairment of health-related quality of life at 1 year are very frequent (21). Our results go in line with others that shows a high proportion of ongoing symptoms as well as a substantial new disability and reduced health quality of life in critically ill COVID-19 survivors (23). Moreover, our results show no differences in the prevalence of symptoms or post-COVID syndrome between discharged patients and those who needed to continue the follow-up in the unit. This highlights the need for a more precise definition of post-COVID syndrome (24). In our cohort, symptoms such as dyspnea and fatigue were explained by DLCO and FACYT score measurements, while the other symptoms were not. This result should be interpreted with caution because it could be explained by the overlap of ARDS sequelae (25, 26), the so-called postintensive care syndrome (PICS) (27) and the post-COVID syndrome (28). Interestingly, a study performed by Hodgson and colleagues (29, 30) showed that COVID-19 and non-COVID-19 PICS at 6 months after ICU admission are at least phenotypically

similar, with similar post-ICU care. Be that as it may and consequently, these critical survivors have a high consumption rate of health resources (31) that must be managed in an adequate post-COVID care unit.

There are some limitations to our study. First, it is a small cohort from a single city which may reduce the external validity and generalizability of the findings. Second, due to the clinical nature of this consultation, we were not able to describe the pulmonary and functional evaluation of all patients who required a critical COVID-19 admission at 12 months. However, we have described the real clinical practice and the follow-up of these patients in a post-COVID unit.

In conclusion, in a single center post-COVID critical care unit, 32% of patients need to continue follow-up beyond 1 year due to the high proportion of patients with abnormal DLCO and chest CT. Many comorbidities (related to COVID-19 or not) were diagnosed during the follow-up. Finally, persistent symptoms and post-COVID syndrome are very common, which leads to high health care consumption.

DATA AVAILABILITY STATEMENT

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 Medical Ethics Committee (CEIC/2273). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JG, MZ, IB, DG-C, GT, JB, SG, SB, RF, AC, LF, AMot, JR, RM, DG-G, OP, GL, JC, CB, AT, and FB contributed to the study concept and design. MA, SS, RV, OM, FS, AM-M, AMon, and CG-P contributed to the data acquisition. JG, MZ, IB, DG-C, AM-M, and CG-P contributed to the data analysis and interpretation. FB was the guarantor of the manuscript, had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the manuscript draft, critically revised the manuscript for important intellectual content, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.897990/full#supplementary-material

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MINI REVIEW
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Exercise Intolerance in Post-Acute Sequelae of COVID-19 and the Value of Cardiopulmonary Exercise Testing- a Mini-Review

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Coronavirus disease (COVID-19) is an infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with systemic organ damage in the most severe forms. Long-term complications of SARS-CoV-2 appear to be restricted to severe presentations of COVID-19, but many patients with persistent symptoms have never been hospitalized. Post-acute sequelae of COVID-19 (PASC) represents a heterogeneous group of symptoms characterized by cardiovascular, general, respiratory, and neuropsychiatric sequelae. The pace of evidence acquisition with PASC has been rapid, but the mechanisms behind it are complex and not yet fully understood. In particular, exercise intolerance shares some features with other classic respiratory and cardiac disorders. However, cardiopulmonary exercise testing (CPET) provides a comprehensive assessment and can unmask the pathophysiological mechanism behind exercise intolerance in gray-zone PASC. This mini-review explores the utility of CPET and aims to provide a comprehensive assessment of PASC by summarizing the current evidence.

Keywords: post-acute sequelae COVID-19, cardiopulmonary exercise testing, autonomic dysfunction, exercise intolerance, hyperventilation

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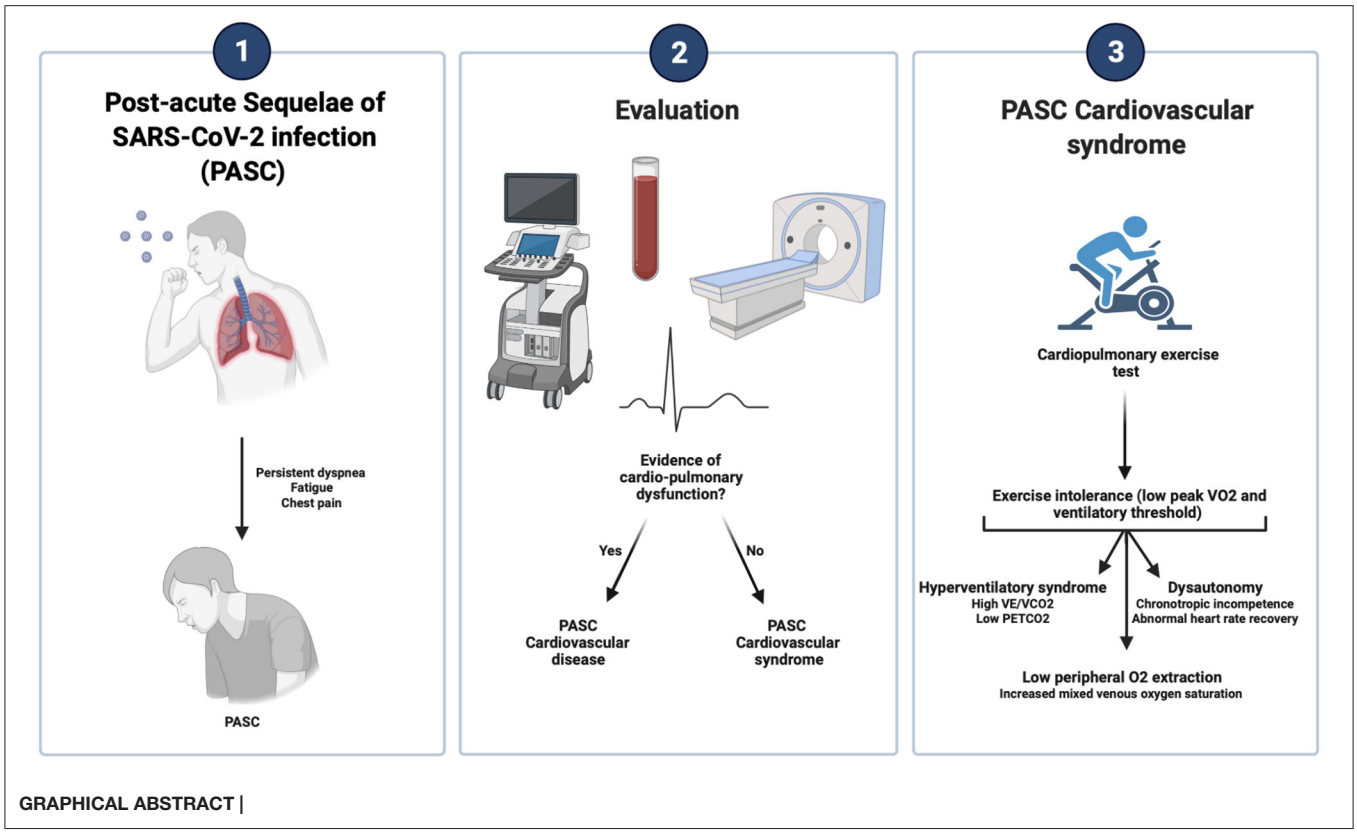
INTRODUCTION

Long Coronavirus disease 2019 (COVID-19) or post-acute sequelae of severe acute respiratory syndrome coronavirus 2 infection (PASC) is expected to increase in prevalence and become a public health problem (1, 2). PASC is a heterogeneous clinical syndrome. The growing scientific evidence recognizes PASC as one of the conditions that cause exercise intolerance (2), but the relationships between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and exercise capacity remain unclear. A diminished exercise capacity has been associated with a poor quality of life and higher mortality in other conditions. Therefore, understanding the mechanism behind the limitation in exercise capacity of these patients is a fundamental step in improving patient outcomes.

A hallmark of exercise intolerance is dyspnea and fatigue upon exertion. Although it is intuitive to think that patients with PASC would be limited primarily by the cardiopulmonary system, studies indicate that most of these patients have (2) cardiac and pulmonary testing within normal values (3, 4). Exercise is dependent on the balance between oxygen supply, oxygen consumption, and



GRAPHICAL ABSTRACT |

clearance of toxic metabolites. These processes rely on the cardiovascular and pulmonary systems to achieve optimal exercise performance. Therefore, by studying external respiration in response to exercise, it is possible to address the functional competence of the organ systems by coupling external adjustments to cellular respiration. Cardiopulmonary exercise testing (CPET) offers the opportunity to study the cellular, cardiovascular, and ventilatory systems' responses simultaneously, providing an objective evaluation of exercise capacity (5, 6).

This contemporary review focuses on the essential role of CPET in the evaluation of patients with PASC and the potential mechanism behind exercise intolerance. Therefore, we explore and summarize the currently available evidence to increase awareness of this entity and improve the quality of care.

Post-Acute Sequelae of SARS-CoV-2 Infection

PASC represents the long-term sequelae of COVID-19 and it is classified according to the time frame of symptom persistence into a subacute (4–12 weeks) or chronic phase (>12 weeks) (1). PASC occurs in a heterogeneous group of patients with different clinical presentations (2), but it is characterized by a systemic involvement with the ability to impair patients' quality of life (4). Recent studies have looked at risk factors contributing to PASC observing an association with symptom burden during an active infection, female gender, and COVID-19 severity (7, 8).

The American College of Cardiology classifies PASC into two groups whether there is objective evidence of cardiovascular disease. Accordingly, PASC-cardiovascular disease is characterized by myocardial, pericardial, vascular, and/or arrhythmic conditions that appear beyond 4 weeks from the initial SARS-CoV-2 infection. Whereas, the term PASC-cardiovascular syndrome (PASC-CVS) is defined by the absence of cardiovascular disease, but on the contrary, also by the persistence of cardio-pulmonary symptoms. The two most commonly reported symptoms are fatigue and dyspnea, regardless of PASC time (9). Both are common in non-COVID patients with other cardiopulmonary conditions (10, 11) and in the convalescence phase of any critical illness (12), where exercise intolerance is also a characteristic feature (10, 11). Therefore, we should expect a high prevalence of exercise intolerance among COVID-19 survivors. Data regarding the pathophysiologic mechanism behind PASC-CVS are scarce, but it is not yet fully understood how this translates into reduced exercise capacity.

Evidence of Exercise Capacity in PASC-CVS

Despite there are no dedicated guidelines on the evaluation of PASC-CVS, patients should undergo a CPET evaluation to identify limiting factors for decreased maximal exertion

Abbreviations: COVID-19, coronavirus disease 2019; CPET, cardiopulmonary exercise test; HR, heart rate; PASC, postacute sequelae of COVID-19; PASC-CVS, postacute sequelae of COVID-19 cardiovascular syndrome; Vo_2, oxygen consumption; V_T, anaerobic or ventilatory threshold; VE/VCO_2, minute ventilation/CO_2 output.

that is usually found during the convalescence phase of any critical illness (12). Otherwise, the lack of evidence of residual cardiopulmonary damage may cause a delay in the diagnosis. Exercise capacity is evaluated through the assessment of oxygen consumption (Vo_2) at peak exercise if a sustained maximal effort has been reached. Maximal performance is also age-, sex- and weight-adjusted to be reported as a percentage predictive of peak Vo_2. Moreover, ventilatory threshold (V_T) is effort-independent providing a more accurate assessment of aerobic efficiency and reflecting the Vo_2 at submaximal exercise levels when supply does not match requirements triggering an anaerobic environment (5, 6).

Follow-up studies on SARS-CoV survivors observed a diminished exercise capacity (13, 14), but similar findings were observed during hospital discharge because of COVID-19 (15). To date, there is only scarce observational data on exercise capacity with PASC-CVS during follow-up. A small single-center retrospective study found during short-term follow-up a diminished exercise capacity (16). Similar findings were reported by a multi-center retrospective study of 200 patients, in which those with PASC (56%) had a lower peak Vo_2 (25.8 $\pm$ 8.1 vs. 28.8 $\pm$ 9.6 mL/min/kg; $p = 0.017$) and a smaller chance of achieving the V_T (OR: 0.38; 95% CI 0.20–0.72) (17). On the contrary, mid-to-long-term retrospective studies identify borderline exercise capacity in PASC patients (18) that was not different from controls with unexplained dyspnea (19).

These data were reproduced by prospective studies during short-term follow-up (20–24) and when stratified by COVID-19 severity no differences were found between groups (25); however, other studies reported that previous critical care unit admission, need for mechanical ventilation and a longer hospital stay were independently associated with peak Vo_2 (26). The incorporation of unexplained dyspnea in PASC-CVS into the design and analysis of recent studies have yielded similar findings during follow-up. A single-center prospective study of 70 consecutive patients observed that PASC-CVS patients with persistent dyspnea (59%) experienced a smaller exercise capacity (78 vs. 99% of predicted peak Vo_2; $p < 0.001$) than asymptomatic COVID-19 survivors (27). Accordingly, a multicenter prospective study that evaluated 156 patients also reported among PASC patients with persistent dyspnea (47%) a diminished exercise capacity (76 $\pm$ 16 vs. 89 $\pm$ 18% of predicted peak Vo_2; $p = 0.009$) (28).

An unexplored scenario is the possibility of an immediate improvement in the functional capacity of PASC patients. A prospective study that monitored the persistence of exercise intolerance in PASC with serial CPET evaluation reported an improvement between 3-and-6 months of peak Vo_2 (18 vs. 20.5 mL/kg/min; $p = 0.001$) and V_T (9.7 vs. 10.4 mL/min/kg; $p = 0.018$). However, these improvements were not observed in all patients and were less evident when compared to healthy controls (29). Findings from other prospective studies confirm that exercise intolerance is also observed during mid-to-long term follow-up in PASC (30–35).

In general, low peak Vo_2 is common among patients with PASC-CVS during follow-up (**Table 1**), but application and interpretation of CPET results are challenging. Peak Vo_2 is defined by the Fick equation as the product of cardiac output and arteriovenous oxygen difference [C (a–v) O2]. This is important because cardiac output is the product of stroke volume times heart rate (HR) and arteriovenous oxygen difference reflects the peripheral oxygen tissue extraction (5, 6). Consequently, abnormalities in any of these variables can contribute to exercise intolerance in PASC-CVS.

Contributors of Exercise Intolerance in PASC-CVS

Identification of patterns during CPET may identify the organ systems involved in the exercise intolerance referred by PASC patients as we cannot rely exclusively on a decreased peak Vo_2 and V_T (see **Graphical Abstract**). Therefore, CPET can be combined with the invasive and non-invasive tests to further phenotype more accurately PASC-CVS. However, given the systemic nature of COVID-19, we may expect a cardiac, ventilatory, peripheral, and/or pulmonary gas exchange limitation at exercise.

Cardiovascular Limitation

Cardiovascular limitation to exercise intolerance in PASC-CVS patients may be explained by several factors, but electrocardiographic changes and a pathological blood pressure response during exercise have not yet been reported. Moreover, low CO could explain exercise intolerance in most PASC patients; however, no left ventricular dysfunction has been reported in the studies that evaluated cardiac function at rest (15, 21, 26, 27, 29). Similarly, two prospective studies that evaluated cardiac function at rest and during CPET concluded that cardiac function was within normal values, regardless of previous COVID-19 severity (20, 31). However, Szekely et al. also observed a reduced stroke volume with a blunted peak HR and a higher peak arteriovenous difference among PASC patients (20), raising the possibility of a cardiac autonomic dysregulation as a major cause of exercise intolerance.

Modulation of the HR during exercise is a dynamic process tightly regulated by the autonomic nervous system and its imbalance may manifest during exercise as chronotropic incompetence or inadequate HR recovery. Some of the studies reported chronotropic incompetence (16, 20), while others observed an abnormal HR recovery (29, 30, 32). Interestingly, both were more commonly observed among PASC patients with evidence of ventilatory inefficiency.

Ventilatory and Pulmonary Vascular Limitation

Lung mechanical-related mechanism because of significant reduction of pulmonary function should be, in theory, the expected primary cause of exertional dyspnea in PASC. Contrary to that, most of the studies did not observe a correlation between abnormal lung functions and persistent dyspnea regardless of COVID-19 severity. This is further supported by normal breathing reserve among PASC patients (18, 19, 23, 27, 28, 30, 33). However, some studies reported a significant decrease in D_{LCO} showed some discordant findings concerning peak V_{O2} (21, 22, 26, 29, 31, 33).

TABLE 1 | Most Relevant Studies evaluating exercise capacity in Post-acute sequelae of COVID-19 with cardiopulmonary exercise test.

References	Study design	Sample size	Follow-up	Main findings
Baratto et al. (15)	Single center	36	Hospital discharge	COVID-19 patients had at the time of discharge a smaller exercise capacity (59 vs. 90% of predictive peak Vo_2; $p < 0.001$) and peripheral oxygen extraction (0.66 vs. 0.81; $p = 0.006$) compared to controls[a]. COVID-19 patients had ventilatory inefficiency (VE/VCO_2 slope 32 vs. 28 mmHg; $p = 0.007$) likely explained by hyperventilation. Ca_{O2} (tau = 0.58, $p = 0.012$) and hemoglobin ($R = 0.46$, $p = 0.002$) were positively correlated with peak Vo2. No differences were found in cardiac echocardiography at rest between groups.
Mohr et al. (16)	Single center retrospective	10	3 months	PASC patients with persistent dyspnea showed a mean 72.7% of predictive peak Vo_2, 78.1 ± 7.3% of predictive heart rate, 96 ± 15.5% of predictive Vo_2/HR and a mean lactate post-exercise of 5.6 ± 1.8 mmol/L.
Barbagelata et al. (17)	Multicenter retrospective	200	3 months	PASC patients[b] showed a lower peak Vo_2 (25.8 ± 8.1 vs. 28.8 ± 9.6 mL/min/kg; $p = 0.017$) but with a similar % of predicted peak Vo_2 (89.7 ± 19.9 vs. 92.9 ± 18.7 %; $p = 0.257$) compare to asymptomatic post-COVID patients. Ventilatory efficiency was similar between groups (VE/VCO_2 slope 33.1 ± 5.9 vs. 32.5 ± 5.5; $p = 0.521$). Most common reported symptom during CPET was dyspnea[c] (93%), particularly in PASC patients (97 vs. 75%; $p = 0.008$). PASC was associated with smaller V_T (OR: 0.38; 95% CI 0.2–0.72) and a greater chance of symptoms during CPET (OR: 7.0; 95% CI: 3.5–16.2).
Debeaumont et al. (18)	Single center retrospective	23	6 months	Persistent dyspnea[c] was significantly associated with peak Vo_2 (rho = −0.49). PASC was associated with a diminished % of peak Vo_2 (84 ± 19%), particularly in ICU survivors (77 ± 15 vs. 87 ± 20%). Ventilatory efficiency was low (VE/VCO_2 slope 32 ± 5) in the global cohort, but higher in ICU survivors (VE/VCO_2 slope 34 ± 5). Hemoglobin and pulmonary function test were within normal reference values.
Alba et al. (19)	Single center retrospective	36	8 months	PASC patients with persistent dyspnea[b] had comparable peak Vo_2 (20 vs. 19.5 mL/min/kg; $p = 0.8$), % of predicted peak Vo_2 (85.5 vs. 85%; $p = 0.9$), anerobic threshold and ventilatory efficiency (VE/VCO_2 slope 29.8 vs. 28.4; $p = 0.15$) compare to controls[d]. PASC patients with abnormal CPET where mostly characterized by low O2 pulse with a normal cardiac function suggestive of a peripheral limitation. One patient underwent iCPET that showed a high mixed O2 venous content. Hemoglobin and pulmonary function test were within normal values.
Szekely et al. (20)	Single center prospective	106	3 months	PASC patients (67%) had a lower V_T (12.3 ± 3.6 vs. 15.4 ± 5.7 mL/min/kg; $p = 0.02$) and Vo_2 (1.6 ± 0.5 vs. 2.24 ± 0.9 L/min; $p = 0.03$) compared to controls[e] (33%). PASC patients had a smaller CO (9.8 ± 2.7 vs. 14 ± 4.2 L/min; $p < 0.0001$) and greater A-Vo_2 difference (0.18 ± 0.05 vs. 0.13 ± 0.04; $p = 0.004$) compared to controls[e] suggesting a cardiac limitation. PASC patients with persistent dyspnea showed ventilatory inefficiency (VE/VCO_2 slope 30.5 ± 4 mmHg) and chronotropic incompetence.
Ribeiro Baptista et al. (21)	Single center prospective	105	3 months	35% of patients with previous severe COVID-19 had a diminished exercise capacity defined by <80% of predicted peak Vo_2. Impaired exercise capacity was associated with decrease lung volumes and D_{LCO}, but with a preserved breathing reserve at peak Vo_2. Cardiac dysfunction at rest was not observed at rest, but those with diminished exercise capacity had a smaller % predicted Vo_2 /HR (66 ± 9.6 vs. 96.6 ± 14.7; $p < 0.0001$) suggestive of peripheral limitation.
Clavario et al. (22)	Single center prospective	200	3 months	59% of patients complained about dyspnea[d] and the global cohort showed a median of 85 (74–98) % of predicted peak Vo_2. Main causes of exercise limitation were non-cardiopulmonary (50.8%) among patients with <85% of predicted peak Vo_2 (50.5%). Pulmonary lung function in the entire cohort, but those <85% of predicted peak Vo_2 showed a smaller % of predicted D_{LCO} (70 vs. 85%; $p < 0.001$). Predicted FEV1 (95% CI: 0.73–9.85, $p = 0.023$), D_{LCO} (95% CI: 2.49–10.13, $p = 0.001$), and dominant leg extension maximal strength (95% CI: 3.83–24.35, $p = 0.008$) were independently associated with peak Vo_2.
Rinaldo et al. (23, 25)	Single center prospective	75	3 months	Most common reported symptom was dyspnea[b] (52%). Average peak Vo_2 was 20 mL/min/kg that corresponded to 83 ± 15% of the predicted peak Vo2, no differences were observed irrespective of previous COVID-19 severity ($p = 0.895$). Average VE/VCO_2 slope was 28.4 ± 3.1 and the median alveolar–arterial gradient for oxygen was 26 (18–31) mmHg. Pulmonary lung function test was within normal mean values, but D_{LCO} was diminished irrespective of the exercise capacity (74 ± 14 vs. 69 ± 13% $p = 0.175$). Mean hemoglobin level was 15.0 ± 1.5 g/dL.
Jahn et al. (24)	Single center prospetive	35	3 months	Pulmonary function and $D_L$co were normal with values ≥80% of predicted in 66% of patients despite previous severe COVID-19[f]. 46% of patients had ≥82% of predicted peak Vo_2 and 54% had <81% of predicted peak Vo_2. Patients with a < 82% of predicted peak Vo_2 had a smaller % of predicted D_{LCO} (80 ± 13 vs. 96 ± 18%; $p = 0.06$). Exercise limitation due to neuromuscular impairment was considered unlikely given the normal maximal inspiratory (99.4% of predicted) and expiratory (79.9% of predicted) pressures.

(Continued)

TABLE 1 | Continued

References	Study design	Sample size	Follow-up	Main findings
Motiejunaite et al. (26)	Single center prospective	114	3 months	Most common reported symptom was dyspnea (40%) and fatigue (32%). Entire cohort had a diminished exercise capacity (71% of predicted peak Vo_2, but those with a D_{LCO} ≤75% (42%) had a smaller % of predicted peak Vo_2 (16.2 vs. 19 mL/min/kg; $p < 0.001$) and V_T (39 vs. 45%; $p = 0.014$). Median VE/VCO_2 slope was 33, and irrespective of $D_{LCO} \leq 75\%$ (VE/VCO_2 slope 34 vs. 32; $p = 0.105$), suggesting a ventilatory inefficiency. Inappropriate hyperventilation was observed in 24% of all patients. No differences were observed in resting echocardiography. Age, ICU admission, mechanical ventilation and length of hospital stay were independently associated with % predicted peak Vo_2.
Aparisi et al. (27)	Single center prospective	70	3 months	Persistent dyspnea[g] was associated with a diminished QoL ($p < 0.001$), exercise capacity (77.8 vs. 99% of predictive peak Vo_2; $p < 0.001$) and ventilatory inefficiency (VE/VCO_2 slope 32 vs. 29.4 mmHg; $p = 0.022$). Need of hospital admission was not associated with a greater rate of persistent dyspnea ($p > 0.05$) during follow-up. No differences were observed between groups in resting echocardiography, laboratory makers and pulmonary lung function. No signs of pulmonary embolism or fibrosis among those who underwent CT-scans.
Skjørten et al. (28)	Multicenter prospective	156	3 months	Patients with persistent dyspnea[a] had a lower % of predictive peak Vo_2 compared to asymptomatic patients (76 ± 16 vs. 89 ± 18 %; $p = 0.009$), but without abnormalities in lung function, breathing reserve, peripheral O2 and $D_{LCO.}$ Patients with persistent dyspnea were characterized by ventilatory inefficiency mostly due to circulatory limitation (38%) and dysfunctional breathing pattern (46%). Those with previous ICU admission showed during follow-up a smaller exercise capacity (82 ± 15% vs. 90 ± 17% of predictive peak Vo_2; $p = 0.004$) compared to non-ICU patients.
Cassar et al. (29)	Single center prospective	88	6 months (serial assessment)	PASC patients (previous history of moderate-severe COVID-19) had a significant smaller exercise capacity during 3 (peak Vo_2 of 18 vs. 28 mL/kg/min; V_T of 9.7 vs. 11.9 mL/min/kg) and 6 (peak Vo_2 of 20.5 vs. 28 mL/min/kg; V_T of 10.4 vs. 11.9 mL/min/kg) months follow-up compare to controls[h]. Ventilatory response was abnormal (VE/VCO_2 slope >30) regardless the time-frame compare to controls. Heart rate recovery was impaired at 3 months (16.6 vs. 21.9 bpm; $p = 0.018$), but improve at 6 months (22.2 vs. 21.9 bpm; $p = 0.67$) compare to controls. No differences during serial cardiac imaging were observed. Hemoglobin was within normal values. There was no correlation between the extent of lung abnormalities on MRI, lung function parameters and dyspnea.
Dorelli et al. (30)	Single center prospective	28	6 months	Patients with ventilatory inefficiency (28.6%) had a smaller HR recovery (17.5 ± 7.6 vs. 24.4 ± 5.8; $p = 0.015$), but with similar peak Vo_2 (32.9 ± 13.1 vs. 27.6 ± 5.2; $p = 0.137$) to those without ventilatory inefficiency. No differences were observed in pulmonary lung function between groups. Ventilatory inefficiency was inversely correlated with HR recovery ($r = -0.537$; $p = 0.003$).
Vannini et al. (31)	Single center prospective	41	6 months	Most common reported symptoms were dyspnea (56.1%) and fatigue (51.2%), with a similar prevalence irrespective of exercise capacity. Mean % of predictive peak V_{O2} was 73.6 ± 15.6 %, without differences according to previous disease severity ($p > 0.05$) despite severe pneumonia and ARDS presented lower D_{LCO} in comparison to mild pneumonia (6.85 vs. 7.72 vs. 9.35 mmol/min*kPa; $p = 0,04$ and $p = 0.033$). Basal and stress test echocardiographic findings were within normal values. 36.5% of the patients exhibit an abnormal ventilatory response (VE/VCO_2 slope >30) to exercise without significant desaturation or pathological Vd/V_T increase.
Ladlow et al. (32)	Single center	205	6 months	25% of the patients met the criteria for dysautonomia[i], this group had lower Vo_2 at V_T (12.6 ± 2.1 vs. 14.1 ± 3.2 mL/kg/min; $p = 0.001$) and peak exercise (30.6 ± 5.5 vs. 35.8 ± 7.6 mL/kg/min; $p = 0.001$). PASC patients with dysautonomia had a higher HR at rest (95 ± 12 vs. 81 ± 12 bpm; $p < 0.001$) and in the first V_T (114 ± 15 vs. 107 ± 17 bpm; $p = 0.017$), but smaller HR at peak exercise (170 ± 13 vs. 177 ± 15 bpm; $p = 0.003$) and attenuated HR recovery (17 ± 4 vs. 31 ± 17 bpm; $p < 0.001$). Patients with dysautonomia showed a lower ventilatory efficiency (VE/VCO_2 slope 29.9 ± 4.9 vs. 27.7 ± 4.7 mmHg; $p = 0.005$) and a higher breathing frequency.
Vonbank et al. (33)	Single center prospective	100	6 months	Lung function was within normal values, but D_{LCO} was lower in PASC with previous severe disease (74.8 ± 18.2 vs. 85 ± 14.8; $p = 0.01$). Compared to controls, PASC with previous mild and severe disease had a significant smaller % of predictive peak Vo_2 and V_T. Patients with previous severe COVID-19 showed a smaller % of predictive D_{LCO} (74.8 vs. 85%; $p = 0.01$), but other lung function parameters were comparable and within normal values. Ventilatory inefficiency (higher VE/VCO_2) was evident among PASC compared to healthy controls[k] at V_T and peak exercise. Younger age, male sex, lower BMI, higher D_{LCO} and lower breathing reserve were associated with a higher peak $Vo_{2.}$

(Continued)

TABLE 1 | Continued

References	Study design	Sample size	Follow-up	Main findings
Mancini et al. (34)	Single center prospective	41	9 months	PASC patients had an average 77 ± 21% of predicted peak Vo_2 and 10.6 ± 2.8% of predicted Vo_2 at V_T. Those with peak Vo_2 <80% of predicted had a circulatory limitation to exercise. 88% of PASC patients had dysfunctional breathing, ventilatory inefficiency (increased VE/VCO_2 slope) and/or hypocapnia ($PetCO_2$ < 35 mmHg). 46% of the patients met the criteria for myalgic encephalomyelitis/chronic fatigue syndrome.
Singh et al. (35)	Single center prospective	20	11 months	COVID-19 survivors had a smaller exercise capacity (70 ± 11 vs. 131 ± 45% of predictive peak Vo_2; $p = 0.001$) and a greater degree of ventilatory inefficiency (VE/VCO_2 slope 35 ± 5 vs. 27 ± 5 mmHg; $p = 0.01$) compared to controls[f]. COVID-19 survivors showed a greater peak exercise mixed venous oxygen saturation (50 ± 10% vs. 22 ± 5%; $p < 0.0001$) and peak Vo_2 content (33 ± 6 vs. 27 ± 5 mmHg; $p = 0.01$) suggesting a peripheral limitation to aerobic exercise. No differences were observed in terms of right atrial pressure, left-side filling pressure and total pulmonary. resistance at peak exercise between groups

BMI, body mass index; CT, computed tomography; FEV1, forced expiratory volume in 1 second; MRI, magnetic resonance imaging; Ca_{O2}, content of oxygen in arterial blood; CO, cardiac output; CPET, cardio-pulmonary exercise test; D_{LCO}, diffusion carbon monoxide capacity; ICU, intensive care unit; QoL, quality of life; V/Q, ventilation/perfusion; HR, heart rate; Vo_2, oxygen consumption; Vo_2 /HR, oxygen pulse; V_T, Ventilatory threshold; VE/VCO_2 slope, slope of minute ventilation to CO_2 production. [a] Matched age, sex and body mass index healthy controls in 1:1 ratio with COVID-19 patients. [b] Defined as dyspnea or fatigue persisting for at least 45 days after symptom onset. [c] Defined as mMRC >1. [d] Matched controls also complained about unexplained dyspnea. [e] Historical matched age, sex, weight, height, hypertension and diabetes controls. [f] Severe COVID-19 was defined if ≥2 of the following criteria were met: respiratory rate >30 bpm, peripheral oxygen saturation <93% while breathing ambient air, C-reactive protein levels >75 mg/L, ground glass opacities or diffuse infiltrates on CT scan, or rapid progression of CT findings >50% within 24–48 h. [g] Defined as NYHA > II. [h] Negative SARS-CoV-2 controls matched for age, sex, body mass index and risk factors (smoking, diabetes, and hypertension) without previous hospitalization. [i] Patients with dysautonomia met the following criteria: (1) resting HR of >75 bpm; (2) increase in HR during exercise of <89 bpm; and (3) HR recovery of < 25 bpm in the first 60 s after cessation of exercise. [j] Symptomatic normal individuals with a normal peak Vo_2 and peak CO of ≥80% predicted in invasive CPET. [k] Healthy controls matched for age, sex, body mass index.

Under normal conditions, ventilation increases proportionally to CO_2 production (36) but a common finding from the CPET of PASC patients is the ventilatory inefficiency (increased VE/VCO_2 slope) suggesting an abnormal response (15, 17, 18, 20, 26–28, 30, 33, 35). Multiple mechanisms can explain it, but PASC may present with a characteristic pattern observed in pulmonary vascular or interstitial diseases (36). Pulmonary hypertension is typically seen with a diminished partial pressure of end-tidal CO_2 (37), which has also been reported in PASC patients with ventilatory inefficiency (27, 34, 35). However, several findings argue against this hypothesis. First, despite COVID-19 being associated with pulmonary embolism or right ventricular dysfunction (38), none of the studies reported such findings in PASC patients (26, 27, 29, 31). Second, those studies with stress test echocardiogram or invasive CPET did not observe signs of exercise-induced pulmonary hypertension with exception of a few isolated cases (34, 35). Third, an increase in the physiological dead space/tidal volume ratio was not observed (15, 18, 30, 31, 34, 35) when a raise is expected with severe ventilation-perfusion mismatching (37). Finally, no peripheral oxygen desaturation was reported even in those with pathological D_{LCO} (21–26, 33).

More recently, hyperventilation syndrome has been suggested to occur in PASC patients (26) given the increase VE/VCO_2 and low $PETCO_2$ observe during exercise without clear evidence of cardio-pulmonary diseases (39). Therefore, dysfunctional breathing characterized by exercise-induced hyperpnea may explain the persistence of symptoms in PASC-CVS.

Peripheral Limitation

The peripheral limitation has also been postulated as contributing to PASC. Alba et al. (19) found that a great number of PASC patients with abnormal CPET showed a low O_2 pulse with a normal cardiac function, suggesting a peripheral limitation. In the same way, Ribeiro Baptista et al. (21) didn't observe cardiac dysfunction at rest, but those with diminished exercise capacity had a smaller predicted O_2 pulse suggestive of peripheral limitation. As already mentioned, according to the Fick equation (peak V_{O2} is defined as the product of cardiac output and arteriovenous oxygen difference), a depressed peak V_{O2} can be the result of a blunted cardiac output response (impair oxygen delivery), and impaired peripheral oxygen extraction (diffusion defect) or both (5, 6).

In this sense, Singh et al. (35) performed invasive cardiopulmonary exercise testing on 10 patients who had recovered from COVID-19. These patients, in contrast to the control group, showed an increased peak exercise mixed venous oxygen saturation and peak venous O_2 content. The authors concluded that the impaired oxygen extraction was attributed primarily to reduced oxygen diffusion in the peripheral microcirculation, exhibiting a peripheral limitation to aerobic exercise.

Underlying anemia can contribute to both reduced systemic oxygen delivery and extraction (5, 6). Several studies collected hemoglobin levels, with the mean being within normal ranges (18–20, 23, 27, 35), ruling out the presence of anemia as a contributing factor to reduced peak V_{O2} found in these patients. However, one study from Baratto et al. (15) found underlying anemia in patients who had recovered from COVID-19 at the time of hospital discharge. This reduction in hemoglobin levels

leads to reduced arterial O_2 content and therefore to a lower O_2 delivery and reduced peak V_{O2}.

DISCUSSION

PASC is a disorder that occurs irrespective of previous disease severity and is characterized by a myriad of conditions and symptoms. Data suggest that dyspnea, fatigue, and exercise intolerance are the most common referred symptoms during outpatient follow-up. PASC cardiovascular disease is associated with structural or functional cardiovascular abnormalities that may explain the persistence of symptoms, but a non-negligible number of patients have no objective evidence of organ involvement. Therefore, PASC-CVS represents a heterogeneous group of patients with persistent symptoms that generally present a normal cardiopulmonary function (9). Because of a paucity of data, not much attention has been given to CPET despite its application can accurately evaluate PASC-CVS and improve the quality of care for these patients.

PASC-CVS is associated with an objective reduction of the exercise capacity during CPET (15–35). A long list of conditions can lead to poor physical conditions referred by PASC-CVS patients. Among all the potential causes and CPET variables, the following stand out: chronotropic incompetence, abnormal heart rate recovery, ventilatory inefficiency (high VE/VCO_2 and low $PETCO_2$), and diminished peripheral oxygen extraction. Patients presenting with pulmonary vasculopathy or interstitial lung disease demonstrate similar findings during CPET (36). PASC-CVS may share a common mechanism, but data from follow-up studies do not support that hypothesis as most show no evidence of cardio-pulmonary sequelae (15–35).

Therefore, given all key factors in determining oxygen availability (5, 6), it seems that the cornerstone of PASC-CVS may involve a peripheral limitation. This is further supported by invasive CPET findings, where a diminished peripheral tissue extraction during exercise led to a decreased exercise capacity (35). Notably, hyperventilation leads to a leftward shift of the hemoglobin oxygen affinity that is translated into a decreased O_2 unloading and impaired diffusion (5, 6). However, such impaired diffusion could also be explained by direct damage to the endothelium (40) leading to exercise intolerance as observed in chronic fatigue syndrome (41). Similarly, endothelial dysfunction has been reported in PASC with and without chronic fatigue syndrome (42). Interestingly, all the aforementioned factors may be linked to autonomic dysregulation (43, 44).

Dysfunctional breathing has been widely described among PASC patients (45), with hyperventilation being characterized by a decreased exercise capacity and signs of ventilatory inefficiency without evidence of cardio-pulmonary dysfunction. Interestingly, a high respiratory rate causes sympathetic activation and vagal withdrawal leading to exercise intolerance not only through an impaired O_2 diffusion but also through a diminished O_2 delivery (43). The impairment in O_2 delivery is supported by the evidence of cardiac autonomic dysfunction among PASC patients (32, 46, 47), where ventilatory inefficiency was also a common finding (16, 20). However, PASC-CVS may also manifest as dysfunctional breathing with a chaotic ventilatory pattern with normal peak VO_2, $PETCO_2$, and VE/VCO_2 during CPET (48).

Finally, evidence of autonomic dysfunction in PASC is further supported by recent studies suggesting that some patients present with signs and symptoms suggestive of postural orthostatic tachycardia syndrome (49). Indeed, postural orthostatic tachycardia syndrome can explain the CPET findings as it is associated with sympathetic stimulation, vasoconstriction, and hyperpnea (50).

Thus, the most appropriate hypothesis seems to be cardiac and peripheral autonomic dysregulation creating a vicious cycle that alters the exercise capacity in PASC-CVS. Nevertheless, it is difficult to draw any definitive conclusions, as the observations might be time-sensitive. In addition, none of the studies reported the baseline physical activity and physiological status of individuals before getting COVID-19, which raises the possibility of a cause-effect bias.

Prognostic Utility of CPET in PASC-CVS

PASC-CVS is expected to become a major challenge as most recent findings suggest that it shares some features with chronic fatigue syndrome (50). Although younger age and shorter time since COVID-19 have been recently described as potential predictors of submaximal CPET in PASC (51), there are no published studies examining the long-term prognostic value unless some ideas are extrapolated from previous studies. In particular, in heart failure patients a peak Vo_2 >14 ml/kg/min is associated with smaller 1-year mortality (52). Similarly, a high VE/VCO_2 slope is also associated with the worst clinical outcomes among cardiac and pulmonary patients (53) with recent studies suggesting that a high VE/VCO_2 in PASC-CVS is an independent predictor for endothelial dysfunction (54). Endothelial dysfunction has been associated with the worst outcomes in other medical conditions (44). Therefore, the presence of a diminished peak Vo_2 or high VE/VCO_2 slope could be associated with an increased risk of death during follow-up.

Interestingly, there is growing evidence that autonomic dysfunction might a fundamental factor in the observed symptoms in PASC-CVS (49). Theoretically, we could speculate about the potential utility of HR dynamics assessment in this group of patients during maximal effort and recovery. Previous studies have noted that chronotropic incompetence is associated with poor outcomes in heart failure patients (55). Furthermore, the detection of a heart rate recovery of ≤12 beats per minute is a strong predictor of all-cause mortality (RR: 2; 95% CI 1.5–2.7; $p < 0.001$) irrespective of previous cardiovascular risk factors and even in the absence of heart failure or myocardial perfusion defects (56). Nevertheless, risk stratification for PASC-CVS is limited. Thus, future studies with CPET both at baseline and follow-up are expected and will provide a more reliable estimation of long-term clinical outcomes in these patients with a special emphasis on previously known prognostic factors.

CONCLUSIONS

Our current understanding of PASC is vague, but exercise limitation is a common finding despite the absence of objective cardio-pulmonary sequelae in PASC-CVS. Physiological assessment with CPET may provide valuable information about the functional status of these patients and identify the potential pathogenic mechanism. Autonomic dysfunction might be the missing link. Future studies evaluating predictors of exercise intolerance and long-term prognosis are warranted, as it could have a positive effect on disease evolution and clinical outcomes.

AUTHOR CONTRIBUTIONS

ÁA, JT, and JASR contributed to conception of the work. ÁA wrote the first draft of the manuscript. ÁA, RL, and CY-F wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Antiplatelet therapy for patients with COVID-19: Systematic review and meta-analysis of observational studies and randomized controlled trials

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Background: Hyperinflammation and coagulopathy are hallmarks of COVID-19 and synergistically contribute to illness progression. Antiplatelet agents have been proposed as candidate drugs for COVID-19 treatment on the basis of their antithrombotic and anti-inflammatory properties. A systematic review and meta-analysis that included early observational studies and recent randomized controlled trials (RCTs) was performed to summarize and compare evidence on this issue.

Methods: PubMed, Embase, and the Cochrane Central Register of Controlled Trials (CENTRAL) were searched to identify studies published up to Nov 7, 2021, and the results of registered clinical trials were followed up to Mar 30, 2022. We included RCTs and observational studies assessing the effect of antiplatelet therapy in adult patients with COVID-19. Data on baseline patient characteristics, interventions, controls, and outcomes were extracted by two independent reviewers. The primary outcome was mortality. Data were pooled using a random-effects model.

Results: Twenty-seven studies were included, of which 23 observational studies were pooled in a meta-analysis, and the remaining four RCTs (ACTIV-4B, RECOVERY, ACTIV-4a, and REMAP-CAP) were narratively synthesized. Based on 23 observational studies of 87,824 COVID-19 patients, antiplatelet treatment favors a lower risk of mortality [odds ratio (OR) 0.72, 95% confidence interval (CI) 0.61–0.85; $I^2 = 87.0\%$, $P < 0.01$]. The narrative synthesis of RCTs showed conflicting evidence, which did not support adding antiplatelet therapy to the standard care, regardless of the baseline illness severity and concomitant anticoagulation intensity.

Conclusion: While the rationale for using antiplatelet treatment in COVID-19 patients is compelling and was supported by the combined result of early observational studies, evidence from RCTs did not confirm this approach.

Several factors that could explain this inconsistency were highlighted alongside perspectives on future research directions.

KEYWORDS

coronavirus disease 2019 (COVID-19), thromboembolism, antiplatelet therapy, aspirin, clopidogrel, systematic review, meta-analysis

Introduction

Dysregulated inflammation and coagulopathy are hallmarks of severe COVID-19 and contribute to an increased risk of thromboembolic complications and mortality (1–4). Platelets are anucleate cell fragments derived from megakaryocytes that are not simply involved in thrombosis and immune response but also exert a hub function bridging these two processes as a new mechanism, termed immunothrombosis (5–7). The multifaceted role of platelets in immunothrombosis has been well-documented in previous literature and further highlighted in the current COVID-19 situation (8–11). A hyperactive platelet phenotype, as characterized by increased platelet surface markers [e.g., P-selectin, platelet Factor 4 (PF4), and CD40L], platelet-derived soluble mediators [e.g., thromboxane B2 (TxB2) and 5-hydroxytryptamine (5-HT)], and platelet homotypic and heterotypic aggregates, has been extensively identified in COVID-19 patients (9, 12–16). Likewise, data from transcriptome and proteome analyses indicate that platelet hyperactivity is a predominant cellular signature in response to SARS-CoV-2 infection (17, 18), thus suggesting a possible role of platelets in this novel viral disease. Moreover, activated platelets can trigger the formation of neutrophil extracellular traps (NETs) (19, 20), which have recently been recognized as pivotal players in thrombosis (21–23). Autopsy reports of COVID-19 patients revealed microthrombi with platelet and NET deposition in inflamed lung tissues, along with endothelial disruption (23–25).

Given the possible role of hyperactive platelets in the pathological mechanism of COVID-19, antiplatelet agents, such as aspirin and P2Y12 inhibitors, have been proposed as a potential treatment strategy for COVID-19 patients on the basis of their antithrombotic and anti-inflammatory properties (26–28). Additionally, significant thrombotic events have been observed despite anticoagulant treatment in clinical trials, implying that antiplatelet agents could be potential candidates for additional adjunctive antithrombotic therapy (29–31). In fact, an association between antiplatelet drug use and improved outcomes for COVID-19 patients has been reported in early observational studies (32–34). However, recently completed RCTs failed to confirm the effectiveness of antiplatelet treatment in preventing COVID-19 progression. While RCTs are considered to be more reliable than observational studies in evaluating interventions, the latter has helped us establish an initial foundation, which is particularly significant in an urgent situation (35). In the present circumstances, there is a need for findings to be assessed in the context of existing evidence in order to ensure reasonable interpretation of all studies (36). Here, we perform a systematic review and meta-analysis that included both RCTs and observational studies to provide an overview of existing evidence on antiplatelet therapy in patients with COVID-19. Furthermore, several study elements (e.g., baseline illness severity, the timing of antiplatelet therapy, and concomitant anticoagulation intensity) that might contribute to discrepancies among current lines of evidence and should be taken into consideration in future research are discussed.

Methods

This systematic review was performed following the PRISMA statement (37). The study protocol is provided in Supplementary material 1. Briefly, PubMed, Embase, and Cochrane CENTRAL were searched to identify studies published up to Nov 7, 2021, and the results of registered clinical trials were followed up to Mar 30, 2022. Details of the search strategies are provided in Supplementary material 1. The inclusion criteria were adult COVID-19 patients confirmed by laboratory testing, administration of antiplatelet therapy at any time or dose, comparison between patients with and without antiplatelet therapy, and availability of English or Chinese full texts. Studies involving patients with a particular illness or emergency conditions were excluded (e.g., cancer and pregnancy). When studies had significant overlapping data, the most comprehensive study was included.

The results of observational studies and RCTs were separately synthesized and compared (35). For pooled analysis, we selected all-cause mortality as the primary outcome for

Abbreviations: 5-HT, 5-Hydroxytryptamine; CI, Confidence intervals; COVID-19, Coronavirus Disease; GP Ib, Glycoprotein Ib; HR, Hazard ratio; LMWH, Low-molecular-weight heparin; NETs, Neutrophil extracellular traps; NOS, Newcastle–Ottawa Scale; OR, Odds ratio; PARs, Protease-activated receptors; PF4, Platelet Factor 4; RCTs, Randomized controlled trials; RR, Relative risk; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SGLT2, Sodium-glucose cotransporter-2; TxB2, Thromboxane B2.

effect estimates. Considering that all the included studies for quantitative pooled analysis were retrospective in design, the odds ratio (OR) was used as the common measure of association across studies. Hazard ratios (HRs) and relative risks (RRs) were directly considered as ORs. A random-effect model was selected to account for clinical heterogeneity. Heterogeneity across studies was assessed using the Q statistic with its *P*-value and I^2 statistic (38). Subgroup analyses were conducted to investigate variation in estimates according to original effect size, study center, illness severity, antiplatelet drugs, the timing of drug administration, and concomitant anticoagulant use. Sensitivity analysis was performed on the primary outcome by omitting one study at a time to assess the robustness of the results (39). A funnel plot was drawn to assess publication bias. The quality of the included observational studies for meta-analysis was evaluated following the Newcastle–Ottawa scale (NOS) by two independent reviewers (40). Studies with NOS scores of 8 or 9, 6 or 7, and < 6 were judged as having a low, medium, and high risk of bias, respectively. Discrepancies in data extraction and quality assessment were resolved through discussion with a third author. Statistical analyses were performed using RStudio software.

Results

Study identification

Our search yielded 1,228 records. After initial screening and full-text review, 23 observational studies (41–63) and 4 RCTs (64–67) (ACTIV-4B, RECOVERY, ACTIV-4a, and REMAP-CAP) were finally included for evidence synthesis and comparison (Figure 1). Observational studies were mostly performed in the first half of 2020, and RCTs were subsequently conducted between late 2020 and early 2021. For observational studies, the overall risk of bias was determined to be

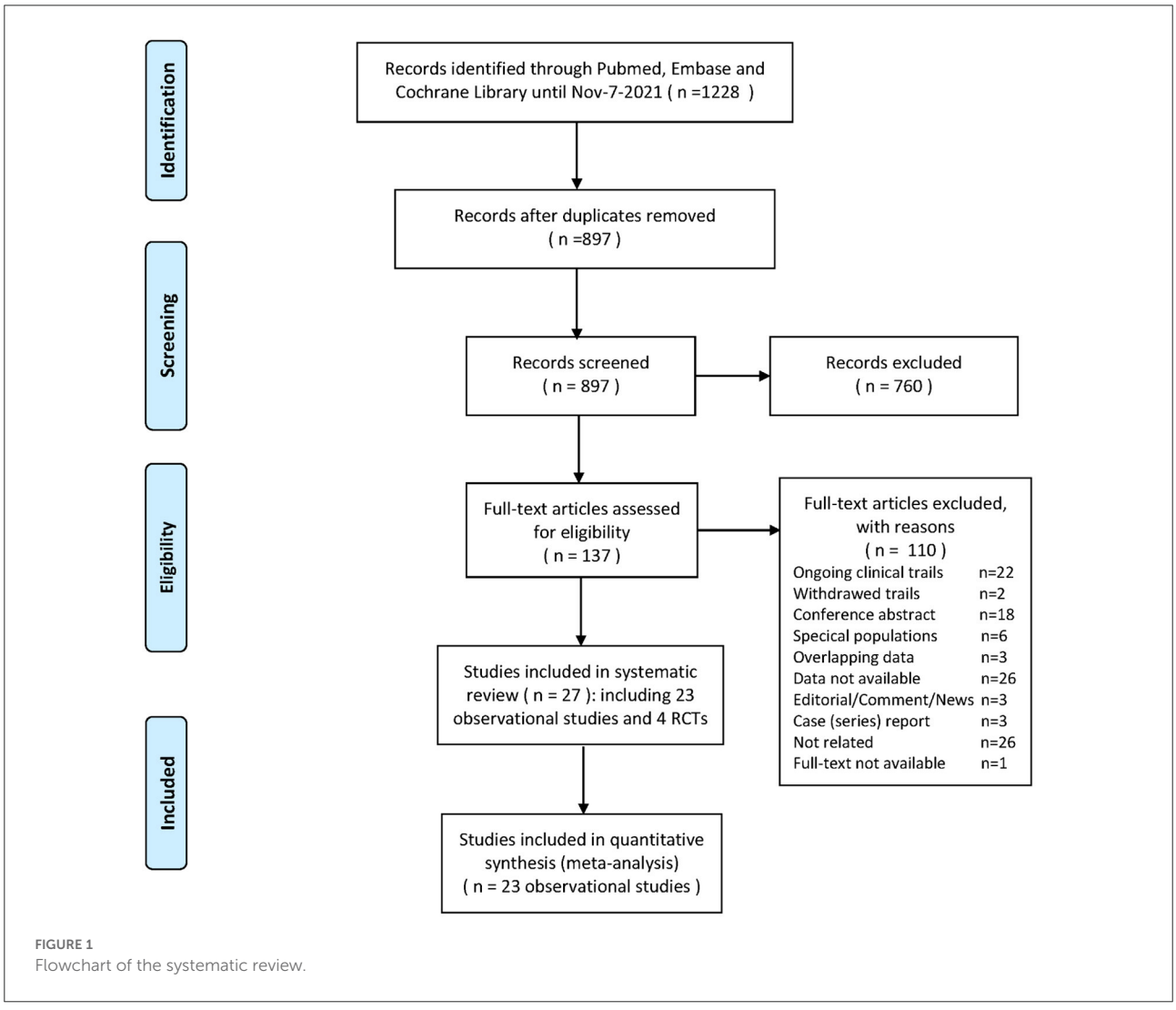

FIGURE 1
Flowchart of the systematic review.

medium (Supplementary Table 1). Adjusted estimates could be determined for all observational studies even though the adjusted factors were slightly different. Among all studies, aspirin is the most common antiplatelet drug. Tables 1, 2 show details of the included observational studies and RCTs, respectively.

Meta-analysis of observational studies

We first performed a meta-analysis of the 23 included observational studies and obtained a combined OR of 0.72 (95% CI: 0.61–0.85), suggesting that antiplatelet treatment favors a lower risk of mortality in patients with COVID-19 (Figure 2). Although significant heterogeneity was evident ($P < 0.01$, $I^2 = 87\%$), the combined estimates were consistent in the random- and fixed-effect models, and the sensitivity analysis suggested that our result was stable (Supplementary Figure 1), probably driven by a large number of participants ($n = 87{,}824$). To investigate the variation of combined evidence among observational studies and improve comparability with RCTs, pre-specified subgroup analyses were further conducted. All results of the subgroup analyses are summarized in Figure 3 (forest plots are shown in Supplementary Figures 2–7). The only significant antiplatelet treatment-covariate interaction identified in subgroup analyses was concomitant anticoagulant use, with OR = 0.64 (95% CI: 0.50–0.83) among patients with anticoagulant use (including partial use) and OR = 1.07 (95% CI: 0.94–1.21) among patients without anticoagulation treatment. There was no evidence to suggest a differential treatment effect for any other subgroups. The asymmetric shape of the funnel plot shows some evidence of publication bias among the evaluated studies (Supplementary Figure 8).

RCTs

Currently, one outpatient trial (ACTIV-4B) (64) and three inpatient trials (RECOVERY, ACTIV-4a, and REMAP-CAP) (65–67) have released their results. As there was obvious heterogeneity in the study population, antiplatelet treatment regimen, and concomitant anticoagulation intensity among these studies, their results were narratively synthesized (Table 2). The ACTIV-4B trial aimed to assess whether antiplatelet therapy (aspirin 81 mg) can safely reduce major adverse cardiopulmonary outcomes among symptomatic but clinically stable outpatients with COVID-19. The study was terminated early because of an event rate (0.7%) lower than anticipated and no evidence of efficacy when comparing aspirin with placebo.

RECOVERY (65) is the current largest randomized study investigating the effect of antiplatelet therapy in COVID-19, with 14,892 participants from 171 centers. This study found that in a mixed population of patients with mild, moderate, and severe COVID-19, adding 150 mg aspirin to standard care did not reduce 28-day mortality [relative risk (RR) = 0.96, 95% CI: 0.89–1.04] or the probability of progression to the composite of invasive mechanical ventilation or death (RR = 0.96; 95% CI: 0.90–1.03).

In recently completed multiplatform trials (ATTACC, ACTIV-4a, and REMAP-CAP), therapeutic-dose heparin vs. conventional thromboprophylaxis has been found to improve organ support-free days in hospitalized non-critically ill patients (30) but is not beneficial for critically ill patients (29). Subsequently, the ACTIV-4a trial (66) tested whether the addition of a P2Y12 inhibitor to anticoagulant therapy would further change clinical outcomes in non-critically ill patients hospitalized for COVID-19. After 562 patients completed the trial, no significant differences were found in the primary outcome (the composite of organ support-free days evaluated on an ordinal scale combined with in-hospital death) or in the secondary outcome (the composite of major thrombotic events or death by 28 days).

In parallel with ACTIV-4a, the REMAP-CAP trial (67) aimed to examine the add-on effect of antiplatelet therapy [aspirin, 75–100 mg; $n = 565$ or P2Y12 inhibitors (clopidogrel, 75 mg; ticagrelor, 60 mg; or prasugrel, 60 mg); $n = 455$] alongside prophylactic dose anticoagulation in severe COVID-19 patients. First, this trial observed equivalence between the aspirin and P2Y12 inhibitor groups (OR = 1.00; 95% CI, 0.8–1.27; >90% posterior probability of equivalence). In a subsequent adaptive pooled analysis of the two antiplatelet treatment groups in comparison with controls, the median for organ support-free days was 7 in both the antiplatelet and control groups (median-adjusted OR = 1.02; 95% CI, 0.86–1.23; 95.7% posterior probability of futility). Although a modest benefit on the secondary endpoint of 90-day mortality was determined (HR = 1.22; 95% CI, 1.06–1.40; 99.7% posterior probability of efficacy), the median number of organ support-free days was again equal (14 days) among survivors in both groups. Additionally, the authors reported a small but certain increased risk of major bleeding in the antiplatelet group (2.1 vs. 0.4%; adjusted OR = 2.97; 95% CI, 1.23–8.28; 99.4% probability of harm).

Discussion

This systematic review summarized and compared current evidence regarding antiplatelet treatment for patients with COVID-19. The combined effect estimates of observational studies suggested that antiplatelet therapy favors a lower risk of mortality, and the results were consistent in all pre-specified subgroup analyses in addition to those based on anticoagulant use. Nevertheless, subsequent RCTs did not confirm this association. A series of well-conducted randomized studies found no additional effect when adding antiplatelet therapy

TABLE 1 Details of observational studies included in this meta-analysis of the association between antiplatelet treatment and mortality.

References	Time of patient inclusion	Region	Center	Population	*N*	Age (year)	Male sex	Drugs	Endpoint	Events rate	Adjusted factors	NOS
Aydinyilmaz et al. (41)	Mar to Dec 2020	Turkey	S	Severely ill Inpatients	373	E: 73.9 ± 0.9 C: 69.1 ± 1.9	E: 72.9% C: 58.0%	Aspirin	In-hospital mortality	–	Male gender, diabetes, hypertension	7
Chow et al. (42)	Feb to Apr 2020	United States	M	Inpatients	17,347	E: 72 (64–80) C: 72 (64–80)	E: 54.5% C: 53.3%	Multiple*	In-hospital mortality	20.5%	Age, male, race, BMI, comorbidities, medications	8
Corrochano et al. (43)	Mar to May 2020	Spain	S	Inpatients	1,443	66.5 ± 17.1	53.2%	Multiple	28 d mortality	19.3%	Sex, age, comorbidities	8
Fröhlich et al. (44)	Feb to Apr 2020	Germany	M	Inpatients	5,971	E:79 (69–84) C: 65 (52–79)	E: 63.8% C: 51.1%	Multiple	All-cause mortality or ventilation	27.5%	Age, gender, and comorbidities	8
Gupta et al. (45)	Feb to May 2020	United States	S	Inpatients	2,626	–	–	P2Y12 inhibitor	30 d mortality	–	Age, sex, BMI, comorbidity, medications	7
Haji Aghajani et al. (46)	Mar 2019 to Jul 2020	Iran	S	Severely ill inpatients	991	61.6 ± 17.0	54.9%	Aspirin	In-hospital mortality	25.8%	Age, sex, BMI, comorbidity, medications	7
Ho et al. (47)	Feb to Jul 2020	United States	M	Outpatients	27,824	E: 66 (55–77) C: 41 (30–53)	E: 53.0% C: 48.0%	Multiple	Mortality	3.3%	Age, sex, race, BMI, comorbidities	8
Izzi-Engbeaya et al. (48)	Mar to Apr 2020	UK	M	Inpatients	889	65.8 ± 17.5	60.1%	–	Death and/or ICU admission	36.0%	Age, sex, race, comorbidity, Laboratory and clinical parameters, and medications	6
Liu et al. (49)	Jan to Mar 2020	China	S	Inpatients	48	E: 69 (61–76) C: 74 (65–79.5)	E: 58.3% C: 70.8%	Aspirin	30 d mortality	16.7%	Age, sex, comorbidities, Laboratory and clinical parameters, and medications	8
Matli et al. (50)	Apr 2020 to Jan 2021	Lebanon	S	Inpatients	146	E: 66.2 ± 13.8 C: 59.6 ± 17.0	E: 67.4% C: 58.8%	Multiple	In-hospital mortality	14.1%	Age, sex, smoking, weight, comorbidity, medications	8
Meizlish et al. (51)	Mar to Jun 2020	United States	M	Inpatients	638	–	63.3%	Aspirin	In-hospital mortality	–	Age, sex, max D-dimer, comorbidities, medications	8
Merzon et al. (52)	Feb to Jun 2020	Israel	M	Inpatients	112	–	–	Aspirin	In-hospital mortality	6.3%	Age, sex, smoking, comorbidity, medications	7
Mura et al. (53)	–	30 countries	M	Severely ill inpatients	527	–	–	Aspirin	Mortality	31.3%	Age, gender	6
Osborne et al. (54)	Mar to Aug 2020	United States	M	Inpatients	12,600	E: 67.4 ± 10.7 C: 67.2 ± 11.1	E: 95.2% C: 96.6%	Aspirin	30 d mortality	7.4%	Age, gender, and Care Assessment Needs (CAN) score	8

(Continued)

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TABLE 1 (Continued)

References	Time of patient inclusion	Region	Center	Population	*N*	Age (year)	Male sex	Drugs	Endpoint	Events rate	Adjusted factors	NOS
Pan et al. (55)	Mar to Apr 2020	United States	S	Inpatients	762	E: 69.6 ± 12.5 C: 58.5 ± 16.2	E: 60.3% C: 54.3%	Multiple	28 d mortality	∼20%	Age, sex, BMI, smoking, comorbidities	8
Russo et al. (56)	Feb to Apr 2020	Italy	M	Inpatients	192	67.7 ± 15.2	59.9%	Multiple	In-hospital mortality	18.5%	Age, smoke, comorbidities	8
Sahai et al. (57)	Mar to May 2020	United States	M	Outpatients	496	E: 68.5 ± 13.6 C: 69.5 ± 14.1	E: 56.5% C: 59.5%	Aspirin	In-hospital mortality	14.3%	Age, sex, race, smoking, platelets, and comorbidities	7
Santoro et al. (58)	Jan to May 2020	7 countries	M	Inpatients	7,716	64 ± 17	58.0%	Multiple	In-hospital mortality	18.0%	Age, sex, comorbidities, invasive ventilation, medications	8
Sisinni et al. (59)	Feb to Apr 2020	Italy	M	Inpatients	984	72 [62–81]	69.0%	Multiple	30 d mortality or respiratory support upgrade	72.0%	Age, male gender, hypertension, glucocorticoid therapy	8
Soldevila et al. (60)	Mar to Jun 2020	Spain	M	Inpatients	1,306	86.7 ± 7.3	28.7%	Multiple	30 d mortality	24.4%	Age, gender, comorbidities, Barthel score, frailty score, medications	8
Terlecki et al. (61)	Mar to Oct 2020	Poland	S	Inpatients	1,729	63 [50–75]	51.2%	Multiple	In-hospital mortality	12.9%	Age, gender, comorbidities, medications	8
Tremblay et al. (62)	Mar to Apr 2020	United States	M	Inpatients	1,064	E: 61.2 ± 10.9 C: 63.0 ± 12.2	54.9% 57.5%	–	In-hospital mortality	15.0%	Age, sex, race, Charlson comorbidity index and obesity	8
Zhao et al. (63)	Feb 2020 to Mar 2021	United States	M	Severely ill inpatients	2,070	65 ± 16	58.8%	Aspirin	In-hospital mortality	29.0%	Age, sex, smoking, BMI, comorbidity, laboratory indices, vital signs, medications	8

*Two or more antiplatelet drugs were together defined as exposure, with aspirin plus P2Y12 inhibitors being most common among studies. The ages of the study populations were expressed as the mean ± standard deviation or median [interquartile range]. BMI, body mass index; E, exposure group; C, control group; –, Data not reported or not calculable; NOS, Newcastle–Ottawa Scale.

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TABLE 2 Details of RCTs investigating the effect of antiplatelet treatment for patients with COVID-19.

Study	Study time frame	Number of centers/country	Population	*N*	Median/mean age (year)	Male (%)	Antiplatelet treatment	Primary outcome
ACTIV-4B (64)	Sep 2020–Jun 2021	52/USA	Symptomatic but clinically stable outpatients	328	54.0	41.8	Aspirin (81 mg)	A composite of all-cause mortality, thromboembolic events or hospitalization for cardiovascular or pulmonary cause
RECOVERY (65)	Nov 2020–Mar 2021	171/UK, Indonesia, Nepal	Adult hospitalized patients	14 892	59.2	61.8	Aspirin (150 mg)	28 d mortality
ACTIV-4a (66)	Feb–Jun 2021	60/Brazil, Italy, Spain, USA	Non-critically ill hospitalized patients	562	52.7	58.5	P2Y12 inhibitors	Organ support-free days
REMAP-CAP (67)	Oct 2020–Jun 2021	105/Canada, France, Germany, India, Italy, Nepal, the Netherlands, UK	Critically ill hospitalized patients	1 549	57.0	66.4	Aspirin (75–100 mg) or P2Y12 inhibitors	Respiratory and cardiovascular organ support–free days to day 21

to standard care, regardless of baseline illness severity and concomitant anticoagulation intensity. The reason for this inconsistency may be multiple. First, since all observational studies included for pooled analysis were retrospective in design, selection bias might have occurred in the selection of exposed subjects according to an antecedent prescription of antiplatelet medication. Additionally, while adjusted estimates could be determined for all studies, potential cofounding associated with both exposure and outcome cannot be definitively excluded (68, 69).

In addition to the limitations ascribed to the study design *per se*, another noteworthy factor is the timing of antiplatelet treatment. For most observational studies (41–45, 47, 52, 54–57, 59–62) included for evidence synthesis, antiplatelet therapy was initiated before COVID-19 diagnosis in contrast to during hospitalization in RCTs (65–67). Possibly, the baseline suboptimal platelet reactivity due to prior chronic antiplatelet therapy restrains illness progression and aggravation. At the time of hospitalization because of moderate or severe illness, platelet activation may have already reached a maximum level, for which antiplatelet treatment is too late (66). Additionally, as mentioned above, the rationale for antiplatelet medication in COVID-19 is based on the antithrombotic and anti-inflammatory properties. However, there is evidence that the distribution of platelets is not limited only to intravascular compartments but also to alveolar translocation (70–73). Moreover, platelets differentially bind to neutrophils and Treg cells at distinct time points to orchestrate both the initiation and resolution of pulmonary inflammation. These interactions prevent excessive lung damage after infection (70). In sum, platelets still offer a candidate treatment target for infection-related thrombosis, yet the treatment timing may be of great relevance and warrant further investigation at the clinical level.

Among currently completed RCTs, ACTIV-4B is the only outpatient trial. In addition to its negative finding, this trial also reported a markedly low rate of events (a composite of all-cause mortality, thromboembolism, myocardial infarction, stroke, or hospitalization for cardiovascular or pulmonary cause), namely, 0.7% among the study populations, which is much lower than that reported by early epidemiological data (64). The significant decline in adverse event incidence among mildly ill outpatient populations could partially be attributed to aggressive vaccination and progress in medical care since the pandemic outbreak (74, 75). Correspondingly, the recently updated COVID-19 treatment guidelines recommend against the use of anticoagulants and antiplatelet therapy in the outpatient setting, unless the patient has other indications for the therapy (76). Another ongoing trial (OLA COVID; NCT04937088) (77) that tests whether a novel, liquid aspirin formulation can reduce COVID-19-related hospitalizations in old populations will provide more evidence on this issue.

For hospitalized patients with COVID-19, thrombotic complications have been reported to be common

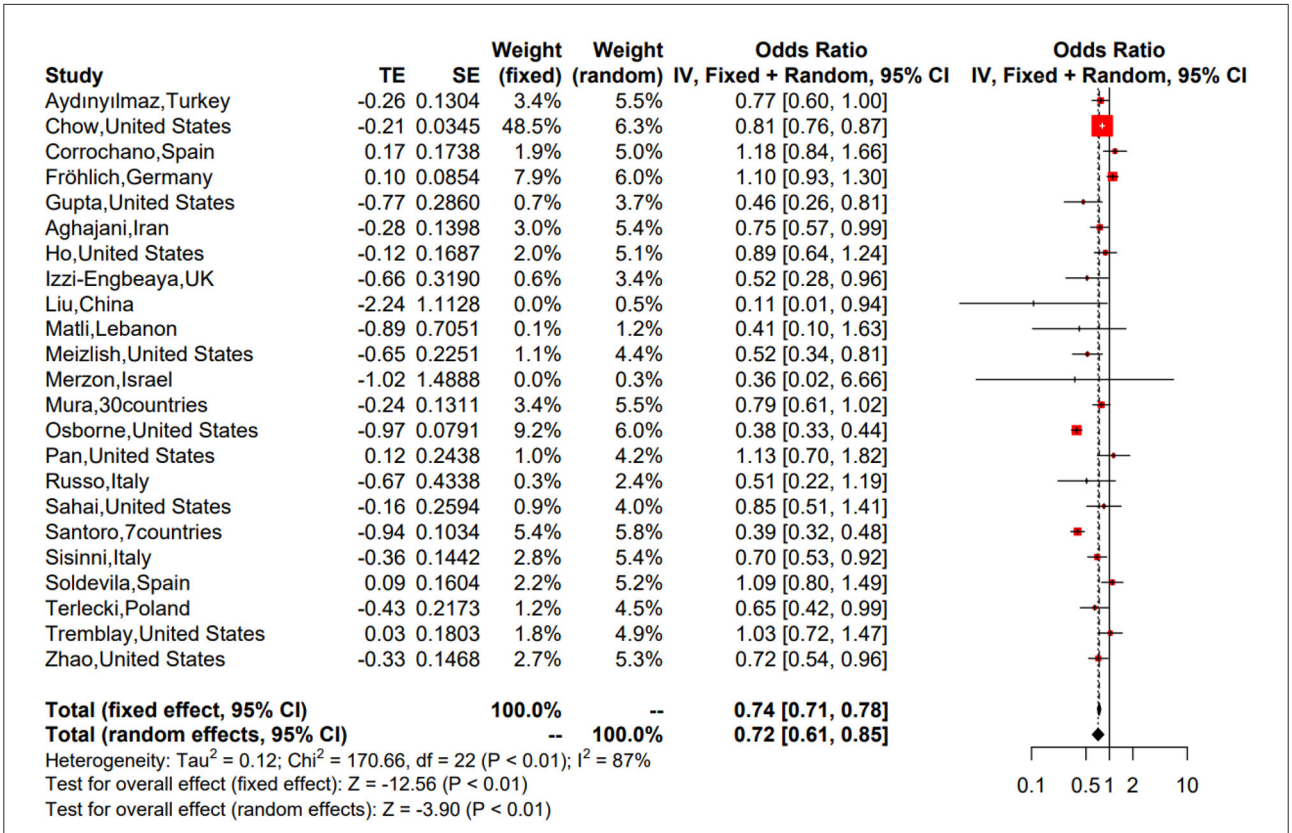

FIGURE 2
Forest plot of the 23 included observational studies.

despite conventional thromboprophylaxis and therapeutic anticoagulation (31). In this setting, the RECOVERY, ACTIV-4a, and REMAP-CAP trials sought to examine the additional effect and risk of antiplatelet treatment on the basis of thromboprophylaxis and anticoagulation therapy. Overall, the results of these well-conducted trials found no additional effect when antiplatelet therapy was added to anticoagulation (mostly Low-molecular-weight heparin, LMWH) in hospitalized patients with COVID-19, despite a slightly increased risk of bleeding. The reason for these negative results is not obvious. A possible explanation is that the anticipated antithrombotic effect of antiplatelet drugs was partially masked by LMWH (78).

While the mechanism of COVID-19-related coagulopathy has not yet been elucidated, a major cause is tissue factor overexpression on the surface of damaged endothelial cells and immune cells, which further initiates coagulation cascades and leads to thrombin generation (8). This opinion can be supported by anticoagulation trials that found the superiority of heparin/LMWH by targeting thrombin. However, thrombin is not only a central enzyme involved in coagulation cascades but also a potent inducer of platelet activation (78, 79). In a more recent study, the TF/thrombin pathway was found to be pivotal for platelet activation in an *ex vivo* SARS-CoV-2 infection model (80). The authors concluded that TF activity from SARS-CoV-2-infected cells activates thrombin, which signals to protease-activated receptors (PARs) on platelets (80). Taken together, it is plausible to speculate that the key upstream pathway that promotes platelet activation during SARS-CoV-2 infection is inhibited by heparin through disturbing thrombin ligation to platelet Glycoprotein Ib (GP Ib) and PARs (78, 80), whereby the anticipated antithrombotic effects of aspirin and P2Y12 inhibitors in the above trials were diluted.

To date, our successful experience in the combined use of heparin and antiplatelet agents is almost confined to thrombotic disease, with most valid evidence in arterial thrombosis, yet, under the premise that antiplatelet treatment *per se* is effective (81). Whether antiplatelet therapy alone can prevent illness progression for hospitalized patients with COVID-19 is still unclear. This question is difficult to clarify in future trials, as it is unethical to abrogate proven beneficial anticoagulation for patients to measure the effect of single antiplatelet therapy. Alternatively, early observational studies could shine a light on this issue. In the subgroup analysis of observational studies by anticoagulant intensity, we identified four studies (43, 44, 47, 62) including 36,302 patients without anticoagulant use. The combined OR of 1.07 (95% CI, 0.94–1.21; $I^2 = 0\%$, $P = 0.32$)

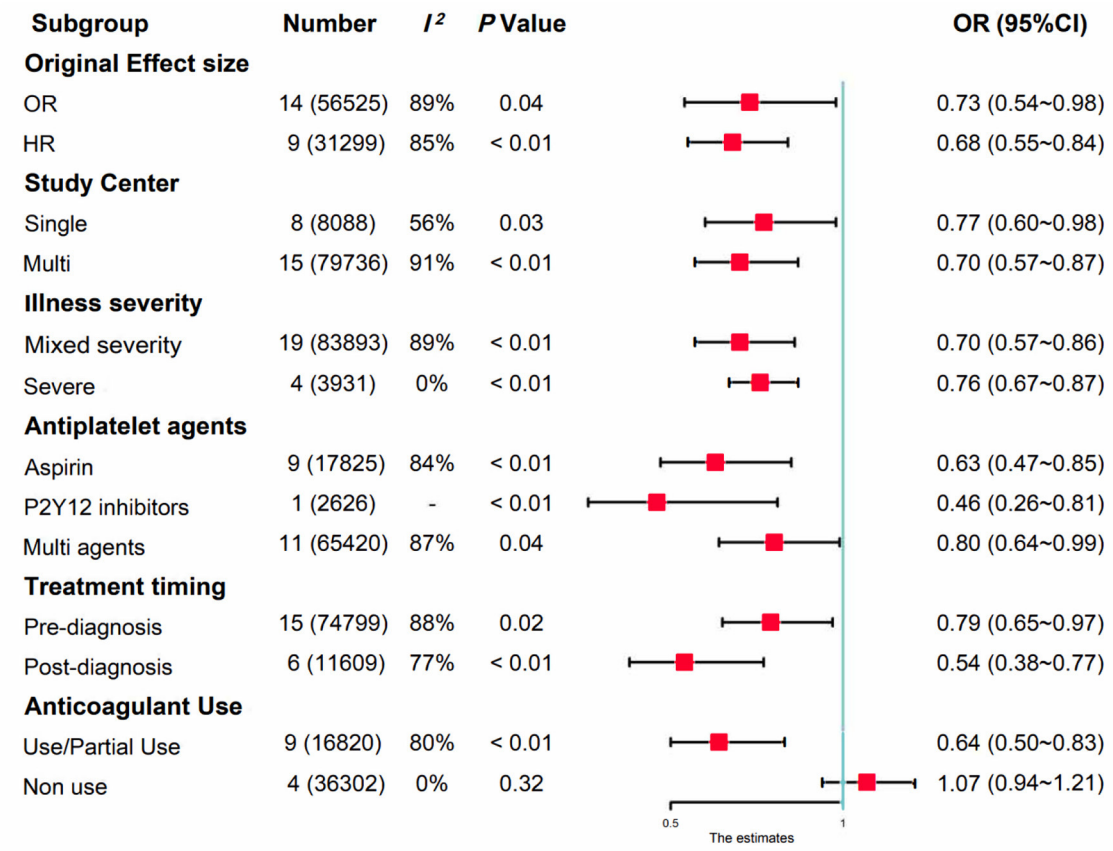

FIGURE 3
Summary of the subgroup analyses in observational studies.

suggested that single antiplatelet treatment is not associated with lower mortality (see Figure 3). However, this result may be limited by the lack of sufficient direct comparisons and should be regarded with extreme caution. In contrast to targeting platelets *per se*, there is an ongoing arm of the ACTIV-4a trial that aims to test whether inhibiting the cross-talk between platelets and immune as well as endothelial cells, by using Crizanlizumab (82, 83) or sodium-glucose cotransporter-2 (SGLT2) inhibitor (84), will further improve the hypercoagulable state of patients with COVID-19, and the results are eagerly anticipated.

Conclusion

This paper provides an overview of existing evidence on antiplatelet therapy for patients with COVID-19. In summary, while the rationale for using antiplatelet drugs to prevent COVID-19 progression is compelling and was supported by combined evidence from early observational studies, recently completed RCTs do not support this approach. The consistent negative results of such RCTs have supplied more valid evidence against adding antiplatelet therapy to standard care for COVID-19 patients in either community or hospital settings. In terms of directions for future study, the optimal antithrombotic regimen for patients with COVID-19 should be individualized (85) and guided by biomarkers, such as urinary 11-dehydrothromboxane B2, platelet reactivity, platelet-platelet aggregates, and platelet-leukocyte aggregates detected by new microscopic techniques (16, 31). Moreover, several factors that could explain the inconsistency among the current evidence and advocate for further investigation were highlighted in the current review.

Data availability statement

The datasets used during the current study are available from the corresponding author upon reasonable request.

Author contributions

XZ contributed to the conception and design of the study, acquisition of data, analysis and interpretation of the data, and drafting of the manuscript. ZL, WW, DW, and ZC reviewed

and revised the manuscript. XW, YL, and ZL contributed to the acquisition as well as to the analysis and interpretation of the data. All authors gave final approval and agree to be accountable for all aspects of the work ensuring integrity and accuracy.

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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.2022.965790/full#supplementary-material

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Synergistic effect of myocardial injury and mid-regional proAdrenomedullin elevation in determining clinical outcomes of SARS-CoV-2 patients

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Objective: Coronavirus disease 2019 (COVID-19) is a systemic disease induced by SARS-CoV-2 causing myocardial injury. To date, there are few data on the correlation between mid-regional proAdrenomedullin (MR-proADM) and myocardial injury. The aim of this study was to evaluate whether the association of myocardial injury and elevated mid-regional proAdrenomedullin values could predict mortality of SARS-CoV-2 patients, to offer the best management to COVID-19 patients.

Materials and methods: All patients hospitalized for SARS-CoV-2 infection at the COVID-19 Center of the Campus Bio-Medico of Rome University were included between October 2020 and March 2021 and were retrospectively analyzed. Myocardial injury was defined as rising and/or fall of cardiac hs Troponin I values with at least one value above the 99th percentile of the upper reference limit (≥15.6 ng/L in women and ≥34.2 ng/L in men). The primary outcome was 30-day mortality. Secondary outcomes were the comparison of MR-proADM, CRP, ferritin, and PCT as diagnostic and prognostic biomarkers of myocardial injury. Additionally, we analyzed the development of ARDS, the need for ICU transfer, and length of stay (LOS).

Results: A total of 161 patients were included in this study. Of these, 58 (36.0%) presented myocardial injury at admission. An MR-proADM value ≥ 1.19 nmol/L was defined as the optimal cut-off to identify patients with myocardial injury (sensitivity 81.0% and specificity 73.5%). A total of 121 patients (75.2%) developed ARDS, which was significantly more frequent among patients with myocardial injury (86.2 vs. 68.9%, $p = 0.015$). The overall 30-day mortality was 21%. Patients with myocardial injury presented significantly higher mortality compared to those without the same (46.6 vs. 6.8%, $p < 0.001$). When dividing the entire study population into four groups, based on the presence of myocardial injury and MR-proADM values, those patients with both myocardial injury and MR-proADM ≥ 1.19 nmol/L presented the highest mortality (53.2%, $p < 0.001$). The combination of myocardial injury and MR-proADM values ≥ 1.19 nmol/L was an independent predictor of death (OR = 7.82, 95% CI = 2.87–21.30; $p < 0.001$).

Conclusion: The study is focused on the correlation between myocardial injury and MR-proADM. Myocardial injury induced by SARS-CoV-2 is strongly associated with high MR-proADM values and mortality.

KEYWORDS

myocardial injury, mid-regional proAdrenomedullin, COVID-19, Troponin I (tni), SARS-CoV-2

Introduction

Coronavirus disease 2019 (COVID-19) is a systemic disease induced by Severe Acute Respiratory Distress Syndrome *Coronavirus* 2 (SARS-CoV-2) causing widespread endothelial damage primarily involving the pulmonary and cardiovascular systems (1–4).

Acute cardiac injury in COVID-19 patients is present in upto 15–50% of critically ill patients and is represented by myocardial injury, endothelitis, heart failure, Takotsubo cardiomyopathy, acute coronary syndromes, pulmonary thromboembolism, and arrhythmias (2, 5–8).

Myocardial injury is defined as an increase in myocardial enzyme levels (Troponin) with at least one value above the 99th percentile upper reference limit in absence of myocardial ischemia and can be caused by several mechanisms (9). Myocardial injury occurs due to indirect or direct myocardial damage with a mortality of 60% (8).

Indirect myocardial injury evidenced by the increase of Troponin is present in up to 36% in the early course of SARS-CoV-2 infection and it is associated with an increased risk of requiring mechanical ventilation, fatal ventricular arrhythmias, and a 59.6% of risk mortality (10–15).

A direct myocardial injury affects hs Troponin I in case of acute coronary syndrome and could affect adrenomedullin expression that is expressed by cardiomyocytes, pericytes, cardiofibroblasts, endothelial cells, epicardial adipose cells, vascular endothelial cells, smooth muscle cells, and migratory angiogenic cells (16, 17).

Currently, the understanding of the underlying physiopathological mechanisms of the onset of myocardial injury is still limited and there are only little data on the correlation between myocardial injury and MR-proADM. This biomarker helps clinicians in identifying those patients with severe disease and at higher risk of death (4, 18–22).

The aim of this study was to evaluate whether the association of myocardial injury and elevated mid-regional proAdrenomedullin values could predict mortality of SARS-CoV-2 patients, to offer the best management to COVID-19 patients.

Materials and methods

Patient selection and characteristics

All patients hospitalized with SARS-CoV-2 infection at the COVID-19 Center of the Campus Bio-Medico of

Abbreviations: ADM, adrenomedullin; ARDS, acute respiratory distress syndrome; AUC, areas under the curve; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; MR-proADM, mid-regional-proAdrenomedullin; PCT, procalcitonin; ROC, receiver operating characteristic; SARS-CoV-2, severe acute respiratory distress syndrome coronavirus 2; SOFA, sequential organ failure assessment.

TABLE 1 Normal range and methodology for biomarkers assessment.

Biomarker	Sample	Methodology	Sex	Normal range values	Unit
hs Troponin I	Plasma	Chemiluminescence	Female	0–15.6	pg/mL
hs Troponin I	Plasma	Chemiluminescence	Male	0–34.2	pg/mL
MR-proAdrenomedullin	Plasma	TRACE* assay		0.00–0.50	nmol/L
C-reactive protein	Plasma	Turbidimetric	Female/Male	≤0.5	mg/dL
Ferritin	Serum	Chemiluminescence	Female	4.63–204	ng/mL
Ferritin	Serum	Chemiluminescence	Male	21.81–264.66	ng/mL
Procalcitonin	Plasma	TRACE* assay		0.00–0.50	ng/mL
Neutrophils-absolute value	Whole blood	Flow cytometric		2.00–7.00	$\times 10^3/\mu L$
Lymphocytes-absolute value	Whole blood	Flow cytometric		1.00–3.00	$\times 10^3/\mu L$

*TRACE, time-resolved amplified cryptate emission technology assay.

TABLE 2 Characteristics of patients.

Variable	Overall population (*n* = 161)	Myocardial injury (*n* = 58)	Absence of myocardial injury (*n* = 103)	*P-value*
Age [years (IQR)]	73 (62–81)	79 (73–83)	67 (58–80)	<0.001
Male sex [*n* (%)]	99 (61.5)	32 (55.2)	67 (65.0)	0.216
Cardiovascular risk factors [*n* (%)]				
Diabetes mellitus	48 (29.8)	20 (34.4)	28 (27.2)	0.331
Hypertension	106 (65.8)	44 (75.9)	62 (60.2)	0.044
Dyslipidemia	61 (37.9)	26 (44.8)	35 (34.0)	0.173
Smoking habit	26 (16.1)	12 (20.6)	14 (13.6)	0.240
BMI > 30 kg/m^2	25 (15.5)	13 (22.4)	12 (11.7)	0.070
Coronary artery disease [*n* (%)]	34 (21.1)	18 (31.0)	16 (15.5)	0.021
Chronic pulmonary disease [*n* (%)]	30 (18.6)	16 (27.6)	14 (13.6)	0.029
Chronic kidney disease [*n* (%)]	27 (16.8)	16 (27.9)	11 (10.7)	0.006
Chronic liver disease [*n* (%)]	9 (5.6)	2 (3.4)	7 (6.8)	0.375
Active cancer [*n* (%)]	24 (14.9)	9 (15.5)	15 (14.6)	0.870
Laboratory [median (IQR)]				
hs Troponin I [ng/l]	11 (10–49)	83 (42–226)	10 (10–10)	<0.001
MR-proADM [nmol/l]	1.12 (0.78–1.91)	1.90 (1.24–3.83)	0.88 (0.66–1.29)	<0.001
CRP [mg/dl]	6.6 (2.3–11.9)	10.9 (6.8–15.7)	3.6 (1.4–8.0)	<0.001
Ferritin [ng/ml]	802 (279–1540)	1403 (621–2230)	635 (247–1299)	<0.001
PCT [ng/ml]	0.07 (0.04–0.37)	0.21 (0.07–0.83)	0.06 (0.03–0.10)	<0.001
Leukocytes [unit/μl]	9800 (7120–12300)	12275 (8440–16040)	9080 (6470–11420)	<0.001
Neutrophils [unit/μl]	8230 (5280–10920)	10275 (7110–13960)	6910 (4510–9490)	<0.001
Lymphocytes [unit/μl]	930 (560–1460)	765 (430–1110)	1020 (610–1560)	0.013
Neutrophil/Lymphocyte ratio	9.68 (4.22–15.58)	12.97 (7.00–26.50)	7.98 (3.33–13.07)	<0.001
PaO2/FiO2	216 (108–327)	145 (85–281)	252 (130–357)	<0.001
ICU admission [*n* (%)]	41 (25.5)	17 (29.3)	24 (23.3)	0.401
Median hospital stay [days (IQR)]	14 (7–23)	15 (8–26)	12 (7–21)	0.387
ARDS [*n* (%)]	121 (75.2)	50 (86.2)	71 (68.9)	0.015

Rome University were included between October 2020 and March 2021 and were retrospectively analyzed. The COVID-19 Center includes the Medicine Department with a sub-intensive care unit.

We included all patients with a positive reverse transcription polymerase chain reaction test (RT-PCR) for SARS-CoV-2, with hs Troponin I and MR-proADM assessment. We excluded patients < 18 years old and pregnant women.

The study was approved by the Ethical Committee of the University Campus Bio-Medico of Rome.

All methods were performed in accordance with the relevant guidelines and regulations available at that moment.

The control group consisted of patients with SARS-CoV-2 infection without increased hs Troponin I or acute coronary syndrome (ACS), pericarditis, or myocarditis admitted to the COVID-19 center in the same period.

Clinical outcomes and definitions

Primary outcome of the study was 30-day mortality. Secondary outcomes were the comparison of MR-proADM, CRP, ferritin, and PCT as diagnostic and prognostic biomarkers of myocardial injury. Additionally, we analyzed the development of ARDS, the need for ICU transfer, and length of stay (LOS).

Myocardial injury was defined by the rise and/or fall of cardiac hs Troponin I values with at least one value above the 99th percentile of the upper reference limit; ARDS was defined according to the Berlin definition (9, 10, 23).

The following data were collected at inclusion: demographic characteristics (age and gender), onset symptoms, relevant comorbidities, immune status (active malignancy or other causes of immunosuppression), concomitant antimicrobial, use of antiretroviral medication, immunosuppressive treatments, and clinical presentation.

All patients received a complete physical examination including body temperature, blood pressure, heart and respiratory rate, cardiac, pulmonary, abdominal, and neurological evaluation, electrocardiogram, and chest tomography, while an echocardiogram was performed only if clinically needed.

Laboratory tests performed at inclusion were complete blood count (CBC), hs Troponin I, MR-proADM, CRP, ferritin, PCT, D-Dimer, INR, TTPA, liver function test, creatinine, arterial blood gases, and serum lactate.

All patients received standard of care based on disease severity and need for oxygen support. When needed, patients received low-molecular weight heparin, remdesivir, and steroid therapy.

All included patients were followed until death or 30-day follow-up, whichever came first.

Laboratory markers

Diagnosis of COVID-19 was performed by molecular testing through RT-PCR on a nasopharyngeal swab and/or endotracheal aspirate, detecting spike three SARS-CoV-2 genes (S, N, and E or S, RdRP, and N genes) (4).

Myocardial injury was considered when hs Troponin I was ≥15.6 ng/L in women and ≥34.2 ng/L in men.

Ferritin, hs Troponin I, and CRP were measured by Alinity c (Abbott, diagnostics) following the manufacturer's instruction. Normal ranges are shown in **Table 1**. CBC was performed on a whole blood sample by Sysmex XE-9000 (Dasit, Italy) following the manufacturer's instruction. NLR was calculated by the ratio between absolute values of neutrophils and lymphocytes. MR-proADM and PCT plasma concentrations were measured on an automated Kryptor analyzer, using a time-resolved amplified cryptate emission (TRACE) technology assay (Kryptor PCT; Brahms AG; Hennigsdorf, Germany), with commercially available immunoluminometric assays (Brahms) (24–27).

Statistical analysis

As appropriate, continuous variables were reported as mean (standard deviation) or as median (interquartile range). Categorical variables were reported as frequencies and percentages. Comparisons between continuous variables were performed using Student's *t*-test or the Mann-Whitney *U*-test. Comparison between categorical variables was evaluated using the Fisher exact test or the Pearson chi-square test, as appropriate. The normal distribution of continuous variables was tested with the Shapiro-Wilk test. Correlation between continuous variables was assessed using the Spearman rank test. A receiver operating characteristic (ROC) curve analysis was used to test the ability of laboratory values to discriminate between patients with and without myocardial injury and patients who died and did not during the hospital stay. The optimal cutoff point was calculated by determining the value that provided the greatest sum of sensitivity and specificity. All baseline clinical features were evaluated in univariate analysis for the association with myocardial injury and death using logistic regression. Only variables with a p-value < 0.05 were considered for the final multivariable logistic regression models, providing odds ratios (ORs) and 95% confidence intervals (CI). Statistical analysis was performed using Stata/IC version 14.0 (STATA Corp., College Station, TX, USA), and p-values < 0.05 (2-tailed) were considered significant.

Results

Study population

A total of 161 patients were included in this study. Of these, 58/161 (36%) presented myocardial injury at admission. The characteristics of the patients are shown in **Table 2**. A total of 99/161 (61.5%) patients were males. Among them, 32/99 (32%) developed myocardial injury ($p = 0.21$).

Patients with myocardial injury were significantly older (79 [IQR = 73–83] vs. 67 [IQR = 58–80] years old, $p < 0.001$) than those without myocardial injury. These patients had a more frequent history of Hypertension (75.9 vs. 60.2%, $p = 0.044$), coronary artery disease (31 vs. 15.5%, $p = 0.021$),

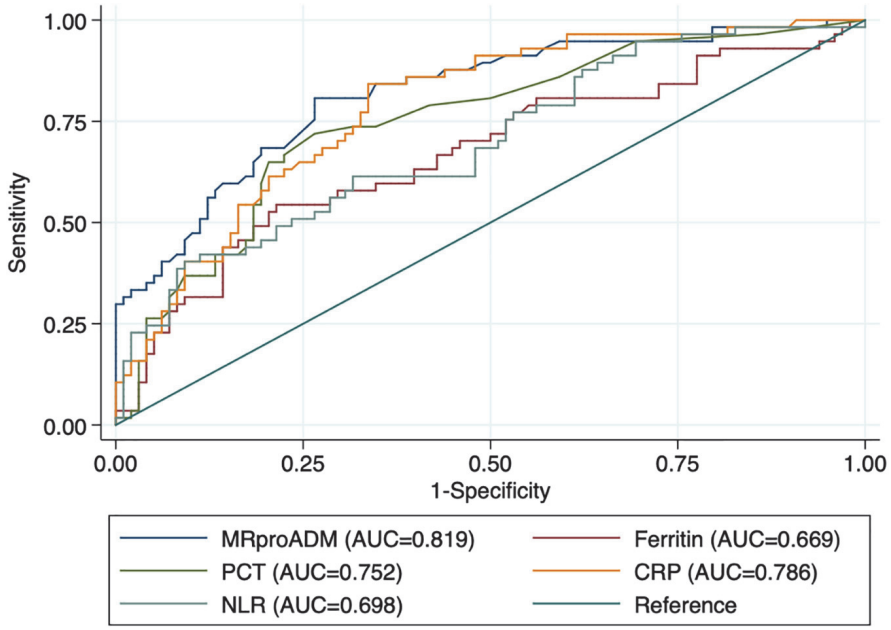

FIGURE 1
Receiver operating characteristic curves (ROC) of biomarkers in patients with myocardial injury.

chronic pulmonary disease (27.6 vs. 13.6%, $p = 0.029$), and chronic kidney disease (27.9 vs. 10.7%, $p = 0.006$). Among the patients with myocardial injury, 3/58 (5.17%) had acute coronary syndrome.

In the overall population, 41/161 patients (25.5%) were admitted to the intensive care unit during the hospitalization, and the median hospital stay was 14 (7–23 IQR) days. A total of 121/161 patients (75.2%) developed ARDS, which was significantly more frequent among patients with myocardial injury (86.2 vs. 68.9%, $p = 0.015$). In-hospital mortality was 21% (34/161 of the overall population) and 46.6% (27/58 of patients with myocardial injury).

Myocardial injury

A significant correlation was found between hs Troponin I and MR-proADM levels (Spearman $r = 0.569$, $p < 0.001$). An MR-proADM value $\geq$ 1.19 nmol/L was defined as the optimal cut-off to identify patients with myocardial injury. This cut-off had 81.0% of sensitivity and 73.5% of specificity.

As reported in Table 2, patients with myocardial injury at admission showed significantly higher values of MR-proADM, CRP, ferritin, PCT, and neutrophil/lymphocyte ratio. At ROC curve analysis, all laboratory markers were able to discriminate between patients with and without myocardial injury (Figure 1 and Table 3). However, MR-proADM showed the greatest area under the curve ([AUC] 0.818, 95% CI = 0.750–0.875; $p < 0.001$). Pairwise comparison showed that the AUC of MR-proADM was significantly greater than the AUC of ferritin ($p = 0.010$) and neutrophil/lymphocyte ratio (0.021), but similar to that of CRP and PCT.

Predictors of myocardial injury

At univariate analysis (Table 4), age, hypertension, a history of coronary artery disease, chronic pulmonary disease, chronic kidney disease, and MR-proADM $\geq$ 1.19 nmol/L were significantly associated with an increased risk of myocardial injury. In the multivariate analysis (Table 4), an MR-proADM value of $\geq$1.19 nmol/L was an independent predictor of increased risk of myocardial injury (OR = 7.25, 95% CI = 2.93–17.9, $p < 0.001$).

Predictors of death

Overall, 30-day death occurred in 34 (21.1%) patients and was significantly more frequent among those with myocardial injury (46.6 vs. 6.8%, $p < 0.001$). In the ROC curve analysis, MR-proADM was able to discriminate between patients who died and those who did not (AUC = 0.822, 95% CI = 0.751–0.877; $p < 0.001$; optimal cut-off $\geq$ 1.19 nmol/L). Among patients with MR-proADM values $\geq$ 1.19 nmol/L ($n = 72$), the incidence of death was significantly higher compared with those patients with low MR-proADM values (40.3 vs. 5.9%, $p < 0.001$). Also,

TABLE 3 Receiver operating characteristic (ROC) curves of laboratory markers for myocardial damage and pairwise comparison between mid-regional proAdrenomedullin (MR-proADM), C-reactive protein, ferritin, procalcitonin, and neutrophil/lymphocyte ratio.

	AUC	95% CI	*P-value* (vs MR-proADM)	Optimal cut-off
MR-proADM	0.818	0.750–0.875	–	1.19 nmol/L
CRP	0.786	0.713–0.858	0.386	5.67 mg/dL
Ferritin	0.669	0.578–0.761	0.010	1,403 ng/mL
PCT	0.752	0.672–0.832	0.131	0.1 ng/mL
Neutrophil/Lymphocyte ratio	0.698	0.611–0.783	0.021	12.67

TABLE 4 Logistic regression analysis for myocardial injury.

	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P-value*	OR	95%CI	*P-value*
Age	1.06	1.03–1.10	< 0.001	1.03	0.99–1.07	0.123
Hypertension	2.08	1.01–4.27	0.046	1.07	0.42–2.76	0.882
Coronary artery disease	2.45	1.13–5.29	0.023	1.18	0.45–3.10	0.743
Chronic kidney disease	3.19	1.36–7.45	0.008	1.28	0.47–3.51	0.631
Chronic pulmonary disease	2.42	1.08–5.42	0.031	1.54	0.56–4.26	0.400
MR-proADM ≥ 1.19 nmol/l	11.4	5.21–25.14	< 0.001	7.25	2.93–17.9	< 0.001

OR, odds ratio; CI, confidence interval.

when only considering patients with myocardial injury, MR-proADM was able to discriminate between patients who died and those who did not (AUC = 0.690, 95% CI = 0.551–0.820; p = 0.007; optimal cut-off ≥ 4.01 nmol/L). This cut-off had 40.7% of sensitivity and 89.7% of specificity.

When dividing the entire study population in four groups based on the presence of myocardial injury and MR-proADM values, 75 patients (46.6%) had no myocardial injury and MR-proADM < 1.19 nmol/L, 11 patients (6.8%) had a myocardial injury and MR-proADM < 1.19 nmol/L, 28 patients (17.4%) had no myocardial injury and MR-proADM ≥ 1.19 nmol/L, and 47 patients (29.2%) had a myocardial injury and MR-proADM ≥ 1.19 nmol/L. Death occurred in 3/75 (4.0%), 2/11 (18.2%), 4/28 (14.2%), 25/47 (53.2%), respectively (p < 0.001; Figure 2).

In the univariate analysis (Table 5), age, ARDS, myocardial injury, MR-proADM values of ≥1.19 nmol/L, and the combination of myocardial injury and MR-proADM values of ≥1.19 nmol/L were significantly associated with an increased risk of death.

When myocardial injury and MR-proADM values of ≥1.19 nmol/L were entered separately in the same multivariate model, myocardial injury (OR = 4.87, 95% CI = 1.63–14.52, p = 0.005) and ARDS (OR = 10.58, 95% CI = 1.26–88.95, p = 0.030) were independent predictors of increased risk of death (Table 5). In a separate model, the combination of myocardial injury and MR-proADM values ≥ 1.19 nmol/L was an independent predictor of death with an OR of 7.82 (95% CI = 2.87–21.30, p < 0.001; Table 5).

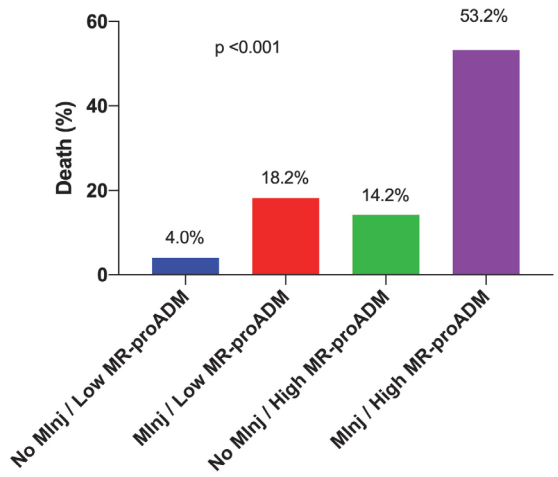

FIGURE 2
Incidence of in-hospital death according to the presence of myocardial injury and mid-regional proAdrenomedullin (MR-proADM) values of ≥1.19 nmol/L.

Discussion

The epidemiological data on myocardial injury in the literature is discordant. In the same way, the pathophysiological mechanism of myocardial injury onset is still unclear.

Consistent with the published data, 36% of the study population developed myocardial injury (11, 12). Among this

TABLE 5 Logistic regression analysis for death.

	Univariate analysis			Multivariate analysis			Multivariate analysis		
	OR	**95%CI**	***P-value***	**OR**	**95%CI**	***P-value***	**OR**	**95%CI**	***P-value***
Age	1.06	1.02–1.10	0.003	1.03	0.98–1.08	0.317	1.04	0.99–1.09	0.122
ARDS	14.93	1.97–113.21	0.009	10.58	1.26–88.95	0.030	10.74	1.27–91.10	0.029
Myocardial injury	12.50	4.93–31.68	< 0.001	4.87	1.63–14.52	0.005			
MR-proADM ≥ 1.19 nmol/L	10.79	3.90–29.89	< 0.001	3.39	0.96–12.02	0.058			
MInj/MR-proADM ≥ 1.19 nmol/L	14.31	5.82–35.18	< 0.001				7.82	2.87–21.30	< 0.001

OR, odds ratio; CI, confidence interval; MInj, myocardial Injury.

group of patients, 27/58 (46.6%) died, in comparison with the 60% described in the literature (11, 12).

The mortality of patients with myocardial injury with both elevated values of hs Troponin I and MR-proADM ≥ 1.19 nmol/L reached 53.2% vs. mortality of 14.8% in the case of the elevated value of hs Troponin I only. Furthermore, the elevation of both biomarkers allowed the identification of patients with myocardial injury at higher mortality risk. In fact, if they were both negative the mortality was only 4%, but if both of them were positive, the mortality reached 53.2%.

These results agree with previous reports, where MR-proADM ≥ 2 nmol/L identified those patients affected by moderate/severe COVID-19 with high mortality risk related to multiple organ dysfunction syndrome, while values ≥3 nmol/L were predictive for ARDS development (4).

While an MR-proADM value of ≥1.19 nmol/L allows identifying patients with myocardial injury with high sensitivity and specificity, an MR-proADM value of ≥4.01 nmol/L allows identifying patients with myocardial injury at high risk of death with high specificity.

Therefore, the dosage of MR-proADM allows stratifying patients with myocardial injury at high risk of death by identifying patients who may also benefit from therapy with adrecizumab.

The median value of hs Troponin I in case of myocardial injury resulted in 83 vs. 11 ng/L of the overall population. Some studies had reported an optimal cut-off of 17 ng/L for Troponin T to predict mortality and of 0.03 μg/L for Troponin I in COVID-19 patients with cardiovascular disease (15, 28). These data suggest a role of hs Troponin I, not only as a marker of ischemia but also as a relevant biomarker of global stress for myocardial injury. In this way, hs Troponin I could be used to guide the prognosis and clinical management of the patients.

Our study shows that MR-proADM ≥ 1.19 nmol/L expresses myocardial injury with high diagnostic accuracy (sensitivity 81% and specificity 73.5%) when compared to ferritin and NLR ratio.

Of all bio-markers, MR-proADM was found to be the most specific of myocardial injury and SARS-CoV-2-related mortality.

MR-proADM ≥ 1.19 nmol/L has been shown to be an independent predictor of increased risk of myocardial injury and it has been significantly associated with risk factors of myocardial injury such as age, hypertension, history of coronary artery disease, and chronic pulmonary or kidney disease.

Age ≥ 65 years, male sex, and multicomorbidities increase the possibilities for developing severe SARS-CoV-2 infection, while pre-existing cardiovascular diseases, such as hypertension, diabetes mellitus, coronary artery disease, and heart failure, are associated with a worse prognosis (10, 14, 29–31).

Myocardial injury and MR-proADM ≥ 1.19 nmol/L were independent predictors of death ($p < 0.001$).

According to the literature, myocardial injury was also a predictor of in-hospital mortality.

Also, considering that acute cardiac injury in patients who died of COVID-19 has been reported in 35%, with detection of SARS-CoV-2 within the myocardium in 47% of post-mortem studied hearts (2, 5–7). Furthermore, one-third of severely ill COVID-19 patients develop acute kidney failure. Many of them require hemodyalitic procedures. This complication could weaken the diagnostic accuracy of Troponin value in the assessment of cardiac injury (10). It would be desirable to evaluate in further studies the combined dosage of hs Troponin I and MR-proADM, which could allow us to estimate with greater accuracy the real incidence of myocardial injury also in the absence of chest pain, troponin assessment, or evaluation of myocardial contractility.

The study has the limitation of being a single-center study and therefore the data obtained should be further confirmed by multicentric studies.

To our knowledge, this is one of the few studies that focused on the correlation between myocardial injury and MR-proADM. Values of MR-proADM ≥ 1.19 nmol/L correlate with myocardial injury and widespread endothelitis severity.

A myocardial injury might occur during SARS-CoV-2 infection as a consequence of myocardial, pulmonary, and endothelial damage. The mechanisms involved are represented by hypoxia that induces a decreased oxygen supply to the heart, causing modest or massive elevation of Troponin concentration, which is not necessarily correlated with deterioration of systolic

left ventricular function but could be associated with right ventricular dysfunction due to acute right ventricular overload secondary to parenchymal or vascular lung disease resulting in subendocardial damage of the right ventricular myocardium in 19% of cases and by cytokine-induced injury (10, 15, 28, 32–35).

Adrenomedullin (ADM) is a protein that is released by endothelial and vascular smooth muscle cells following volume overload with the aim to preserve the endothelial barrier function. It binds to receptors prevalently found in cardiovascular and pulmonary systems (36–38). ADM induces vasodilatation, with consequent blood flow increase by reducing vasoconstriction acting as an inhibitor of the renin–angiotensin–aldosterone system (RAS). Furthermore, ADM contributes to endothelial integrity decreasing vascular permeability and acts as a bronchodilator.

Hypoxia, inflammatory cytokines, bacterial or viral products, shear stress, and vascular leakage represent stimuli for ADM up-regulation as it happens during SARS-CoV-2 infection, contributing to the failure of the ADM regulation (4, 39–41).

Disruption of the ADM system leads to (1) decrease of vascular resistance and capacitance vessels determining blood flow increase with hypoxic cardiac ischemia. (2) RAS activity reduces vasoconstriction, which leads to vascular leakage, increasing inflammation, and activation of the coagulation cascade. Additionally, RAS activation increases edema, oxidation, proliferation, and fibrosis, resulting in hypoxic cardiac ischemia and diffuse endothelitis that can lead to multiorgan failure (4, 42–50).

The mid-regional proAdrenomedullin (MR-proADM) is a peptide derived from ADM in a 1:1 ratio that can be used as a biomarker of organ failure, disease severity, and mortality in patients with COVID-19 (4, 51).

The alterations in endothelial cell lining are adaptive or maladaptive depending on disease extension, the time elapsed from disease onset, long-lasting viral shedding, and the host's genetic heritage that expresses more or less ADM receptors, determining the extent of the immune-metabolic-inflammatory response. Instead, SARS-CoV-2 loads or variants have not so far indicated to influence the extent of organ damage (1, 52, 53).

Therefore, the role of ADM in COVID-19-related organ damage might suggest the use of new therapeutic agents, such as monoclonal antibodies. Adrecizumab, a humanized, monoclonal, non-neutralizing ADM-binding antibody could be used to improve vascular integrity, tissue congestion, and thereby clinical outcomes (18, 19).

Furthermore, the high incidence of myocardial injury caused by SARS-CoV-2 corresponds to that observed in other viral infections, such as Influenza, in which myocardial damage was detected as asymptomatic cardiac involvement in 0–53% of cases, with the presence of electrocardiogram alterations on roughly 50% of patients or highlighted post-mortem by the presence of myocarditis, pericarditis or acute coronary syndrome (14, 15, 54–58). Viral infections, indeed, can determine endothelial dysfunction up to apoptosis rousing coronary vasoconstriction and procoagulant state causing activation of plaque to hemodynamic instability (59).

Vaccination represents the best preventive method for both adults and children with effectiveness rates of 65–95 vs. 50–60% for Influenza, respectively, mostly in high-risk patients (>65 years, young children, presence of comorbidities, and immunocompromised patients), and it could be useful to prevent cardiovascular damage reducing mortality (59–65).

Conclusion

Myocardial injury induced by SARS-CoV-2 is relevant.

The elevation of hs Troponin I and MR-proADM allows the identification of patients with myocardial injury at higher mortality risk.

An MR-proADM value of ≥1.19 nmol/L identifies patients with myocardial injury, and a MR-proADM value of ≥4.01 nmol/L identifies patients with myocardial injury at high risk of death.

Therefore, the dosage of MR-proADM allows stratifying patients with myocardial injury at high risk of death to offer the best management to critically ill COVID-19 patients.

Data availability statement

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 the Ethical Committee of the University Campus Bio-Medico of Rome. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

SS led the study design, data collection, data analysis, data interpretation, and manuscript writing. FM, GD'A, and DL assisted with data collection and analysis of the validation dataset. FM performed the statistical

analysis. FS assisted the patients. MF, LL, DL, and GB assisted with computer queries, data analysis, and manuscript preparation. CB, AA, JM, and RM assisted with data collection and analysis of the development dataset as well as study design, data interpretation, and manuscript writing. CB, JM, AA, SC, and SA assisted with chart review, data analysis, and supervised all aspects of the investigation, as well as assisting with study design, data interpretation, and manuscript writing. All authors contributed to manuscript revision, read, 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.

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Circulating tissue inhibitor of metalloproteinases 1 (TIMP-1) at COVID-19 onset predicts severity status

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Background: Systemic biomarkers for severity of SARS-CoV-2 infection are of great interest. In this study, we evaluated a set of collagen metabolites and extracellular matrix remodeling biomarkers including procollagen type III amino terminal propeptide (PIIINP), tissue inhibitor of metalloproteinases 1 (TIMP-1) and hyaluronic acid (HA) as prognostic indicators in COVID-19 patients.

Methods: Ninety COVID-19 patients with the absence of chronic liver diseases were enrolled. Serum PIIINP, TIMP-1, and HA were measured and correlated with inflammatory indices and clinical variables. Patients were stratified for disease severity according to WHO criteria in two groups, based on the requirement of oxygen support.

Results: Serum TIMP-1, but not PIIINP and HA was significantly higher in patients with WHO score ≥5 compared to patients with WHO score <5 [PIIINP: 7.2 (5.4–9.5) vs. 7.1 (4.5–9.9), p = 0.782; TIMP-1: 298.1 (20.5–460) vs. 222.2 (28.5–452.8), p = 0.01; HA: 117.1 (55.4–193.7) vs. 75.1 (36.9–141.8), p = 0.258]. TIMP-1 showed moderate correlation with CRP (r = 0.312, p = 0.003) and with LDH (r = 0.263, p = 0.009). CRP and serum LDH levels were significantly higher in COVID-19 patients with WHO score ≥5 compared to the group of patients with WHO score < 5 [15.8 (9–44.5) vs. 9.3 (3.4–33.8), p = 0.039 and 373 (282–465) vs. 289 (218–383), p = 0.013, respectively].

Conclusion: In patients with COVID-19, circulating TIMP-1 was associated with disease severity and with systemic inflammatory index, suggesting that

TIMP-1 could represent a promising non-invasive prognostic biomarker in COVID-19 patients. Interestingly, our results prompted that serum TIMP-1 level may potentially be used to select the patients for therapeutic approaches targeting matrix metalloproteases pathway.

KEYWORDS

COVID-19, fibrosis, TIMP-1, collagen metabolites, extrcellular matrix remodelling biomarkers

Introduction

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the infective agent responsible for Coronavirus Disease 2019 (COVID-19). SARS-CoV-2 stimulates the immune system leading to cytokine storm (1) with markedly increased levels of several cytokines as IL–1α, IL-1β, IL-6, and TNF-α (2). In addition, an increase of neutrophils count and decreased count of lymphocytes have been observed (3). COVID-19 infection also leads to ROS generation (4) and coagulation cascade favoring the risk of thrombosis (5). Some subjects infected by SARS-CoV-2 developed a broad range of pathologies including not only pneumonia, acute respiratory distress syndrome (ARDS), respiratory failure but also systemic inflammation and multiorgan failure (1). Severe COVID-19 was associated with massive alveolar damage with loss of lung architecture, leading to ventilatory failure. A recently published article (6) reported two types of lung fibrosis after COVID-19. The first with a diffuse fibrotic alveolar damage is characterized by extracellular matrix deposition resulting in fibrosis; these patients require intubation, mechanical ventilation and/or extracorporeal membrane oxygenation (ECMO). The second is the post-COVID pulmonary fibrosis, diagnosed by the combination of clinical, radiological, and pathological information. In about 25% of patients with severe COVID-19 disease (WHO Severity Grade 3 and 4), a restrictive ventilatory defect was revealed. Thus, there is a compelling clinical need to identify circulating fibrosis markers in COVID-19. Ideally, these markers should be non-invasive, able to mirror the extent of fibrosis and to reflect disease progression and therapeutic response. SARS coronavirus induced up-regulation of Type I collagen, leading to pulmonary pro-fibrotic responses (7). Thus, collagen metabolism plays a key role in COVID-19 clinical picture. Several blood parameters have been evaluated as predictors of COVID-19 severity. However, at present, still no validated biomarkers are reliably used in routine clinical practice.

Procollagen type III amino terminal propeptide is the peptide released during the biosynthesis and depositing of type III collagen (8). TIMP-1 is an inhibitor specific for extracellular matrix (ECM) degradation enzymes (9). HA is a glycosaminoglycan engaged in the formation of ECM (10).

Elevated serum levels of PIIINP, HA or TIMP-1 were found to be increased in other diseases, such as in patients with systemic sclerosis (SSc) (11). High levels of PIIINP and HA were demonstrated to be unfavorable predictors for survival in SSc suggesting that these markers could be useful to predict other fibrotic lesions (12).

In this study we investigated the potential role of PIIINP, HA and TIMP-1 as prognostic markers in COVID-19 patients.

Materials and methods

Patients

We enrolled 90 adult hospitalized patients with a diagnosis of SARS-CoV-2 infection, confirmed by molecular analysis (RT-PCR) of the nasopharyngeal swab (13).

Patients were stratified for COVID-19 disease severity based on WHO scale (14). According to this classification patients were classified as: (1), asymptomatic, not hospitalized (2), symptomatic, not hospitalized, independent; (3), symptomatic, not hospitalized, assistance needed; (4), hospitalized, not requiring supplemental oxygen; (5), hospitalized, requiring oxygen by non-invasive mechanical ventilation (mask or nasal prongs); (6–9), hospitalized, requiring high-flow oxygenation and/or invasive mechanical ventilation; and 10, death.

Our study population was divided according to the severity of COVID-19 at the time of sampling into the following groups: (1) hospitalized COVID-19-positive patients requiring no respiratory support or oxygen support only (WHO $\leq$5); (2) hospitalized COVID-19-positive patients requiring invasive or non-invasive mechanical ventilation (WHO $>$ 5).

The study was conducted in compliance with the Declaration of Helsinki. The protocol was approved by the Ethical Committee of the University Federico II of Naples (prot. no. 140/20). Informed consent was obtained from all individuals. At the time of sampling, laboratory parameters, clinical and demographic data were recorded.

Biomarkers

Fasting blood samples were obtained. Sera were frozen and stored at −80°C until measurements. Samples were assayed in an automated analyzer that performs magnetic separation enzyme immunoassay tests (ADVIA Centaur; Siemens Healthcare Diagnostics, Tarrytown, NY, United States) for Hyaluronic acid (HA), amino-terminal propeptide of type-III-procollagen (PIIINP) and tissue inhibitor of metalloproteinase type-1 (TIMP-1).

Statistical analysis

All statistical analyses were performed using the R platform version 4.1.2. Standard descriptive statistics were used to describe the cohort: mean ± standard deviation (range) or median (25th; 75th percentile) (range) in case of numerical variables and absolute frequency with percentages for categorical factors. Accordingly, between-group comparisons were assessed using the *t*-test for independent samples, the Mann-Whitney U-test and the Chi-square test (or the Fisher exact test when appropriate). Median regression with bootstrapped standard errors was used to adjust the analysis for potential confounding factors.

Results

A total of 90 COVID-19 patients (43 female and 47 male) were enrolled and classified for disease severity based on World Health Organization (WHO) stage. 68 (75.6%) COVID-19 patients with a WHO score ≤5 and 22 (24.4%) with a WHO score >5. Demographic and clinical features are showed in **Table 1**. Mean age was 58.6 ± 15.4 (range: 38–62) years, patients with WHO score >5 were significantly older than patients with WHO score ≤5 (p = 0.013); no differences in comorbidities at baseline were observed between the two groups. Median disease duration (time length to negativization) was 23 days (range 5–72 days) days with a longer disease duration in patients with a WHO score >5. In the overall cohort, 37 (41.6%) patients had a time length of negativization ≤21 days and 52 (58.4%) >21 days.

Patients were classified also for High-Resolution Computed Tomography (HRCT) score, resulting in 54 subjects (60%) with a score ≤10 and 36 (40%) >10. Of note, lymphocyte number was significantly lower in patients with HRCT score >10 [670 (270–2540) vs. 880 (260–3350); p = 0.026).

Serum TIMP-1 levels were significantly higher in patients with WHO score >5 than in those with a WHO score ≤5 [TIMP-1: 222.2 (20.5–460) vs. 298.1 (28.5–452.8), p = 0.010] and the difference was confirmed after adjusting the analysis for the age of patients through median regression (p = 0.003). On the contrary, no statistically significant difference was observed in serum PIIINP and HA between patients with severe and mild disease [PIIINP: 7.2 (1.1–18.4) vs. 7.1 (1.2–47.5), p = 0.782; HA: 117.1(4.7–331.1) vs. 75.1 (8.3–1345.9), p = 0.258; **Figure 1**].

As shown in **Table 2**, LDH and CRP values were significantly higher in patients with severe disease [LDH: 373 (193–670) vs. 289 (111–741), p = 0.013; CRP: 15.8 (1.3–222.5) vs. 9.3 (0.3–132.6), p = 0.039]. **Table 3** showed that serum PIIINP, HA and TIMP-1 positively correlated with LDH levels (PIIINP: r = 0.264, p = 0.009, HA: r = 0.267, p = 0.008; TIMP-1: r = 0.263, p = 0.009).

Serum PIIINP and HA negatively correlated with albumin values (PIIINP: r = −0.362, $p < 0.001$; HA: r = −0.387, $p < 0.001$); circulating TIMP-1 levels positively correlated with CRP values (r = 0.312, p = 0.003).

Discussion

Our study highlighted the significant positive correlation between changes of TIMP-1 and disease severity based on WHO classification, suggesting that TIMP-1 could serve as a non-invasive biomarker for prognosis in COVID-19.

Metzemaekers et al. (15) reported significantly higher levels of plasmatic tissue inhibitor of metalloproteinase 1 (TIMP-1) and of TIMP-1/MMP-9 complexes and significantly lower circulating total MMP activity in COVID-19 patients at intensive care unit (ICU) admission.

Our data showed that serum TIMP-1 in SARS-CoV-2 infected patients correlates with the WHO score and the CRP values, but not with HRCT score and time length of negativization.

These findings may reflect peculiar aspect of the involvement of TIMP-1 in the fibrotic process: TIMP-1 represent decreased collagen degradation and was a strong predictor of early fibrosis (16).

Considering the short disease duration and moderate disease severity of most of our study population, our data indicated that TIMP-1 could be a useful marker of fibrotic burden and disease prognosis in patients with COVID-19 at initial diagnosis. Several molecular mechanisms involving matrix metalloproteases pathway have been identified as relevant players in the clinical picture of COVID-19 (17). It has been recently shown that matrix metalloproteinase-9 (MMP-9) gene expression is increased in subjects infected with SARS-CoV-2 (18) and circulating MMP-9 levels were significantly associated with the risk of respiratory insufficiency (19) and with severity in COVID-19 patients (20). In fact, some authors previously demonstrated that metalloproteinases (MMPs) seem to play a key role in lung disease (21, 22). Severe COVID-19 shared many characteristics with sepsis (23) and plasma MMP-9 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) have been also proposed as septic biomarkers (24, 25).

Other authors showed that periodontitis and diabetes have been associated with COVID-19 poor outcomes and both these

TABLE 1 Clinical and demographical characteristics of the study cohort stratified according to the World Health Organization (WHO) score at baseline.

	Overall (n = 90)	WHO score ≤5 (n = 68; 75.6%)	WHO score >5 (n = 22; 24.4%)	P-value
Age (years)	58.6 + −15.4 (20.6–93.9)	57.3 + −16.1 (20.6–86.9)	65.8 + −12.6 (39.3–93.9)	**0,013**
COPD	8 (11.9)	5 (10)	3 (17.6)	0,684
Diabetes	17 (25.4)	12 (24)	5 (29.4)	0,749
Hypertension	17 (25.4)	12 (24)	5 (29.4)	0,749
Arhythmias	3 (4.5)	2 (4)	1 (5.9)	1
Time to negativization; days	23 (19; 33) (5–72)	22 (18; 31) (5–72)	30 (21; 41) (9–51)	**0,045**
HRCT score				0,176
≤10	54 (60)	44 (64.7)	10 (45.5)	–
>10	36 (40)	24 (35.3)	12 (54.5)	–

Data are expressed as mean ± standard deviation (range); median (25th; 75th percentile) (range) or absolute frequency (percentage). COPD, chronic obstructive pulmonary disease; HRCT, High Resolution Computed Tomography. Bold values indicate the strong correlation.

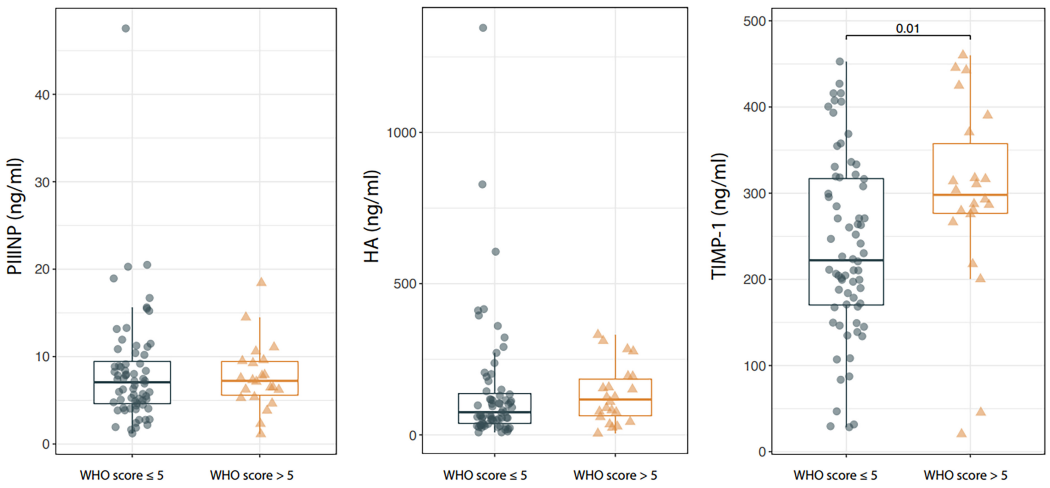

FIGURE 1
Box-plot showing serum procollagen type III amino terminal propeptide (PIIINP), Hyaluronic acid (HA), and tissue inhibitor of metalloproteinases 1 (TIMP-1) levels in patients with Coronavirus Disease 2019 (COVID-19) stratified according to the WHO score at baseline. Boxes are defined by Q1, Median (bold line) and Q3. Whiskers reach the minimum and the maximum of the distribution except for the presence of outliers, defined as data points below Q1−1.5*IQR or above Q3 + 1.5*IQR. To avoid overlapping a small amount of horizontal jitter was added. Q1, First quartile; Q3, Third quartile; IQR = Q3−Q1.

diseases have been correlated with elevated MMP-8 levels (26, 27), further highlighting the role of MMPs as key players in COVID-19 risk and escalation.

Tissue damage during SARS-CoV-2 lung infection is associated with activation of members of the MMPs family (28, 29). Targeting MMPs pathway has been proposed as therapeutic strategy to counterbalance the host marked pro-inflammatory response to the SARS-CoV-2 infection (30). In addition of being MMP-inhibitor, TIMP-1 is independently proinflammatory and pro-growth-factor (31–33). Thus, the measurement of circulating TIMP-1 levels could be useful to assess the prognosis and to adopt a personalized treatment approach.

Serum PIIINP, TIMP-1, and HA are combined to calculate the Enhanced Liver Fibrosis (ELF) score, initially developed from a chronic liver disease cohort (34–36). Thus, it was expected that the algorithm was not readily applicable to COVID-19. Nevertheless, our results suggest the need to derive a COVID-specific algorithm based on the clinical performance of single analytes markers in SARS-CoV-2 infected subjects.

It should also be considered that serum collagen metabolites may be affected by age, diet and disease duration (16, 37). Thus, to prevent results misinterpretation, they could be better used for within-individual changes during follow-up.

This study has several limitations. First, the study population is small, second, data on the correlation of TIMP-1 levels and specific treatment are lacking, third, serial measurements to assess longitudinal modifications of serum collagen markers according to fibrotic changes are not available. Thus, larger samples are needed to obtain a better evaluation of TIMP-1

TABLE 2 Distribution of blood parameters in patients stratified according to their World Health Organization (WHO) score at baseline.

	WHO score ≤5 (*n* = 68; 75.6%)	WHO score >5 (*n* = 22; 24.4%)	*P*-value
Hb g/dL	12.7 + −2.1 (7.7–18.1)	12.6 + −2.5 (7.6–16.1)	0,839
WBC (cells/μl)	6,565 (4,660; 9212.5) (2,380–17,090)	7,765(5682.5; 10147.5) (4210–21,150)	0,131
Neutrophils (N/mmc)	5,525 (3,545; 7,735) (1,000–13,600)	6,200 (4,385; 8007.5) (3,680–19,200)	0,064
Lymphocytes (N/mmc)	845 (602.5; 1267.5) (260–2,970)	635 (465; 1037.5) (270–3,350)	0,069
Platelets (cell/μL)	225,000 (185,500; 282,000) (55,000–363,000)	234,000 (134,750; 295,250) (31,000–607,000)	0,974
Fibrinogen (mg/dl)	581.7 + −173.2 (283–1,000)	563.2 + −187.2 (260–1,000)	0,685
INR	1.1 (1.04; 1.22) (0.8–3.84)	1.1 (1.03; 1.19) (0.8–1.33)	0,622
D-Dimer (mg/l)	1.02 (0.56; 1.69) (0.04–18.99)	0.94 (0.43; 2.38) (0.18–25.56)	0,794
Albumin (g/dl)	3.5 + −0.5 (2.6–4.5)	3.6 + −0.5 (2.5–5)	0,562
Total bilirubin mg/dl	0.65 (0.45; 0.85) (0.21–5.09)	0.74 (0.5; 1.06) (0.26–1.54)	0,289
Direct bilirubin mg/dl	0.29 + −0.13 (0.1–0.76)	0.34 + −0.17 (0.1–0.69)	0,255
Ferritin (ng/ml)	533.5 (267.8; 744.5) (40–2,000)	488 (149; 820) (39–2,000)	0,816
AST U/L	25 (20; 33.5) (9–141)	29 (23.5; 35.5) (8–176)	0,135
ALT U/L	27 (19; 42) (7–177)	35 (21.5; 55) (9–474)	0,318
LDH U/L	289 (218; 383) (111–741)	373 (281.8; 465) (193–670)	**0,013**
hsCRP mg/L	9.3 (3.4; 33.8) (0.3–132.6)	15.8 (9; 44.5) (1.3–222.5)	**0,039**
IL-6 (pg/ml)	13.2 (7.2; 28.5) (2.3–63.4)	19.6 (6.3; 59.9) (3.5–258)	0,276

Data are expressed as mean ± standard deviation (range); median (25th; 75th percentile) (range) or absolute frequency (percentage). Hb, haemoglobin; WBC, white blood cells; INR, international normalized ratio; AST, aspartate transaminase; ALT, alanine transaminase; LDH, Lactate dehydrogenase; hsCRP, high sensitive C-reactive protein; IL-6, interleukin-6. Bold values indicate the strong correlation.

TABLE 3 Correlation among procollagen type III amino terminal propeptide (PIIINP), Hyaluronic acid (HA), and tissue inhibitor of metalloproteinases 1 (TIMP-1) with inflammatory markers.

	HA	PIIINP	TIMP-1
Fibrinogen	−0.162 (0.124)	−0.05 (0.638)	0.151 (0.152)
hsCRP	0.147 (0.175)	0.162 (0.134)	**0.312 (0.003)**
Ferritin	−0.028 (0.816)	0.084 (0.48)	−0.053 (0.657)
IL-6	−0.003 (0.986)	0.008 (0.959)	0.082 (0.61)
albumin	**−0.387 (<0.001)**	**−0.362 (<0.001)**	−0.177 (0.079)
LDH	**0.267 (0.008)**	**0.264 (0.009)**	**0.263 (0.009)**

Bold values indicate the strong correlation.

levels circulating levels as a prognostic biomarker in COVID-19 patients and to investigate its potential role in monitoring therapeutic response in different treatment subgroups of COVID-19 patients.

In conclusion, our study shed new light on the potential clinical utility of serum collagen metabolites and extracellular matrix remodeling as suitable markers of disease severity in COVID-19 patients. We unveil that changes in serum TIMP-1 significantly correlate with changes in clinical outcome. Collagen metabolites and extracellular matrix remodeling markers are worthy of further studies to assess their potential as prognostic and predictive biomarkers in COVID-19 patients. The identification of a COVID-specific index reflecting the fibrotic process in SARS-CoV-2 patients is strongly encouraged for its potential as a disruptive tool for clinical management.

Data availability statement

The data that support the findings of this study are available from the corresponding author DT, (daniela.terracciano@unina.it), upon reasonable request and with permission of AOU Federico II.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethical Committee of the University Federico II of Naples (prot. no. 140/20). The patients/participants provided their written informed consent to participate in this study.

Author contributions

DT and GP: conceptualization and writing—review and editing. SB, BP, LS, MF, VV, and IM: data curation. DB: formal analysis. GP: funding acquisition, methodology, supervision, and validation. SB, DT, MF, and LS: investigation. IG, GP, AC, and MM: resources. DT, IG, AC, MM, and GP: visualization. DT: writing—original draft. All authors have read and agreed to the published version of 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.

Publisher's note

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Persistence of inflammatory and vascular mediators 5 months after hospitalization with COVID-19 infection

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Background and aim: In acute severe COVID-19, patients present with lung inflammation and vascular injury, accompanied by an exaggerated cytokine response. In this study, our aim was to describe the inflammatory and vascular mediator profiles in patients who were previously hospitalized with COVID-19 pneumonitis, months after their recovery, and compare them with those in patients recovering from severe sepsis and in healthy controls.

Methods: A total of 27 different cytokine, chemokine, vascular endothelial injury and angiogenic mediators were measured in the plasma of forty-nine patients 5.0 ± 1.9 (mean ± SD) months after they were hospitalized with COVID-19 pneumonia, eleven patients 5.4 ± 2.9 months after hospitalization with acute severe sepsis, and 18 healthy controls.

Results: Compared with healthy controls, IL-6, TNFα, SAA, CRP, Tie-2, Flt1, and PIGF were significantly increased in the post-COVID group, and IL-7 and bFGF were significantly reduced. While IL-6, PIGF, and CRP were also significantly elevated in post-Sepsis patients compared to controls, the observed differences in TNFα, Tie-2, Flt-1, IL-7 and bFGF were unique to the post-COVID group. TNFα levels significantly correlated with the severity of acute COVID-19 illness (spearman's r = 0.30, $p < 0.05$). Furthermore, in post-COVID patients, IL-6 and CRP were each strongly negatively correlated with gas transfer factor %predicted (spearman's r = −0.51 and r = −0.57, respectively, $p < 0.002$) and positively correlated with computed tomography (CT) abnormality scores at recovery (r = 0.28 and r = 0.46, $p < 0.05$, respectively).

Conclusion: A unique inflammatory and vascular endothelial damage mediator signature is found in plasma months following acute COVID-19 infection. Further research is required to determine its pathophysiological and clinical significance.

KEYWORDS

COVID-19, inflammation, cytokines, vascular injury, post-COVID

Introduction

Clinical outcomes in acute coronavirus disease 2019 (COVID-19) are highly dependent upon the cytokine response in the host (1). The entry of SARS-Cov-2 virions into pulmonary epithelial cells *via* the angiotensin converting enzyme (ACE2), triggers a wave of pro-inflammatory cytokines and chemokines (2). In the healthy immune response infected cells are cleared and this inflammatory cascade recedes. In patients with more severe disease, however, an exaggerated elevation of these mediators has been observed, termed "cytokine release syndrome" (1, 3–5), which may lead to immunopathogenesis by causing tissue damage. These inflammatory pathways are the target of several successful treatments in the acute setting, such as dexamethasone and the anti-IL6R monoclonal antibody tocilizumab (6, 7). In addition, the vascular endothelium is also dysregulated in acute COVID-19 and microvascular thrombosis and endothelial inflammation contribute significantly to the pathology (8–10).

Contrary to the acute effects, our understanding of the longer-term effects of COVID-19 on inflammatory mediators and vascular function remains opaque, and other follow-up studies often have lacked an appropriate control group (11, 12). In this study, we examine levels of cytokine, chemokine and markers of vascular injury and angiogenesis in the peripheral blood of patients recovering from COVID-19 pneumonia many months after their acute infection, and compare their profiles to those of patients recovering from severe sepsis and to those of healthy controls.

Methods

Our post-COVID cohort consisted of 49 patients [aged 60 ± 9 years (mean ± SD), 13 females] from whom venous blood was collected 5.0 ± 1.9 months after hospitalization with acute COVID-19 pneumonia. These patients were recruited from a post-COVID-19 follow-up respiratory clinic, having previously been hospitalized with acute COVID-19 pneumonia in the period between March 2020 and Jan 2021. For comparison, blood was obtained from a group of 11 patients 5.4 ± 2.9 months after hospitalization with severe sepsis (age 66 ± 17 years, seven females) (13) and 18 healthy control participants (age 47 ± 16 years, two females). Plasma was obtained by centrifugation of blood collected in EDTA-lined tubes and stored at −80°C prior to measurement of 27 different cytokine, chemokine, angiogenic and vascular injury markers [Meso Scale Discovery (MSD) V-PLEX multiplex assays using a Meso-Scale Discovery SQ120 device]: Interferon gamma, Interleukin 1B (IL-1B), IL-4, IL-6, IL-10, Tumor Necrosis Factor alpha (TNF alpha), Granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-17A, Interleukin 12 (IL-12/23p40), IL-7, Macrophage Inflammatory protein 1A (MIP-1A), MIP-1B, Monocyte Chemoattractant Protein 1 (MCP-1), MCP-4, Interferon-inducible protein 10 (IP-10), Thymus and activation regulated chemokine (TARC), Vascular Endothelial Growth Factor A (VEGF-A), VEGF-C, VEGF-D, Placental Growth Factor (PIGF), Vascular Endothelial Growth Factor Receptor 1 (Flt-1), Angiopoietin 1 receptor (Tie-2), basic Fibroblast Growth Factor (bFGF), Serum Amyloid A protein (SAA), C-reactive protein (CRP), Vascular Cell Adhesion Molecule 1 (VCAM-1), Intercellular Adhesion Molecule 1 (ICAM-1) (see **Table 1** for more details). All participants provided informed written consent. The studies were approved by the North-West Preston (20/NW/0235) and Oxford C (19/SC/0296) Research Ethics Committees.

Statistical comparisons between groups were performed using Kruskal-Wallis tests for non-normally distributed data and one-way ANOVA for normally distributed data. To allow for multiple testing, false discovery rate (FDR) correction was performed using the Benjamini Hochberg method (FDR-adjusted q-value of 0.05). Where appropriate, pair-wise comparisons were undertaken using Dunn's (or Tukey's) multiple comparison tests. Associations between variables are given as Spearman correlation coefficients. Analyses were undertaken using Prism (version 8) and RStudio (version 1.2.5033).

Results

Table 1 summarizes the assay results for all the mediators tested in the three participant groups, showing a three-way comparison between groups.

In keeping with previous reports of abnormal cytokine profiles months after COVID-19 infection (11, 12, 14), we demonstrate persistent significant elevation of IL-6, TNFα, Tie-2, Flt-1, PIGF, SAA, and CRP in our post-COVID cohort, compared with healthy controls, and persistent significant suppression of IL-7 and bFGF, as shown in **Figure 1**.

We are unable to determine whether these mediators are markers of previous disease in these patients or mediators of ongoing pathology. However, in a recent UK study of >2,000 patients, only 26% of patients felt fully recovered 5 months after COVID-19 infection, and IL-6 and CRP were among the cytokines persistently upregulated in those with more significant impairment (12). In our study, IL-6 and CRP levels in the post-COVID patients correlated positively with the thoracic computed tomography (CT) abnormality score ($r = 0.28$, $p < 0.05$ for IL-6, $r = 0.46$, $p < 0.002$ for CRP) and negatively with gas transfer factor (DLCO %predicted; $r = -0.51$, $p < 0.002$ for IL-6, $r = -0.57$, $p < 0.0005$ for CRP), which were performed at around the same time that blood was obtained for this study. Details of thoracic CT scores and lung function test results (DLCO and spirometry) are shown in **Table 2**. However, there was no correlation between breathlessness measured by the MRC dyspnea score (defined in **Table 2**) and levels of these or other measured mediators. Within the post-COVID group, we found a significant correlation between the severity of the acute illness (as defined in **Table 2**) and levels of TNFα ($r = 0.30$, $p < 0.05$).

Importantly, our study also significantly adds to previous findings by identifying mediators for which expression is persistently abnormal in patients recovering from COVID-19, but not in those recovering from another pathology characterized by acute inflammation, namely severe sepsis. This group of mediators, which therefore constitutes a specific post-infection inflammatory/vascular injury signature of COVID-19, include the angiogenic factors Tie-2, Flt-1, and bFGF, and the inflammatory markers IL-7 and TNFα, as shown in **Figure 1** and **Table 1**.

Discussion

In this study we found persistent elevation of multiple mediators – IL-6, TNFα, SAA, Tie-2, Flt1, PIGF, and CRP – and

TABLE 1 Multiplex assay results.

Plasma marker	Healthy pg/ml, (median, IQR)	Post-Sepsis pg/ml, (median, IQR)	Post-COVID pg/ml, (median, IQR)	Between three groups	COVID vs. Healthy	COVID vs. Sepsis	Sepsis vs. Healthy
Pro-inflammatory							
Interferon gamma	10.18 [7.81, 14.13]	10.24 [9.01, 14.68]	10.41 [7.50, 18.89]	ns			
IL-1B	0.049 [0.013, 0.160]	0.290 [0.074, 0.364]	0.104 [0.053, 0.141]	$p < 0.05$	ns	ns	+
IL-4	0.070 [0.044, 0.099]	0.082 [0.040, 0.112]	0.076 [0.050, 0.102]	ns			
IL-6	0.972 [0.696, 1.607]	2.264 [1.300, 4.058]	1.934 [1.295, 2.834]	$p < 0.0005$	+	ns	+
IL-10	0.654 [0.446, 0.798]	0.558 [0.442, 0.760]	0.630 [0.491, 0.941]	ns			
TNF alpha	***1.405 [0.754, 2.566]***	***1.616 [1.393, 1.938]***	***3.146 [2.109, 5.294]***	***$p < 0.0001$***	+	+	***ns***
Cytokine							
GM-CSF	0.634 [0.329, 1.190]	0.409 [0.260, 1.244]	0.608 [0.340, 0.975]	ns			
IL-17A	6.366 [2.890, 13.330]	8.000 [4.521, 26.170]	8.015 [3.637, 16.320]	ns			
IL-12/23p40	283.1 [198.6, 344.3]	408.4 [297.1, 632.7]	325.0 [197.7, 478.4]	ns			
IL-7	***9.805 [6.689, 11.810]***	***7.785 [4.533, 33.360]***	***5.023 [3.263, 7.026]***	***$p < 0.005$***	–	–	***ns***
Chemokine							
MIP-1beta	136.8 [110.3, 184.8]	229.9 [143.3, 336.7]	132.8 [97.8, 182.4]	$p < 0.005$	ns	–	ns
TARC	140.5 [112.5, 329.1]	340.7 [146.4, 550.3]	122.3 [81.7, 189.7]	$p < 0.005$	ns	–	ns
IP-10	585.9 [460.0, 820.1]	1,077.0 [455.7, 1325.0]	770.0 [520.3, 1117.0]	ns			
MIP-1alpha	50.39 [35.81, 335.20]	122.00 [48.67, 274.10]	45.43 [39.61, 57.28]	ns			
MCP-1	125.8 [184.5, 245.2]	208.5 [178.7, 328.8]	261.3 [214.7, 318.1]	ns			
MCP-4	119.2 [97.4, 155.5]	236.7 [125.6, 309.0]	134.3 [114.1, 184.2]	ns			
Angiogenesis							
VEGF-A	48.31 [34.02, 83.91]	76.94 [51.08, 152.20]	49.01 [30.09, 73.96]	ns			
VEGF-C	1129 [921, 1482]	1716 [1102, 2974]	1063 [782, 1668]	ns			
VEGF-D	726.8 [554.9, 944.3]	861.1 [702.8, 1127.0]	819.2 [684.4, 1092.0]	ns			
Tie-2*	***3434 ± 300***	***3245 ± 302***	***3923 ± 784***	***$p < 0.005$***	+	+	***ns***
Flt-1	***93.49 [82.81, 109.60]***	***97.10 [65.96, 113.80]***	***124.40 [97.24, 157.90]***	***$p < 0.005$***	+	+	***ns***
PIGF	3.010 [2.548, 3.177]	3.968 [2.577, 5.888]	4.260 [3.593, 5.360]	$p < 0.0001$	+	ns	+
bFGF	***22.17 [14.03, 41.34]***	***49.59 [9.19, 117.00]***	***2.06 [1.38, 4.23]***	***$p < 0.0001$***	–	–	***ns***
Vascular injury							
SAA	1266741 [832641, 2592227]	2616109 [1540017, 8354912]	2720091 [1382324, 5395892]	$p < 0.02$	+	ns	ns
CRP	633598 [418901, 1559464]	3550592 [1632105, 6856682]	2253276 [1117127, 5794106]	$p < 0.005$	+	ns	+
VCAM-1	493133 [457268, 538105]	657406 [491048, 878164]	552729 [434409, 729479]	$p < 0.05$	ns	ns	+
ICAM-1	305599 [253191, 381896]	576237 [449555, 743289]	385068 [287088, 480021]	$p < 0.005$	ns	–	+

Five different MSD assay panels, each containing a set of mediators as shown, were used: (i) pro-inflammatory panel, (ii) cytokine panel (cytokines related to immune differentiation and growth), (iii) chemokine panel (cytokines related to monocyte chemotaxis), (iv) angiogenic, and (v) vascular injury panels (related to vascular injury and repair). Between-group analysis was performed by the Kruskal-Wallis test. Values are given as median and interquartile range (25th centile, 75th centile). Statistical significance was achieved if $p < 0.05$ and $p <$ Benjamini-Hochberg critical value, with an FDR-adjusted q-value of 0.05. Where the three-way comparison between groups was significant, pairwise analysis was performed (Dunn test) for "post-COVID vs. healthy," "post-COVID vs. post-sepsis," and "post-sepsis vs. healthy." + denotes significantly higher in 1st vs. 2nd group; – denotes significantly lower in 1st vs. 2nd group. Individual markers shown in bold and italic denote "COVID different from healthy control and different from post-Sepsis." * for SAA, values are given as mean ± SD, and ANOVA test was used for between-group analysis (and Tukey's test for pairwise comparisons) as data were normally distributed.

persistent depression of IL-7 and bFGF in patients recovering from COVID-19 several months following their acute infection. Importantly, we also showed for the first time that persistent changes in Tie-2, Flt-1, bFGF, IL-7, and TNFα were uniquely seen in patients following recovery following COVID-19, but not in patients recovering from severe sepsis.

Our findings suggest that processes of endothelial injury and repair persist months after acute COVID-19 infection. The soluble angiopoietin 1 receptor (Tie-2), which has been previously reported increased following COVID-19 infection (11), is nearly exclusive to endothelial cells and has a critical role in antithrombotic signaling (15). Higher circulating levels of this receptor may reflect

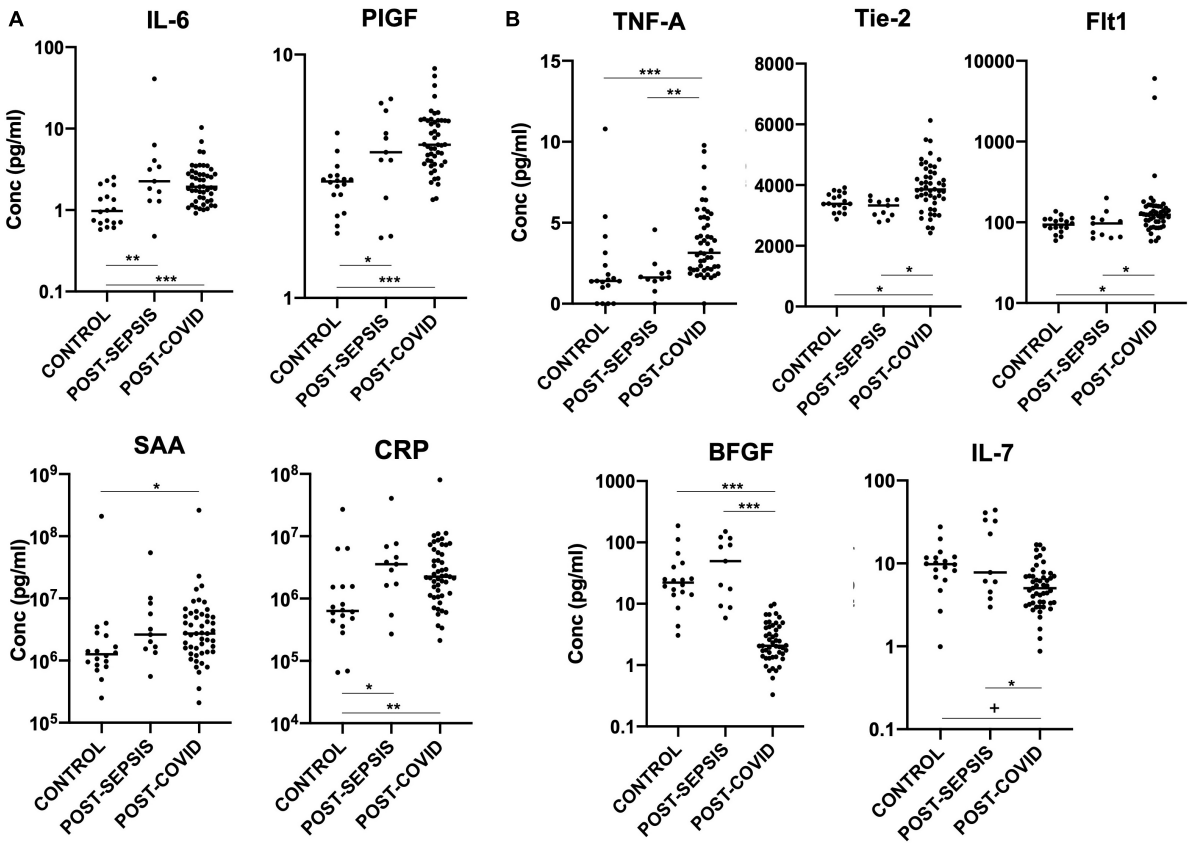

FIGURE 1
(A) Plasma markers elevated in both post-COVID and post-Sepsis groups, compared to healthy controls: IL-6, PIGF, SAA, and CRP. **(B)** Plasma markers uniquely different in post-COVID patients but not in post-Sepsis patients compared with healthy controls: TNFα, Tie-2, and Flt-1 are elevated while bFGF and IL-7 are depressed in post-COVID patients compared to healthy controls and compared to post-Sepsis patients. Horizontal bars indicate medians. $+p < 0.01$, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

a homeostatic response to increased levels of its prothrombotic antagonist, angiopoietin-2 (15). Alternatively, it may reflect loss or cleavage of Tie-2 from the endothelial surface during ongoing inflammatory states. It could be a consequence of ongoing downregulation and shedding of angiotensin converting enzyme 2 (ACE2), the receptor for SARS-CoV-2, and a resulting accumulation of angiotensin 2 which has inflammatory and thrombotic effects when bound to the endothelial receptor AT1R (8, 16, 17). This final suggested mechanism may also explain our finding of increased levels of soluble vascular endothelial growth factor receptor (Flt1). Flt-1, an inhibitor of the vascular endothelial growth factor pathway which promotes endothelial dysfunction, has been shown to be increased acutely in COVID-19 (18) but its persistent elevation in the post-COVID setting is a novel finding of our study. The interaction between angiotensin 2 and the receptor AT1R has also been found to promote local Flt-1 upregulation in hypoxia (19). Therefore, dysregulation of the renin/angiotensin system is a potential unifying mechanism for our findings of increased Tie-2 and Flt-1 in post-COVID-19 patients. Unfortunately, we do not have data on tissue expression of ACE2, angiopoietin-2 or angiotensin 2 to explore this further.

Detrimental inflammatory and cytotoxic effects of angiotensin 2 binding to AT1R might also play a role in our finding of reduced levels of the basic fibroblastic growth factor (bFGF) in post-COVID-19 patients. BFGF is present in basement membranes, activated during wound healing, and has mitogenic effects on endothelial cells (20). Reduced levels of bFGF after COVID-19 could represent reduced production or increased consumption during healing from COVID-19 pneumonitis, or other endothelial injuries. This result, reported also by (11), shows an interesting contrast with the finding of elevated levels of bFGF in a large cohort of young adults with acute COVID who did not require hospitalization (21). It is possible that reduced levels of bFGF in our post-COVID-19 are a finding specific to more severe disease requiring hospitalization and post COVID-19 pneumonitis. It is noted, however, that no correlation is found here between reduced bFGF with raised inflammatory mediators IL-6 or CRP (which are associated with reduced gas transfer factor) or with TNF alpha (which correlates with severity of acute illness within our hospitalized cohort). Further exploration of the role and utility of bFGF in patients with post-COVID syndrome would be of interest.

We also found a persistent elevation of PIGF in post-COVID patients, which in the acute setting has been shown to correlate with in-hospital mortality (22), but did not see any differences (between COVID-19 patients and healthy controls) in levels of the vascular intercellular adhesion molecules ICAM-1 and VCAM-1 (important in inflammatory cell recruitment to the lung), nor in the endothelial growth factors VEGF-A, VEGF-C, or VEGF-D. The acute phase inflammatory proteins serum amyloid A and CRP are elevated in our post-COVID-19 cohort, in common with post-sepsis patients; but there are no differences in the levels of the leukocytic pyrogen

TABLE 2 Severity scores of acute coronavirus disease-2019 (COVID-19) pneumonia and details of follow-up thoracic computed tomography (CT) and lung function tests in the post-COVID patients.

Severity of acute COVID-19 pneumonia	Severity = 0 (n = 24)	Severity = 1 (n = 14)	Severity = 2 (n = 11)
FEV1% predicted (Mean ± SD)	94.2 ± 26.5	93.3 ± 18.7	93.3 ± 19.3
FVC% predicted (Mean ± SD)	94.7 ± 23.3	96.7 ± 23.8	89.3 ± 17.7
DLCO% predicted (Mean ± SD)	76.5 ± 14.4	80.0 ± 14.8	73.8 ± 21.0
CT score No. of patients (%) 0 1 2	 13 (54) 9 (38) 1 (4)	 5 (36) 5 (36) 3 (21)	 0 (0) 3 (27) 8 (73)
MRC dyspnea score Median, IQR	2 [1, 2]	2 [2, 2]	2 [1.5, 3]

Severity of acute coronavirus disease-2019 (COVID-19) pneumonia was classified according to requirement for respiratory support, where 0 indicates simple oxygen therapy, 1 indicated non-invasive respiratory support (e.g., continuous positive airway pressure or high-flow nasal oxygen therapy) and 2 indicates invasive mechanical ventilation. Lung function tests reported were performed at follow-up in the post-COVID clinic, around the same time that blood was obtained for this study. The forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC) and gas transfer factor i.e., diffusing capacity of the lungs for carbon monoxide (DLCO) were expressed as a percentage of the predicted value, using the equations provide by the Global Lung Initiative. The CT thorax abnormality score was based on a review of CT imaging performed around the same time that blood was obtained for this study, and images were classified according to residual abnormality where 0 indicates normal images, 1 indicates mild residual ground glass change, and 2 indicates ground glass changes with additional lung fibrosis. Medical Research Council (MRC) Dyspnea Score is defined as: 1, not troubled by breathlessness except with strenuous exercise; 2, troubled by breathlessness when hurrying on the level or walking up a slight hill; 3, walks slower than most people of same age on the level because of breathlessness or has to stop for breath when walking at own pace on the level; 4, stops for breath after walking 100 yards or after a few minutes on the level; 5, too breathless to leave the house or breathless when dressing or undressing.

and component of the inflammasome complex, IL-1β, from healthy control patients, in contrast with the findings of another study of patients reporting post-acute sequelae of COVID-19 (23).

The observed persistent suppression of IL-7 in our post-COVID cohort is also in keeping with previous reports (11), but contrasts the elevation of IL-7 in the acute setting (24, 25). IL-7 is critical for the development, maturation and survival of lymphocytes, and prevents memory cell apoptosis (26). In a recent study, recombinant IL-7 increased CD8+ and CD4+ T cell proliferation (*ex vivo*) in critically ill COVID-19 patients (27), and IL-7 has been suggested as immunotherapy and/or a vaccine adjuvant for COVID-19 (27–29). Reduced IL-7 after COVID-19 could reflect persistent lymphocyte exhaustion, contributing to inefficient viral clearance and chronic immune stimulation.

Tumor necrosis factor alpha, a pro-inflammatory cytokine, is elevated both in acute COVID-19 and acute sepsis and is linked to more severe disease and worse prognosis (4, 30, 31). In addition to its persistent elevation in our post-COVID patients, we identified a positive correlation between TNFα plasma levels after discharge and acute COVID-19 disease severity score ($p < 0.05$). No such correlation was identified for any other mediator in the current study. Others have found elevated TNFα in those with persistent symptoms many months after mild COVID-19 (14), and suggested it has a role in sustaining macrophage activation and cellular inflammation (14). These findings, as well the distinct TNFα elevation in post-COVID but not in post-Sepsis patients in our study, merit further study and consideration given the availability of established anti-TNF therapies.

Finally, of particular interest is the observation that elevation of IL-6 seen in our post-COVID cohort, and reported by others (11, 12, 14), is not specific to post-COVID but is also seen in a post-sepsis cohort. This has implications when considering anti-IL6 therapies in the post-COVID setting.

As noted above, a strength of our study is the control group consisting of patients recovering from sepsis, as well as a second group of healthy controls. Limitations include the relatively small numbers in the post-sepsis group, and the fact that patients were recruited early in the pandemic, prior to the emergence of later variants of the SARS-CoV-2 virus, such as the omicron variant. Notwithstanding these limitations, our key finding is that COVID-19 appears to be associated with a post-inflammatory signature that persists for at least 5 months, and that is distinct from the profile seen in patients recovering from sepsis. Understanding this signature may be important both for understanding the pathophysiology of the long-term effects of COVID-19, and for development or targeting of effective therapy. Further, more targeted study is required, for example in those who may suffer with prolonged symptoms (post-COVID syndrome), to understand the pathophysiological significance and potential clinical utility of theses uniquely persistent mediators.

Data availability statement

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 the North West Preston (20/NW/0235) and Oxford C (19/SC/0296) Research Ethics Committees. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BR, AM, NT, and NP conceived and designed the study. JM, AA, AM, AF, EF, AK, JK, and NP collected data. JM and NP performed experiments and analyzed data. JM, NT, and NP drafted the manuscript. All authors contributed to the interpretation of data, revision of the manuscript and approved the final version submitted for publication.

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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

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D-dimer trends elaborate the heterogeneity of risk in hospitalized patients with COVID-19: A multi-national case series from different waves

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Introduction: Variable D-dimer trends during hospitalization reportedly result in distinct in-hospital mortality. In this multinational case series from the first and second waves, we show the universality of such D-dimer trends.

Methods: We reviewed 405 patients with COVID-19 during the first wave admitted to three institutions in the United States, Italy, and Colombia, and 111 patients admitted to the U.S. site during the second wave and 55 patients during the third wave. D-dimer was serially followed during hospitalization.

Results: During the first wave, 66 (15%) patients had a persistently-low pattern, 33 (8%) had early-peaking, 70 (16%) had mid-peaking, 94 (22%) had fluctuating, 30 (7%) had late-peaking, and 112 (26%) had a persistently-high pattern. During the second and third waves, similar patterns were observed. D-dimer patterns were significantly different in terms of in-hospital mortality similarly in all waves. Patterns were then classified into low-risk patterns (persistently-low and early-peaking), where no deaths were observed in both waves, high-risk patterns (mid-peaking and fluctuating), and malignant patterns (late-peaking and persistently-high). Overall, D-dimer trends were associated with an increased risk for in-hospital mortality in the first wave (overall: HR: 1.73) and stayed the same during the second (HR: 1.67, $p < 0.001$) and the third (HR: 4.4, $p = 0.001$) waves.

Conclusion: D-dimer behavior during COVID-19 hospitalization yielded universal categories with distinct mortality risks that persisted throughout all studied waves of infection. Monitoring D-dimer behavior may be useful in the management of these patients.

KEYWORDS

COVID-19, D-dimer, variability, in-hospital mortality, heterogeneity

Introduction

Coronavirus disease 2019 (COVID-19) has been reportedly associated with a hypercoagulable state (1). An increase in fibrin degradation products (D-dimer) linked to a thrombotic state is an integral part of the COVID-19 laboratory signature (2). While clinical trials evaluating the benefit of anticoagulation are underway (3), strategies to prevent or mitigate thrombosis in these patients are currently based on limited evidence. COVID-19, however, has a wide range of symptoms and severity, and is demographically, clinically, and pathologically heterogeneous (4). One aspect of such heterogeneity can be represented by the behavior of the D-dimer levels throughout the hospitalization with COVID-19. We recently reported that the variation in D-dimer trends during the hospital course involves specific trends that resulted in distinct patterns of in-hospital mortality. Here, we report a multicenter case series from infection waves during different time points in which we show the universality of such D-dimer trends and their risk (5).

Methods

In a retrospective study protocol, we defined different waves of increased infection rates during the pandemic according to the Center for Disease Control and Prevention statistics as published on their website (6). The curves reported by the CDC for the total number of weekly newly reported cases in New York State were examined. The first three peaks with stable plateaus of low reported number in-between were identified, and the onset and offset of each of these curves were identified as the time threshold for each wave. Figure 1A shows the curves for the first three waves as produced by the CDC website and the time thresholds for each wave. Accordingly, the first wave was defined as the period between 25 March 2020 and 31 June 2020. The second wave of the pandemic was defined as the period between 1 November 2020 and 30 April 2021. The third wave of the pandemic was defined as the period between 1 July 2021 and 31 October 2021. We scanned patients admitted with PCR-confirmed COVID-19 in the first wave period admitted to three different institutions representing three different continents (North America, Europe, and South America) [BronxCare Health System, New York, USA (New York site); IRCCS Istituto Auxologico Italiano, Milan, Italy (Milan Site); and Hospital San Ignacio, Bogota, Colombia (Bogota site)]. Patients who had their D-dimer followed during hospitalization ($\geq$4 levels) until the outcome of the hospitalization (death or discharge) were included in the study. Moreover, patients admitted to the New York site in the second and third wave periods were also included in the study if they had their D-dimer followed during the hospitalization similar to those described earlier.

D-dimers were classified into six different trend categories (Figure 1B) based on the behavior during hospitalization: (a) persistently-low: if D-dimer levels during admissions were $\leq$1,000 ng/ml and stayed below 1,000 ng/ml throughout the hospitalization, (b) early-peaking: D-dimer on admissions was $>$1,000 ng/ml and immediately or progressively normalized to levels $<$1,000 ng/ml and stayed low for the rest of the hospitalization, (c) mid-peaking: D-dimer levels were $<$1,000 ng/ml on admission, however, peaked to levels $>$1,000 ng/ml during the hospitalization, and then immediately decreased and stayed low for the rest of the hospitalization, (d) fluctuating: D-dimer levels were either low or normal during admission, however, with multiple rises and falls $>$1,000 ng/ml during the hospital course, (e) late-peaking: D-dimer levels that were $<$1,000 ng/ml on admission and stayed low throughout the hospitalization, however, exhibited sudden rise to levels $>$1,000 ng/ml at the end of the encounter, and (f) persistently-high: D-dimer levels $>$1,000 ng/ml on admission that stayed high throughout the hospitalization. Figure 2 illustrates examples of patients' D-dimer trends.

Statistical analyses

Categorical data are presented as numbers (%) and were compared using the chi-square test. Continuous data are presented as mean $\pm$ SD. Data were tested for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests, and accordingly, continuous data were compared using the t-test or analysis of variance (ANOVA) if they were normally distributed or the Mann–Whitney U-test if they were not normally distributed. Cox regression models and Kaplan–Meier survival curves were used to test the difference in cumulative in-hospital mortality. Differences were considered statistically significant at $p < 0.05$. All analyses will be performed with commercially available software (SPSS, version 23.0; SPSS, Inc).

Results

During the first wave, 3,203 patients were reviewed (New York site: 1,207, Milan site: 1,160, Bogota site:836 patients) of whom 405 patients had serial D-dimer measurements and were included from the first wave from the three institutions (149 from New York site, 161 from Milan site, and 95 from Bogotá site). Moreover, 700 patients were reviewed from the New York Site during the second wave, with 111 patients having serial D-dimer measurements and being included, and 104 patients were reviewed from the New York Site during the third wave, with 55 patients having serial D-dimer measurements and being included.

Comparisons between the patients from the first, second, and third waves and the populations from the three institutions are summarized in Tables 1, 2. Briefly, patients in the second wave were more likely to be women, had less anticoagulation, more mechanical ventilation, and in-hospital deaths, while admission D-dimer was not different between the three waves. When patients from different institutes included in the first wave were compared, it was found that patients from the New York site had the highest BMI, highest mechanical ventilation, and in-hospital death. In contrast, patients from the Milan site were the oldest, most frequently male subjects, with the longest symptom onset to hospital admission and the longest hospital stay; while patients from Bogota were younger and more frequently female subjects, with the shortest symptom onset to hospital admission, the shortest hospital stay, and the most frequent anticoagulation use. It is important to note that the admission D-dimer from the three sites was not different.

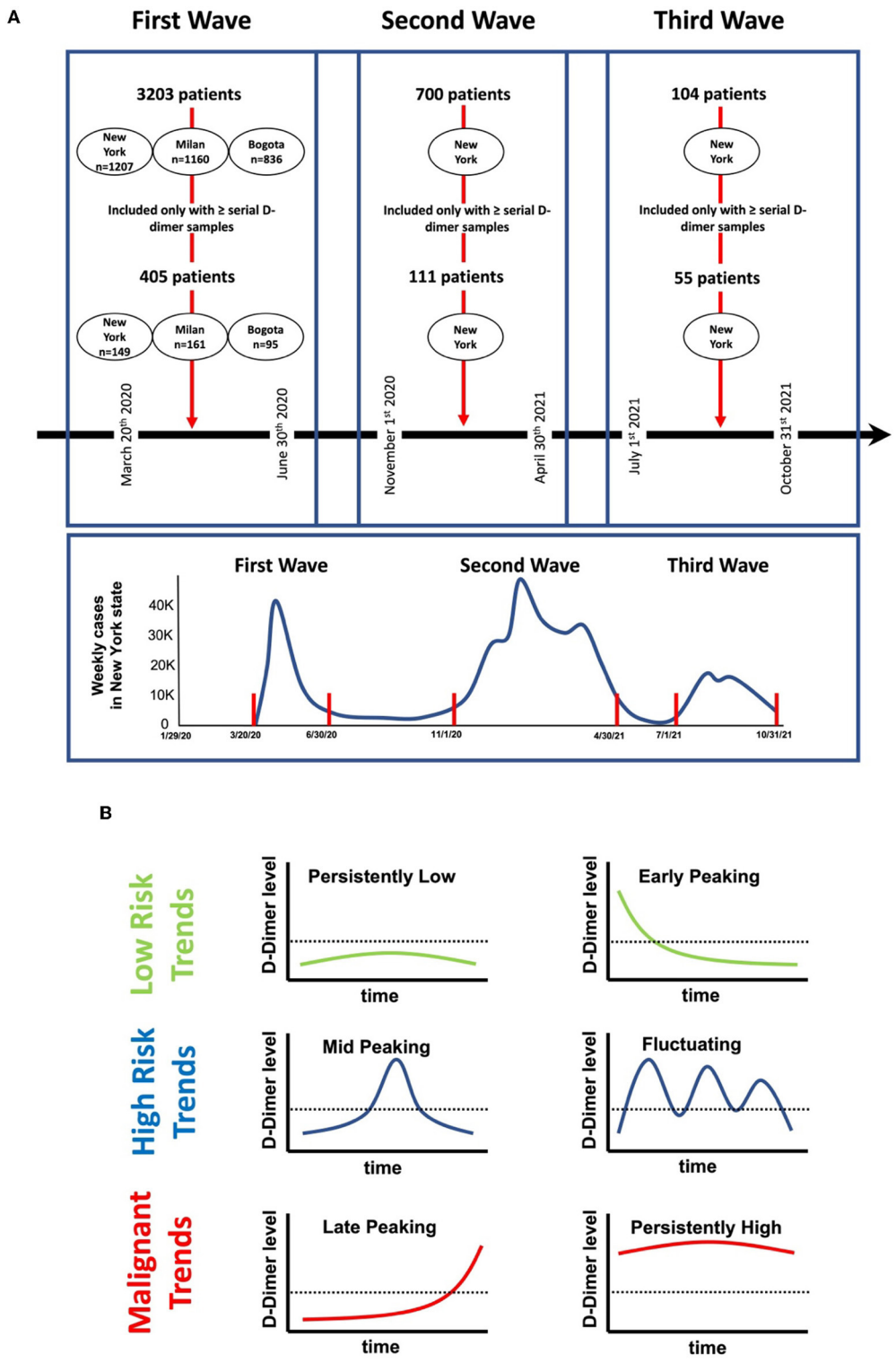

FIGURE 1
Study timelines and protocol. **(A)** Number of patients involved in the study at each center according to the defined time points in the first, second, and third waves. **(B)** Schematic representation of in-hospital D-dimer patterns observed in our study. Persistently-low: if D-dimer levels during admission were below 1,000 ng/ml and stayed below 1,000 ng/ml throughout the hospitalization. Early-peaking: D-dimer levels on admissions were >1,000 ng/ml and immediately normalized to levels <1,000 ng/ml and stayed low for the rest of the hospitalization. Mid-peaking: D-dimer levels were <1,000 ng/ml on admission, however, peaked at levels >1,000 ng/ml during the hospitalization, and then immediately decreased and stayed low for the rest of the hospitalization. Fluctuating: D-dimer levels were either low or normal during admission, however, with multiple rises and falls >1,000 ng/ml during the hospital course. Late-peaking: D-dimer levels were <1,000 ng/ml on admission and stayed low throughout the hospitalization, however, exhibited a sudden rise to levels >1,000 ng/ml at the end of the encounter. Persistently-high: D-dimer levels were >1000 ng/ml on admission which stayed high throughout the hospitalization.

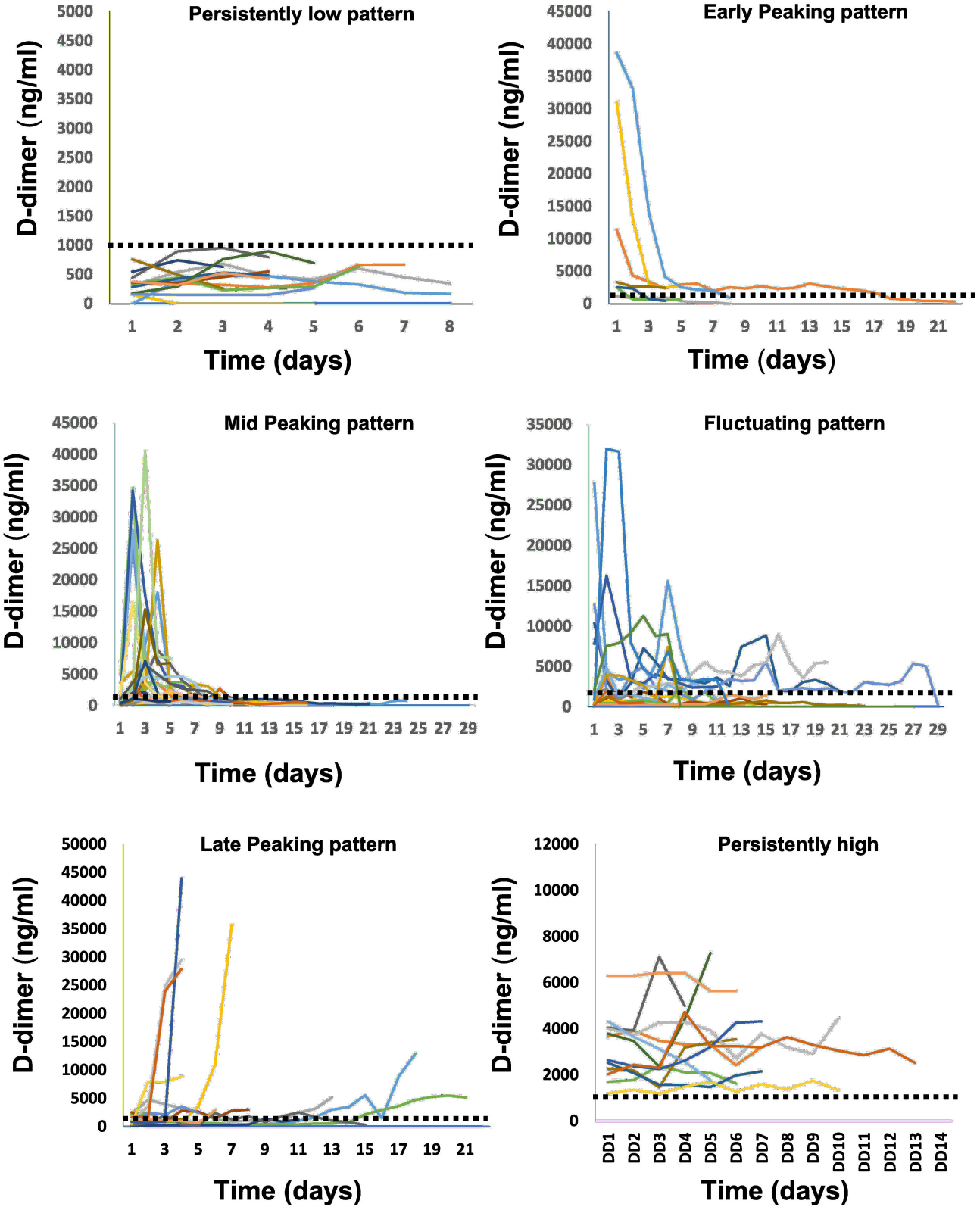

FIGURE 2
Examples of in-hospital D-dimer patterns observed in our study from different patients. Each curve represents D-dimer behavior measured from repeated samples during the hospitalization time for a separate patient.

TABLE 1 Comparisons between different waves.

	First wave ($n = 405$)	Second wave ($n = 111$)	Third wave ($n = 55$)	p-value
Age, year	64.8 ± 16.3	64.1 ± 17	64.4 ± 19.5	0.681
Females, *n* (%)	192 (45)	79 (71)	22 (40)	<0.001
BMI, *n* (%)	29.7 ± 8.4	31 ± 9.8	32 ± 8.7	0.071
Diabetes miletus, *n* (%)	130 (32)	57 (51)	26 (47)	<0.001
Hypertension, *n* (%)	255 (63)	89 (80)	45 (82)	<0.001
Asthma, *n* (%)	28 (7)	2 (2)	6 (11)	0.002
COPD, *n* (%)	55 (14)	1 (1)	10 (18)	0.003
Admission D-dimer, ng/ml	2,714 ± 6,646	3,962 ± 15,685	1,455 ± 2,912	0.253
Length of stay, days	16.2 ± 12.8	15.3 ± 10.6	13.4 ± 7.4	0.467
Therapeutic anticoagulation, *n* (%)	267 (62)	57 (51)	45 (82)	0.003
Mechanical ventilation, *n* (%)	124 (29)	78 (70)	20 (36)	<0.001
In-hospital death, *n* (%)	120 (28)	67 (60)	17 (31)	<0.001
D-dimer trends				0.003
Persistently-low, *n* (%)	66 (15)	22 (20)	12 (22)	
Early-peaking, *n* (%)	33 (8)	12 (11)	5 (9)	
Mid-peaking, *n* (%)	70 (16)	7 (6)	13 (24)	
Fluctuating, *n* (%)	94 (22)	24 (22)	6 (11)	
Late-peaking, *n* (%)	30 (7)	20 (18)	8 (15)	
Persistently-high, *n* (%)	112 (26)	26 (23)	11 (20)	

BMI, body mass index; COPD, chronic obstructive pulmonary disease.

D-dimer trends

According to our definitions for D-dimer trends, of the 405 patients included during the first wave, 66 (15%) patients had a persistently-low pattern, 33 (8%) patients had an early-peaking pattern, 70 (16%) patients had a mid-peaking pattern, 94 (22%) patients had a fluctuating pattern, 30 (7%) patients had a late-peaking pattern, and 112 (26%) patients had a persistently-high pattern (Table 1). During the second wave, 22 (11%) patients had a persistently-low pattern, 12 (11%) patients had an early-peaking pattern, 7 (6%) patients had a mid-peaking pattern, 24 (22%) patients had a fluctuating pattern, 20 (18%) patients had a late-peaking pattern, and 26 (23%) patients had a persistently-high pattern (Table 1). During the third wave, 12 (22%) patients had a persistently-low pattern, 5 (9%) patients had an early-peaking pattern, 13 (24%) patients had a mid-peaking pattern, 6 (11%) patients had a fluctuating pattern, 8 (15%) patients had a late-peaking pattern, and 11 (20%) patients had a persistently-high pattern (Table 1).

During the first wave, patients from the Milan site had the highest number of early-peaking and fluctuating patterns, and patients from the Bogota site had the highest number of persistently-low and persistently-high patterns. Compared to the first wave, the second wave patients showed more frequent persistently-low and late-peaking D-dimer patterns, while the third wave showed more frequent late-peaking patterns (Table 2).

Comparisons between different D-dimer patterns

Comparisons between the different D-dimer trends in all waves are shown in Table 3. In brief, there was no significant difference between the different trends regarding age, sex, BMI, or symptom onset to hospital admission. D-dimer levels on admission were significantly different between groups as can be expected from the classification. Moreover, the longest hospital stay was noted in the fluctuating and late-peaking groups, and the shortest was found for the persistently-low trend. Importantly, the lowest use of AC and mechanical ventilation were observed in the persistently-low pattern. Importantly, no in-hospital deaths were recorded in the persistently-low or the early-peaking groups, while the highest deaths occurred in the late-peaking and the persistently-high groups. Similar results were also observed in the second and third waves (Table 3).

Kaplan–Meier curves revealed that different patterns of D-dimer were highly significantly different in terms of in-hospital mortality (Figure 3). Importantly, the patterns of risk observed were similar in all waves. Based on the curves, we found that the patterns can be classified according to in-hospital mortality risk into low-risk patterns (persistently-low and early-peaking), where no deaths were observed in all waves, high-risk patterns (mid-peaking

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TABLE 2 Comparisons between different study sites during the first wave.

First wave	New York $N = 149$	Italy $N = 161$	Colombia $N = 95$	p-value
Age, year	63.7 ± 14.8	68.6 ± 15.2	59.8 ± 18.8	<0.001
Females, *n* (%)	72 (48)	58 (36)	62 (65)	<0.001
BMI, *n* (%)	31.3 ± 9.3	26.9 ± 4.5	26.9 ± 11	<0.001
Symptom onset till admission, days	7 ± 6.1	11.4 ± 10	5.8 ± 4.1	<0.001
Admission D-dimer, ng/ml	3,441 ± 9,122	2,316 ± 4,520	1,863 ± 1,743	0.168
Diabetes millitus, *n* (%)	92 (62)	26 (16)	12 (13)	<0.001
Hypertension, *n* (%)	119 (80)	96 (60)	40 (42)	<0.001
Asthma, *n* (%)	24 (16)	3 (2)	1 (1)	<0.001
COPD, *n* (%)	24 (16)	18 (11)	13 (14)	0.558
Length of stay, days	16.5 ± 13	18.6 ± 13.1	11.6 ± 11	<0.001
Therapeutic anticoagulation, *n* (%)	113 (76)	66 (41)	88 (93)	<0.001
Mechanical ventilation, *n* (%)	81 (54)	20 (12)	23 (24)	<0.001
In-hospital death, *n* (%)	64 (43)	41 (25)	15 (16)	<0.001
D-dimer trends				<0.001
Persistently-low, *n* (%)	22 (15)	25 (16)	19 (20)	
Early-peaking, *n* (%)	11 (7)	16 (10)	6 (6)	
Mid-peaking, *n* (%)	29 (19)	23 (14)	18 (19)	
Fluctuating, *n* (%)	21 (14)	63 (39)	10 (11)	
Late-peaking, *n* (%)	16 (11)	5 (3)	9 (9)	
Persistently-high, *n* (%)	50 (34)	29 (18)	33 (35)	

BMI, body mass index; COPD, chronic obstructive pulmonary disease.

and fluctuating), and malignant patterns (late-peaking and persistently high).

Cox-regression analysis revealed that, overall, D-dimer trends are associated with an increased risk for in-hospital mortality in the first wave (overall: HR: 1,73, $p < 0.001$; New York site: RR: 1.58, $p < 0.001$; Milan site: RR: 1.82, $p < 0.001$; Bogota site: 1.9, $p = 0.008$) and stayed the same during the second wave (HR: 1.67, $p < 0.001$) and the third wave (HR: 2, $p = 0.002$).

Compared to low and high risk (Figure 4), the malignant risk patterns were associated with a significant RR of in-hospital mortality in the first wave (RR:3.64, $p < 0.001$, New York site: RR: 2.87 $p < 0.001$; Milan site: RR: 3.85, $p < 0.001$; Bogota site: 7.4, $p = 0.009$) as well as the second wave (RR: 3.83, $p < 0.001$), and the third wave (RR: 9.5, $p = 0.001$).

Univariate Cox-regression models were initiated for predictors of in-hospital mortality in all patients from all sites and across all waves (Table 4). It was found that age, hypertension, diabetes, mechanical ventilation, and D-dimer trends were all associated with increased risk for in-hospital mortality. Multivariate regression showed that only D-dimer trends and mechanical ventilation were associated with increased risk for mortality; however, D-dimer trends were a stronger predictor compared to mechanical ventilation (Table 4). When patients were stratified based on mechanical ventilation and malignant D-dimer trends, it was found that patients with malignant D-dimer trends were associated with a higher risk of in-hospital mortality both in those who were mechanically ventilated and those who did not require mechanical ventilation (Figure 4).

Discussion

In this case series, we report D-dimer patterns during hospitalization in patients with COVID-19 that show distinct mortality behavior. Six different patterns were observed (persistently-low, early-peaking, mid-peaking, fluctuating, late-peaking, and persistently-high). While we noted a progressively increasing risk of in-hospital death in these patterns, we also noted that the persistently-low and early-peaking are benign patterns associated with no mortality in our report. Mid-peaking and fluctuating patterns, in contrast, are patterns associated with elevated risk for in-hospital mortality, and late-peaking and persistently-high D-dimer were malignant patterns associated with the highest in-hospital mortality. Patients with the malignant D-dimer trends were noted to have an elevated risk of in-hospital mortality after adjustment for co-variates and regardless of the requirement of mechanical

TABLE 3 Comparisons between different trends in all waves.

	Persistent-low	Early-peaking	Mid-peaking	Fluctuating	Late-peaking	Persistent-high	*p*-value
1st wave							
Number	66	30	70	94	30	112	
Age, years	62.3 ± 15.8	65.8 ± 15.8	60.7 ± 16.6	66.8 ± 17.4	65.1 ± 18.9	66.7 ± 14.9	0.116
Females, *n* (%)	32 (48)	19 (63)	39 (56)	56 (60)	13 (43)	60 (54)	0.711
BMI, kg/m^2	31 ± 11.8	26.7 ± 4	29.7 ± 7.1	28.8 ± 7	31.4 ± 6	30.3 ± 9.9	0.444
Symptoms onset till admission, days	8.6 ± 10.5	7.6 ± 6	8.1 ± 6.6	9.8 ± 9.3	9.6 ± 10.7	6.5 ± 4.9	0.178
Admission D-dimer, ng/ml	926.2 ± 1,387	2787.8 ± 6,825	873 ± 838	2,031 ± 4,169	1,110 ± 1,143	6,388 ± 11,155	<0.001
Length of stay, days	12.7 ± 10	15.5 ± 13	15.9 ± 13	24.2 ± 13.8	17.8 ± 13.2	13.6 ± 11.6	<0.001
Therapeutic anticoagulation, *n* (%)	24 (36)	22 (73)	52 (74)	59 (63)	22 (73)	82 (73)	<0.001
Mechanical ventilation	8 (12)	1 (3)	22 (31)	32 (34)	14 (47)	39 (35)	<0.001
In-hospital death, *n* (%)	0 (0)	0 (0)	13 (19)	32 (34)	14 (47)	51 (46)	<0.001
2nd wave							
Number	22	12	7	24	20	26	
Age, years	57.6 ± 15.4	65.7 ± 13	69 ± 11.5	67.4 ± 15	65 ± 19	68.3 ± 15	0.215
Females, *n* (%)	10 (45)	6 (50)	3 (43)	14 (58)	11 (55)	18 (69)	0.614
BMI, kg/m^2	28.8 ± 8.7	30.5 ± 6.6	31.9 ± 7	31.6 ± 5.9	33.8 ± 12.4	33.9 ± 10.7	0.441
Admission D-dimer, ng/ml	390 ± 247	4,080 ± 4,455	468 ± 242	4,121 ± 10,782	404 ± 182	13,033 ± 32,596	<0.001
Length of stay, days	13.5 ± 8.6	16.4 ± 5.9	16.8 ± 3.6	23.2 ± 12.5	13.4 ± 6.5	17.8 ± 13.2	0.014
Therapeutic anticoagulation, *n* (%)	3 (14)	7 (58)	6 (86)	16 (67)	7 (35)	11 (42)	0.001
Mechanical ventilation	7 (32)	4 (33)	1 (14)	18 (75)	16 (80)	22 (85)	<0.001
In-hospital death, *n* (%)	0 (0)	0 (0)	1 (14)	17 (71)	17 (85)	24 (92)	<0.001
3rd wave							
Number	12	5	13	6	8	11	
Age, years	69.2 ± 19.6	74 ± 11.7	60.3 ± 15	53 ± 26.6	69.5 ± 13.9	61.7 ± 14.1	0.248
Females, *n* (%)	7 (58)	2 (40)	3 (23)	1 (17)	5 (63)	3 (27)	0.216
BMI, kg/m^2	32 ± 8.1	25 ± 3.4	29.3 ± 4.9	33.8 ± 11	38.3 ± 10	31.4 ± 9.1	0.09
Admission D-dimer, *n*g/ml	339 ± 109	2,002 ± 882	426 ± 232	516 ± 231	398 ± 179	5,025 ± 5,255	<0.001
Length of stay, days	16.5 ± 14.9	23.2 ± 19.1	18.6 ± 14.7	12.1 ± 8.6	13.9 ± 5.1	13.4 ± 6.7	0.612
Therapeutic anticoagulation, *n* (%)	5 (42)	4 (80)	12 (92)	5 (83)	7 (88)	11 (100)	0.009
Mechanical ventilation	0 (0)	0 (0)	4 (31)	5 (83)	5 (63)	5 (45)	0.002
In-hospital death, n (%)	0 (0)	0 (0)	3 (23)	1 (17)	6 (75)	6 (55)	0.002

ventilation. Importantly, these patterns and their associated risk seem to be universal among patients from different institutes with expected different genetic and ethnic backgrounds, and similar patterns were also observed in different waves of the pandemic.

D-dimer behavior as an example of COVID-19 heterogeneity

Our observation confirms the clinical and pathological heterogeneity in patients with COVID-19 (4, 7) and provides

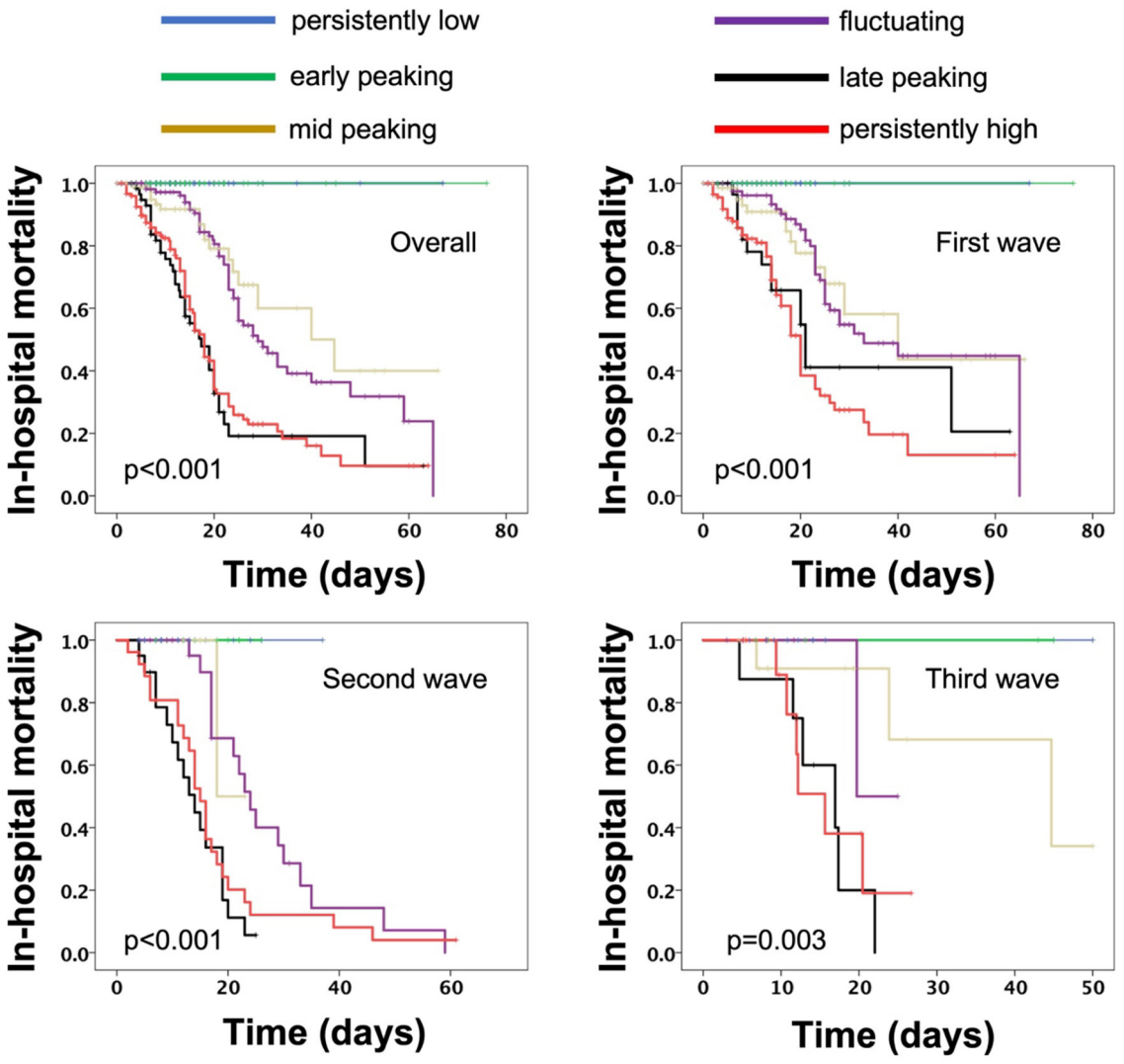

FIGURE 3
Kaplan–Meier curves for in-hospital mortality overall as well as during the first, second, and third waves.

an example of such heterogeneity through in-hospital D-dimer behavior. The laboratory signature of hospitalized patients with COVID-19 indicated that increased D-dimer levels are an integral part of the disease that is associated with worse outcomes and may be linked to a thrombotic state (9). Several studies have suggested the predictive ability of D-dimer in COVID-19 for worse outcomes; however, such studies focused on point measurement of D-dimer, especially during admission (10, 13). Our observation here suggests that elevated D-dimer on admission is not a pre-requisite for poor outcomes in patients with COVID-19. In fact, elevated D-dimer during admission may be associated with benign outcomes if the D-dimer decreases and stays low, while low D-dimer on admission may be associated with worse outcomes if D-dimer elevates once or more during the hospital stay. As such, our observation suggests that worse outcomes of COVID-19 are associated with specific patterns of D-dimer behavior during hospitalization rather than point-time measured values. The patterns observed suggest that worse outcomes are linked to a "later elevation" of D-dimer (during the hospitalization or toward the end of the encounter) or "delayed normalization" of D-dimer, and, vice versa, better outcomes are linked to earlier and continuous normalization of D-dimer.

Clinically, these findings seem of interest at least to guide the decisions in hospitalized patients with COVID-19. The role of D-dimer in the course of management of COVID-19 in all stages (pre-hospitalization, during hospitalization, and after discharge) is expanding, and its use to guide medical therapeutics such as anticoagulation is progressing despite early suspicion (8). In one prior study, it was found that the rate and the magnitude of the rise in D-dimer within the first 10 days in hospitalized patients with COVID-19 are associated with poor outcomes. In that study, this D-dimer behavior was found to be associated with venous thromboembolism but not mortality (12).

Moreover, D-dimer levels during hospitalization have been recently reported to be associated with the risk of worse outcomes in patients with COVID-19 (11). In a recent study,

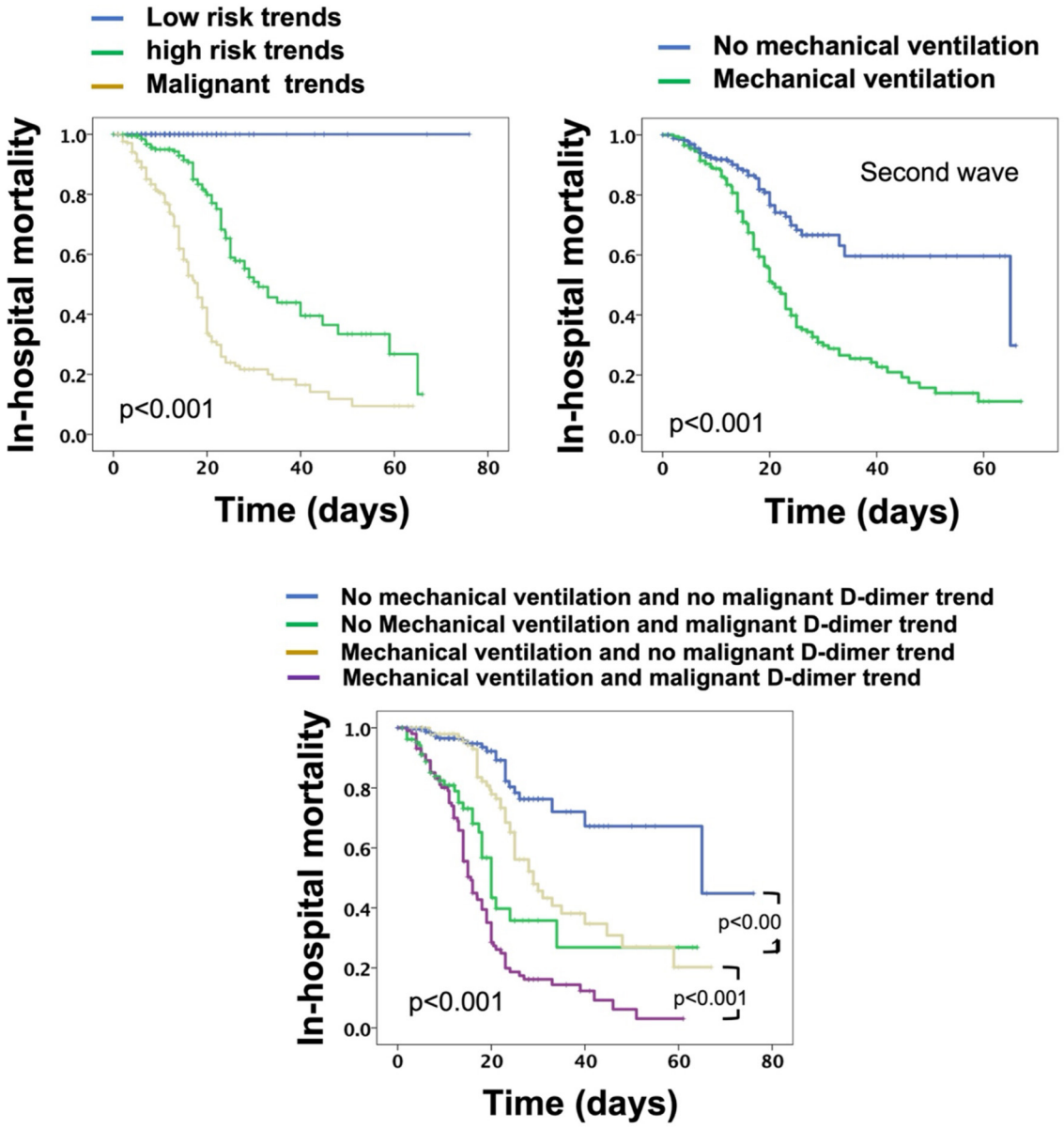

FIGURE 4
Kaplan–Meier curves for overall in-hospital mortality for patients stratified based on D-dimer trends, mechanical ventilation, and both.

the patterns of D-dimer during hospitalization were associated with higher risk than static measurements (11) indicating that an increasing D-dimer trend during hospitalization is associated with worse risk compared to stable or decreasing D-dimer levels. It is to be noted, however, that a clear differentiation of a normal cutoff value was not identified, and the inclusion criteria involved ≥3 D-dimer levels within 21 days of hospital admission which may have led to significant variation in D-dimer levels that can pass undetected between measured samples. Comparatively, in our report, patients were classified based on the lowest cutoff value reported in previous studies (1,000 ng/ml). The in-hospital D-dimer trends noted in our study somewhat differed in patterns and significance. First, because of the more frequent sampling in our study, more changes could be captured allowing for the identification and differentiation of the increasing D-dimer during hospitalization into three different groups (fluctuating D-dimer, mid-peaking, and late-peaking) compared to "increasing levels" in the aforementioned study. Second, in our study, we differentiated stable patterns into persistently-low and persistently-high. Third, the decreasing pattern in our study was a low-risk pattern compared to a higher risk for the same group in the aforementioned study and that can be explained by the immediate normalization of D-dimer in our report. Finally, the group of patients with "persistently increased" D-dimer was the sickest group of patients and was associated with the worst risk of outcomes. While it is unclear whether differences between both studies are reproducible, similarities in patterns do exist, pointing toward a level of heterogeneity among patients with COVID-19 previously underappreciated.

TABLE 4 Overall predictors of in-hospital mortality.

	Univariate			Multivariate		
	HR	*p*-value	95%CI	HR	*p*-value	95%CI
Age, years	1.01	0.015	1.01–1.02	1.01	0.178	0.99–1.02
Females, *n* (%)	0.79	0.117	0.59–1.06	-	-	-
Diabetes mellitus, *n* (%)	1.43	0.012	1.09–1.95	1.21	0.216	0.89–1.6
Hypertension, *n* (%)	1.6	0.007	1.14–2.24	1.36	0.101	0.94-2
Asthma, *n* (%)	0.8	0.481	0.42–1.5	-	-	-
COPD, *n* (%)	0.82	0.454	0.49–1.38	-	-	-
Admission D-dimer, ng/ml	1.01	0.09	0.99–1.02	-	-	-
Therapeutic anticoagulation, *n* (%)	0.96	0.681	0.68–1.29	-	-	-
Mechanical ventilation	2.6	<0.001	1.85–3.56	1.9	<0.001	1.4–2.7
DD trends						
Overall	3.8	<0.001	2.9–5	3.4	<0.001	2.6–4.6
High risk	4.2	<0.001	3.1–5.7	3.6	<0.001	2.6–5

We acknowledge the limitation of the observational nature of our case series report with small sample size, and conclusions should not be drawn until our findings are confirmed in large randomized clinical trials. Moreover, the effect of vaccination on the noted D-dimer trends was not conducted, and the expected taming effect of vaccination on the trends cannot be seen in the current report. It should also be emphasized that D-dimer behaviors noted in our study do not seem to be the governing factor behind the disease's extensive heterogeneity, as a large number of co-variates are suspected. D-dimer behavior is rather just a representation of how stratifying patients in such a manner may uncover previously under-detected effects such as the stratification done for the mechanical ventilation done in our study. While studying the nature and explanation of such heterogeneity is beyond the scope of the current report, it seems that such heterogeneity involves all demographic, clinical, and laboratory aspects of the disease. Accordingly, more in-depth large systematic prospective studies and retrospective meta-analyses taking into consideration the reported finding of D-dimer behavior in addition to other factors contributing to heterogeneity are needed to support our hypothesis. Finally, it is unknown whether the current observations are specific to patients with COVID-19, and further studies should compare D-dimer levels followed in patients between COVID-19 and other causes of elevated D-dimer in hospitalized patients.

Conclusion

Coronavirus disease 2019 is a thrombo-inflammatory disease that is both dynamic and heterogeneous. D-dimer behavior during hospitalization is an important example of such heterogeneity and yielded categories with a distinct risk of in-hospital mortality. Such patterns seem to be universal between different hospitals from different geographic locations despite the use of different anticoagulation approaches and occurred in similar fashions in all pandemic waves. Monitoring D-dimer behavioral categories may be useful in the management of these patients regardless of the need for mechanical ventilation. Further studies are needed to determine whether D-dimer category-guided management improves outcomes in patients with COVID-19.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, upon reasonable written request.

Ethics statement

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

Author contributions

DB, AO, and MP: conceptualization, hypothesis generation, data collection, statistical analysis, manuscript preparation, and revision. SH, HL, GP, VP, AA, MD, CV-T, JC, and SC: statistical analysis, manuscript preparation, and 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.

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Gut distress and intervention *via* communications of SARS-CoV-2 with mucosal exposome

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Acute coronavirus disease 2019 (COVID-19) has been associated with prevalent gastrointestinal distress, characterized by fecal shedding of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA or persistent antigen presence in the gut. Using a meta-analysis, the present review addressed gastrointestinal symptoms, such as nausea, vomiting, abdominal pain, and diarrhea. Despite limited data on the gut–lung axis, viral transmission to the gut and its influence on gut mucosa and microbial community were found to be associated by means of various biochemical mechanisms. Notably, the prolonged presence of viral antigens and disrupted mucosal immunity may increase gut microbial and inflammatory risks, leading to acute pathological outcomes or post-acute COVID-19 symptoms. Patients with COVID-19 exhibit lower bacterial diversity and a higher relative abundance of opportunistic pathogens in their gut microbiota than healthy controls. Considering the dysbiotic changes during infection, remodeling or supplementation with beneficial microbial communities may counteract adverse outcomes in the gut and other organs in patients with COVID-19. Moreover, nutritional status, such as vitamin D deficiency, has been associated with disease severity in patients with COVID-19 *via* the regulation of the gut microbial community and host immunity. The nutritional and microbiological interventions improve the gut exposome including the host immunity, gut microbiota, and nutritional status, contributing to defense against acute or post-acute COVID-19 in the gut–lung axis.

KEYWORDS

COVID-19, SARS-CoV-2, meta-analysis, gastrointestinal symptoms, gut-lung axis, microbiota, nutritional intervention

1. Introduction

The coronavirus disease-19 (COVID-19) first occurred in 2019 and is now a worldwide pandemic with more than 15 million deaths (1). Typically, the presence of gastrointestinal signs or symptoms during COVID-19 has been associated with approximately 35–50% of COVID-19 cases. In a meta-analysis examining 4,243 patients, the pooled prevalence of gastrointestinal symptoms was 17.6% (2). Frequently observed gastrointestinal symptoms include anorexia, diarrhea, vomiting, and abdominal pain (3). With increasing COVID-19 severity, gastrointestinal symptoms were more apparent (4). The pathogenesis of COVID-19, including gastrointestinal symptoms, remains elusive, despite tissue-specific immunofluorescence detection of SARS-CoV-2 binding to a specific receptor such as angiotensin-converting enzyme 2 (ACE2), predominantly expressed in the gastrointestinal tract (5, 6). Numerous cohort studies have reported that patients with COVID-19 and gastrointestinal

symptoms might exhibit an increased risk for worse clinical outcomes (7, 8). Disruption of the intestinal mucosal immune barrier can result in gut commensal microbes and pathogens entering local inner tissues and the vascular system, leading to septicemia and acute respiratory distress syndrome (ARDS) (9). Immune cells induced by various antigens can move between the gut and lungs *via* the lymphatic system or blood vessels, thereby regulating the immune response of target organs. Moreover, humoral factors, including cytokines and hormones, contribute to inter-organ communication (10).

The "gut–lung axis" is defined as the cross-talk between intestinal and pulmonary tissues mediated by microbes, immune cells, immune mediators, and other endogenous humoral regulators (11). SARS-CoV-2-induced distress in the gut–lung axis can be elucidated by several potent mechanisms: 1. Viruses directly cause gastrointestinal distress, resulting in symptoms, such as diarrhea, abdominal pain, and vomiting. 2. Viral infection may excessively trigger tissue injury factors, including proinflammatory cytokines, during a cytokine storm, increasing the risk of sepsis, ARDS, and multiorgan failure. 3. Viral infection may dysregulate the intestinal microbiota, increasing the risk of immunological disorders in the gut–lung axis and the systemic impact. Considering the gut–lung axis, we compared the gastrointestinal exposure and underlying pathogenesis mechanisms, including gut barrier distress, mucosal immune dysregulation, and disruption of the microbial community in the gut. Accordingly, the present review addressed the potential role of the gut–lung axis in the pathogenesis of COVID-19 and microbiota alteration in the immune response to establish effective dietary interventions. Inter-organ communication could provide new insights into gut-based interventions against SARS-CoV-2 infection.

2. Clinical symptom-based association between viral infection and gastrointestinal adverse outcomes

First, we evaluated the clinical evidence using the literature-based symptoms of gut distress in patients with COVID-19. The literature search for this association was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline. To address the clinical association between SARS-CoV-2 infection and gut distress, we performed the meta-analysis by collecting studies reporting the gastrointestinal symptoms or clinician-observed features in patients using laboratory-confirmed methods. To obtain an evidence-based minimum set of items according to the PRSIMA guideline, the gastrointestinal symptom-based case-control studies were selected from PubMed and LitCovid ($n = 244$), ScienceDirect ($n = 759$), and Google ($n = 140$). After de-duplication, all unique citations were independently screened by reviewers. In particular, articles that failed to meet established inclusion criteria were excluded by screening titles and abstracts, scrutinizing, and the consensus decision-making. We included studies with adequately available data on both control and case groups, but excluding case reports and studies of patients with symptoms other than gastrointestinal symptoms or underlying diseases such as cancer, autoimmune disease, and metabolic diseases. Finally, eight articles were evaluated in the meta-analysis (Figure 1). The selected articles covered events in 14,188 patients, comprising 2,800 COVID-19-positive patients and 11,388 control patients from five countries, including the USA, Portugal, China, Italy, and Australia. For efficient data extraction, we combined symptoms of "abdominal pain" and "abdominal distension" into the more prevalent and widely reported symptoms of "abdominal discomfort". Where studies reported one symptom "or" another (e.g., nausea or vomiting), we extracted the prevalence of both. We extracted grouped symptoms (e.g., any gastrointestinal symptoms) without further description or definition, rather than using the sum of all gastrointestinal symptom data to prevent data overlapping between symptoms. The pooled prevalence of each symptom was estimated using the Metaprop package and the variance was normalized using a random-effects model such as Freeman-Tukey arcsine transformation of the prevalence. Statistical heterogeneity was assessed by I2, the proportion of total variation due to inter-study heterogeneity.

2.1. Association of gastrointestinal symptoms with COVID-19

In study ID 1, the pooled odds ratio (OR) was 1.91 (95% confidence interval [Cl]: 1.17–3.12), with a weight of 15.18% (12). In study ID 2, the pooled OR was 2.34 (95% Cl: 1.94–5.23), with a weight of 13.64% (13). In study ID 3, the pooled OR was 1.28 (95% Cl: 0.30–5.48), with a weight of 9.30% (14). In study ID 4, the pooled OR was 1.56 (95% Cl: 1.40–1.73) with a weight of 16.10% (15). In study ID 5, the pooled OR was 1.5 (95% Cl: 0.95–2.56), with a weight of 15.14% (16). In study ID 7, the pooled OR was 2.59 (95% Cl: 1.55–4.32), with a weight of 15.08% (17). In study ID 8, the pooled OR was 1.49 (95% Cl: 1.02–2.17), with a weight of 15.56% (18). Collectively, the pooled OR of 1.76 (95% CI: 1.61–1.93) indicated a significant association between COVID-19 and GI symptoms, while the random-effect meta-analysis revealed a large heterogeneity among studies ($I^2 = 98.1\%$; Figure 1A).

2.2. Association of diarrhea with COVID-19

In study ID 1, the pooled OR was 5.03 (95% Cl: 1.44–17.53), with a weight of 11.16% (12). Study ID 2 was not included (13). In study ID 3, the pooled OR was 1.28 (95% Cl: 0.30–5.48), with a weight of 9.65% (14). In study ID 4, the pooled OR was 1.67 (95% Cl: 1.45–1.93) with a weight of 16.66% (15). In study ID 5, the pooled OR was 0.96 (95% Cl: 0.54–1.72), with a weight of 15.33% (16). In study ID 6, the pooled OR was 2.69 (95% Cl: 1.59–4.56), with a weight of 15.58% (19). In study ID 7, the pooled OR was 2.37 (95% Cl: 1.47–3.82), with a weight of 15.78% (17). In study ID 8, the pooled OR was 1.42 (95% Cl: 0.89–2.24), with a weight of 15.85% (18). Overall, the pooled OR of 1.88 (95% CI: 1.68–2.11) indicated a significant association between COVID-19 and diarrhea, while the random-effect meta-analysis revealed a large heterogeneity among studies ($I^2 = 96.2\%$; Figure 1B).

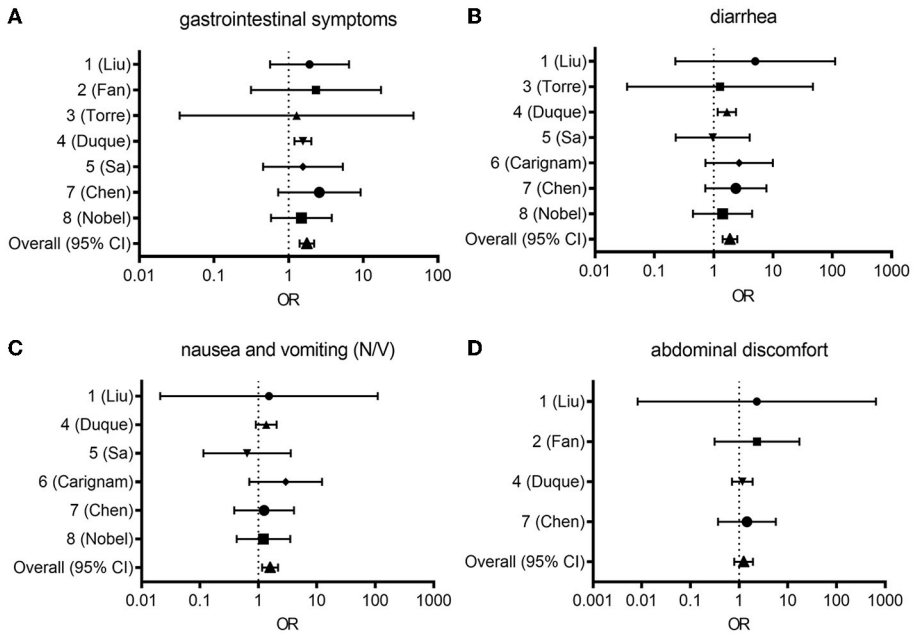

FIGURE 1
Forest plot from random effects analysis: OR for presenting any gastrointestinal symptom **(A)**, diarrhea **(B)**, nausea and vomiting [N/V, **(C)**], abdominal discomfort **(D)** in the COVID-19 group vs. the control group. CI, confidence interval; COVID-19, coronavirus disease 2019; OR, odds ratio.

2.3. Association of nausea and vomiting with COVID-19

In study ID 1, the pooled OR was 1.53 (95% Cl: 0.27–8.50), with a weight of 9.02% (12). Study IDs 2 and 3 were not included in this analysis (13, 14). In study ID 4, the pooled OR was 1.36 (95% Cl: 1.16–1.61), with a weight of 19.27% (15). In study ID 5, the pooled OR was 0.64 (95% Cl: 0.32–1.28), with a weight of 17.09% (16). In study ID 6, the pooled OR was 2.93 (95% Cl: 1.65–5.23), with a weight of 17.80% (19). In study ID 7, the pooled OR was 1.26 (95% Cl: 0.78–2.01), with a weight of 18.31% (17). In study ID 8, the pooled OR was 1.22 (95% Cl: 0.80–1.87), with a weight of 18.51% (18). Overall, the pooled OR of 1.59 (95% CI: 1.40–1.87) indicated a significant association between COVID-19 and diarrhea, while the random-effect meta-analysis revealed a large heterogeneity among studies ($I^2 = 97.9\%$; Figure 1C).

2.4. Association of abdominal discomfort with COVID-19

In study ID 1, the pooled OR was 2.30 (95% Cl: 0.24–22.38), with a weight of 8.60% (12). In study ID 2, the pooled OR was 2.34 (95% Cl: 1.04–5.23) with a weight of 27.98% (13). Study ID 3 was not included (14). In study ID 4, the pooled OR was 1.17 (95% Cl: 0.96–1.42) with a weight of 32.80% (15). Study IDs 5 and 6 were not included in this analysis (16, 19). In study ID 7, the pooled OR was 1.24 (95% Cl: 0.84–2.52), with a weight of 30.62% (17). Study ID 8 was absent (18). Overall, the pooled OR of 1.24 (95% CI: 1.04–1.48) indicated a significant association between COVID-19 and abdominal discomfort, while the random-effect meta-analysis revealed a large heterogeneity among studies ($I^2 = 96.0\%$; Figure 1D).

Owing to the high levels of heterogeneity (96.0–98.1%) among studies, additional subgroup analysis, meta-regression, or sensitivity analysis could clarify the underlying causes behind high heterogeneity between studies. The Newcastle-Ottawa Scale may afford an alternate tool for assessing the quality of case-control studies in meta-analyses (20). Taken all symptoms and prevalence, all pooled OR (95% CI: 1.04-2.24) indicated notable positive associations between COVID-19 and gut distress-associated symptoms despite the heterogeneity between studies. Based on the literature-based assessment of the clinical outcomes, we further evaluated the pathological processes and mechanisms of the lung-gut communications in patients with COVID-19.

3. Viral entry and translocation into the gut–lung axis

3.1. Airway entry and reverse translocation to gut

Coronaviruses are enveloped single-stranded RNA viruses characterized by club-like spikes projecting from their surfaces, with a remarkably large RNA genome. The SARS-CoV-2 genome encodes four major structural proteins: spike (S), nucleocapsid (N), membrane (M), and envelope (E), each of which is essential for composing the viral particle (21). Phylogenetic analysis of the complete genome sequence of SARS-CoV-2 revealed that the new virus shares 89.1% nucleotide sequence identity with SARS-like coronaviruses detected in bats (22). ACE2, the functional

receptor of SARS-CoV-1 and SARS-CoV-2, plays a crucial role in the pathogenesis of COVID-19, as it allows viral entry into human cells (23). Similar to SARS-CoV-1, the viral S protein of SARS-CoV-2 binds to ACE2 as a cellular receptor. Importantly, SARS-CoV-2 is more pathogenic, partly owing to its 10-to-20-fold increased binding affinity for ACE2 (24). This binding leads to viral host cell entry, in parallel with S protein priming by the host cell protease, transmembrane serine protease 2 (TMPRSS2). The S glycoprotein contains two functional domains: an S1 receptor-binding domain (RBD) and a second S2 domain that mediates the fusion of viral and host cell membranes (25). The SARS-CoV-2 S protein initially binds to the ACE2 receptor on the host cell through the S1 RBD. The S1 domain is shed from the viral surface, allowing the S2 domain to fuse with the host cell membrane. This process depends on the activation of the S protein by cleavage at two sites (S1/S2 and S2') *via* the proteases furin and TMPRSS2. Furin-induced cleavage leads to conformational changes in the viral S protein, exposing the RBD and S2 domains. TMPRSS2-mediated cleavage of the SARS-CoV-2 S protein facilitates the fusion of the viral capsid with the host cell to permit viral entry (26). Exposure of the RBD in the S1 protein subunit results in an unstable subunit conformation; thus, during binding, this subunit undergoes conformational rearrangement between two states, known as the up- and down-conformations. The down-state transiently hides the RBD, whereas the up-state exposes the RBD but temporarily destabilizes the protein subunit (27–29). Within the trimeric S protein, only one of the three RBD is present in an accessible conformation for binding with the ACE2 receptor.

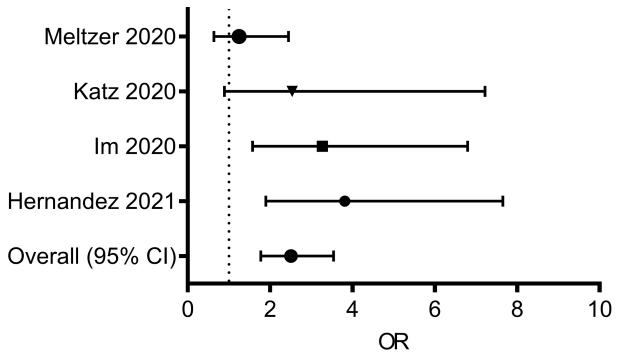

FIGURE 2
Forest plot from random effects analysis: Vitamin D status (Low serum 25OHD, daily dietary intake) and COVID-19 infection rate. ORs of having vitamin D deficiency in the COVID-19 group vs. the control group. 25OHD, 25-hydroxyvitamin D; COVID-19, coronavirus disease 2019; ORs, odds ratio.

ACE2 is detected in the nasal and bronchial epithelial cells. In addition to the upper respiratory tract, ACE2 is abundantly expressed on the surface of alveolar type II pneumocytes, which co-express several other genes involved in the regulation of viral reproduction and transmission, including TMPRSS2. Type II pneumocytes are well-known to produce surfactants, maintain their self-renewal ability, and exert immunoregulatory functions. Importantly, these cells share the same basement membrane as the closely juxtaposed capillary endothelial cells, which also express high ACE2 levels. Therefore, type II pneumocytes, along with the neighboring capillary endothelium, could be primary sites for SARS-CoV-2 entry, resulting in damage to the alveolocapillary membrane with reactive hyperplasia of type II pneumocytes. As type II pneumocytes are known targets of viral entry and replication, this may lead to a vicious cycle of persistent alveolar wall destruction and repair, eventually culminating in

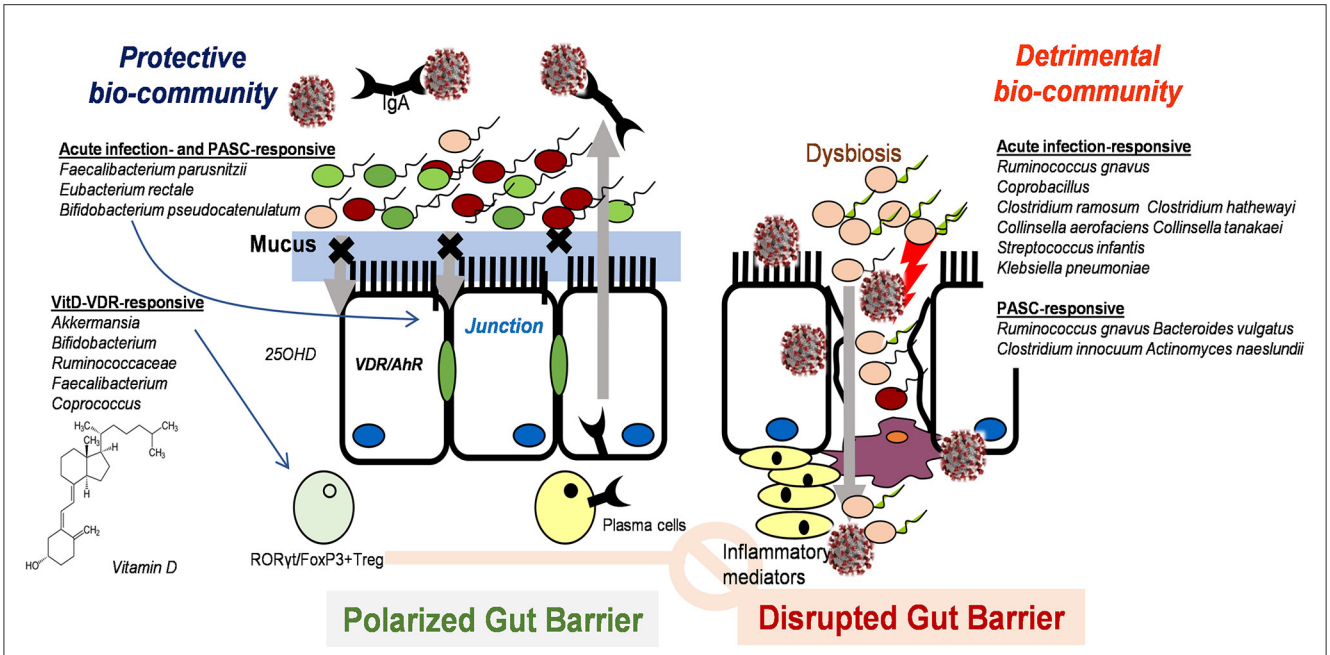

FIGURE 3
Postulated scheme of vitamin D-induced intervention against SARS-CoV-2 infection in the gut mucosa *via* modulation of microbiota and subsequent immune regulation.

progressive, severe diffuse alveolar damage. Upregulated ACE2 expression has been documented in the airways of patients with chronic respiratory disease who are smokers, which, together with disturbed ciliary movement and abnormal mucus viscosity, may increase disease vulnerability (30). However, clinical evidence indicates that smoking does not necessarily lead to increased vulnerability (31). Recently, a healthy human donor-based evaluation suggested that the virus could exploit goblet and ciliated cells in the nasal epithelia as entry portals, a plausible primary infection site (32). Considering the variant-mediated adverse outcomes, Omicron is known to cause relatively mild symptoms compared with other variants of concern. The Omicron variant can enter epithelial cells through different binding proteins such as cathepsins and display lower replication competence than other variants (33), potently contributing to attenuated severity of the clinical outcomes.

Airway particles, including viral particles, are entrapped in the airway mucosa and cleared *via* mucociliary transport. However, the clearance system can be damaged following SARS-CoV-2 infection *via* dedifferentiation of multiciliated cells and subsequent attenuation of cilial movement, as shown in a reconstructed human bronchial epithelium model (34). As guardians of the airway, alveolar macrophages can play crucial roles in removal *via* phagocytosis or translocation from the peripheral lung to the larynx, with subsequent passage through the gut and fecal excretion (35). In addition to gastrointestinal translocation from the airway, the virus can enter the water and food supply systems directly, ultimately reaching the gastrointestinal tract in humans (36, 37). Viral particles that successfully reach the alveolar vasculature or translocate into the gut can systematically affect extra-airway tissues including the gut if they escape the immune system in circulation.

3.2. Vascular translocation and circulation of SARS-CoV-2

ACE2 receptors are also expressed in endothelial cells. It remains unknown whether vascular derangements in COVID-19 can be attributed to endothelial cell involvement mediated by the virus. Intriguingly, SARS-CoV-2 can directly infect engineered human blood vessel organoids *in vitro* (38). In this *in vitro* experiment, to verify the possibility of COVID-19 transmission through the endothelial tissue, the authors used human capillary organoids from induced pluripotent stem cells infected with SARS-CoV-2 (39). Notably, human recombinant secretory ACE2 could inhibit infection in organoids mimicking human capillaries with CD31 and PDGFR.

An initial study has suggested that the SARS-CoV-2 S protein can bind to CD147 on the cell surface and subsequently enter blood cells, such as platelets and megakaryocytes. Megakaryocytes and platelets actively take up SARS-CoV-2 virions, possibly through an ACE-2-independent mechanism. Based on *in vitro* antiviral tests, meplazumab, an anti-CD147 humanized antibody that blocks the interaction between the S protein and the CD147 cell surface receptor, could significantly inhibit viral cell entry into circulation. CD147 is a SARS-CoV-2 surface entry receptor, leading to inflammation and thrombosis, which differs from the common cold coronavirus. Moreover, given that elevated blood sugar levels could upregulate CD147 expression, diabetes could be a potential risk factor for poor prognosis in patients with COVID-19 (40). Vasculature-translocated surviving viral particles are available for the secondary tissue infection and subsequent inflammatory outcomes in the gut.

3.3. Gut entry *via* fecal–oral transmission

Owing to intestinal viral RNA shedding, there have been growing concerns that SARS-CoV-2 could be transmitted *via* the fecal–oral route, given that viral RNA has been detected in patient stool samples (41). It has been suggested that the presence of gastrointestinal symptoms is a likely indicator of viral RNA in the stool (2, 42). In contrast, studies have failed to establish a statistically significant correlation between viral RNA and increased gastrointestinal symptom intensity (41, 43). However, it has been suggested that stool samples may be positive for viral RNA even when the virus is undetectable in respiratory samples (2, 44). It is well-established that viruses can enter the gut, but most cannot survive in the digestive tract, owing to the low pH of gastric fluid and the harsh intestinal environment comprising bile and digestive enzymes. Therefore, no infectious virus was recovered from the fecal samples of patients with COVID-19. Although stool is unlikely to contain infectious viruses (45), confirmative assessments are warranted to comprehensively establish the risk of fecal–oral transmission during infection and its significance in the food system (46).

Theoretically, SARS-CoV-2 directly invades the gastrointestinal epithelium through ACE2 receptor. ACE2 is highly expressed in the esophageal upper and stratified epithelium, as well as in absorptive enterocytes derived from both the ileum and colon (5). In approximately 50% of COVID-19 cases, viral RNA was detected in fecal samples, even in the absence of gastrointestinal tract manifestations and after clearance of respiratory infection, thereby suggesting an asymptomatic SARS-CoV-2 infection in the gut and the possibility of fecal–oral transmission (47). However, considering the limited data available, a fecal–oral transmission route clarifying enteric symptoms in patients with COVID-19 is yet to be proposed. Moreover, it is also challenging to rationalize that SARS-CoV-2, as an enteric virus, passes through the stomach and reaches the intestine to infect the intestinal cells. For successful infection *via* fecal–oral transmission, the virus must overcome biological barriers, such as stomach acid and intestinal bile salts after ingestion. Coronavirus can undergo complete inactivation at pH 2.26 and 4.38 at 37 °C (48). Although the virus can survive under wet or dry conditions for up to 3 days, it was found to survive at pH 2.2 for up to 1 h only at high concentrations (49). Bile salts are one of the various mechanisms that mediate host defense, exerting detergent action against the lipid layer integrity of infectious agents (50). SARS-CoV-2 contains an outer lipid-containing membrane and is an enveloped virus (23). Bile acid is known to be effective against viruses with lipoproteins, but envelope-deficient variants are resistant to its detergent action. Considering all the evidence, in addition to the airway viral infection, the oral ingestion of surviving viral particles contributes to the gastrointestinal distress.

4. Impact of SARS-CoV-2 on mucosal defense

4.1. SARS-CoV-2-mediated gut barrier distress

The gut is divided into several anatomical barriers, each of which plays a vital role in serving as a barrier against foreign materials, such as pathogens and other noxious stimuli. The mucus layer is the first line of defense, composed of mucus, antibodies, and other antimicrobial factors (51). It functions as a physical barrier protecting epithelial cells from microbes (bacteria, fungi, and virus) and large molecules, such as food particles (52). The second layer, beneath the mucus layer, comprises highly glycosylated proteins, glycocalyx, lining the epithelial cell surface. These cell membrane-bound glycoproteins, such as the mucus layer, act as a physical barrier that prevents pathogenic microorganisms from communicating with the gut epithelial cellular monolayer and invading the submucosal tissues (53). The epithelial cell barrier is another defense mechanism against gut microbes and luminal antigens *via* modulation of the epithelial junctional molecules or transmitting danger signals to the underlying mucosal immune system while facilitating the transport of nutrients and water (54). Epithelial cells have pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which allow the recognition of microbial antigens. Enterocytes (or intestinal epithelial cells) are the most common cell type in the mucosal epithelial layer, accounting for 90% of cells (55). Enterocytes are well-known absorption sites and important components of the gut barrier. Gut epithelial cells can also interact with SARS-CoV-2 through the highly expressed ACE2 (24, 56, 57). SARS-CoV-2 has been shown to infect intestinal organoids (58). Furthermore, TMPRSS2, which is also highly expressed in enterocytes in the ileum and colon (57, 59), reportedly participates in priming the SARS-CoV-2 S protein and facilitates viral entry into cells (24). Accordingly, ACE2 and TMPRSS2 are promising targets for intervention against SARS-CoV-2 (60) despite limited evidence on the efficacy of blockers targeting the two proteins (61). Intestinal viral infections may damage the epithelial barrier. For example, Middle East respiratory syndrome-related coronavirus was shown to disrupt the gut epithelial barrier in an animal model (62). Mechanistically, SARS-CoV infection can lead to the redistribution of the PALS1 protein, a tight junction protein, and subsequent disruption of epithelial integrity in the gut and lungs. Moreover, SARS-CoV-2 RNA and viral nucleocapsid protein were persistent in mucosal tissues and cells, including the gut epithelium and $CD8^+$ T cells of patients with inflammatory bowel disease nearly 7 months after SARS-CoV-2 infection (63). Consistent with the airway infection, the Omicron variants showed reduced levels of cytotoxicity- and damage-associated markers in infected gut organoids, compared with the wild type virus and delta variants (33). In contrast, delta variant-infected mini-gut exhibited active clustering of infected gut cells and relatively high levels of the replication efficacy. Since active invasion by the Omicron variant was extremely scarce and lumen-restricted in the gut model, the variant is not assumed to affect the submucosa parts. Therefore, different strains may have different relative tissue tropisms and invasiveness, potently leading to strain-specific clearance rates and clinical symptoms in the gut.

Fecal SARS-CoV-2 RNA has been detected in 50% of patients experiencing gastrointestinal symptoms, such as abdominal pain, nausea, and vomiting, within the first week after diagnosis (64). In particular, 12.7% (8.5–18.4%) of subjects displayed persistent fecal shedding of SARS-CoV-2 RNA even after 4 months of diagnosis, without ongoing shedding of oropharyngeal SARS-CoV-2 RNA. Although the above-mentioned study failed to link mucosal viral antigens with the severity of acute COVID-19, it is necessary to address the roles of mucosa-persistent antigens in mucosal defense, recurrence, and disease progression as post-acute sequelae of COVID-19 (PASC). After acute COVID-19, most patients with inflammatory bowel disease presented persistent presence of SARS-CoV-2 antigens in their gut mucosa, irrespective of inflammation levels, potentially contributing to PASC symptoms (63). Despite the lack of mechanistic evidence, it has been proposed that SARS-CoV-2 may increase intestinal permeability, potentially by damaging enterocytes and the epithelial layer (65), necessitating further molecular investigation.

4.2. Mucosal and systemic innate immunity to SARS-CoV-2

Coronaviruses are known to cause airway damage and lead to pneumonia with imbalanced and hyper-immune responses (22). Increased proinflammatory cytokines and lymphocytopenia have been associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (66). An unbalanced immune response and excessive inflammatory cytokine secretion, known as a "cytokine storm," have been associated with disease severity and worse prognosis in patients with COVID-19, including multiorgan failure (67, 68). Of 197 patients, approximately 34.5% presented neutrophilia (69), which is known to trigger ARDS and sepsis growth in patients with COVID-19. Secondary hemophagocytic lymphohistiocytosis (SHLH), an underrecognized hyperinflammatory syndrome, could also be a significant factor in the development of COVID-19, given that SHLH can cause hypercytokinemia-related fatal and fulminant multiorgan failure (70).

SARS-CoV-2 can spread *via* respiratory droplets, contact, and the fecal–oral route. Viral replication commences in the nasopharynx and upper respiratory tract and continues through the lower respiratory tract and gastrointestinal mucosa (5). Monocytes, macrophages, and dendritic cells (DCs) can serve as primary hallmarks of SARS-CoV-2 infection, given that they link innate and adaptive immunity and play an important role in the antiviral response (71–73). Although the precise correlation between DCs and SARS-CoV-2 in the mucosa has been poorly explored, SARS-CoV-2 accelerates the activation of PRR-linked signaling, including NLRP3 inflammasome or occasionally leads to the cytokine release syndrome (CRS) *via* robust production of proinflammatory mediators, such as interleukin (IL)-6, granulocyte-macrophage colony-stimulating factor, IL-1β, and tumor necrosis factor (TNF)-α during the CRS (74). Therapeutic agents, such as anti-IL-6R, which can target macrophage-related activity, could be crucial interventions against the cytokine storm that occurs during severe SARS-CoV-2 infection (33). In addition to the phagocytic system, natural killer (NK) cells have been associated with a severely

poor prognosis of SARS-CoV-2 infection in the presence of functional exhaustion. Among the various cytokines produced during early severe COVID-19, interferon (IFN)-α expression markedly correlated with the severity of COVID-19 (75, 76). According to single-cell transcriptomic analysis based on two COVID-19 cohorts, IFN-α directly suppressed IFN-γ production by NK cells (76). Moreover, exhausted NK cells reportedly express CD94/NK group 2 member A(NKG2A), which functions as an inhibitory receptor that reduces the production of CD107a, IFN-γ, IL-2, granzyme B, and TNF-α. Therefore, improving NK cell-mediated defense might be a promising defense mechanism during early severe cases of SARS-CoV-2 infection (77, 78). Active NK cells recognize viral infection and transmit death signals into the infected cells in the mucosa. Moreover, NK cells may facilitate mucosal phagocyte-induced viral clearance *via* production of anti-virus cytokines including type I interferons. However, exhausted NK cells would fail to defend against SARS-CoV-2 in the mucosa.

4.3. Acquired immunity and mucosal vaccination against SARS-CoV-2

In addition to direct infective actions of SARS-CoV-2, respiratory virus-responsive mucosal and systemic acquired immune responses would affect the disease progression in the extra-airway tissues. Cytotoxic CD8+ T cells directly neutralize infected cells or CD4+ T cells initiate a humoral response by cooperating with B cells (79, 80). During severe SARS-CoV-2 infection, lymphopenia is accompanied by a marked reduction in CD4+ T and CD8+ T cells, along with elevated neutrophil counts (81–83). An increased neutrophil-to-lymphocyte ratio and elevated levels of IL-6 can indicate poor prognosis and disease severity. Increased serum levels of proinflammatory cytokines, such as IL-6, IL-7, IL-1β, IL-2, and IL-10, can induce a cytokine storm and cause serious damage, more destructive than the coronavirus itself. Elevated proinflammatory cytokine levels have been linked to viral sepsis, respiratory failure, shock, and even death if severe (84). Therefore, addressing lymphopenia and cytokine storm could prevent severe complications associated with coronavirus.

Following the appearance of COVID-19 symptoms, the antibody response increases after 4–8 days, and IgM becomes predominant (85), followed by 10–18 days of persistent IgA and IgG production. IgA is crucial in mucosal defense by neutralizing SARS-CoV-2 and weakening the inflammatory risk (86). The antigen can attach to intestinal epithelial cells or microfold (M) cells, followed by transport into lymph nodes and IgA-secreting B cell activation in the lymphoid tissue (87, 88). Considering SARS-CoV-2, the antigen amount and quality critically impact neutralization. Antibodies should be specific to the S protein and must be detected in the serum for 2–3 weeks post-infection (89, 90). Human convalescent serum transfer has been proposed as a potential strategy to prevent and treat severe cases of COVID-19, with its therapeutic value documented in several clinical trials (84, 91–94). An important challenge in overcoming COVID-19 is viral elimination from the mucosa through antibody-associated shedding. Given that infectious agents trigger mucosal immunity (95), mucosal vaccination could be a promising strategy to evoke IgA antibodies at both the mucosal surface and the systemic immune system (96). Importantly, mucosal vaccination may facilitate IgA-virus complex formation in the mucosa of respiratory and intestinal tissues (97). As current modes of COVID-19 vaccination are predominantly based on systemic antigen exposure, efficient strategies are needed to develop promising mucosal vaccination against continuously evolving SARS-CoV-2.

5. Involvement of gut microbial community in SARS-CoV-2 pathogenesis

Following initial lung infection, SARS-CoV-2 invades the gut mucosal immune barrier, directly impacting the intestinal physiology. Moreover, intestinal tissue damage may facilitate gut dysbiosis. It has been reported that commensal microbiota in the lung and gut can counterbalance viral infection by modulating immune responses in a homeostatic manner (98, 99). For instance, viral infection-induced changes in pulmonary tissues and other microenvironments may alter the structure and function of the gut microbiota (98). In a mouse model, seasonal influenza infection of the respiratory tract increased the number of *Enterobacteria* in the gut microbiota and decreased the number of *Lactobacillus* and *Lactococcus* (99). Furthermore, intestinal dysbiosis has been associated with increased mortality following respiratory infections, probably due to deregulated airway immune responses. Inflammatory dysbiosis of the gut microbiota and epithelial damage reportedly enhance ACE2 levels, increasing the risk of SARS-CoV-2 infection in the gastrointestinal tract, as well as dissemination to other sites *via* circulation (5, 100).

5.1. Microbiota-linked prediction of adverse outcomes

Various studies have revealed how SARS-CoV-2 infection can alter gut microbiota and its association with adverse outcomes in humans. In particular, viral infection-altered gut communities were shown to be associated with inflammatory status in patients with COVID-19. Serum-based proinflammatory biomarkers positively correlated with increased levels of some consortia, including *Ruminococcus gnavus*, during viral infection, whereas *Clostridia* was negatively correlated (101). Moreover, disease severity could be correlated with the abundance of *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi* (102). It has been reported that approximately 50% of patients with COVID-19 display stool positivity for SARS-CoV-2 even in the absence of gastrointestinal manifestations and after recovery of respiratory SARS-CoV-2 infection (47), indicating the presence of persistent gut infection. Based on viral infectivity prediction using metagenomic analysis of the fecal SARS-CoV-2 genome, patients with COVID-19 demonstrate an increased functional capacity for nucleotide and amino acid biosynthesis and carbohydrate metabolism (47). An in-depth assessment demonstrated an evident correlation between viral infection signatures and the enrichment of gut pathogens, including *Collinsella aerofaciens*, *Collinsella tanakaei*, *Streptococcus*

infantis, and *Morganella morganii*, even in the absence of gastrointestinal manifestations (47). Although the Omicron variant is known to cause relatively mild symptoms with marginal invasiveness in humans and gut models, all SARS-CoV-2 variants of concerns remarkably disrupted the mouse gut microbiota (103). Surprisingly, the Omicron variant infection led to long-lasting instability in the gut microbiota and a notable depletion in *Akkermansia muciniphila*, even in the absence of severe lung pathology. In addition to host markers or disease severity, the fecal viral footprint was notably associated with dysbiosis-linked alterations in gut bacterial communities, paving the way for novel diagnostic tools for potent relapse or chronic adverse outcomes in post-COVID or long-term COVID conditions, potently with differential responses to SARS-CoV-2 variants.

In addition, SARS-CoV-2 infection can alter the gut virome community. Although patients with COVID-19 presenting reduced abundance exhibit an under-representation of RNA virus and multiple bacteriophage lineages (DNA viruses), they have notable gut enrichment of environment-derived eukaryotic DNA viruses, mainly including crAs-like phages, *Myoviridae*, and *Siphoviridae* families, even after of 30 days of symptom resolution (104, 105). Viral genes involved in bacteriophage integration, DNA repair, metabolism, and virulence are predicted to contribute to host stress and inflammation; however, some viral consortia are inversely associated with blood levels of proinflammatory proteins, white cells, neutrophils, and disease severity (104, 105). These resident enteric viruses maintain a low level of immune stimulation and are responsible for protective and regulatory effects in the intestine (106). However, given the limited data on the effects of viral composition on microbiota composition and activity during SARS-CoV-2 infection, advanced interkingdom associations need to be addressed to improve the integrated prognosis and intervention against adverse outcomes in patients with post-COVID or long COVID.

5.2. Microbiota-based probiotic counteraction against infection

In patients with COVID-19, reduced beneficial commensals were directly correlated with disease severity and complications (107). It is speculated that a decline in probiotic intestinal microbiota would fail to effectively control excessive proinflammatory immune reactions, leading to the subsequent progression of SARS-CoV-2 infection. Considering the immunomodulatory cytokine production in response to beneficial commensal bacteria, the abundance of *Lactobacillus* species decreased in correlation with anti-inflammatory IL-10 levels during SARS-CoV-2 infection (108). Therefore, serum IL-10 can be employed as a diagnostic indicator to assess disease progression and severity in high-risk patients with COVID-19 (108). Moreover, disease severity is inversely correlated with the abundance of *Faecalibacterium parusnitzii,* an anti-inflammatory bacterium (102) and subjects with low levels of viral infectivity features presented a relatively high abundance of short-chain fatty acid-producing beneficial bacterial communities, including *Parabacteroides*, *Bacteroides*, *Alistipes,* and *Lachnospiraceae*, even in the absence of gastrointestinal manifestations (47). Furthermore, several gut immune-modulating commensal bacteria, including *Faecalibacterium prausnitzii, Eubacterium rectale*, and *bifidobacteria*, were inversely associated with levels of proinflammatory mediators, tissue injury markers (lactate dehydrogenase, aspartate aminotransferase, and gamma-glutamyl transferase), and disease severity (109). Accordingly, these immune-modulating bacteria can potentially counteract proinflammatory and toxic insults during viral infection, providing novel insights into interventions against adverse outcomes during PASC conditions. Patients with PASC tended to display high levels of *Ruminococcus gnavus and Bacteroides vulgatus* and low levels of *Bifidobacterium pseudocatenulatum* and *Faecalibacterium prausnitzii* (110). Considering the inflammatory states due to reduced levels of probiotic commensal community, patients with COVID-19 are speculated to be remarkably susceptible to infection by opportunistic bacteria, such as *Klebsiella pneumoniae*, *Streptococcus*, and *Ruminococcus gnavus,* particularly during the hospitalization period (102). Likewise, patients with PASC were found to be markedly susceptible to nosocomial gut pathogens, such as *Clostridium innocuum* and *Actinomyces naeslundii* (110). These opportunistic bacteria can potentially trigger the production of proinflammatory cytokines, such as IFN-γ and TNF-α (102). Overall, the reduced abundance of probiotic gut bacteria can be associated with severe inflammatory responses *via* the excessive production of proinflammatory cytokines and severe complications in high-risk patients with COVID-19. Therefore, remodeling or supplementation with beneficial microbial communities are promising interventions against the gut mucosal distress in patients with COVID-19.

6. Effects of nutritional status on susceptibility to COVID-19

6.1. Association of nutritional deficiency with disease severity during viral infection

Considering the gastrointestinal involvement in SARS-CoV-2 infection, dietary components, including nutrients, bioactive natural products, and probiotics, were assumed to contribute to immune regulation in response to viral infections. In the French NutriNet-Santé cohort study assessing 7,766 adult patients with anti-SARS-CoV-2 antibodies, dietary intake of vitamin C, vitamin B9, vitamin K, fibers, and fruit vegetables was associated with lower susceptibility to SARS-CoV-2 infection, whereas dietary intake of calcium and dairy products did not contribute to the infection risk (111). The beneficial effects of vitamin C have been well-documented in various *in vitro* and *in vivo* studies. Exposure to high doses of vitamin C can induce antiviral actions against various viruses (112). In clinical trials, treatment with a high dose of intravenous (IV) vitamin C decreased vasopressor requirements and improved mortality in patients with septic shock (113). In addition to intervention against non-communicable chronic diseases *via* regulation of inflammation and complications, various dietary components, including vitamin C treatment, can contribute to the supportive clinical management of infectious diseases, such as COVID-19 (114).

In addition to vitamin C, multiple lines of evidence suggest a potential link between vitamin D and SARS-CoV-2 infection (115–118). Vitamin D is an essential lipid-soluble nutrient absorbed from dietary sources in the proximal small intestine, contributing to skeletal management, intestinal calcium absorption, and immune regulation (119). Although vitamin D deficiency was associated with respiratory distress in patients hospitalized for pneumonia (120), the association between low vitamin D intake and disease severity in COVID-19 cases remains poorly explored (121). A retrospective cohort study revealed that vitamin D deficiency status was positively associated with an increased COVID-19 risk (115). Another retrospective case-control study assessed the possible influence of vitamin D status on disease severity in hospitalized patients with COVID-19 (116). Serum 25-hydroxyvitamin D (25OHD) levels were lower in hospitalized patients with COVID-19 than those in population-based controls, and these patients presented a higher prevalence of vitamin D deficiency (116). Severe vitamin D deficiency (based on a cut-off of ≤10 ng/dL) was noted in 24.0% of patients in the COVID-19 group when compared with 7.3% in the control group (117). Another study by the University of Florida revealed that patients with vitamin D deficiency were five times more likely to be infected with COVID-19 than those without deficiency after adjusting for age groups (118). Taken together, dietary status, such as vitamin D deficiency, may present a risk factor for COVID-19 susceptibility and severity (Figure 2). Moreover, the association of the amount, duration, and interval of nutrient intake with disease severity and prevalence needs to be examined. In addition, specific pathophysiological mechanisms of dietary factor-linked protection should be examined to clarify adverse outcomes in patients.

6.2. Nutritional intervention against gut defense deterioration during viral infection

Vitamin D may counteract gut distress by improving the mucosal and epithelial barriers. Vitamin D supplementation and activation of its nuclear receptor (vitamin D receptor [VDR]) can improve epithelial barrier integrity by enhancing the expression of VDR-associated intracellular junction proteins, including occludin, claudin, and zonula occludens, in the distressed gut (122, 123). Conversely, vitamin D deficiency may compromise the mucosal barrier (124), leading to an increased susceptibility to mucosal damage and infection risk in patients with COVID-19. Moreover, the synthesis and secretion of antimicrobial peptides were elevated *via* vitamin D metabolite-linked VDR activation or subsequent activation of TLR1/2 signaling in the mucosa (125, 126), thereby regulating the excessive commensal bacteria and pathogens by the epithelium or mucosal immune system. Moreover, vitamin D supplementation can activate non-canonical pathways involving the aryl hydrocarbon receptor (AhR), facilitating epithelial tight junctions and mediating anti-inflammatory and antioxidant actions in the injured gut barrier (127). Collectively, vitamin D and the activation of its nuclear receptors, including VDR or AhR, could improve the gut mucosal and epithelial barrier during SARS-CoV-2 infection.

6.3. Nutritional intervention against gut dysbiosis during viral infection

In addition to the direct effects of vitamin D on gut cell physiology, nutritional supplementation is speculated to act on the gut microbial community as another mucosal exposome during SARS-CoV-2 infection. In various experimental models and human studies, notable correlations have been documented between vitamin D and gut microbiota (128, 129). Vitamin D supplementation in healthy individuals significantly increases gut microbial diversity, with an increased ratio of the phylum *Bacteroidetes* to *Firmicutes* (128). Moreover, vitamin D supplementation could remarkably enhance the abundance of health-promoting probiotic taxa, including *Akkermansia, Bifidobacterium, Ruminococcaceae*, *Faecalibacterium*, and *Coprococcus*, while a significant decrease in *Bacteroides acidifaciens* was observed in non-responders. In particular, some probiotic genera, such as *Lactobacillus reuteri*, can metabolize vitamin D to 7-dehydrocholesterol *via* bile salt hydrolase, subsequently contributing to the pools of circulating 25OHD (130). Moreover, supplementation with 25OHD reportedly attenuates inflammatory responses in experimental models of inflammatory bowel disease, accompanied by gut microbial regulation (131). Mechanistically, compared with vitamin D-deficient subjects, vitamin D-sufficient animals displayed enhanced levels of gut microbe-responsive RORγt/FoxP3+ regulatory T cells in the colon. Notably, the number of anti-inflammatory regulatory T cells positively correlated with the abundance of Bacteroides and Clostridium XIVa. Overall, vitamin D status was predicted to shape the gut microbial community, which can facilitate the bioactive metabolic conversion of vitamin D and regulatory responses against inflammation during SARS-CoV-2 infection (Figure 3).

7. Conclusions

Gastrointestinal symptoms are reportedly associated with poor outcomes in patients with acute and post-acute COVID-19. Moreover, persistent remaining viral antigens in the gut mucosal tissue present a risk of recurrent, chronic COVID, and post-acute COVID complications. Based on the findings of a meta-analysis, gastrointestinal symptoms, such as diarrhea, nausea, vomiting, and abdominal discomfort, were notably associated with SARS-CoV-2 infection. In addition to gastrointestinal translocation from the airway in the gut–lung axis, the virus can transmit to water and food supply systems directly and ultimately reaches the gastrointestinal tract in humans *via* fecal–oral transmission. Despite the lack of mechanistic evidence, SARS-CoV-2 could disrupt the mucosal and epithelial barrier and reach the circulation and systemic immune system. Moreover, the prolonged presence of viral antigens and disruption of mucosal immunity may increase gut microbial and inflammatory risks, leading to pathological outcomes and post-acute COVID-19 symptoms. In addition to host immune cell regulation, SARS-CoV-2 infection may alter the gut microbial community, potentially shaping the immunological profile during infection. Generally, patients with COVID-19 exhibit lower bacterial diversity and a higher relative abundance of opportunistic pathogens, such as *Klebsiella pneumoniae*, *Streptococcus*, and

Ruminococcus gnavus in their gut microbiota than healthy controls. Despite the dysbiotic changes during infection, enhancing specific bacterial communities, such as *Lactobacillus* and *Faecalibacterium parusnitzii*, may counteract adverse inflammatory outcomes in the gut and other organs. Moreover, nutritional status, such as vitamin D deficiency, has been associated with disease severity in patients with COVID-19 *via* regulation of the gut microbial community and mucosal immunity. Vitamin D is predicted to improve the gut mucosal and epithelial barrier by activating its nuclear receptors during SARS-CoV-2 infection. Moreover, vitamin D status is predicted to shape the gut microbial community, which can facilitate the bioactive metabolic conversion of vitamin D and immune regulatory responses against infection-induced inflammatory storms. Herein, the collated evidence provides systemic insights into nutritional and microbiological interventions against acute or post-acute COVID-19 in the gut–lung axis.

Author contributions

YM contributed to supervision, conceptualization, methodology, formal analysis, visualization, writing, review, and editing.

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Conflict of interest

The author declares 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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