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COVID-19 and thrombo-inflammatory responses

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COVID-19 and thrombo-inflammatory responses

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Editorial: COVID-19 and thrombo-inflammatory responses

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Editorial on the Research Topic
COVID-19 and thrombo-inflammatory responses

Introduction

The Coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome Coronavirus-2 (SARS-CoV-2) presents with varying clinical symptoms between individuals. Severe COVID-19 causes pneumonia, acute respiratory distress syndrome (ARDS), cytokine storm, and multi-organ failure (1, 2). Despite the end of the epidemic, COVID-19 is still prevalent, transitioning from a lethal phase to one in which people can become infected without experiencing major symptoms or being hospitalized. COVID-19 is associated with a significant increase in the risk of venous and arterial thromboembolic events in hospitalized patients (3, 4), which is associated with vascular barrier dysfunction, edema, endotheliitis, thrombosis, and inflammatory cell infiltration. Although multiple organ failure in COVID-19 is caused by several mechanisms (5, 6), a hypercoagulation state with the development of micro- and macro- circulatory thrombosis plays a prominent role.

This Research Topic is intended to further understanding of COVID-19 and COVID-19-associated thrombo-inflammatory responses. The current issue has 11 articles, most of which are on thrombocytopenia, thromboprophylaxis regimens, Long COVID, and vaccine-induced immune thrombotic thrombocytopenia (VITT). Many of the articles were written from a clinical standpoint to increase insights into COVID-19 pathophysiology in the setting of prothrombotic response.

Platelets and COVID-19

Platelets are well-known for their critical contributions to thrombosis and hemostasis (7). During infection, activated platelets adhere to the sub-endothelium, and their hyperactivity results in thrombus formation, leading to arterial ischemia and even

pulmonary embolisms. Acetylcholine is known to reduce platelet activation via α7 nicotinic acetylcholine receptors (α7nAChR) (8–10). Accumulating evidence suggests that stimulated platelets generate choline products, which activate the α7nAChR, resulting in a positive anti-inflammatory and anti-thrombotic impact. In this issue, Jankauskaite et al. reviewed platelet functions in COVID-19-associated thrombosis and α7nAChR-mediated anti-inflammatory pathway. Nevertheless, *in vivo* studies are required to validate the significance of α7nAChR in platelet function and whether it might be a possible therapeutic target for reducing platelet hyperreactivity during infection, particularly in COVID-19.

Transforming growth factor-β1 (TGF-β1) functions in maintaining a healthy microvasculature by regulating inflammation, clotting, and wound healing. Platelets are the most abundant source of human TGF-β1 (40–100 times more than other cells), which is stored in its latent form in platelet granules (11). In this issue, Arguinchona et al. have summarized, with caveats, the role of TGF-β1 in thrombosis, inflammation, and immune dysregulation in various diseases, including SARS-CoV-2 infection.

Platelet volume indices (PVI), including mean platelet volume (MPV), platelet distribution width (PDW) and platelet-large cell ratio (P-LCR), are considered useful predictors of thrombotic events (12, 13). In the issue, Daniels et al. systematically evaluated the usefulness of PVI as clinical biomarkers for COVID-19 prognosis and as early predictors for severity and mortality in COVID-19. They found that due to the variability in results, it was difficult to conclude whether COVID-19 patients with elevated PVI are more likely to develop severe illness or are at higher risk of mortality.

Platelets are activated during COVID-19 and participate in thrombo-inflammatory responses (14–16). RNA-Seq has indicated both direct and indirect impacts of SARS-CoV-2 infection (e.g., mediators, aberrant antibodies) on the platelet transcriptome of critically ill COVID-19 patients (16). Due to conflicting reports (17, 18), the exact molecular mechanisms behind the direct activation of platelets during SARS-CoV-2 infection remain largely unknown and are likely multifactorial. Cappelletto et al. screened >3,000 FDA/EMA approved drugs and identified Niclosamide and Clofazimine as the most effective at suppressing Spike-induced TMEM16 activation. Spike induced a marked procoagulant phenotype in platelets, by enhancing Ca^{2+} flux, phosphatidylserine externalization on the platelet outer cell membrane, and thrombin generation which were inhibited by Niclosamide and Clofazimine.

Fibrin and COVID-19

Coagulation results in an insoluble clot of crosslinked fibrin. Fibrin monomers (FM) have been proposed as a diagnostic marker of DIC (19) and a predictor of thrombosis and/or a hypercoagulable state earlier than D-dimer (20). In healthy individuals, FM levels are very low in peripheral blood, often below the detection limit. In this issue, Smadja et al. evaluated the relationship between FM and COVID-19 mortality in hospitalized patients. FM levels >7 μg/ml were used as lower cut-off and were monitored during initial hospitalization to predict COVID-19 outcomes. During the first 9 days of hospitalization 37% of patients had positive FM at least once; these patients had higher in-hospital mortality ($p = 0.001$), indicating that monitoring fibrin monomers might be a prognostic tool in moderate-to-critically ill COVID-19 patients.

Thrombocytopenia and prophylactic regimens in COVID-19

High-quality evidence from meta-analyses and randomized controlled trials exploring the clinical outcomes of several preventive regimens in critically ill patients has resulted in contradictory findings (21–23). The anticipated benefit of increasing the anticoagulant dosage is still debated. Rychlíčková et al. in a case study, reported that Fondaparinux can be considered a reasonable and affordable anticoagulant, without a high risk of bleeding, in patients on extracorporeal membrane oxygenation (ECMO). Similarly, Alrashed et al. performed a retrospective cohort analysis (811 patient records) on standard, intermediate, and high anticoagulation dosage regimens in critically ill COVID-19 patients. There were no statistically significant differences in overall in-hospital mortality between the standard-dose and the intermediate-dose groups [51 vs. 53.4%; aHR = 1.4 (95% CI: 0.88–2.33)] or standard-dose and high-dose groups [51 vs. 61.1%; aHR = 1.3 (95% CI: 0.83–2.20)]. The intermediate- and high-dose groups experienced the same frequency of major bleeding episodes as the standard dose group. Thus, these findings recommend standard-dose as the preferred regimen for COVID-19-patients.

Long COVID-19

The older the patients, the more likely they are to succumb to COVID-19 due to immunological dysfunction (24) and comorbidities (25), such as obesity. Many cohort and case-control studies have shown high body mass index (BMI) as a risk factor for disease severity and mortality in COVID-19 patients (26). In this issue, Xiang et al. review suggest that the intersection of obesity and Long COVID, and persistent viral presence, long-term inflammation, micro clots, and hypoxia may contribute to the development of persistent symptoms, and that patients with obesity are uniquely susceptible to Long COVID.

Jaeger et al. in their perspective, propose that acute and Long COVID patients may benefit from treatment with Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis, which has been in clinical use for 37 years. H.E.L.P removes microthrombi without causing bleeding, enhances oxygen supply to the capillaries, lowers cytokine storm, and removes precursors of the procoagulant and fibrinolytic cascade.

Vaccines and thrombosis in COVID-19

Vaccination has been the most promising strategy for combating the COVID-19 pandemic. Antibodies that recognize platelet factor 4 (PF4, also known as CXCL4) bound to platelets caused VITT (27). Cari et al. conducted a meta-analysis of several adenovirus-based vaccinations and their incidence of VITT, non-VITT thrombosis, and arterial events. Although rare, recipients of the Vaxzevria and Jcovden vaccinations had a greater incidence of VITT compared to Comirnaty, implying a link between these occurrences and the adenovirus-based vaccines. The authors hypothesize that the venous and arterial thromboses observed with adenovirus-based vaccines and in absence of thrombocytopenia are due to the combination of at least three triggering factors, all of which may be involved in vascular inflammation and coagulation and suggest that it is independent of anti-PF4 antibodies. Likewise, Jevtic et al. present an update on the clinical diagnosis of VITT and a comprehensive assessment of VITT epidemiology, and similarities and differences between HIT and VITT. According to the review, HIT and VITT antibodies bind to distinct locations on PF4. Furthermore, diagnostic tests established for HIT frequently produce false-negative findings for VITT and should not be employed as a VITT diagnostic test.

Conclusions

In conclusion, this special issue (perspective, case report, research findings, selective reviews, and meta-analysis) highlights the importance of COVID-19 and COVID-19-associated thrombocytopenia, thromboprophylaxis regimens, Long COVID, and VITT. Over the past three years, our understanding of COVID-19-associated prothrombotic mechanism is slowly resolving. COVID-19 severity is heavily influenced by co-morbidities. Accumulating evidence reveals that even after recovery, those who had COVID-19 experience ongoing cardiovascular issues such coagulopathy or bleeding disorders. Researchers are also learning more about how new variants could potentially affect Long COVID. We are still investigating to what extent certain groups are at higher risk, and if different groups of people tend to experience different types of Long COVID complications. Further close monitoring of post-COVID conditions will aid in our understanding of Long COVID and how healthcare providers might treat or support people suffering from these long-term impacts.

Author contributions

SS, CR, PPK, and LS are topic editors of this special issue and contributed to writing and revising of this editorial. SS drafted the editorial. 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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A comparison of three thromboprophylaxis regimens in critically ill COVID-19 patients: An analysis of real-world data

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Introduction: Thrombotic complications of coronavirus disease 2019 (COVID-19) have received considerable attention. Although numerous conflicting findings have compared escalated thromboprophylaxis doses with a standard dose to prevent thrombosis, there is a paucity of literature comparing clinical outcomes in three different anticoagulation dosing regimens. Thus, we investigated the effectiveness and safety profiles of standard, intermediate, and high-anti-coagulation dosing strategies in COVID-19 critically ill patients.

Methodology: This retrospective multicenter cohort study of intensive care unit (ICU) patients from the period of April 2020 to August 2021 in four Saudi

Arabian centers. Inclusion criteria were age ≥ 18 years, diagnosis with severe or critical COVID-19 infection, and receiving prophylactic anticoagulant dose within 24–48 h of ICU admission. The primary endpoint was a composite of thrombotic events, with mortality rate and minor or major bleeding serving as secondary endpoints. We applied survival analyses with a matching weights procedure to control for confounding variables in the three arms.

Results: A total of 811 patient records were reviewed, with 551 (standard-dose = 192, intermediate-dose = 180, and high-dose = 179) included in the analysis. After using weights matching, we found that the standard-dose group was not associated with an increase in the composite thrombotic events endpoint when compared to the intermediate-dose group {19.8 vs. 25%; adjusted hazard ratio (aHR) =1.46, [95% confidence of interval (CI), 0.94–2.26]} or when compared to high-dose group [19.8 vs. 24%; aHR = 1.22 (95% CI, 0.88–1.72)]. Also, there were no statistically significant differences in overall in-hospital mortality between the standard-dose and the intermediate-dose group [51 vs. 53.4%; aHR = 1.4 (95% CI, 0.88–2.33)] or standard-dose and high-dose group [51 vs. 61.1%; aHR = 1.3 (95% CI, 0.83–2.20)]. Moreover, the risk of major bleeding was comparable in all three groups [standard vs. intermediate: 4.8 vs. 2.8%; aHR = 0.8 (95% CI, 0.23–2.74); standard vs. high: 4.8 vs. 9%; aHR = 2.1 (95% CI, 0.79–5.80)]. However, intermediate-dose and high-dose were both associated with an increase in minor bleeding incidence with aHR = 2.9 (95% CI, 1.26–6.80) and aHR = 3.9 (95% CI, 1.73–8.76), respectively.

Conclusion: Among COVID-19 patients admitted to the ICU, the three dosing regimens did not significantly affect the composite of thrombotic events and mortality. Compared with the standard-dose regimen, intermediate and high-dosing thromboprophylaxis were associated with a higher risk of minor but not major bleeding. Thus, these data recommend a standard dose as the preferred regimen.

KEYWORDS

thromboprophylaxis doses, critically ill patients, COVID-19, mortality, thromboprophylaxis

Introduction

In addition to pulmonary manifestations of coronavirus 2019 (COVID-19) (1, 2), there is increasing concern about COVID-19-related extra-pulmonary complications, including thrombotic complications (3). Following emerging data, investigators paid more attention to the hypercoagulability state seen in COVID-19 cases that can result in the development of microthrombi in pulmonary microvasculature, deep vein thrombosis (VTE), and pulmonary embolisms (PE) (4). Initial studies from the early days of COVID-19 found a trend of thrombotic events in COVID-19 patients which prompted a flood of research in this area (5, 6). The incidence of thrombotic events was broadly inconsistent, with reports claiming that up to 69% of COVID-19 patients in a specific population were affected, despite the use of thromboprophylaxis (5–8). Compared to non-COVID-19 patients, previous studies showed COVID-19 patients have a greater risk of venous thromboembolism (VTE) 11.7 vs. 4.8% (9). According to meta-analyses, the incidence of VTE in intensive care unit (ICU)-admitted patients is higher than in those admitted to the general ward, with rates of 31 and 7%, respectively (10, 11). Such coagulation dysfunction has been associated with poor prognosis and negative outcomes, with 40% dying in those who developed VTE in the ICU (12, 13). Given high VTE occurrence reports and its unfavorable prognosis post-COVID-19 infection (14), some experts, agencies, and scientific committees advocated for increased thromboprophylaxis doses to be considered despite the absence of randomized evidence (15–18).

A comprehensive understanding of COVID-19 pathogenesis is still unclear. However, with available data related to the pathology of venous thromboembolism, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has an affinity binding

to angiotensin-converting-enzyme (ACE) 2 receptor, which exists in different tissues including, but not limited to, arterial and venous endothelial cells (19). As per Virchow's triad, a possible higher risk of developing thromboembolic events is expected due to vascular injury. Another possible explanation of VTE is that the inflammatory reaction caused by viral, bacterial and fungal infection results in the activation of host defense systems. This eventually contributes to the up-regulation of coagulation factors and thrombin formation pathways (20, 21). In addition to the risk factors of VTE that critically ill patients carry as a result of venous stasis (22–25), other factors that increase the risk of hypercoagulability may exist. The pro-coagulant profile, particularly D-dimer, is speculated to be a predictor of VTE development with sensitivity and specificity of 85 and 88.5%, respectively (12, 26, 27).

The anticipated benefit of increasing the anticoagulant dose is still debated. A meta-analysis of 23 retrospective observational studies found a favorable mortality reduction with the escalated dose of prophylactic anticoagulant when compared to the standard dose (28). However, high quality evidence of meta-analyses and randomized controlled trials (RCTs) investigating the clinical outcomes of various prophylactic regimens in critically ill patients have revealed conflicting results (29–33). Generally, previous studies were notably limited by poor study designs (6, 34), diversity of study populations (34–37), small sample sizes (6, 36, 38), variations in treatment settings (ICU vs. non-ICU) (39), heterogeneity of thromboprophylaxis dosing regimen definition (40), and unadjusted pooled crude estimates (34). Furthermore, numerous studies compared the effectiveness and safety of just two thromboprophylaxis regimens. However, one open question about comparing three different regimens of thromboprophylaxis simultaneously in critically ill COVID-19 patients is whether they are comparable in terms of effectiveness and coagulopathy. Obviously, limiting comparisons to two thromboprophylaxis regimens (standard and intermediate/high) may cast doubt on whether observed associations of thrombotic events or bleeding are causal or simply artifacts of more complex interrelationships between the disease itself, outcome, interventional dose, and covariates. Thus, our study aimed to assess how three different thromboprophylaxis dose regimens affect the rate of thrombotic events in critically ill COVID-19 patients.

Methods

Study design and setting

This retrospective analysis of the cohort study was conducted at 4 centers in Saudi Arabia, with patients hospitalized in ICUs of tertiary specialty referral hospitals: King Fahad Medical City (KFMC) in Riyadh, King Saud Medical City Hospital (KSMC) in Riyadh, Prince Mohammed Bin Abdulaziz hospital (PMAH) in Riyadh, and Almoosa Specialized Hospital in Al-Ahsa. Study approval was granted by the Institutional Review Boards at KFMC and PMAH (IRB: 20-666), KSMC (IRB:H1RI-16-Nov20-01), Almoosa hospital (IRB: ARC-20-12-4). Due to the retrospective design, informed consent was waived, as it was considered exempt. Our report adopted the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) Statement checklist (41).

Participant selection

Lists of critically ill COVID-19 patients admitted to ICU between April 2020 and August 2021 were obtained from the health informatics officers. We used a random-selection technique to screen patients for eligibility. Random selection avoids sampling bias in giving each patient's record an equal chance of selection and coding (42). Inclusion criteria were age $\geq$18 years, diagnosis of critical SARS-CoV-2 infection by real-time polymerase chain reaction (RT-PCR) from the nasopharyngeal swab and receiving prophylactic anticoagulant within 24–48 h of ICU admission. Patients were excluded if pregnant, diagnosed with VTE or atrial fibrillation during COVID-19 admission, patients with chronic anticoagulants at admission, had a contraindication to anticoagulants including active bleeding, platelet counts $< 25 \times 10^9$/L, and fibrinogen <0.5 g/L, or if they were on VTE-induced medications (oral contraceptives, tamoxifen, etc.,).

Intensities of anticoagulant dose

This study looked at three different anticoagulant prophylactic dosing strategies: standard, intermediate, and high. The "standard dose" was defined as enoxaparin 40 mg subcutaneous (SC) daily, or 30 mg in renal failure patients, heparin 5,000 units SC twice or thrice daily, or fondaparinux 2.5 mg SC daily. The "intermediate dose" included patients treated with enoxaparin 1 mg/kg SC daily or enoxaparin 40 mg SC twice daily, or heparin 7,500 units SC twice or thrice daily. The high dose was enoxaparin 1 mg/kg SC twice daily or heparin infusion. Patients received the prescribed dose of prophylactic regimens within 24–48 h of ICU admission until hospital discharge, developing of thrombotic events, or death.

Data collection

The study data were collected and managed using REDCap (Research Electronic Data Capture) a secure, web-based data capture application (43). Electronic case report forms (eCRFs) were developed, pilot tested, and revised accordingly. Data were manually extracted from electronic health records (EHRs)

and entered into the REDCap system in a de-identified manner. A trained team of data managers were recruited to be responsible for delivering a complete, clean, and accurate dataset. Clinical data managers performed various levels of data validation following data collection, known as edit checks, until it was considered "clean" enough to support analysis. During this step, they used categories to define the essential checks which included missing data, simple range checks, logical inconsistencies, cross-form checks, and protocol violations. Edit check specification (data validation procedure) was used to ensure that all data was the same edited consistently throughout the study. Extracted data include demographic characteristics [e.g., age, gender, weight, height, body mass index (BMI)], clinical characteristics [e.g., history of VTE, diabetes, hypertension, cancer, and cerebrovascular and cardiovascular disease, renal failure, renal dialysis, post-surgery, mechanical ventilation, acute respiratory distress syndrome (ARDS), APACHE score, use of a sedative agent or paralytic agents, recent use of oral contraceptive, steroid intake, vascular access device (VAD), regimen for COVID-19 treatment], lab parameters [D-dimer, prothrombin time (PT) and activated partial thromboplastin time (APPT), international normalized ratio (INR), fibrinogen, and platelet count], clinical outcome [composite thrombotic events (PE, DVT, ischemic stroke, myocardial infarction, systemic arterial embolism)] and any bleeding (minor or major), and death.

Study outcomes

The primary outcome was the occurrence of any component of composite thrombotic events in COVID-19 patients admitted to ICU who received standard, intermediate, or high anticoagulant doses for VTE prevention. Mortality rate and occurrence of major and minor bleeding were secondary outcomes of interest.

Definitions

We defined severe and critical cases of COVID-19 based on the World Health Organization (WHO) (44). Severe manifestation was defined as fever plus symptoms $\geq$1 of the following: respiratory rate $\geq$30/min, dyspnea, respiratory distress, $SpO_2 \leq 93\%$ on room air, PaO_2/FiO_2 ratio <300 or lung infiltrate $>50\%$ of lung field within 24–48 h. Critical illness was evidenced by symptoms $\geq$1 of the following: ARDS, septic shock, altered consciousness, and/or multi-organ failure.

We defined thrombotic events as a composite outcome where at least one of the following occurred: symptomatic acute PE, DVT, ischemic stroke, myocardial infarction, or systemic arterial embolism (45). Three distinct justifications for adopting composite endpoints as the primary outcome: the rate of individual response was expected to be low; the full effect could not be captured meaningfully by a single outcome; and thrombotic events were expected to present in various manifestations of the same disease. Typically, PE diagnosis was determined by computed tomography pulmonary angiography (CTPA) or a combination of high pretest clinical probability of PE with high probability ventilation-perfusion (V/Q) lung scan. DVT diagnosis was confirmed by compression ultrasonography (CUS) with doppler of lower extremities. Myocardial infarction was diagnosed by clinical means, ECG changes and cardiac criteria according to the European Society of Cardiology (ESC) (46). Ischemic stroke was confirmed if suspected patients had brain computed tomography (CT) scan and/or magnetic resonance imaging (MRI), as well as the radiology consultant's report indicated the final diagnosis in the patient file. Mortality was defined as death that occurred during hospitalization for any reason.

Major bleeding was identified by the International Society on Thrombosis and Hemostasis (ISTH) as fatal bleeding and/or symptomatic bleeding in critical areas or organs (such as intracranial, intraspinal, intraocular, retroperitoneal, intraarticular or pericardial, or intramuscular bleeding with compartment syndrome) or documented bleeding causing a decrease in hemoglobin (Hgb) level of 2 g/dL or more, and transfusion of 2 or more units of packed red blood cells (PRBCs). In contrast, minor bleeding was all bleeds that were not considered major or non-major bleeding events (47).

Sample size calculation

We calculated the sample size using Power Analysis and Sample Size (PASS) 11® and G*Power (Version 3.1.9.7) software. Because of a wide variety of thrombotic events at the time of study inception, we assumed a −15% risk difference between the standard dose, intermediate dose, and high dose arms. The total sample size was calculated to be 549 with an estimated ratio of 1:1:1 ($N = 183$ in each arm) as needed to provide an 80% power and significance level (α, type 1 error rate) of <0.05 to assess the hypothesis.

Statistical analysis

The three treatment groups' demographic and baseline data were compared using standard descriptive statistics. Where appropriate, continuous data were presented using means with standard deviations (±SDs) and medians with interquartile ranges (IQRs). ANOVA and Kruskal Wallis tests were used to compare normally and non-normally distributed quantitative variables between treated groups, respectively. To compare categorical variables, the chi-squared test was performed, and the results were reported as frequencies and percentages.

Missing data handling

Multiple Imputation by Chained Equations (MICE) approach with Nelson Aalen estimator was used to handle missing data, which was regarded as missing at random (MAR) (48, 49). To accommodate for uncertainty, multiple dataset predictions (5 imputed datasets with 10 iterations) were constructed for each missing value in this method, resulting in decreased variability and more accurate standard errors. Only variables with <15% missing data were considered. Convergence and density plots were visually checked for missing variables. All estimates were pooled across the generated datasets.

Matching weights procedure and survival analysis

Due to the challenge of multiple covariates and multiple treatment arms in this observational study, we applied the matching weights method, an extension of inverse probability of treatment weighting (IPTW), as a sensitivity analysis to estimate the average treatment effect (ATE) outcome across multiple treatment groups (50). The approach reweights all standard, intermediate, and high groups to simulate a propensity score-matched population. Multinomial logistic regression was used to fit all propensity scores of the covariates of interest. The use of this methodology is explained by Yoshida et al. (50). Post-weighting balance assessment is used to find the optimal balance with absolute standardized mean differences of <0.2 for all covariates, indicating better covariate balance (50). Propensity score distribution and overlap was visually inspected *via* a mirror diagram. A covariate balance check was demonstrated *via* a Love plot. To estimate the probability of survival of the three groups, we used an adjusted Kaplan-Meier (KM) model with weighted data. Time 0 was the time of anticoagulation initiation. Censoring was considered for patients with no events that were discharged alive or were still admitted patients at the time of data collection. The stratified log-rank test was used to compare the survival distributions of three samples. In order to draw weighted KM curves, we used "svykm" function from the "survey" package in R which assumes weights as sampling weights to account for the matching design. This would allow the estimation of robust standard errors for survival. For the relative effect, we fitted the Cox regression model. The assumptions were adequately checked by using Schoenfeld residuals and visual plots. We implemented robust variance estimation to estimate standard errors. In the case of low to no events, we could not estimate hazards ratios.

Statistical software

R Core Team (51) software (R Foundation for Statistical Computing, Version 4.0.1, Vienna, Austria) was used. The following packages in the R interface were used to conduct the analyses: survival (52), survey (53), mice (48), MatchThem (54), cobalt (55) and tableone (56).

Results

Baseline characteristics

During the study period, 811 patients were screened for eligibility criteria (Figure 1). A total of 551 patients met inclusion criteria for analysis, with the remaining patients being excluded due to lack of ICU admission (n = 142), late start of thromboprophylaxis regime >48 h (n = 57), anticoagulant contraindications (n = 6), mild/moderate COVID-19 cases (n = 10), and on VTE-induced medicine (n = 6). Table 1 summarizes the main baseline characteristics of the standard dose group (n = 192), intermediate-dose group (n = 180), and high dose group (n = 179). A total of 112 (58%) patients out of the 192 standard dosing patients received heparin; whereas only 16 (8.9%) received heparin intermediate dosing and only 1 patient received heparin high dosing. None of this cohort received fondaparinux. For the pre-covariate balance of original data, there were explicit differences in the baseline characteristics of age ($P = 0.032$), gender ($P = 0.017$), ethnicity ($P = 0.006$), kidney function ($P < 0.001$) APACHE II score ($P = 0.009$), WHO severity ($P < 0.001$), mechanical ventilation ($P = 0.001$), and medications such as intravenous steroid ($P = 0.001$) and carbapenem ($P = 0.021$). Additionally, groups differed in the following laboratory variables: d-dimer ($P = 0.014$) and APTT ($P < 0.001$). Missing data were highest for fibrinogen (37.9%), followed by APACHE II score and D-dimer (7–10%) (Table 1, footnote).

After applying the weights matching procedure, all covariates of interest were adequately balanced (Table 2; Figure 2). We illustrated the propensity score distribution using a Love plot for absolute standardized mean difference (SMD) distribution (Figure 2). Most patients (>60%) had D-dimer >1.5 μg/ml (Supplementary Table S1).

Thrombotic events and mortality outcomes

After weights matching, the risk of a composite of thrombotic events was not significantly different between the standard-dose and intermediate-dose groups {21.6 vs. 25.2%; adjusted hazard ratio (aHR) = 1.4, [95% confidence of interval (CI), 0.88–2.33]} (Table 3; Figure 3A). Also, the standard-dose group was associated with a similar hazard of thrombotic events to the high-dose group [21.6 vs. 28.8%; aHR = 1.3 (95% CI, 0.83–2.20)] (Table 3; Figure 3A). Furthermore, each component of the composite outcome has comparative events in the three different prophylactic anticoagulant intensities

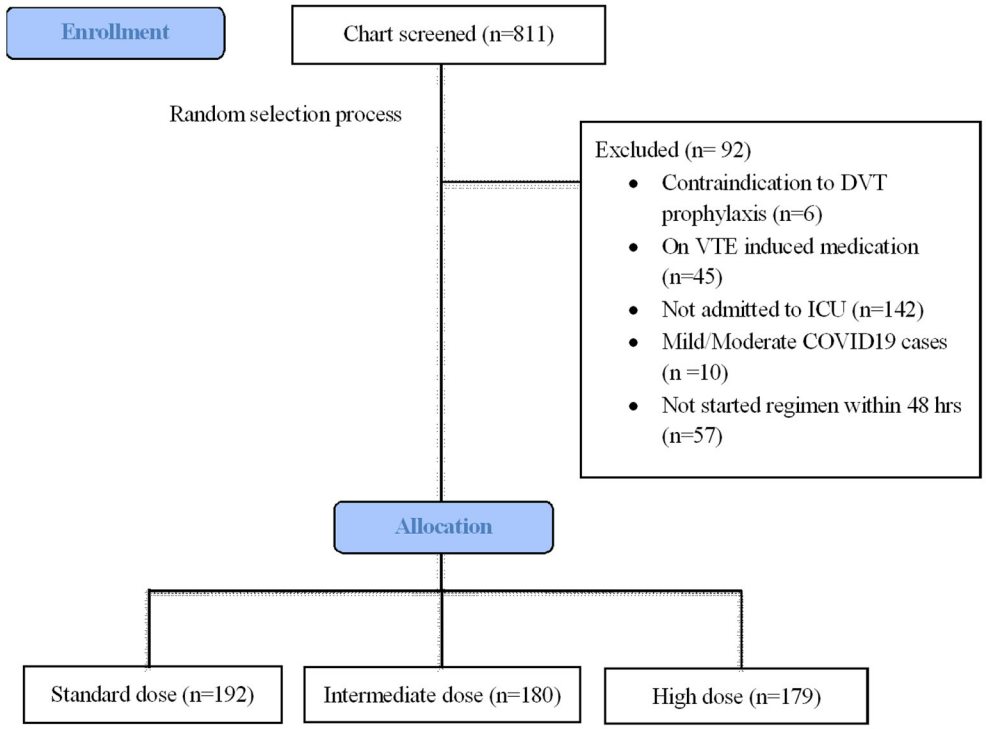

FIGURE 1
Patients selection flowchart.

(Table 3). A subgroup of patients having a D-dimer of >1.5 vs. <1.5 g/ml were associated with similar composite thrombotic events (Supplementary Table S1). For secondary outcome, patients on standard-dose and intermediate-dose arms had comparable overall in-hospital mortality [51.0 vs. 53.4%; aHR = 1.2 (95% CI, 0.88 to 1.72)] (Table 3; Figure 3B). Similarly, we found in-hospital mortality occurred more frequently in the high-dose groups (61.1%) than in the standard-dose group (51.0%), although the findings were not statistically significant [aHR = 1.3 (95% CI, 0.92–1.74)] (Table 3; Figure 3B). When the unadjusted hazard ratio was considered, analyses revealed no differences in primary outcome and mortality (Supplementary Figures S1A,B).

Bleeding outcome

Minor bleeding in the intermediate-dose group (12.6%) was higher compared to the standard-dose group (4.9%) [aHR = 2.9 (95% CI, 1.26–6.80)] (Table 3; Figure 3C). Also, higher proportion of patients experienced minor bleeding in high-dose group (17.3%) vs. the standard-dose group (4.9%) [aHR = 3.9 (95% CI, 1.73–8.76)] (Table 3; Figure 3C). No significant differences regarding major bleeding were observed between the 3 study groups (standard: 4.9 vs. intermediate: 2.9 vs. high: 8.6%) (Table 3; Figure 3D). The unadjusted analysis can be seen in Table 3 and Supplementary Figures S1C,D).

Discussion

This retrospective cohort study compared three different thromboprophylactic regimens in COVID-19 ICU patients. We found that these three regimens (standard-dose, intermediate-dose, and high-dose groups) had comparable primary composite of thrombotic events, including the major components. Furthermore, no significant differences were observed between the three study groups with respect to hospital mortality. Those who received intermediate-dose and high-dose groups had a similar frequency of major bleeding events as those who received standard-dose. However, there were more minor bleeding events in the intermediate-dose and high-dose groups compared with the standard-dose group.

The effectiveness of anticoagulant dose escalation in COVID-19 ICU patients continues to be debated, even among RCT studies. Our findings are in line with the hypothesis that high-dose thromboprophylaxis has a similar composite of thrombotic events in critically ill patients with COVID-19 as intermediate-dose and standard-dose thromboprophylaxis regimens (29, 31, 32). In addition, parallel to observational and RCT studies (29, 31, 32, 57, 58),

TABLE 1 Baseline characteristics.

Variable	Standard ($n = 192$)	Intermediate ($n = 180$)	High ($n = 179$)	*P* value
Age, mean (SD)	59.2 (15.0)	56.4 (13.8)	55.6 (13.1)	0.032
Female, n (%)	61 (31.8)	39 (21.7)	36 (20.1)	0.017
Ethnicity				0.006
Middle eastern	113 (58.9)	75 (41.7)	89 (49.7)	
Southeast/east Asian	8 (4.2)	14 (7.8)	12 (6.7)	
South Asian	26 (13.5)	46 (25.6)	39 (21.8)	
Central Asian	8 (4.4%)	9 (5.0)	0 (0.0)	
African	15 (7.8)	7 (3.9)	12 (6.7)	
Other/Unknown	23 (12.0)	28 (15.6)	27 (15.1)	
Weight (kg), median (IQR)	77.5 (67.5–90.0	78.6 (70.0–90.0)	80.0 (70.0–90.0)	0.141
BMI (kg/m^2), median (IQR)	27.9 (24.8–31.8	28.0 (24.8–31.8	28.0 (24.8–32.4)	0.849
Scr (mg/dl), median (IQR)	1.4 (0.8–2.6)	1.0 (0.7– 2.1)	1.0 (0.8–1.4)	0.003
CKD EPI (mL/min/m^2), median (IQR)	53.0 (22.5–93.6)	79.3 (32.9–101.2)	79.4 (47.9–99.4)	<0.001
CKD stage, n (%)				<0.001
Stage 1	54 (28.1)	73 (40.6)	64 (35.8)	
Stage 2	30 (15.6)	37 (20.6)	47 (26.3)	
Stage 3a	17 (8.9)	8 (4.4)	23 (12.8)	
Stage 3b	21 (10.9)	13 (7.2)	18 (10.1)	
Stage 4	43 (22.4)	27 (15.0)	15 (8.4)	
Stage 5	20 (10.4)	15 (8.3)	4 (2.2)	
Unknown	7 (3.6)	7 (3.9)	8 (4.5)	
APACHE II score, median (IQR)	18.0 (12.0–23.0)	15.0 (10.0–22.0)	14.0 (10.0–21.0)	0.009
PT (s), median (IQR)	14.2 (13.0–16.0)	14.0 (12.9–15.7)	14.6 (13.2–16.1)	0.058
INR, median (IQR)	1.2 (1.0–1.3)	1.1 (1.0–1.3)	1.1 (1.1–1.3)	0.229
APTT (s), median (IQR)	34.0 (29.9–47.0)	32.0 (28.0–38.0)	35.0 (29.4–42.4)	<0.001
Platelet (per 109/L), median (IQR)	229.0 (145.0–323.0)	249.0 (184.0–340.0)	244.0 (184.0–340.0)	0.074
Fibrinogen (g/L), median (IQR)	4.9 (3.3–6.7)	4.9 (3.2–6.6)	5.3 (3.2–7.4)	0.395
D-Dimer (μg/ml), median (IQR)	1.9 (1.0–4.4)	2.0 (0.9– 6.0)	2.8 (1.3–7.4)	0.014
Respiratory disease, n (%)	28 (14.6)	21 (11.7)	20 (11.2)	0.559
Established Cardiovascular disease, n (%)	20 (10.4)	17 (9.4)	22 (12.3)	0.674
Diabetes, n (%)	110 (57.3)	96 (53.3)	95 (53.1)	0.655
Hypertension, n (%)	108 (56.2)	82 (45.6)	90 (50.3)	0.117
Dyslipidemia, n (%)	11 (5.7)	10 (5.6)	15 (8.4)	0.476
Liver disease, n (%)	5 (2.6)	1 (0.6)	3 (1.7)	0.313
HIV status, n (%)	3 (1.6)	1 (0.6)	0 (0.0)	0.331
Mechanical ventilation, n (%)	144 (75.0)	117 (65.0)	90 (50.3)	<0.001
ECMO, n (%)	28 (14.6)	26 (14.4)	10 (5.6)	<0.001
WHO severity				
Severe	47 (24.5)	70 (38.9)	91 (50.8)	<0.001
Critical	145 (75.5)	110 (61.1)	88 (49.2)	<0.001
Sedative agent, n (%)	173 (90.1)	165 (91.7)	162 (90.5)	0.911
COVID-19 regimen during hospital stay, n (%)				
Favipiravir	20 (10.4)	28 (15.6)	31 (17.3)	0.132
Remdesivir	3 (1.6)	6 (3.3)	3 (1.7)	0.140
Hydroxychloroquine	4 (2.1)	5 (2.8)	4 (2.2)	0.939

(Continued)

TABLE 1 Continued

Variable	Standard ($n = 192$)	Intermediate ($n = 180$)	High ($n = 179$)	*P* value
Azithromycin	3 (1.6)	6 (3.3)	12 (6.7)	0.037
Lopinavir/ritonavir	7 (3.6)	8 (4.4)	3 (1.7)	0.341
Hydroxychloroquine+azithromycin	0 (0.0)	2 (1.1)	2 (1.1)	0.398
Azithromycin + Favipiravir	2 (1.0)	2 (1.1)	5 (2.8)	0.482
Azithromycin + Favipiravir + Tocilizumab	2 (1.0)	5 (2.8)	9 (5.0)	0.075
Hydroxychloroquine+Azithromycin+Tocilizumab	0 (0.0)	5 (2.8)	0 (0.0)	0.007
Lopinavir/ritonavir+Interferon-B+Ribavirin	5 (2.6)	9 (5.0)	9 (5.0)	0.043
IV steroid use, n (%)	162 (84.4)	168 (93.3)	175 (97.8)	<0.001
ACEI or ARB, n (%)	33 (17.2)	22 (12.2)	33 (18.4)	0.234
Beta blocker, n (%)	64 (33.3)	51 (28.3)	72 (40.2)	0.057
Calcium Channel Blocker, n (%)	65 (33.9)	55 (30.6)	59 (33.0)	0.783
Aspirin, n (%)	52 (27.1)	46 (25.6)	52 (29.1)	0.757
Insulin, n (%)	141 (73.4)	132 (73.3)	122 (68.2)	0.443
Statin, n (%)	68 (35.4)	54 (30.0)	48 (26.8)	0.191
Thiazide diuretic, n (%)	2 (1.0)	2 (1.1)	4 (2.2)	0.609
Loop diuretic, n (%)	82 (42.7)	94 (52.2)	107 (59.8)	0.004
Cefepime, n (%)	19 (9.9)	12 (6.7)	23 (12.8)	0.143
Vancomycin, n (%)	112 (58.3)	92 (51.1)	100 (55.9)	0.366
Carbapenems, n (%)	103 (53.6)	105 (58.3)	121 (67.6)	0.021
Piperacillin/Tazobactam, n (%)	107 (55.7)	103 (57.2)	82 (45.8)	0.061
Metronidazole, n (%)	6 (3.1)	4 (2.2)	2 (1.1)	0.445

BMI, body mass index. Scr, serum creatinine. CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration. The Acute Physiology and Chronic Health Evaluation (APACHE II), Established cardiovascular disease was defined as a documented history of stable angina, unstable angina, percutaneous coronary intervention (PCI), coronary artery bypass graft surgery, or myocardial infarction (MI), heart failure or cerebrovascular disease included transient ischemic attack (TIA) or stroke. Respiratory disease, asthma or chronic obstructive pulmonary disease (COPD). HIV, human immunodeficiency virus. ECMO, extracorporeal membrane oxygenation. ACEI, angiotensin-converting enzyme inhibitors. WHO, World health organization. ARB, angiotensin receptor blockers. CCB, calcium channel blockers. IV, intravenous. Carbapenems included meropenam and imipenam.
Missing data: <1% (paralytic agent, INR, vasopressor use, BMI, steroid use, weight in kg). 1–5% (treatment regimen during hospitalization, PT, APTT, platelet count, Scr, CKD EPI). 7–10% (APACHE II score, D-dimer). Fibrinogen (37.9%).

different thromboprophylaxis doses did not eliminate the risk of overall mortality. This cast doubt on other studies that show a superior survival rate with escalated doses (37, 59, 60). Of note, a meta-analysis of observational studies, has also confirmed that increasing the anticoagulation regimen to the therapeutic dose, resulted in an increase in bleeding events (28).

To the best of our knowledge, only one study investigated the efficacy of three different anticoagulation dose regimens in COVID-19-infected critically ill patients (60). It concluded that high-dose thromboprophylaxis was associated with a lower risk of cumulative incidence of thromboembolic events and fewer bleeding events compared with lower doses. However, the study's findings should be interpreted with caution because of study limitations that may lead to misleading estimates of treatment effect. This includes small sample size and that almost half (45.4%) of patients underwent dose adjustment of the anticoagulant during ICU stay (60).

There are several possible explanations for the high incidence of venous thromboembolism (VTE) in the ICU population. One possible explanation is that the bioavailability of subcutaneous thromboprophylaxis is reduced, especially in edematous patients or those who receive vasoactive medications concurrently, thereby potentially providing reduced efficacy (61). Another possibility is low cardiac output in a population with pre-existing cardiovascular disease (62). Furthermore, significant VTE occurrence was observed not only with COVID-19, but also with other epidemic respiratory virus infections. When relevant data from previous virus infections is reviewed, it provides a lesson from the past about the magnitude of coagulation disorders' severity when compared to COVID-19 infection. Critically ill patients with H1N1 virus and SARS-CoV-1 showed substantial VTE rates of 44 and 30%, respectively (63, 64).

Some studies speculated that abnormal coagulation parameters, such as elevated D-dimer in COVID-19 (D-dimer > 1.5 μg/ml), were predictors of ICU admission, mortality

TABLE 2 Covariate balance post weight matching procedure and multiple imputations.

Covariate	Pre-covariate balance				Post-covariate balance			
Data	Original data				Weight matching			
Arm	Standard $n = 192$	Intermediate $n = 180$	High $n = 179$	Absolute SMD	Standard $n = 102$	Intermediate $n = 103$	High $n = 104$	Absolute SMD
Age, mean (±SD)	59.2 (14.98)	56.4 (13.79)	55.6 (13.12)	0.169	56.43 (14.99)	57.07 (13.66)	58.06 (12.75)	0.079
Female, *n* (%)	61 (31.8)	39 (21.7)	36 (20.1)	0.179	23.7 (23.4)	26.0 (25.3)	24.4 (23.4)	0.030
Ethnicity, *n* (%)				0.401	29.18 (6.54)	29.35 (6.06)	29.11 (6.31)	0.070
Middle Eastern	113 (58.9)	75 (41.7)	89 (49.7)		56.2 (55.3)	56.0 (54.6)	53.9 (51.8)	
Central Asian	7 (3.6)	9 (5.0)	0 (0.0)		0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	
East/Southeast Asian	8 (4.2)	14 (7.8)	12 (6.7)		6.3 (6.2)	5.7 (5.6)	5.4 (5.2)	
South Asian	26 (13.5)	46 (25.6)	39 (21.8)		17.8 (17.6)	18.9 (18.4)	21.2 (20.4)	
African	15 (7.8)	8 (4.4)	12 (6.7)		6.6 (6.5)	7.0 (6.9)	7.0 (6.7)	
Unknown/other	23 (12.0)	28 (15.6)	27 (15.1)		14.7 (14.4)	15.0 (14.6)	16.6 (16.0)	
BMI (kg/m^2), mean (SD)	28.95 (6.37)	28.85 (6.02)	29.25 (6.08)	0.043	29.18 (6.54)	29.35 (6.06)	29.11 (6.31)	0.025
CKD EPI (ml/min/m^2) mean (SD)	61.54 (40.50)	73.53 (39.79)	77.84 (43.87)	0.263	70.25 (40.70)	71.17 (40.48)	67.70 (34.65)	0.061
Respiratory diseases, *n* (%)	28 (14.6)	21 (11.7)	20 (11.2)	0.068	13.2 (12.9)	14.0 (13.6)	12.6 (12.1)	0.030
Established cardiovascular diseases, *n* (%)	20 (10.4)	17 (9.4)	22 (12.3)	0.061	12.2 (12.0)	11.2 (10.9)	11.3 (10.9)	0.032
Type 1 or 2 diabetes, *n* (%)	110 (57.3)	96 (53.3)	95 (53.1)	0.057	50.7 (49.7)	55.5 (50)	57.5 (55.3)	0.036
Hypertension, *n* (%)	108 (56.2)	82 (45.6)	90 (50.3)	0.143	50.7 (49.7)	51.5 (50)	54.5 (52.4)	0.032
Liver disease, *n* (%)	5 (2.6)	1 (0.6)	3 (1.7)	0.112	0.7 (0.7)	1.0 (1.0)	2.1 (2.0)	0.078
Mechanical ventilation, *n* (%)	144 (75.0)	117 (65.0)	90 (50.3)	0.350	70.5 (69.0)	68.5 (66.8)	71.9 (61.1)	0.036
ECMO, *n* (%)	28 (14.6)	26 (14.4)	10 (5.6)	0.201	11.0 (10.8)	9.6 (9.3)	9.9 (9.5)	0.034
WHO critical category, *n* (%)	145 (75.5)	110 (61.1)	88 (49.2)	0.374	64.8 (36.5)	67.7 (66.0)	67.4 (64.7)	0.032
D-dimer (μg /mL), mean (SD)	5.11 (10.37)	7.00 (14.62)	7.60 (12.74)	0.136	5.20 (11.30)	5.49 (10.60)	6.00 (8.44)	0.053
APACHE II score, mean (SD)	18.45 (8.42)	16.70 (8.46)	15.70 (8.56)	0.217	16.70 (8.48)	16.57 (8.64)	16.95 (8.99)	0.029
ACEI or ARB, *n* (%)	33 (17.2)	22 (12.2)	33 (18.4)	0.115	17.3 (17.0)	16.1 (15.7)	18.0 (17.3)	0.029
Aspirin, *n* (%)	52 (27.1)	46 (25.6)	52 (29.1)	0.052	28.7 (28.1)	28.4 (27.6)	29.7 (28.6)	0.013
Favipiravir, *n* (%)	20 (10.4)	28 (15.6)	31 (17.3)	0.134	14.3 (14.1)	12.8 (12.4)	15.4 (14.8)	0.046
Remdesivir, *n* (%)	3 (1.6)	7 (3.9)	1 (0.6)	0.156	1.5 (1.5)	0.8 (0.8)	1.0 (1.0)	0.044
Steroid, *n* (%)	164 (85.4)	170 (94.4)	176 (98.3)	0.333	97.3 (95.4)	100.5 (97.9)	101.1 (79.2)	0.084
Azithromycin, favipiravir and tocilizumab, *n* (%)	2 (1.0)	5 (2.8)	9 (5.0)	0.159	2.0 (2.0)	1.6 (1.6)	2.0 (1.9)	0.021

BMI, body mass index. Scr, serum creatinine. CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration. Established cardiovascular disease was defined as a documented history of stable angina, unstable angina, percutaneous coronary intervention (PCI), coronary artery bypass graft surgery, or myocardial infarction (MI). Heart failure and cerebrovascular disease included transient ischemic attack (TIA) or stroke. Respiratory disease: asthma or chronic obstructive pulmonary disease (COPD). VTE, venous thromboembolism. ECMO, extracorporeal membrane oxygenation; ARB, angiotensin receptor blockers; WBC, White blood cells; Hgb, Hemoglobin; Pao_2, partial pressure of oxygen; FiO2, fraction of inspired oxygen; ALT, Alanine transaminase; ALP, Alkaline phosphatase; CRP, c-reactive protein; IQR, Interquartile range; SMD: Standardized mean difference. Percentages were rounded.

Prior Weight matching, missing data were imputed using Multivariate Imputation by Chained Equations (MICE) equations that included Nelson–Aalen estimator.

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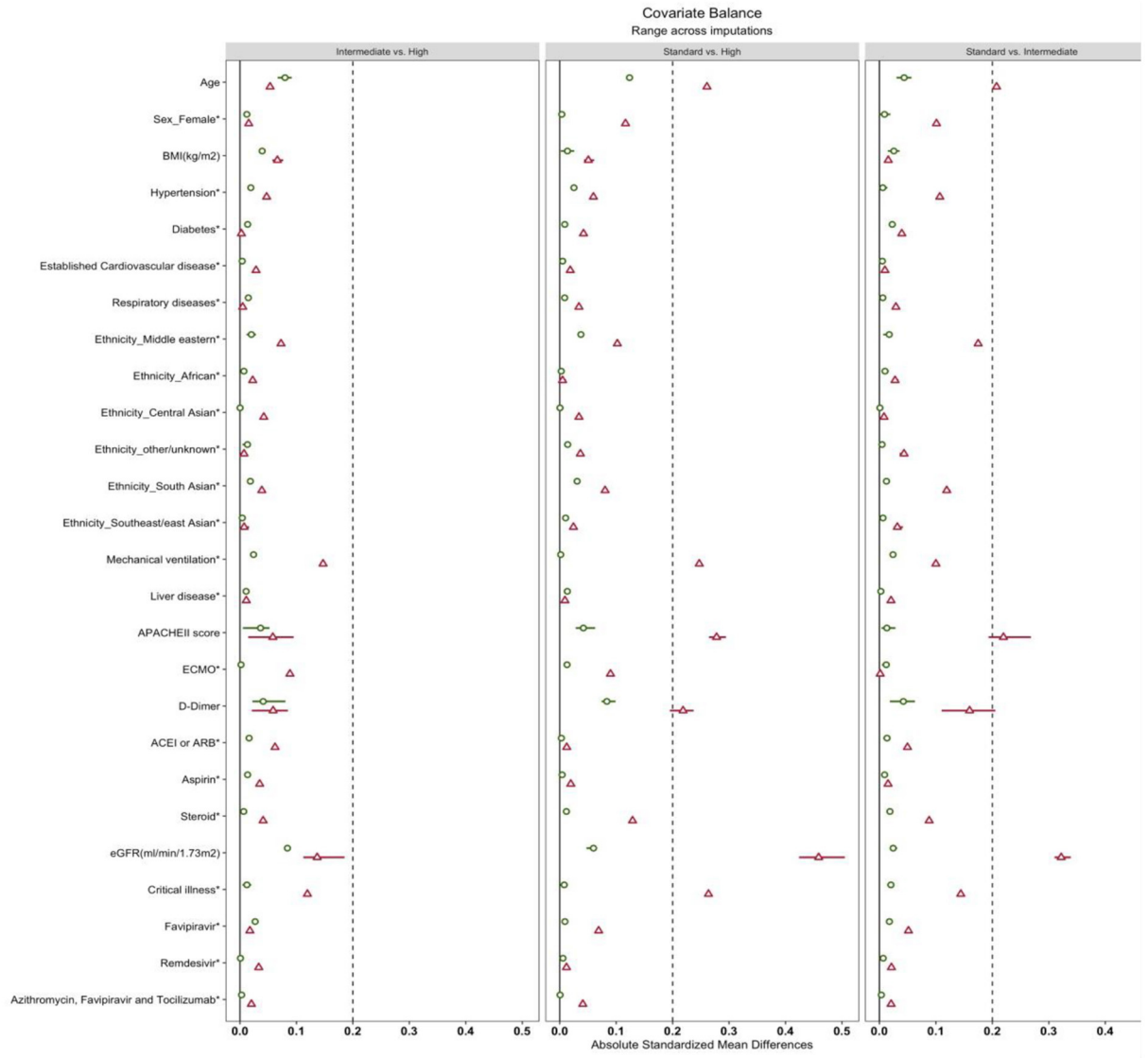

FIGURE 2
Love plots of covariate balance. BMI, body mass index. Established cardiovascular disease was defined as a documented history of stable angina, unstable angina, percutaneous coronary intervention (PCI), coronary artery bypass graft surgery, myocardial infarction (MI), heart failure or cerebrovascular disease included transient ischemic attack (TIA) or stroke. Respiratory disease: asthma or chronic obstructive pulmonary disease (COPD), the Acute Physiology and Chronic Health Evaluation (APACHE II). ECMO, extracorporeal membrane oxygenation; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; eGFR, estimated Glomerular Filtration Rate.

and the development of VTE (12, 13, 65–67). However, the generalizability of these results is subject to certain limitations. First, those studies were limited by a small sample size, lack of serial D-dimer monitoring, absence of laboratory methodology details for the D-dimer assay, and lack of validation. Also, in one study, continuous D-dimer data was empirically categorized (levels of $\leq$0.5 μg/ml, >0.5 to $\leq$1 μg/ml, and >1 μg/ml) instead of using receiver operating characteristic (ROC) analysis to determine the optimal cutoff predictive value of D-dimer for poor prognosis and mortality (67). Second, the D-dimer assay test is limited by low specificity and high rate of false-positive results in a variety of non-thrombotic conditions, such as inflammation, infection, sepsis, female gender, black race, increased aging, active malignancy, sickle cell disease, lupus, chronic liver disease, trauma or surgical status (68). Third, compared to the current and previous pandemic and epidemic viruses, we noticed that D-dimer levels were elevated in both severely infected COVID-19 and SARS-CoV-1 patients, at 59.6 and 45%, respectively (69, 70). Thus, using D-dimer levels as a marker to inform anticoagulant

TABLE 3 Clinical outcomes results.

Outcome, n (%)	Standard (n = 192)	Intermediate (n = 180)	HR (95%CI)†	High (n = 179)	HR (95%CI)†
(A) Unadjusted outcomes					
Composite endpoint	38 (19.8)	45 (25.0)	1.46 (0.94–2.26)	43 (24.0)	1.31(0.85–2.04)
Pulmonary embolism	25 (13.0)	28 (15.6)	1.37 (0.79–2.35)	30 (16.8)	1.42 (0.83–2.43)
Deep venous thrombosis	13 (6.8)	5 (2.8)	0.49 (0.17–1.40)	5 (2.8)	0.40 (0.14–1.15)
Stroke	0 (0.0)	5 (2.8)	0.91(0.30–2.77)	4 (2.2)	0.92 (0.30–2.78)
Myocardial infarction	|3 (1.6)	7 (3.9)	2.87 (0.74–11.15)	9 (5.0)	3.63 (0.98–13.47)
Systemic arterial embolism	0 (0.0)	0 (0.0)	–	1 (0.6)	–
Hospital death	112 (58.3)	93 (51.7)	1.18 (0.85–1.66)	104 (58.1)	
Bleeding					
Minor	10 (5.2)	22 (12.2)	2.66 (1.26–5.64)	31 (17.3)	3.73 (1.82–7.63)
Major	6 (3.1)	6 (3.3)	0.23 (0.41–3.93)	18 (10.1)	3.81 (1.51–9.65)
Outcome, n (%)	**Standard (*n* = 102)**	**Intermediate (*n* = 103)**	**HR (95%CI)†**	**High (*n* = 104)**	**HR (95%CI)†**
(B) Matching weights procedure					
Composite endpoint	22 (21.6)	26 (25.2)	1.4 (0.88–2.33)	30 (28.8)	1.3 (0.83–2.20)
Pulmonary embolism	13 (12.7)	16 (15.3)	1.3 (0.71–2.46)	22 (21.2)	1.7 (0.90–3.02)
Deep venous thrombosis	8 (7.8)	3 (2.9)	0.4 (0.12–1.29)	3 (2.9)	0.3 (0.10–1.12)
Stroke	0 (0.0)	3 (2.9)	1.2 (0.33–4.05)	3 (2.9)	0.8 (0.23–2.98)
Myocardial infarction	2 (2.0)	5 (4.9)	3.2 (0.68–0.53)	5 (4.8)	2.8 (0.61–12.59)
Systemic arterial embolism	0 (0.0)	0.0 (0.0)	–	1.0 (1.0)	–
Hospital death	52 (51.0)	55 (53.4)	1.2 (0.88–1.72)	64 (61.1)	1.3 (0.92–1.74)
Bleeding					
Minor	5 (4.9)	13 (12.6)	2.9 (1.26–6.80)	18 (17.3)	3.9 (1.73–8.76)
Major	5 (4.9)	3 (2.9)	0.8 (0.23–2.74)	9 (8.6)	2.1 (0.79–5.80)

Occurrence of any composite endpoint defined as symptomatic acute pulmonary embolism (PE), deep-vein thrombosis, ischemic stroke or myocardial infraction. Follow up day is to the first event occurred.
† All comparisons were against standard as reference.
[a]Odds ratios were estimated.
Bleeding score according to International Society on Thrombosis and Haemostasis bleeding scale.
HR, Hazard ratio; CI, Confidence intervals.

dosing regimens in ICU patients may be inadequate for clinical decision-making.

The definition of prophylactic anticoagulation intensity played a vital role in determining the extent to which study results would be affected (71). Notably, dosing regimens of anticoagulant varied widely across different studies, where it was defined according to local site protocols or trial protocol (28, 33). The therapeutic dose in the large REMAP-CAP/ACTIV-4a/ATTACC multiplatform trial was defined as to meet the target for aPTT of 1.5 to 2.5 times the upper limit of normal (for unfractionated heparin) or therapeutic anti-Xa levels (for enoxaparin). (32). The study concluded that therapeutic dose was associated with significantly lower rates of VTE and higher rates of major and minor bleeding (32). However, these results were inconsistent with what we found. Considering that thrombotic events were not regularly screened during hospital stay in our institutions, but rather, were prompted by the treating physician upon suspicion. This would underestimate the rate of thrombotic events in our cohort. However, given local protocol-based practice, this would be in line with current COVID-19 treatment guidelines panel recommendations (71).

In addition, we noted in our study significant variations in heparin and enoxaparin proportions between groups. Comparing how each anticoagulant type might affect the magnitude of clinical outcomes was beyond the scope of this study. Additionally, practical constraints might prevent designing different study groups with various anticoagulant dosing regimens and types. Obviously, a still open question is whether the anticoagulant type will affect the efficacy and safety outcomes.

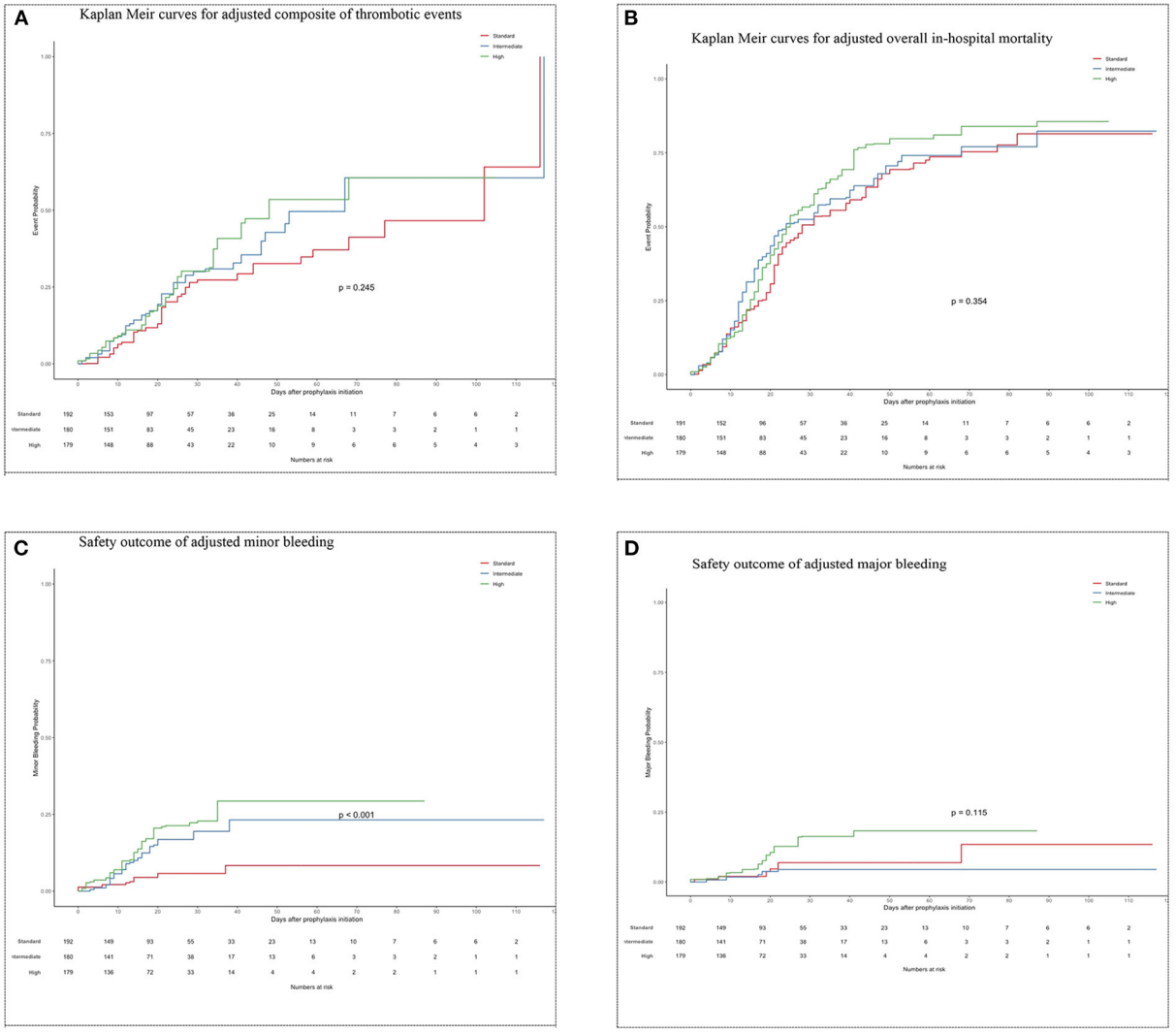

FIGURE 3
(A) Kaplan Meir curves for adjusted composite of thrombotic events. **(B)** Kaplan Meir curves for adjusted overall in-hospital mortality. **(C)** Safety outcome of adjusted minor bleeding. **(D)** Safety outcome of adjusted major bleeding.

Many studies compare just two different thromboprophylaxis regimens. Our study shares many features with others, but the combination used is unique. First, it represents a comprehensive examination of the most frequent three different thromboprophylaxis dosing regimens used in ICU admitted COVID-19 patients. Being familiar with the same efficacy of three prophylactic-dose regimes empowers clinicians to make decisions and recommend a standard dose of thromboprophylaxis in non-obese ICU COVID-19 patients. Second, this study was conducted at 4 centers (multicenter) in two Saudi Arabian cities. Third, in our analysis, we considered all possible covariates that may influence the thrombotic events and mortality findings for ICU patients when developing the Cox regression prediction model. Fourth, this study offers valuable insight into high-risk patients, such as those with elevated D-dimer levels, with patients receiving standard, intermediate, and high-dose thromboprophylaxis, having mean D-dimer baselines of 5.65, 6.44, and 7.54 μg/ml, respectively.

The American Society of Hematology guidelines and Saudi Critical Care Society practice guidelines suggest using the standard prophylactic dose for adults with critical COVID-19 who had no clinical suspicion of VTE (72, 73). However, our findings may counter the uncertainty about using standard prophylactic dose in patients with high suspicion of VTE, namely those with D-dimer level >1.5 μg/ml (in >60% of our cohort). More research using randomized controlled trials is needed to investigate the efficacy and safety of three different anticoagulation regimens

in critically ill COVID-19 patients with D-dimer levels >1.5 μg/ml.

The present study was subject to a several potential weaknesses. First, it was an observational cohort study, which could have included unmeasured confounding factors that could not be accounted for without a randomized study design. Second, despite many of our patients having D-dimer levels above 1.5 μg/ml, around 30–35% of each group had D-dimer levels <1.5 μg/ml, which gives further caution on the generalizability of these findings in this populations of patients. Lastly, missing data could bias our estimates, despite using the powerful statistical tool MICE.

Conclusion

This current study shows that standard, intermediate, high anticoagulation dose targets for thromboprophylaxis of critically ill COVID-19 patients have a comparable composite of thrombotic events and mortality. An escalated dose of thromboprophylaxis (intermediate and high dose) could increase the rate of minor bleeding but not major bleeding when compared to the standard dose. Thus, these data recommend the standard-dose as preferred regimen.

Data availability statement

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

Ethics statement

The study approval was granted by the Institutional Review Boards at KFMC and PMAH (IRB: 20-666), KSMC (IRB: H1RI-16-Nov20-01), and Almoosa Hospital (IRB: ARC-20-12-4). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization was done by AAlr, YM, and AAlam. Statistics, methodology, software, and interpretation of the results were done by AAlam and AAlr. Study supervision was done by PC. Consultation on statistics was provided by PC and IA. The first draft of the paper was done and the abstract was drafted by AAlr. All authors contributed to the final 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.978420/full#supplementary-material

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Thrombotic events with or without thrombocytopenia in recipients of adenovirus-based COVID-19 vaccines

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COVID-19, the severe acute respiratory syndrome, is one of the major emergencies that have affected health care systems. Drugs and oxygen are only partially effective in saving lives in patients with severe COVID-19, and the most important protection from death is vaccination. The widespread use of COVID-19 adenovirus-based vaccines has provided evidence for the occurrence of rare venous thrombotic events including cerebral venous thrombosis and splanchnic venous thrombosis in recipients of Vaxzevria and Jcovden vaccines and the review focus on them. One year ago, thromboses in Vaxzevria recipients have been associated with thrombocytopenia in the presence of antibodies to platelet factor 4 and have been called vaccine-induced immune thrombotic thrombocytopenia (VITT). The incidence of VITT is equal to 9-31 events per one million doses of vaccines as evaluated by health agencies worldwide and is higher in female and young vaccine recipients. More recently, by using the European EudraVigilance database, it has been demonstrated that the incidence of thrombosis in recipients of adenovirus-based vaccines is 5–10 fold higher than that of VITT and 7–12 fold higher than observed in the recipients of Comirnaty, an mRNA-based vaccine, suggesting that adenovirus-based vaccines cause not only VITT but also thrombosis without thrombocytopenia (non-VITT thrombosis). The incidence of the vaccine-dependent non-VITT thrombosis is different in the adenovirus-based vaccines and the VITT/non-VITT incidence ratio depends on the severity of thrombosis and is inversely related to the age of the recipients. The possible causes and clinical implications of non-VITT thrombosis in vaccine recipients are discussed.

KEYWORDS

COVID-19 vaccines, adenovirus-based vaccines, VITT, thrombosis, thrombocytopenia, inflammatory response

Efficacy and adverse events of COVID-19 vaccines

COVID-19 vaccines protect against severe COVID-19

COVID-19, the severe acute respiratory syndrome caused by the SARS-CoV-2 virus, is one of the major emergencies that have affected health care systems and society in recent decades. The clinical signs of COVID-19 depend on the severity of the disease and can be classified as mild, severe, and critical (1). Severe COVID-19 is characterized by oxygen saturation <90% on room air, signs of pneumonia, and trouble breathing. Exacerbation of the disease is defined by hypoxemic respiratory failure, sepsis, septic shock, and impairment of multiple organs, particularly the lungs (pneumonia and acute respiratory distress syndrome), heart (arrhythmias, chest pain), brain (dizziness, headache, impaired consciousness), kidneys, and liver. Many symptoms derive from the inflammatory response to the SARS-CoV-2 virus and the activation of coagulation. The Spike (S) protein may be implicated in the development of both phenomena (2–5).

In patients with a critical disease, drugs and life-sustaining treatments are only partially effective in saving lives (1, 6, 7), and the most important protection from death is represented by vaccination (8–14).

The mRNA-based vaccines Comirnaty (BNT162b2) and Spikevax (mRNA1273) and the adenovirus-based vaccines Vaxzevria (ChadOx1 nCoV-19) and Jcovden (AD26.COV2.S) were the first approved vaccines. Several studies have shown that these vaccines provide important protection against infection, severe COVID-19, and death (11–15). With the advent of omicron, protection against symptomatic disease is relatively poor, ranging from 20 to 80% after the primary vaccine series, but rapidly decreasing over time (0–30% of effectiveness 4–5 months after the last dose) (16–18). Despite the presence of major differences in efficacy following vaccination, as shown by several studies evaluating the same vaccine, it seems reasonable to conclude that protection is slightly lower after vaccination with adenovirus-based vaccines (18). On the contrary, the effectiveness of the vaccines against severe disease and hospitalization is high, ranging from 80 to 100% after the booster dose and remaining constant over time (18).

Adverse events of COVID-19 vaccines

The adverse events (AEs) observed following vaccination are largely mild or moderate in severity and include fever, chills, headache, flu-like symptoms, muscle pain, and lymphadenopathy (19–22). These events are observed soon after the injection, are not life-threatening and, except for extremely rare anaphylactic reactions, do not require additional treatments. On the other hand, severe AEs (SAEs), although rare, may occur even several days after vaccination and can be life-threatening.

Some SAEs were described for all vaccines, such as myocarditis and pericarditis; in a recently published study (23), the number of these events in young vaccinated males was 11.5, 11.7, and 15.4 events per million doses administered (OMD) of Comirnaty, Spikevax, and Vaxzevria, respectively. These events were reversible and no deaths were observed in under-40 vaccine recipients (23).

Some SAEs occur more frequently with only one/some vaccines, such as Bell's palsy, caused by Comirnaty and Spikevax (24), and Guillain-Barré syndrome, caused by Jcovden (25). Other SAEs associated with specific COVID-19 vaccines are the venous and arterial thromboses observed in recipients of adenovirus-based vaccines. Our review focuses on these SAEs and their association with thrombocytopenia.

Venous thrombosis in recipients of adenovirus-based vaccines

Vaxzevria causes vaccine-induced immune thrombotic thrombocytopenia

The widespread use of COVID-19 vaccines has provided evidence for the occurrence of rare venous thrombotic events in recipients of the Vaxzevria vaccine (26). After two studies demonstrated that, in Vaxzevria recipients, venous thrombotic events [cerebral venous thrombosis (CVT), splanchnic venous thrombosis, and/or other thromboses] are observed in association with platelet aggregation, thrombocytopenia and antibodies to platelet factor 4 (PF4) (27, 28), such event was called vaccine-induced immune thrombotic thrombocytopenia (VITT). VITT is similar to heparin-induced thrombocytopenia (HIT) and is considered an adverse event caused by Vaxzevria (26). Vaccine-induced anti-PF4 antibodies are thought to be responsible for these thrombotic complications because anti-PF4 antibodies were rarely found in CVT patients in the past (i.e., before VITT) (29, 30).

The role of anti-PF4 antibodies in adenovirus-based vaccines was recently confirmed (31, 32). Baker et al. performed a computational simulation showing the electrostatic interaction of adenoviruses with PF4, which was experimentally confirmed by surface plasmon resonance. Greinacher et al. showed that vaccine components can form antigenic complexes with PF4 on platelet surfaces, which become targets of anti-PF4 antibodies in patients with VITT. In addition, they demonstrated that approximately 50% of the protein content of the Vaxzevria vaccine belongs to T-REx-293, the cells in which it is produced, and that EDTA, an excipient of the

Vaxzevria vaccine, causes local vascular leakage that favors the systemic dissemination of the vaccine components (32). Finally, a confirmation of the pathogenesis of VITT comes from a washed platelet aggregation-based assay (32).

Incidence of vaccine-induced immune thrombotic thrombocytopenia in Vaxzevria recipients

After the discovery that Vaxzevria causes VITT, the incidence of VITT was classified as very rare by the European Medicines Agency (EMA) (26) but with the accumulation of data, regulatory agencies stated that in Vaxzevria recipients, 1-3 SAEs per 100,000 vaccinations are VITT. In particular, EMA, the Medicines and Healthcare products Regulatory Agency (MHRA, UK), Health Canada, and the Australian Technical Advisory Group on Immunisation (ATAGI) reported 9.3, 15.1, 21.9, and 27–31 VITT/OMD, respectively (33–36).

By evaluating SAEs from each patient report and considering only patients in whom both thrombosis and thrombocytopenia were reported, we showed that the VITT incidence in the European EudraVigilance database (EEd) was 19.4 SAEs/OMD in Vaxzevria recipients (37). The frequency of VITT reports is about 40 folds greater than that shown in Comirnaty recipients. Similar results were found in South Korea (38) even if the incidence of VITT in Vaxzevria recipients was lower than that observed in EEd.

Study findings and the variable incidence of VITT observed worldwide by regulatory agencies may be due to several factors, including genetics and co-morbidity of vaccinated populations, besides the fact that the sex and age of Vaxzevria recipients may also play a role. For example, we found that the incidence of CVT in Vaxzevria recipients was much higher in young people and women of childbearing age than in adult males and the elderly (39). In particular, a sixfold difference in CVT incidence was found between 18 and 24 years and 60–69 years Vaxzevria recipients (39). Thus, the difference in the incidence of VITT after the Vaxzevria vaccine in South Korea compared with Europe may be due, at least in part, to the age of vaccinee. In fact, in South Korea only 18.3% of the vaccinated people were under 50 years of age, whereas in Europe, these were 37.1% of the population (38, 39).

The incidence of thrombosis after Vaxzevria is much higher than that of vaccine-induced immune thrombotic thrombocytopenia

In Vaxzevria recipients, the incidence of thrombosis including CVTs, splanchnic vein thromboses, thromboembolic diseases, and "other" venous thrombotic events (with or without thrombocytopenia) was about 5 fold more (110.6 SAEs/OMD) than the above-reported incidence of VITT (37). Moreover, in the same recipients, the incidence of thromboses and thrombosis-related deaths was much higher than that observed in Comirnaty recipients, used as a baseline reference (37). Two more studies demonstrated an increased risk of venous thrombosis in Vaxzevria than in Comirnaty and Spikevax recipients (40, 41).

Given clinicians' knowledge of VITT and the recommendation of regulatory authorities to check platelet count in the presence of thrombosis (42), underreporting of low platelet count is unlikely, and data suggest that most thromboses observed in Vaxzevria recipients, despite being caused by vaccination, occur without thrombocytopenia (non-VITT thrombosis). Non-VITT thrombosis following Vaxzevria vaccination has been reported by case studies and shows a persistently normal platelet count and can occur both in the presence and in the absence of anti-PF4 antibodies (43–46).

In Vaxzevria recipients, the VITT/non-VITT thrombosis incidence ratio is severity-dependent and inversely correlated with age

In Vaxzevria recipients, relevant differences in the association between thrombosis and thrombocytopenia are observed depending on the severity of thrombosis. Indeed, thrombocytopenia is associated with 18% of thrombotic SAEs and 48% of thrombosis-related deaths (37). Therefore, the non-VITT/VITT thrombosis incidence ratio is equal to 4.6 in thrombotic SAEs and 1.1 in thrombosis-related deaths. Moreover, thrombocytopenia is associated with 49% of CVT, one of the most severe thrombosis, and 77% of CVT-related deaths (37).

In addition, the incidence of the thrombocytopenia/CVT association is even higher in young-adults representing 83% of Vaxzevria recipients who died because of CVT (37) (**Figure 1A**). Data are consistent with the results published by Greinacher et al. that considered deadly Vaxzevria-dependent CVT to be almost always associated with thrombocytopenia (27). Therefore, thrombocytopenia is more frequent in life-threatening thromboses than in non-fatal ones and in young-adults than in the elderly.

Incidence of vaccine-induced immune thrombotic thrombocytopenia in Jcovden recipients

Cerebral venous thrombosis (CVT) has also been observed in Jcovden recipients (47, 48), making VITT one of the severe AEs of Jcovden (49). Notably, the incidence of VITT in

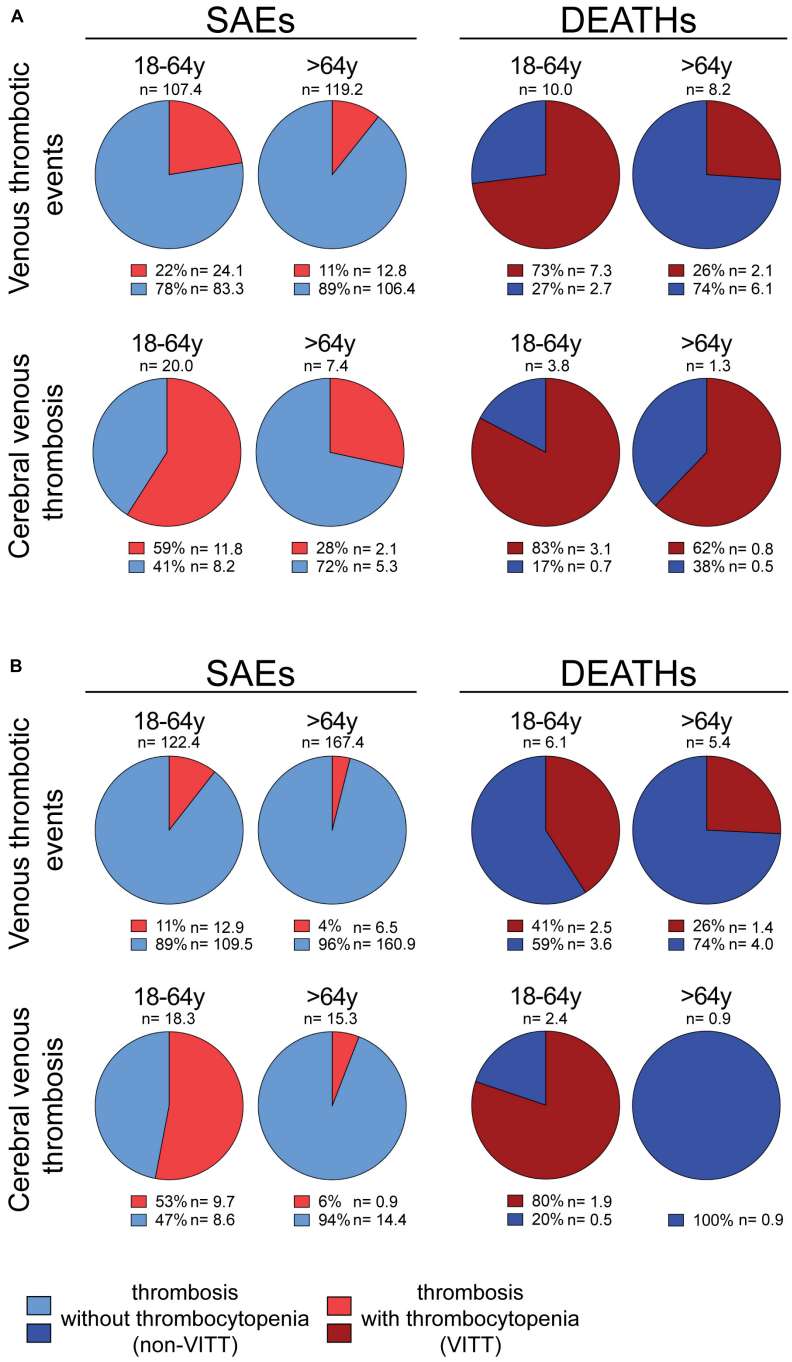

FIGURE 1
Percentage of thrombotic events associated or not with thrombocytopenia in Vaxzevria and Jcovden recipients. **(A, left panel)** All venous thrombotic events (cerebral venous thromboses, splanchnic vein thromboses, thromboembolic diseases, and "other" venous thrombotic events) or only cerebral venous thromboses, reported as SAEs in the EudraVigilance database following the Vaxzevria vaccine are shown. The number of events/OMD (n) and the percentage of individual SAEs associated (light red) or not (light blue) with thrombocytopenia are reported [data from (37)]. **(A, right panel)** The deaths due to all venous thrombotic events (cerebral venous thromboses, splanchnic vein thromboses, thromboembolic diseases, and "other" venous thrombotic events) or cerebral venous thromboses in the EudraVigilance database following the Vaxzevria vaccine are shown. The number of events/OMD (n) and the percentage of deaths associated (dark red) or not (dark blue) with thrombocytopenia are reported [data from (37)]. **(B, left panel)** All venous thrombotic events (cerebral venous thromboses, splanchnic vein thromboses, thromboembolic diseases, and "other" venous thrombotic events) or only cerebral venous thromboses, reported as SAEs in the EudraVigilance database following the Jcovden vaccine are shown. The number of events/OMD (n) and the percentage of individual SAEs associated (light red) or not (light blue) with thrombocytopenia are reported [data from (37)]. **(B, right panel)** The deaths due to all venous thrombotic events (cerebral venous thromboses, splanchnic vein thromboses, thromboembolic diseases, and "other" venous thrombotic events) or only cerebral venous thrombosis in the EudraVigilance database following the Jcovden vaccine are shown. The number of events/OMD (n) and the percentage of deaths associated (dark red) or not (dark blue) with thrombocytopenia are reported [data from (37)].

Jcovden recipients is lower than that observed in Vaxzevria recipients (37). The lower incidence of VITT in Jcovden as compared to Vaxzevria recipients is observed both in young-adults (12.9 vs. 24.1 SAEs/OMD) and in the elderly (6.5 vs. 12.7 SAEs/OMD) (37). Even VITT-related deaths are less frequent in Jcovden as compared to Vaxzevria recipients (37).

The incidence of thrombosis after Jcovden is much higher than that of vaccine-induced immune thrombotic thrombocytopenia

As in the case of Vaxzevria, the incidence of Jcovden-dependent thrombosis is much higher (131 SAEs/OMD) than the incidence of VITT (11.2 SAEs/OMD) (37), suggesting that most vaccine-dependent thromboses are unrelated to thrombocytopenia. The incidence of VITT decreases with the age of the vaccinee (37). Indeed, thrombocytopenia is associated with 11% and 4% of thrombotic SAEs in young-adults and elderly, respectively (**Figure 1B**) (39) and, in those who die, thrombocytopenia is associated with about 41 and 26% of the thrombotic SAEs in young-adults and elderly, respectively (**Figure 1B**) (39). Moreover, the incidence of VITT increases with the severity of SAEs (**Figure 1B**) (37). An exception to the trend of VITT incidence is the virtual absence of VITT in the elderly with CVT or dying of CVT (**Figure 1B**) (37). One case report described the occurrence of non-VITT thrombosis in Jcovden recipients (50).

Differences between Vaxzevria- and Jcovden-dependent thrombosis

The VITT/non-VITT incidence ratio in Vaxzevria and Jcovden recipients is different, although both are adenovirus-based vaccines. While Vaxzevria recipients show a twice higher incidence of VITT than Jcovden recipients, the incidence of non-VITT thrombosis is higher in Jcovden recipients than in Vaxzevria recipients (120 and 91 SAEs/OMD, respectively) (37). In addition, the incidence of thrombosis and thromboembolic diseases is similar in Vaxzevria and Jcovden young-adult recipients and is higher in Jcovden than Vaxzevria elderly recipients (37).

Arterial thrombosis in recipients of adenovirus-based vaccines

The incidence of arterial thrombotic events [myocardial infarction, ischemic stroke, and non-cardiac, non-cerebral arterial thrombotic events (37)] in Vaxzevria and Jcovden recipients has been compared with that in Comirnaty, used as baseline control (37). The incidence of myocardial infarction reported in EEd in young-adult recipients of adenovirus-based vaccines is about sixfold higher, compared with Comirnaty, and an even higher incidence was observed in other arterial SAEs (**Figure 2A**), suggesting that Vaxzevria and Jcovden vaccines not only cause venous thrombosis events but also cause arterial thrombosis. Specifically, in Jcovden elderly recipients, the excess of ischemic stroke over Comirnaty is 106 SAEs/OMD, causing an excess of 10.5 deaths/OMD (37). A recent study, confirms that Vaxzevria and Jcovden vaccines are associated with myocardial infarction (relative incidence, 1.29 and 1.75, respectively), even if significance concerning the SAE due to Jcovden was not reached, probably due to the low number of vaccinations with Jcovden (50).

Regarding arterial SAEs, Jcovden causes more SAEs and SAE-dependent deaths than Vaxzevria (**Figure 2B**).

Discussion

Are VITT and non-VITT thromboses caused by different mechanisms?

It can be hypothesized that non-VITT thrombosis can occur in two ways. First, thrombosis is caused by anti-PF4 antibodies that activate platelets only at specific sites and do not cause thrombocytopenia [thrombosis with anti-PF4 antibodies without thrombocytopenia syndrome (T4-noTS)]. T4-noTS would mean that platelets, activated by low concentrations of anti-PF4 antibodies, aggregate mainly at sites where predisposing factors (e.g., pro-inflammatory environment, blood stasis) are present without causing massive consumption of platelets and thrombocytopenia. According to this hypothesis, T4-noTS would be caused by the same mechanisms leading to VITT. The hypothesis seems to be confirmed by the higher incidence of thrombocytopenia in vaccine recipients who die due to thrombosis as compared to those who survive.

The second way to envision non-VITT thrombosis is to hypothesize that anti-PF4 antibodies are not involved when thrombosis is not associated with thrombocytopenia [thrombosis without anti-PF4 antibodies without thrombocytopenia syndrome (T-no4TS)]. T-no4TS could be caused by factors promoting platelet aggregation, such as an inflammatory response favored by adenovirus-based vaccines and local predisposing factors. The hypothesis seems to be confirmed by the higher incidence of arterial thrombotic events in adenovirus-based vaccine recipients, the different incidence of thrombosis and VITT between Vaxzevria and Jcovden

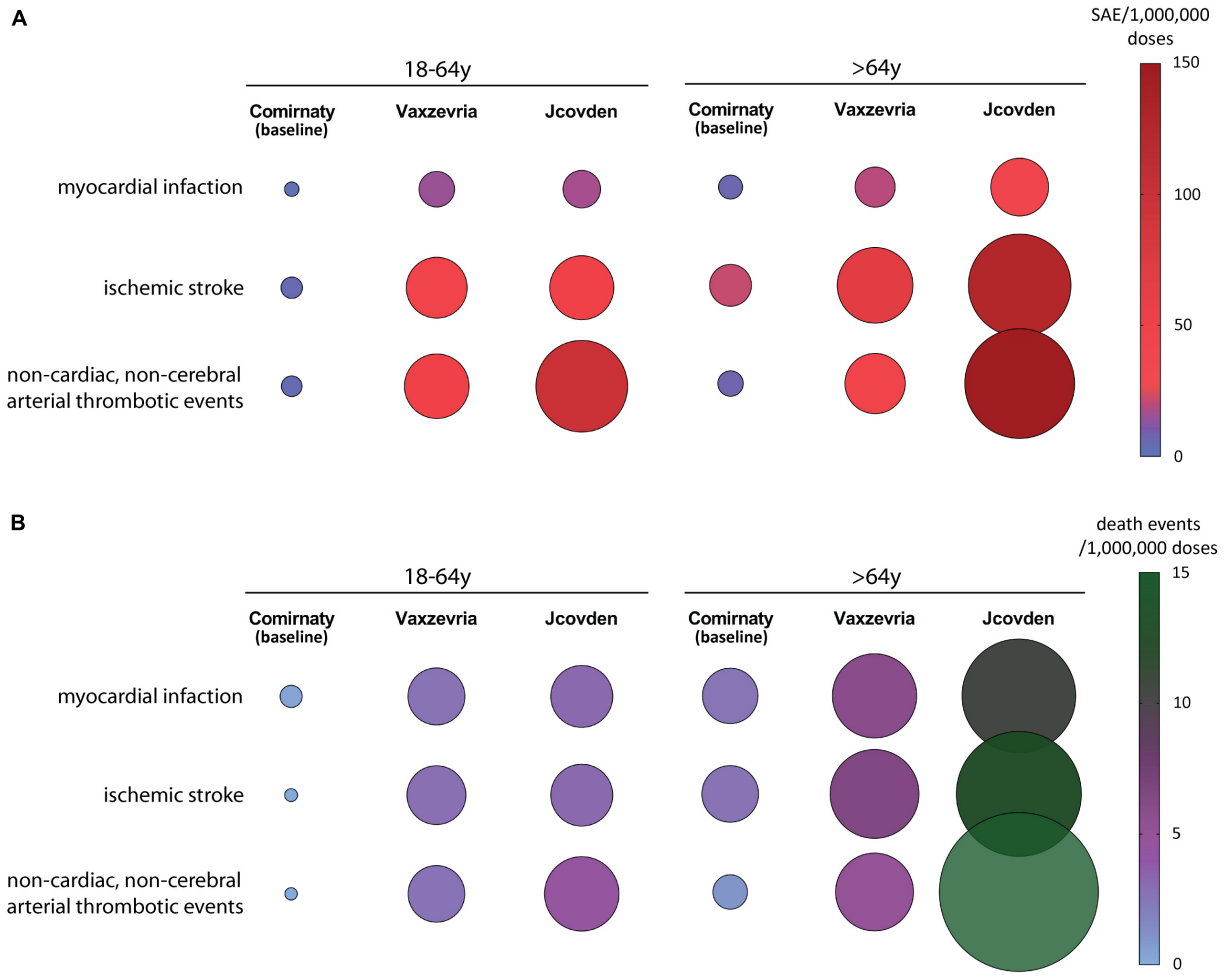

FIGURE 2
The incidence of arterial thrombotic events in the EudraVigilance database following Vaxzevria and Jcovden vaccines are shown. The incidence of events following Comirnaty is reported for comparison. **(A)** Incidence of individual SAEs/OMD related to arterial thrombotic events in young-adult **(left panel)** and elderly **(right panel)** vaccine recipients [data from (37)]. **(B)** Incidence of death events/OMD related to arterial thrombotic events in young-adult **(left panel)** and elderly **(right panel)** vaccine recipients [data from (37)].

recipients, and the different ages at which VITT and non-VITT thrombosis are observed.

How can vaccines cause pro-thrombotic effects independent of anti-PF4 antibodies?

Azzarone et al. hypothesized that in vaccine recipients suffering from local inflammatory reaction, an early event is represented by the production of interleukin 6 (IL-6) (3) which favors endothelial cells reactivity and induces plasminogen activator inhibitor-1 (51, 52), the main physiological inhibitor of the plasminogen activators in the bloodstream (53). A confirmation of the hypothesis came from an observational study demonstrating that 24-48h following Vaxzevria vaccination, the IL-6 levels were almost doubled (54).

Interestingly, another observational study demonstrated that in healthy young-adults vaccinated with Vaxzevria, hemostatic changes were still present after about one month from vaccination (55). In particular, the levels of Von Willebrand Factor (VWF) and active VWF, indicating endothelial activation, were increased 39% and 24%, respectively. Moreover, faster thrombin generation (i.e., more active coagulation system) and lower coagulations levels (use of coagulation factors) were observed, suggesting that the hemostatic system of vaccinee is shifted to a more procoagulant state compared to unvaccinated controls. Therefore, non-VITT thrombo-embolic events may be the clinical expression of procoagulant coagulation profile, consequent to a pro-inflammatory

trigger, represented by vaccination with adenovirus-based vaccines.

How do vaccine components determine pro-inflammatory/pro-thrombotic events?

In search of potential pro-inflammatory and pro-thrombotic triggering factors present in virus-based vaccines, we found three factors: the S protein (2, 4, 56, 57), the adenovirus particles (3, 31), and the cellular debris present in vaccine preparation (32).

The presence of S protein in blood vessels and tissues other than the muscle into which the vaccine is injected can be hypothesized. The S protein may induce pro-inflammatory cytokines and chemokines in macrophages (56, 57) and T cells (58), directly damage endothelium (4), activate platelets (5), and autoimmune response, the latter triggered by antigenic epitopes of S protein shared with molecular chaperones (59). However, given the lower incidence of thrombosis and arterial SAEs in Comirnaty recipients, S protein appears to be not sufficient to cause such events, at least at the concentration determined by the Comirnaty vaccine.

Viral blood dissemination was detected either in patients treated with infusions of adenoviral vectors in the tumor or the right hepatic artery (51, 60). Indeed, after the injection of Vaxzevria into mice, sporadic small amounts of the virus were found in other tissues (61). Adenoviral particles seem to not promote platelet aggregation (3), but the receptors mediating the binding of adenoviral particles to cells are expressed by human endothelial cells, platelets, and erythrocytes (3, 31).

Finally, it should also be considered that the Vaxzevria and Jcovden vaccines contain residual cellular proteins from the cell lines by which adenoviruses are produced likely causing platelet activation (32).

If the reasons why adenovirus-based vaccines cause non-VITT thrombosis and arterial events might be those mentioned above, it is currently impossible to say which is the main culprit. Their synergism is likely crucial.

Why is the incidence of severe adverse events different in recipients of Vaxzevria and Jcovden?

The different incidences of VITT, non-VITT thromboses, and arterial events in recipients of Vaxzevria and Jcovden may be explained by the differences between the two vaccines, which can be summarized as follows. First, the type of adenovirus is different and it has been demonstrated that chimpanzee adenovirus (Vaxzevria) has a stronger negative charge than the human Ad26 virus (Jcovden) (62). Moreover, molecular simulations suggest that the Vaxzevria's adenovirus charge and shape could allow it to bind to the positively charged PF4 protein (31). Second, the amount of infectious units in the Jcovden vaccine is 3.3-fold higher than that in the Vaxzevria vaccine (49, 63), possibly implying a higher S protein and adenoviral protein burden. Third, the human cell lines by which adenoviruses are produced are different: T-REx-293 cells for Vaxzevria and PER.C6 TetR cells for Jcovden (64). Furthermore, approximately 50% of the protein content of the Vaxzevria vaccine belongs to T-REx-293 (32), while Jcovden seems to be less contaminated by cell line debris (64). Finally, EDTA is present in the Vaxzevria vaccine but not in the Jcovden vaccine (49, 63) and may favor the development of inflammation at the vaccine inoculation site.

Concluding remarks

The incidence of VITT, non-VITT thrombosis, and arterial events, although rare, is much higher in recipients of Vaxzevria and Jcovden vaccines than in recipients of the Comirnaty vaccine (37, 38), suggesting a causal relationship between these events and the adenovirus-based vaccines. We hypothesize that the venous and arterial thromboses observed with adenovirus-based vaccines and observed in absence of thrombocytopenia are due to the combination of at least three triggering factors, all of which may be involved in vascular inflammation and coagulation, and several arguments suggest that it is independent of anti-PF4 antibodies (T-no4TS). However, further studies are needed to confidently exclude that non-VITT thromboses are due to low levels of anti-PF4 antibodies.

In Vaxzevria recipients with thrombosis, the incidence of VITT is very high when they die due to CVT. However, in the presence of thrombosis other than CVT, the incidence of VITT is lower, and it is further reduced in the elderly and young adults who do not die. Therefore, in recently vaccinated individuals, the possibility that a thrombosis or arterial event is caused by adenovirus-based vaccines should be considered even in the absence of thrombocytopenia, favoring a correct diagnosis and providing important information for the management of treatment.

Author contributions

GN with the contribution of LC, MNA, AB, SPa, SPi, and GS: conceptualization. GN, LC, and GS: writing—original draft. All authors contributed to the article and approved the submitted version.

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The potential of heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.)-apheresis for patients with severe acute or chronic COVID-19

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Patients with long COVID and acute COVID should benefit from treatment with H.E.L.P. apheresis, which is in clinical use for 37 years. COVID-19 can cause a severe acute multi-organ illness and, subsequently, in many patients the chronic illness long-COVID/PASC. The alveolar tissue and adjacent capillaries show inflammatory and procoagulatory activation with cell necrosis, thrombi, and massive fibrinoid deposits, namely, unsolvable microthrombi, which results in an obstructed gas exchange. Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis solves these problems by helping the entire macro- and microcirculation extracorporeally. It uses unfractionated heparin, which binds the spike protein and thereby should remove the virus (debris). It dissolves the forming microthrombi without bleeding risk. It removes large amounts of fibrinogen (coagulation protein), which immediately improves the oxygen supply in the capillaries. In addition, it removes the precursors of both the procoagulatory and the fibrinolytic cascade, thus de-escalating the entire hemostaseological system. It increases myocardial, cerebral, and pulmonary blood flow rates, and coronary flow reserve, facilitating oxygen exchange in the capillaries, without bleeding risks. Another factor in COVID is the "cytokine storm" harming microcirculation in the lungs and other organs. Intervention by H.E.L.P. apheresis could prevent uncontrollable coagulation and inflammatory activity by removing cytokines such as interleukin (IL)-6, IL-8, and TNF-α, and reduces C-reactive protein, and eliminating endo- and ecto-toxins, without touching protective IgM/IgG antibodies, leukocyte, or platelet function. The therapy can be

used safely in combination with antiviral drugs, antibiotics, anticoagulants, or antihypertensive drugs. Long-term clinical experience with H.E.L.P. apheresis shows it cannot inflict harm upon patients with COVID-19.

KEYWORDS

H.E.L.P. apheresis, PASC, COVID-19, long COVID, SARS-CoV-2, heparin, fibrinogen, rheology

Introduction

In COVID-19 pandemic, the key question is: which therapeutic approach should be favored in order to save seriously sick patients? What kind of approach is suitable to prevent looming acute lung failure involving microthrombi and inflammation of the endothelium (1–5) as a result of an excessive immune response of the body when the host's first lines of defense have already failed? We know that SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) receptor and the transmembrane serine protease 2 (TMPRSS2) as gateways (6–8) to infect cells of the alveolar epithelium (1–4) and endothelial cells in the lungs, heart, kidneys, intestines, and liver (5). This is why patients with coronary artery disease (9–12), hypertension (3, 13), diabetes (3, 13), or obesity (3, 13) exhibit a higher mortality risk as their receptor density is up-regulated (14). Moreover, the binding of the SARS-CoV-2 spike protein inhibits and down-regulates ACE2 function which in turn promotes the inflammatory response (6–8). Diabetes for instance increases thrombogenicity and hyperactivates platelets, and so does hypertension by increasing shear stress in the vessels (15–17).

Histological studies confirmed the presence of the virus in both cell types: alveolar epithelium and endothelial cells (1–5). Alveolar tissue and adjacent capillaries reveal massive inflammatory and procoagulatory activation together with cell necrosis, thrombi, and massive fibrinoid deposits (1–5, 18, 19). It results in the clinical picture of an obstructed gas exchange. The enlargement of the diffusion barrier limits the benefits of artificial ventilation and extracorporeal membrane oxygenation (ECMO) (20–23). In addition, the latter promotes the formation of radicals as a side effect (20–23).

The application of H.E.L.P. apheresis could significantly contribute to the restoration of microcirculation in the lungs and other affected organs. The method, developed by Seidel and Wieland in 1984, primarily for patients with severe hyperlipidemia or familial homozygous hypercholesterolemia (24–30), has not only been proven beneficial as an ultima ratio treatment of arteriosclerosis and its atherothrombotic sequelae, it also has been successfully applied in coronary heart disease (24–27, 31–33) to prevent and treat graft vessel disease following heart transplantation (33–39), acute thrombotic graft occlusion following aortocoronary bypass surgery (40), preeclampsia (41, 42), strokes (43–46), unstable angina pectoris (47), and hyperlipoproteinemia (a) (32). It exhibits anti-inflammatory effects in chronic, and also acute inflammatory processes of the endothelium in the micro- and macrocirculation (26–36, 40, 48, 49) and has anticoagulant and anti-inflammatory properties (25, 50, 51).

Methodology

During H.E.L.P. apheresis, blood cells are first separated from plasma in the extracorporeal circuit, then 400.000 units of unfractionated heparin are added to the plasma, and the pH is lowered to 5.12 using an acetate buffer. That is the isoelectric point for the optimal precipitation of the apolipoproteins from LDL cholesterol, lipoprotein (a) [Lp(a)], and VLDL, which are precipitated in the precipitation filter together with fibrinogen. The excess heparin is adsorbed, and bicarbonate dialysis balances the pH again. The blood cells of the patients are reinfused in parallel with a saline solution (24, 50). The duration of treatment-−2 h on average—can be shortened or extended to meet individual needs (50).

Rationale for H.E.L.P. apheresis

Patients with acute and long COVID-19 most probably will benefit from H.E.L.P. apheresis due to the following reasons:

1. It has no allocation problem and allows direct access to the entire macro- and micro-circulation owing to its extracorporeal access.
2. It uses 400.000 units of unfractionated heparin in the extracorporeal circuit, which was shown of being capable to bind SARS-CoV-2 spike protein (19, 52), and thereby could directly remove the virus and viral debris during viraemia.
3. The large quantity of unfractionated heparin allows the desolvation of forming microthrombi without a bleeding risk due to the heparin adsorber (50).
4. Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis removes about 50–70% of fibrinogen, the most important coagulation protein, within 2–3 h, that in turn immediately improves oxygen supply in the capillaries (50, 51).
5. In addition, it partially removes the precursors of both the procoagulatory and the fibrinolytic cascade by 35–50%,

thus de-escalating the entire haemorheologic system (50). However, antithrombin III is only eliminated by 25% (50) ensuring minimized bleeding risk complications.

6. From the very beginning, H.E.L.P. apheresis is rheologically effective (30, 31, 33, 53): it increases myocardial (30, 53), cerebral (54), pulmonary blood flow rates, and coronary flow reserve (53). These effects facilitate oxygen exchange in the capillaries sustainably (51).
7. It removes cytokines such as interleukin (IL)-6, IL-8, and TNF-α, and reduces C-reactive protein (CRP) concentrations by more than 50% (41, 48, 49). The heparin adsorber completely eliminates endo- and ecto-toxins (48), so that the excessive inflammatory response, the so-called "cytokine storm", can calm down (18, 19, 48, 49).
8. Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis has already been successfully applied for septic multi-organ failure in pilot studies by Bengsch et al. (48, 49). In modified form, it showed a successful outcome in the enterohaemorrhagic *E.coli* (EHEC) epidemic in patients suffering from the hemolytic-uraemic syndrome (HUS) (55).
9. Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis is an established, commercially available system (B. Braun AG, Melsungen, Germany) that has been in clinical use for 37 years. It is easy to handle and was shown to reduce complication rates in acute and chronic cardiac patients very effectively by 82–97% (27, 29, 30, 32, 34, 36). The long-term clinical experience with H.E.L.P. apheresis suggests, with a probability close to certainty, that it cannot inflict harm upon patients with COVID-19.
10. It does not remove protective IgM or IgG antibodies and does not affect leukocyte or platelet function. In the past, the therapy has been shown to be well-tolerated and safe during treatment with antiviral drugs, antibiotics, anticoagulants, or antihypertensive drugs.

Background

In patients who are suffering from severe COVID-19, the computed tomography (CT) scan of the lungs shows ground-glass-like interstitial thickening (5), (which presumably leads to acute respiratory distress syndrome (ARDS). As a result of an excessive immune response, it appears uncontrollable. The advanced disease stage develops after the initial antiviral defense lines of the innate immune system—such as protective effects of interferons and secretory IgA on alveolar epithelium—have failed to eliminate the virus. The presence of SARS-CoV-2 viraemia is the prerequisite for humoral antibody synthesis of IgM and IgG subtypes. They could lyse virus-infected cells in the presence of complement factors. As far as we know, the nature and extent of the cellular immune response to viral antigens are almost entirely dependent on T-lymphocytes (56). The cell-mediated antibody-dependent cytotoxicity is T-cell-dependent and, currently, is being the subject of intensive virological and cell biological research.

In principle, intervention in the inflammatory cascade takes place as early as possible before the onset of the "cytokine tsunami" in order to prevent uncontrollable coagulation and inflammatory activity (18, 19) harming microcirculation in the lungs and other organs. This may be the case in COVID, for example, as this cytokine storm likely results in the presence of microthrombi found in patients suffering from COVID-19 (57). These microthrombi have the ability to block microcapillaries and hence, inhibit oxygen exchange and supply at various organs, resulting in the various symptoms of long COVID such as muscle fatigue, breathlessness, sleep impairment, and anxiety or depression (58). The phenomenon of a "cytokine storm" was first described in 1973 in graft-vs.-host disease (GvHD) following organ transplantation, and later in ARDS, sepsis, Ebola, avian flu H5N1, smallpox, systemic inflammatory response syndrome (SIRS), and now in COVID-19 (59).

Cytokines are proteins that coordinate and modulate cellular immune responses: they guide and activate leukocytes–in particular, T-lymphocytes and monocytes–to the site of inflammation where cytokine secretion is regulated by positive feedback. During a "cytokine storm", leukocytes are activated to such an extent that the immune response seems inexorable. High concentrations of IL-1ß, IL-6, and IL-8 are expressed (18, 19, 59–61). Furthermore, IL-1ß and IL-6, together with TNF-α—the latter being mainly expressed by macrophages-direct systemic inflammatory effects such as the increase in body temperature and blood flow, capillary permeability, and leakage. Due to the complexity of regulation and orchestral functions, IL-6 plays a key role in the transition of mechanisms of innate to acquired immunity (60, 62). The CRP triggers IL-6 (61) and IL-6 links procoagulatory activation, especially triggering fibrinogen production in the liver [51]. Whenever the body's defense is not able to clear the virus from all sites, the inflammation may persist in macrophages, in vascular beds, or in the brain stem and chronify, as recently reviewed by Proal and VanElzakker (63) with the consequence of a wide range of long-lasting clinical symptoms and impaired host immunity. In recent years, Pretorius and Laubscher (64) proved the persistence of insoluble clots containing excess alpha2-Antiplasmin bound plasminogen fibrinogen and amyloid proteins, which results in hindered fibrinolysis in long COVID patients.

Discussion: Effects of HELP apheresis

The anti-inflammatory effects of H.E.L.P. apheresis had been intensively investigated by Bengsch et al. (35, 36) in the nineties.

It has been applied by them in pilot studies to successfully treat sepsis and septic shock patients with multiple organ failures. In 2012, we were able to rescue a patient with EHEC-induced HUS from her comatose state within hours, and from kidney failure within 2 days (55).

In the case of COVID-19, H.E.L.P. apheresis could be of immediate benefit because this extracorporeal system can reduce the trigger and effector of the overwhelming immune response in a simultaneous manner. The SARS-CoV-2, circulating cytokines, CRP, on top fibrinogen are reduced drastically, the latter by 50% within 2 h. As a result, the rheology of the pulmonary microcirculation will immediately be relieved—without reduction of the erythrocyte concentration. Fibrinogen is the effector of plasmatic coagulation and decisive determinant in microcirculation, plasma viscosity, and erythrocyte aggregability (51). Owing to the use of unfractionated heparin, the antithrombotic effect is maximal.

Previous studies using positron emission tomography in heart transplant patients showed that the median coronary blood flow rate remains significantly increased by 17.5% for 24 h after a single 2-h apheresis procedure. It increases by 27% under simulated exposure to the administration of adenosine (33). In principle, the decreased fibrinogen concentration causes rheologically significant effects and facilitates oxygen exchange. Plasma viscosity is reduced by an average of 19%, and erythrocyte aggregability is significantly decreased by 60% (33). In addition, the vascular endothelial growth factor (VEGF) and nitric oxide (NO) release are favorably influenced (33). The improvements have also been demonstrated for cerebral blood flow in the cardiac patients, where they profit from a 63% increase in the CO_2 reserve capacity (54).

Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis reduces LDL cholesterol and Lp(a) concentrations with similar efficacy as fibrinogen (24, 25), thereby improving endothelial function (33, 53, 54). With regards to LDL reduction through apheresis, it remains unclear whether SARS-CoV-2 resembles delta coronavirus, which uses cholesterol as a vector due to its lipid envelope (65).

For practical reasons it is important to mention that H.E.L.P. apheresis is not restricted to a 2-h treatment time. The system can be recirculated for many hours—until the precipitate filter is saturated. The precipitate filter however can also be exchanged during the procedure, so the fibrinogen concentration theoretically could be reduced by up to 99.9999%. In-depth preliminary studies into the influence of H.E.L.P. apheresis on the kinetics of the procoagulation and fibrinolytic cascades have shown that the precursors of both cascades are also reduced by 35–50% within 2 h—with the exception of antithrombin III, which is reduced by 25% (50). Taking together, H.E.L.P. apheresis thus de-escalates the coagulation situation of both arms without any bleeding risk due to the complete adsorption of unfractionated heparin (50).

The heparin adsorber also has the ability to eliminate endo- and exo-toxins regardless of viral or bacterial origin (48, 49, 55). Recent data from Carlo Brogna indicate that the SARS-CoV-2 virus acts as a bacteriophage on the microbiome of the lungs and the guts of infected patients, thereby inducing the bacteria to produce neurotoxic "conotoxins". These so-called conotoxins might also be eliminated by means of H.E.L.P. apheresis (64).

The use of H.E.L.P. apheresis should be considered for the treatment of patients with acute and long COVID in order to restore the vascular homeostasis, remove inflammatory and thrombogenic mediators, and to avoid unnecessary suffering. Our first experiences with patients with long COVID are promising and summarized in the corresponding article. Meanwhile, we could successfully treat hundreds of patients with long COVID with this method.

Data availability statement

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

Author contributions

BJ created the working hypothesis and wrote the paper. HA helped in editing, proofreading, and discussing the theory. WK-M helped brainstorm, discuss the theory, and refine it. DS was the inventor of the HELP apheresis helped with the theoretical hypothesis and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors BJ and DS filed a patent of the use of HELP Apheresis for long COVID to avoid misuse.

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

Publisher's note

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Effect of stimulated platelets in COVID-19 thrombosis: Role of alpha7 nicotinic acetylcholine receptor

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Since early 2020, SARS-CoV-2-induced infection resulted in global pandemics with high morbidity, especially in the adult population. COVID-19 is a highly prothrombotic condition associated with subsequent multiorgan failure and lethal outcomes. The exact mechanism of the prothrombotic state is not well understood and might be multifactorial. Nevertheless, platelets are attributed to play a crucial role in COVID-19-associated thrombosis. To date, platelets' role was defined primarily in thrombosis and homeostasis. Currently, more focus has been set on their part in inflammation and immunity. Moreover, their ability to release various soluble factors under activation as well as internalize and degrade specific pathogens has been highly addressed in viral research. This review article will discuss platelet role in COVID-19-associated thrombosis and their role in the cholinergic anti-inflammatory pathway. Multiple studies confirmed that platelets display a hyperactivated phenotype in COVID-19 patients. Critically ill patients demonstrate increased platelet activation markers such as P-selectin, PF4, or serotonin. In addition, platelets contain acetylcholine and express α7 nicotinic acetylcholine receptors (α7nAchR). Thus, acetylcholine can be released under activation, and α7nAchR can be stimulated in an autocrine manner and support platelet function. α7 receptor is one of the most important mediators of the anti-inflammatory properties as it is associated with humoral and intrinsic immunity and was demonstrated to contribute to better outcomes in COVID-19 patients when under stimulation. Hematopoietic α7nAchR deficiency increases platelet activation and, in experimental studies, α7nAchR stimulation can diminish the pro-inflammatory state and modulate platelet reactiveness *via* increased levels of NO. NO has been described to inhibit platelet adhesion, activation, and aggregation. In addition, acetylcholine has been demonstrated to decrease platelet aggregation possibly by blocking the e p-38 pathway. SARS-CoV-2 proteins have been found to be similar to neurotoxins which can bind to nAChR and prevent the action of acetylcholine. Concluding, the platelet role in COVID-19 thrombotic events could be explained by their active function in the cholinergic anti-inflammatory pathway.

KEYWORDS

COVID-19, SARS-CoV-2, platelets, inflammation, alpha7 nicotinic acetylcholine receptor (α7nAchR), thrombosis

Introduction

Since early 2020, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-induced infection resulted in global pandemics with high morbidity, especially in the adult population (1, 2). Coronavirus disease 19 (COVID-19) presented with a specific organ and system involvement, such as severe acute respiratory syndrome (SARS) which was already observed in other viral infections (3). In addition, this infection demonstrated very specific SARS-CoV-2-unique pathological phenotypes which raised a lot of concern and unanswered questions with regard to evidence-based management options (4, 5). Those clinical phenotypes do differ in patient clinical data on admission, complications, comorbidities, and clinical outcomes; thus, treatment might be tailored based on the clinical course and previous risk (5). Moreover, it emerged that COVID-19 is a highly prothrombotic condition associated with subsequent multiorgan failure and lethal outcomes (6–8). Multiorgan failure is still under investigation, yet different mechanisms such as endothelial cell damage, immune response, dysregulation of the renin-angiotensin-aldosterone system, and thromboinflammation have been involved (9, 10). A new type of COVID-19-associated multiorgan failure—a multisystem inflammatory syndrome (MISC) was described in children (11, 12). It closely resembles Kawasaki disease, known for several decades for its coronary complications (13). Up to 68% of affected children are treated in the pediatric intensive care unit (PICU) (14). Also, the increasing incidence of MISC is reported in young adults (15, 16). In most cases, MISC is characterized and investigated with the main focus on hyperinflammation, meanwhile, coagulation and thrombosis are less understood. Still, a study by Buonsenso et al. found D-dimers (fibrin degradation products) as an independent predictor of the outcomes of MISC (17). From the beginning of the COVID-19 pandemics, various data revealed that 20–50% of all COVID-19 hospitalized cases show abnormal coagulation results (18). An increase in D-dimer concentrations has been shown in a high percentage of severe COVID-19 cases. Elevated D-dimer values are associated with more severe diseases course and unfavorable outcomes of COVID-19 (8, 19–22). Platelets are another important marker in COVID-19. The most common finding in severe SARS-CoV-2-induced infection is thrombocytopenia. A meta-analysis by Jiang et al. demonstrated that lower platelet counts were detected in severe COVID-19 cases compared to milder ones (23). Thrombocytopenia has been reported to be associated with an increased risk of severe disease (24–26). Also, more studies analyse platelet activation role in the prothrombotic phenotype of COVID-19 patients. The exact mechanism of the prothrombotic state is not well understood and might be multifactorial. Nevertheless, platelets are attributed to play a crucial role in COVID-19-associated thrombosis. In this review, we will summarize the platelet role in COVID-19-associated thrombosis. Moreover, we will provide more insight into the role of the platelet alpha7 nicotinic acetylcholine receptor (α7nAChR) in the COVID-19-associated inflammation leading to thrombotic events.

COVID-19 inflammation and thrombotic events: Clinical picture

The global pandemic of COVID-19 caused by SARS-CoV-2 started in 2020 and continues nowadays with the new disease entities. Initially, COVID-19 was thought to cause mainly respiratory symptoms which for the most affected were mild, subsequently, it had shown to be associated with a higher number of different complications.

Thrombosis plays a crucial part in the pathogenesis of COVID-19. In the beginning, SARS-CoV-2 infection induces a tremendous inflammatory reaction leading to uncontrolled or disrupted anti-inflammatory response (27). Interaction between SARS-CoV-2 and host cells, and prolonged inflammation cause endothelial damage and dysfunction with the result of excessive prothrombotic factor production contributing to an increased coagulation state. Moreover, COVID-19-induced hypoxia can further stimulate thrombosis through blood viscosity and hypoxia-inducible transcription factors (28). Nevertheless, DNA and histones from neutrophil extracellular traps (NETs) can additionally contribute to pro-thrombotic pathway activation (9, 29).

Up to 4.7% of severe COVID-19 cases progress to critically ill patients (30), and a significant number—approximately 79% result in severe thrombotic complications associated with a high mortality rate (8, 31, 32). Despite prophylactic anticoagulation treatment, almost one-third of the patients experience thrombotic events as demonstrated by the study of Lodigiani et al. (33). Moreover, a systematic review by Alahyari et al. revealed that thromboembolic events, such as deep vein thrombosis (DVT) or pulmonary embolism are most frequent of all the COVID-19-associated hematologic complications (34). Globally, a wide spectrum of incidence (10.9–58%) of DVT in COVID-19 patients was reported by several studies (33, 35, 36) with a higher percentage in critically ill patients (37). When compared to non-COVID acute respiratory distress syndrome (ARDS), COVID-19 ARDS demonstrated higher rates of pulmonary embolism (2.1 vs. 11.7%, respectively) (38, 39). A post-mortem study by Wichmann et al. revealed an important interplay between COVID-19 and venous thrombosis events (37). Most importantly, the unique feature of COVID-19-induced thrombosis is that it can be, both arterial and venous (32). Arterial thrombotic complications are less common (40), nevertheless, they can cause severe and devastating outcomes even with prescribed prophylactic anticoagulation therapy (41). A plethora of thrombotic complications are affecting cardiovascular and cerebrovascular systems (9, 10), myocardial infarction being the most prominent event (40, 42), and reaching

21% in the most recent meta-analysis study (43). Few studies suggested that ischemic stroke affects COVID-19 diseased younger people (6, 44, 45). Furthermore, the latest study by Xie et al. showed significantly higher cardiovascular outcomes after COVID-19 exposure (46), and cardiac complications have been linked to poor outcomes (43). Additionally, mesenteric ischemia is being reported in 1–5% of the cases with COVID-19 (38, 47). Besides macrovascular complications, more evidence demonstrates COVID-19-associated microvascular thrombotic events. Alveolar-capillary microthrombi have been found in severe COVID-19-induced ARDS cases (48–50). Nevertheless, more evidence shows that microangiopathy can cause complications in COVID-19 asymptomatic patients or patients with mild respiratory symptoms. An international study in perinatology recently demonstrated that pregnant women with mild COVID-19 symptoms resulted in placentitis leading to widespread placental insufficiency with subsequent fetal hypoxia and even lethal outcomes (51). In 37% of the examined placenta samples, multiple intervillous thrombi formations were identified and suggested as one of the contributing mechanisms to severe placental malperfusion. Another hypothesis of the possible presentation of SARS-CoV-2-induced microangiopathy was reported in several studies of case series showing increased incidence of "chilblains like" skin lesions during COVID-19 (52, 53). Moreover, this was supported by histological reports of skin biopsies where microthrombi were detected (54–56). The underlying hypothesis was SARS-CoV-2-associated epithelial damage, and secondary ischemia leading to the microangiopathic lesions (54). However, the clear confirmed pathogenesis and association of these skin lesions to COVID-19, especially in asymptomatic forms of the disease, is still under debate (57, 58). In general, the cause of various thrombosis in SARS-CoV-2 infection is closely related to coagulopathy, inflammation, platelet hyperactivity, thrombocytopathy, and endotheliopathy (9).

Role of the platelets in the immune system, inflammation, and COVID-19-associated thrombosis

For a long time, platelets have been known as cells playing role in thrombosis and hemostasis. It is noteworthy that recently they have been attributed a significant role as immune mediators (59, 60). Platelets are a nucleate blood cells derived from megakaryocytes that reside primarily within the bone marrow (61). Additionally, studies have shown that the lung can be a potential site for platelet biogenesis. In the lung, platelets function as antiseptic cells when released in the vicinity of potential pathogen entry (62).

Patients with acute COVID-19 tend to be in a prothrombotic state and have severe inflammation (63). COVID-19 thrombosis encompasses both arterial and venous thromboembolic events, and they frequently co-occur with thrombocytopenia (32, 39). Systemic inflammation often leads to sepsis and septic shock and may present with increased platelet-leukocyte aggregates and thrombocytopenia (64–66). Genes encoding transcription factors involved in hematopoiesis and megakaryocyte biogenesis, such as Runt-related transcription factor 1 (RUNX1), GATA-binding factor 1 (GATA1), and others, have an impact on variations in platelet count (67–69). The liver produces thrombopoietin (TPO), which activates the TPO receptor in megakaryocytes to cause the creation of platelets through a process that is triggered by thrombocytopenia (70). In the final stage of platelet production, some of the transcription factors play a negative feedback role on TPO (71). Numerous cytokines can initiate megakaryopoiesis (e.g., interleukins 3, 6, and 11 (IL-3, IL-6, IL-11), fibroblast growth factor 4 (FGF4), and others) (72). Viruses can activate the host's cytokine profile to alter platelet formation through hepatic TPO synthesis. The simian immunodeficiency virus (SIV), which increases tumor growth factor (TGF), causes the synthesis of TPO (73); human papilloma virus-6 (HPV6) may prevent the development of TPO-induced megakaryocytic colonies (74). Meanwhile, SARS-CoV-2 *via* its spike protein may trigger the production of antibodies that cross-react with human TPO, to induce thrombocytopenia (75). Conversely, SAR2-CoV-2 stimulates angiotensin-converting enzyme (ACE) expression, which leads to induced inflammation *via* angiotensin II (Ang II) resulting in IL-6 stimulated TPO augmentation (76). In inflammation, IL-6 raises the levels of TPO to promote the creation of platelets (77), therefore, it can be considered that an inflammatory environment is required for COVID-19-induced thrombosis.

Currently, more studies demonstrate direct viral-platelet interaction in platelet thrombotic and inflammatory function modulation (78, 79). Platelets do express pattern recognition receptors (PRRs), such as Toll-like receptors (TLR), Nod-like receptors, or C-type lectin receptors (80). Those receptors are crucial in damage-associated molecular patterns (DAMPs) and exogenous pathogen-associated molecular patterns (PAMPs) recognition. DAMPs and PAMPs are referred to as virus-associated molecular patterns (80–83). The attachment to DAMPs and PAMPs can initiate different intracellular pathways resulting in various pro-inflammatory cytokine production (84–87). In addition, platelet expression of functional TLR2 can further contribute to thrombotic pathway activation (84) (Figure 1). In the case of flu, the influenza virus has been proven to activate platelets *via* TLR-7 and Fcγ receptor IIa (FcγRIIa). The platelet expression of FcγRIIa leads to the activation of immune complexes (88). Antibodies against self-antigens, such as antiphospholipidic antibodies, have been reported in COVID-19 patients (89). However, thrombus formation was seen in COVID-19 patients' serum that had low levels of antiphospholipidic antibodies (90). Nevertheless, aberrant glycosylation of anti-SARS-CoV-2 spike immunoglobulin G(IgG) complexes was found to be a significant factor in the

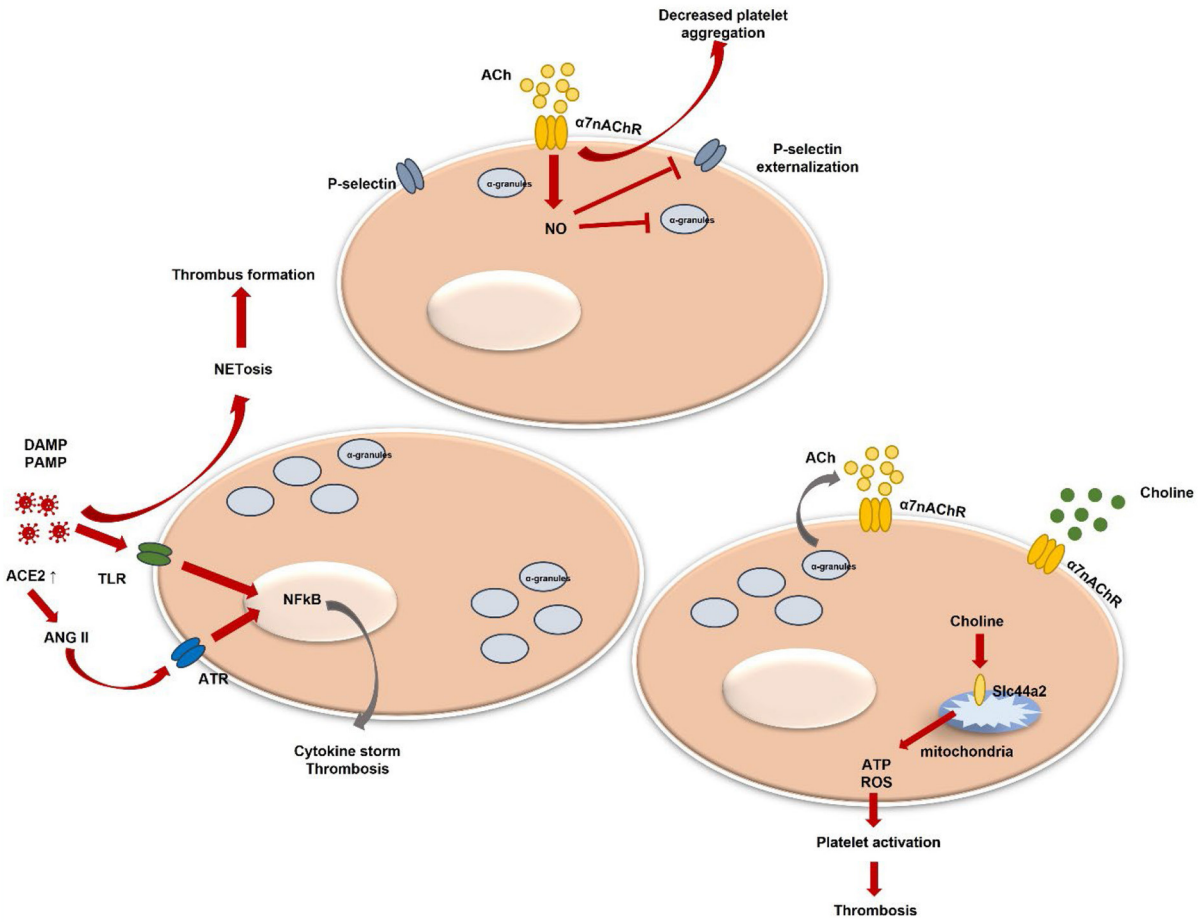

FIGURE 1
Platelet activation and α7nAChR involvement in thrombosis regulation. DAMPs, danger associated molecular patterns; PAMPs, pathogen associated molecular patterns; TLR, toll-like receptor; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; ACh, acetylcholine; NETosis, neutrophil extracellular trap formation; NO, nitric oxide; ACE2, angiotensin-converting enzyme-2; ANG II, angiotensin II; ATR, ANG II receptor; ATP, adenosine triphosphate; ROS, reactive oxygen species; α7nAChR, alpha7 nicotinic acetylcholine receptor.

ability of these complexes to increase thrombus formation (91). Activation of TLR-7 evokes platelet degranulation, platelet-leucocyte aggregation, and NETosis stimulation leading to thrombus formation (79, 92) (Figure 1). Plasma from hospitalized COVID-19 patients demonstrated increased circulating platelet-neutrophil aggregates (93). Additionally, the autopsy of COVID-19 patients showed that microvascular thrombi composed of platelets and neutrophil extracellular traps were present (94). Also, recent studies show that platelets can internalize virus particles, and after viral ssRNA, dsRNA, or CpG DNA attachment to TLRs downstream signaling is initiated leading to platelet activation, platelet granule release, and P-selectin exposure (95, 96). P-selectin is a platelet receptor that has been linked to platelet activation. Platelets can bind to leukocytes *via* the P-selectin glycoprotein ligand-1 to mediate neutrophil rolling and intracellular leukocyte signaling (97, 98). Their depletion or blocking of the P-selectin-mediated interaction with neutrophils may reduce lung injury in COVID-19 (99). P-selectin, soluble CD40 ligand and others are released under platelet stimulation (100, 101). Their increased levels are observed in COVID-19 patients and P-selectin stimulates monocyte tissue factor (TF) expression contributing to a prothrombotic phenotype (102, 103).

Systemic levels of pro-inflammatory cytokines, such as TNFα, IL-1, and IL-6, are markedly elevated in severe COVID-19 (104). Moreover, the expression of pro-inflammatory cytokines, including TNFα, and IL-6 is dependent on Ang II (105, 106) (Figure 1), which amount is increased in SARS-CoV-2 infection (107). Angiotensin II (Ang II) contributes to endothelial dysfunction and the development of microvascular thrombosis (108), it stimulates TF expression, which is the physiological initiator of blood coagulation (109).

Also, Ang II triggers platelet-derived growth factors (PDGF) production (110) and increases platelet aggregation (111). In addition, P-selectin expression levels are enhanced by activated platelets and by endothelial cells during Ang II stimulation (112). The relationship between Ang II and α7nAchR has been determined when activation of α7nAChR alleviated Ang II-mediated vascular smooth muscle senescence (113). Furthermore, it was suggested that decreased expression of α7nAchR might contribute to TNFα-induced vascular tissue inflammation, which was previously described as related to Ang II-mediated microvascular thrombosis (114). α7nAchR and the cholinergic system have been already studied regarding their beneficial role in COVID-19-induced hyperinflammation and disease outcomes (115, 116). Moreover, it has been shown that vagal stimulation *via* α7nAchR improves COVID-19-induced lung infection and inflammation, as well as systemic hyperinflammation (117–119). Additionally, patients lacking α7nAchR levels presented with higher C-reactive protein (CRP) values, more pronounced lymphopenia, extended pulmonary lesions, and increased expression of the TNFα pathway (115). Despite that α7nAchR role in platelets is still not widely studied and not well summarized, thus, we will analyse currently present data and platelet α7nAchR role in COVID-19-induced thrombosis.

The cholinergic system, α7nAChR, and platelet role in COVID-19-induced hyperthrombosis

More and more data suggest that the autonomous nervous system plays a crucial role in inflammation *via* a cholinergic anti-inflammatory pathway (CAP). CAP is mainly composed of the parasympathetic nerves with the vagal nerve being most important together with acetylcholine (ACh) and its receptors (120). This pathway bridges the autonomic nervous system and immune system. Recently, the alleviating effect of COVID-19-induced hyperinflammation has been widely described in several studies (115, 117, 121–123). Under direct activation of the afferent vagal nerve, the main neurotransmitter ACh is released which further stimulates α7nAChR (124, 125) resulting in an anti-inflammatory response. Non-neuronal ACh was demonstrated to have an anti-inflammatory potential as well. α7nAChR is widely present on different immune cells and a variety of other cells, such as neuronal, endothelial cells, and platelets (126–128). Increased levels of TNFα, IL-6, and CRP have been detected in α7nAChR knockout mice (124, 129). Moreover, endothelial cell activation as well as leucocyte recruitment can be inhibited *via* α7nAChR stimulation (126). In addition, platelet function can be modulated during the inflammation process (128, 130).

Several studies have shown that platelets do contain some components of a non-neuronal cholinergic system, e.g., ACh, choline acetyltransferase, and acetylcholinestares (131–133). It is known that acetylcholinestares (AChE), for instance, hydrolyses the neurotransmitter ACh in the nervous system. Under AChE excess, an inflammatory process can be promoted (134). Three C-terminal variants of AChE have been identified (135). One of them—is a read-through transcript which is formed through the continuous transcription through intron I-4. This variant has been demonstrated to play an active role in the hematopoietic system and could be linked with its regulation under specific conditions, such as development or stress (132). Moreover, few studies found RNA signals of nAChR subunits, as well as α7 subunit in platelets (136, 137). Schedel et al. described functional α7nAChR Ca^{2+} channels in human platelets and in the megakaryocytic lineage and proposed an autocrine regulation mechanism *via* released stored ACh (128). Platelets are known to store various molecules in their granules. Those different cargo molecules are released under stimulation and contribute to coagulation, inflammation, or facilitating adhesion to other cells (138, 139). Nevertheless, ACh could be presented *via* other cells, such as endothelial cells, which are in close contact with platelets (131). A study by Bennett et al. indicated that endogenous ACh produced by platelets does inhibit platelet activation (140). It was demonstrated that *via* elevated nitric oxide (NO), ACh inhibits platelet degranulation, inhibits P-selectin externalization, and glycoprotein IIb IIIa (GPIIbIIIa) activation (141, 142) (Figure 1). Moreover, platelets express nitric oxide synthase 3 (NOS3) which may regulate platelets by an endogenous NO pathway (143). Few studies have shown that inhaled NO downregulates P-selectin, platelet aggregation, and fibrinogen binding in severe ARDS (144, 145). Kooijman et al. confirmed that mice lacking α7nAChR showed increased platelet aggregation *ex vivo* (130). Still, the hypothesis by authors has been raised that only a lack of both, platelet and endothelial α7nAChR could be associated with a significant impact on inflammation. Afterall, the role of endothelial cells in a cholinergic anti-inflammatory pathway is not well studied. Platelet and endothelial cell interaction is clearly described and shown to be important in various inflammatory conditions. Endothelial cell disruption during COVID-19 and released cytokines can be a possible mechanism of thrombosis (146). In addition, it has been demonstrated that platelets do play a crucial role in hypercoagulation during COVID-19 (147). Additionally, few studies revealed that monomeric C-reactive protein (mCRP) is linked with platelet activation which is mediated *via* p38 mitogen-activated protein kinase (MAPK) and Jun N-terminal kinase (JNK) (148). Interestingly, ACh has been shown to block mCRP binding and related pro-inflammatory action (149). MAPK is highly important in platelet activation, aggregation, and thrombus formation (150, 151). Moreover, the involvement and activation

of p38 MAPK has already been widely described in SARS-CoV-2 (152). In addition, p38 MAPK can facilitate viral entry *via* ACE2 (153).

Interestingly, α7nAChR can be activated *via* choline as well (154). Choline is a precursor of ACh and phosphocholine (PC). It can efficiently act and is a relatively selective α7nAChR agonist (155). In COVID-19 patients, choline has been found to be downregulated, particularly in severe cases (156). Meanwhile, the intermediate product phosphatidylcholine has been detected to be upregulated. The possible underlying mechanism could be macrophage polarization associated with pathogen presentation (157). This further results in various cytokine secretion as a response to a COVID-19 infection. Another study showed that higher choline levels in pregnant women were associated with protective action against COVID-19 in fetal brain development (158). A recent study identified choline's role in platelet activation and thrombosis. The genetic loci including Slc44a2 have been already studied in thrombosis (159). Slc44a2 was demonstrated to mediate choline transport into mitochondria which results in mitochondrial oxygen consumption and ATP production (159). Mitochondrial dysfunction induces ATP decrease which results in decreased ATP release from platelets. Moreover, decreased ADP causes lower activation of platelets. Slc44a2 was already associated with venous thromboembolism (160, 161). In addition, Slc44a2 is well defined as a human neutrophil antigen (162). Moreover, it was proven to directly interact with platelet integrin $\alpha_{IIb}\beta_3$ and trigger NETosis leading to thrombosis (163, 164) (Figure 1). PC is nAChR agonist as well (165). Studies showed its inhibitory potential for IL-1beta release from monocytes in α7nAChR dependent manner (166). Furthermore, PC epitopes are exposed on various pathogens and their interaction with host proteins, such as platelet-activating factor receptors (167, 168) leads to pathogen adhesion to the surface of the host cell and cell invasion (169, 170). Nevertheless, less is known regarding PC function and excretion from platelets and involvement in SARS-CoV-2 or other viral pathogen-induced inflammation and/or thrombosis. To date, only one study defined that platelets could release choline metabolites under stimulation (171).

Conclusion

Concluding, the prothrombotic state of COVID-19 is multifactorial, nevertheless, platelets do play an important role in inducing COVID-19 hypercoagulation and thrombosis. Due to their different secretory factors which induce coagulation and inflammation, they participate in thrombosis induction *via* different pathways. One of the possible and less studied is the cholinergic system and platelet α7nAChR which has been less studied but could be a very significant part in SARS-CoV-2-induced infection. As previously shown, nervus vagus stimulation can benefit COVID-19-associated hyperinflammation, thus, *via* platelet α7nAChR it might decrease coagulation and thrombotic process together with decreased inflammatory factors (which additionally activate platelets) and benefit COVID-19 patients. Different studies show that platelets can produce choline products under stimulation, thus, α7nAChR could be stimulated leading to its beneficial anti-inflammatory and possible anti-thrombotic effect. However, more studies are necessary to confirm this hypothesis.

Author contributions

LJ: hypothesis, editing, visualization, and supervision. LJ, MM, and AS: analysis, writing original draft, and review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Are platelet volume indices of clinical use in COVID-19? A systematic review

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Background: The incidence of thrombotic complications is high in COVID-19 patients with severe disease. As key regulators of thrombus formation, platelets likely play a crucial role as mediators of severe acute respiratory syndrome coronavirus 2 associated pathogenesis. Studies have reported that parameters reflecting platelet size, known as platelet volume indices (PVI), are raised in patients with thrombosis and can predict poor outcomes. This systematic review evaluates the potential for PVI to be used as a predictor of COVID-19 morbidity and mortality.

Methods: English and Chinese databases were searched electronically to identify studies reporting data on mean platelet volume, platelet distribution width or platelet-large cell ratio in COVID-19 patients. Included articles underwent a quality rating and descriptive narrative analysis.

Results: Thirty-two studies were included in the systematic review. The results show a general trend for PVI to be raised in severe COVID-19 patients and non-survivors, with 14 studies reporting significant differences of baseline PVI between severe and mild disease. Nonetheless, longitudinal studies showed varying PVI trends over the course of the disease and evidence for PVI to be associated with disease progression was limited. The quality rating of 12 studies was poor, 16 were rated fair and four were good. Most studies were retrospective in design, used small study populations and did not consider confounding factors that influence platelet volume. Studies also contained technical flaws in PVI measurement, limiting the reliability of the results.

Conclusion: The evidence on the clinical usefulness of PVI is greatly limited by the lack of prospective evaluation, together with technical problems in measuring PVI. Carefully designed prospective studies are warranted.

Systematic review registration: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=304305, identifier CRD42022304305.

KEYWORDS

COVID-19, mean platelet volume, platelet distribution width, platelet large cell ratio, thrombosis

Background

Coronavirus disease 2019 (COVID-19) disease is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). While most cases of COVID-19 are mild, some develop severe viral pneumonia with respiratory failure, that can result in death. Severe disease is predominantly observed in the elderly and those with underlying health conditions such as hypertension, diabetes and coronary heart disease (1). An unexpectedly high incidence of thrombosis has been reported (2, 3), and severity of COVID-19 disease is associated with elevated inflammatory markers and markers of coagulation such as D-dimer, fibrinogen and von Willebrand factor (1, 4). Moreover, COVID-19 autopsies have shown evidence of widespread microthrombosis in the lungs and other organs (5).

Circulating platelets play a central role in hemostasis and thrombosis, and platelets significantly contribute to immune responses during viral infection in a process termed "immunothrombosis" (6). Platelet hyperreactivity may contribute to immunothrombosis often seen in patients with COVID-19 (7). COVID-19 patients have higher levels of P-selectin expression in resting and activated platelets, elevated circulating platelet-leukocyte aggregates, increased aggregation, and thromboxane generation (8, 9). In addition, mild thrombocytopenia is observed in COVID-19 patients, and a progressive decline of platelet counts (PLT) was significantly associated with increased mortality (10). Moreover, pulmonary megakaryocytes are increased in COVID-19 patients with acute lung injury (11). Since the lung is considered an active site of megakaryopoiesis, a prothrombotic status leading to platelet activation, aggregation and consumption may trigger a compensatory pulmonary response (11).

Platelet activation markers are considered to be useful tools in evaluating risk factors of thrombosis in a variety of clinical conditions (12). While there are many methods used to test platelet activation for research purposes, most of the existing techniques are expensive, require trained personnel and take time to perform, limiting their use in clinical practice (12).

Circulation of larger, younger platelets reflect platelets activity and considered to be useful predictors of thrombotic events (13, 14). Platelet size can be assessed during a routine clinical blood test using automated hematology analyzers. Platelet volume indices (PVI) are a group of parameters that are routinely measured using automated hematology analyzers and include mean platelet volume (MPV), platelet distribution width (PDW) and platelet-large cell ratio (P-LCR). Their wide availability and low cost makes them appealing biomarkers for clinical research. Moreover, increased MPV is associated with thrombocytopenia, a hematological change often exhibited in COVID-19 patients (15). It has been proposed that megakaryocytes increase the production of large immature platelets as a compensatory mechanism for platelet consumption resulting from pulmonary microthrombi formation (16).

The aim of this systematic review is to evaluate the usefulness of PVI as clinical biomarkers for COVID-19 disease prognosis.

Abbreviations: CBC, Complete Blood Count; CENTRAL, Cochrane Central Register of Controlled Trials; CNKI, China Knowledge Resource Integrated; COVID-19, Coronavirus disease 2019; EDTA, Ethylenediaminetetraacetic acid; fL, Femtoliters; ICU, Intensive care unit; IQR, Interquartile range; LMR, Lymphocyte/mean platelet volume ratio; MPV, Mean platelet volume; MPR, Mean platelet volume/platelet count ratio; PDW, Platelet distribution width; P-LCR, Platelet-large cell ratio; PLT, Platelet count; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; PVI, Platelet volume indices; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

Methods

Search strategy

A review protocol was published on PROSPERO (ID: CRD42022304305). The review is reported in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. We carried out a systematic search of the literature from Medline, Embase, PubMed, Web of Science, the Cochrane Central Register of Controlled Trials (CENTRAL) for all literature published up to 8th October 2021. Searches were limited to English language. Relevant studies were identified for all reported studies of associations between COVID-19 and platelet indices reflecting platelet size using the terms: "covid" OR "coronavirus" OR "ncov" OR "sars" OR "sars-cov" AND "mean platelet volume" OR "platelet distribution width" OR "platelet large cell ratio". As an emerging research field a search of the preprint databases, MedRixv and BioRixv, was also conducted for all literature published from 1st January 2020 to 8th October 2021' using phrase terms for "mean platelet volume," "platelet distribution width," and "platelet large cell ratio." The China Knowledge Resource Integrated (CNKI) database was searched for literature up to 18th October 2021, using the search terms "血小板(platelet)" AND "COVID-19". Hand searching was also performed in the reference lists of relevant articles to identify additional eligible studies. See Supplementary material 1 for details of the search strategy.

Inclusion/exclusion criteria

Studies were included in this review if they met the criteria as follows:

Inclusion criteria: (1) Adult patients with laboratory-confirmed COVID-19; (2) PVI biomarker (i.e., MPV, PDW and/or P-LCR); (3) investigation of an association between a PVI and disease severity and/or mortality in COVID-19;

(4) blood tests performed at baseline for prognosis of severe disease or mortality [can be reported as 0–3 days of hospital or intensive care unit (ICU) admission or first test taken]; (5) we included only studies reporting PVI as continuous measures collected at baseline, comprising means or medians with measures of precision, e.g., standard deviation, standard error, confidence intervals or interquartile range (IQR); (6) original (experimental) research including randomized controlled trials, case-control studies, cohort studies, cross-sectional, case reports and series of cases; (7) articles in English or Chinese language.

Exclusion criteria: (1) under 18-year-olds (2) reviews, meta-analyses, conference abstracts, editorials, guidelines, commentaries, protocols (3) animal-based experiments; (4) *in vitro* studies; (5) unrelated studies; (6) studies focused on specific patient populations, e.g., diabetic or cancer patients; (7) no details of time of blood test; (8) no blood test taken at hospital or ICU admission; (9) articles with incomplete PVI data.

Study selection

All records identified by the database search were screened by title and abstract. Chinese literature was translated into English by the Chinese speaking reviewer, HW. A random sample of 20% of the title/abstracts were screened from the literature and discussed between two authors (SD and HW), and the remaining abstracts were screened independently by SD. Studies considered relevant were evaluated in full text according to the prespecified inclusion and exclusion criteria. A random sample of 20% of the full text articles were screened and discussed between two authors (SD and HW), and the remaining full text articles were screened by SD.

Data extraction

One reviewer (SD) extracted data from each study and compiled summary tables. A second reviewer (HW) randomly selected about 50% of the data extraction to check the accuracy. Any discrepancies identified were discussed and resolved between SD and HW and reflected in the remaining 50%. For all included studies, the following data was extracted: lead author, publication year, country, study design, study population (including age and % females), sample number, severity definition, day/time of blood test, subject exclusion if anti-platelet medication taken $\leq$ 10 days prior to test (yes or no), baseline PVI measurements and measures of effect.

Quality assessment

All included articles were quality assessed using the National Institutes of Health's Quality Assessment Tools for Observational Cohort and Cross-Sectional Studies (17). The quality assessment was independently conducted by one reviewer (SD). A second reviewer (HW) randomly selected about 50% of the data extraction to check the accuracy. Any discrepancies identified were discussed and resolved between SD and HW and reflected in the remaining 50%. Each study was rated as poor, fair or good based on the details that were reported and consideration of the concepts for minimizing bias.

Data synthesis and analysis

Given the heterogeneity of the clinical outcomes and PVI measurement, and the poor-fair quality of most studies (see section Results), we chose not to conduct a meta-analysis, and instead the study results are presented as a descriptive narrative analysis. Detailed evidence tables were created, and studies summarized by the reviewers. The technical limitations of the included studies are discussed.

Results

Study characteristics

We identified a total of 236 records from the OVID (Medline, Embase, and CENTRAL) search, 132 records from the Web of Science database, 133 records from PubMed, 196 records from the MedRxiv database, 107 from CNKI and five records from references searches. Of these, 329 were duplicates. Four-hundred and twenty-five records were title and abstract screened, and 96 were taken to full-text review. Sixty-four studies were excluded at the full-text review stage and 32 studies were included in the systematic review (Figure 1).

The baseline characteristics of the included studies are presented in Table 1. Fourteen studies were retrospective cohort, and five were prospective cohort studies. Six were cross-sectional and seven were longitudinal, observational studies. The largest number of studies were from Turkey ($n = 10$), followed by China ($n = 8$), and most were single-center studies ($n = 27$). Wu et al. (18) was an international multi-center study, but we extracted data for patients recruited from one center. Two studies were identified from preprint databases (19, 20) and three were published as letters (21–23). The disease outcomes for 21 studies were severity of COVID-19, and 12 studies assessed mortality. Two studies provided a comparison of patient groups combining severity and mortality (21, 24). Many studies classified disease severity as patients who were admitted to an ICU or presented with at least one of the clinical manifestations listed in national

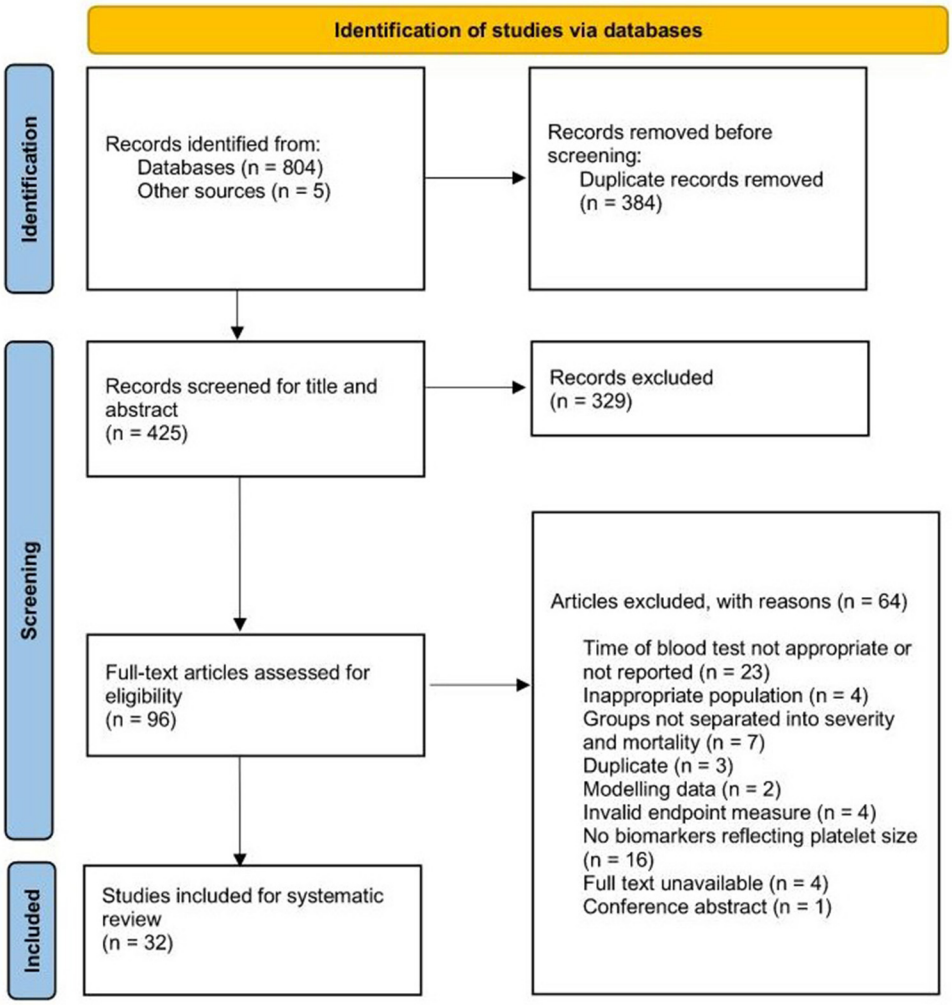

FIGURE 1
PRISMA diagram of search strategy.

guidelines for severe or critical diagnosis of COVID-19 (19, 24–36). Only three studies reported subjects with similar ages between groups ($p > 0.05$) (30, 37, 38), and seven studies reported significantly different male:female ratios ($p < 0.05$) (18, 22, 37, 39–42). Only two excluded patients on antiplatelet drugs > 10 days prior to the blood test (31, 34) and another study adjusted for antiplatelet therapy in the data analysis (21). Values for platelet indices reported at hospital or ICU admission are listed in Tables 2, 3.

Although there was a trend toward lower PLT in the severe and non-survivor groups for most studies, it was reported to be significantly lower in five studies of severe patients vs. mild disease (18, 27, 31, 40, 42) and four studies of non-survivors vs. survivors (20, 22, 24, 39). A longitudinal study reported a higher PLT in non-survivors compared to survivors at the early stages of the disease, while the opposite was evident at the end of the follow-up (46). Only one study reported a mean PLT for severe COVID-19 patients that was within the mild thrombocytopenia range (100–150 $\times$ 10^9) (18). In non-survivors, Rolla et al. (22) reported mild thrombocytopenia and Kilercik et al. (24) reported moderate thrombocytopenia (50–99 $\times$ 10^9).

Quality assessment

Twelve studies were rated poor, 16 were rated fair and four were good (Supplementary material 2). Most studies clearly specified the location, study time period and demographics of the selected participants. Nonetheless, there are several reasons for rating the studies based on potential bias. Most studies were retrospective in design. Consequently, most researchers had no control over the exposure assessment, therefore many

TABLE 1 Study characteristics.

Study					Clinical outcome		Non-severe/survivor			Severe/non-survivor			Comparison of the two groups (*P*-value)		Quality rating
References	year	Country	Study design	Time of blood test	Non-severe/ survivor	Severe disease/ non-survivor	No.	Age	% female	No.	Age	% female	Age	% female	
Ak et al. (25)	2021	Turkey	Retro., cohort, single center study	Admission	Non-severe according to findings evaluated during hospitalization	Severe survivors. Severe disease defined according to the national COVID-19 guidelines	380	61.15 ± 16.5	46.8	73	64.82 ± 12.1	41.1	<0.001*	0.498*	Fair
					Severe survivors (defined as above)	Non-survivors (from the severe survivors group)	73	64.82 ± 12.1	41.1	22	49.52 ± 14.9	36.4	<0.001*	0.498*	
Al-Nimer et al. (26)	2021	Iraq	Retro., cross., single center study	Admission	Recovered from illness and discharged	Non-survivors from the ICU	45	NR	NR	64	NR	NR	NR	NR	Fair
Alnor et al. (27)	2021	Denmark	Retro., cohort, dual center study	Admission	Non-severe	ICU admission and/or death	58	NR	NR	16	NR	NR	NR	NR	Fair
Asan et al. (28)	2021	Turkey	Retro., cohort, single center study	<24 h of admission	Non-ICU admission	ICU admission according to listed criteria	668	41.0 [5.7]	52.7	27	69.0 [21.0]	44.4	<0.001	NR	Fair
Barrett et al. (21)	2020	USA	Pros., cohort, single center study (letter)	<24 h of admission	Hospitalized patients without thrombotic event or death	Hospitalized patients who had a thrombotic event or died	68	63.5 [48.5–73.0]	39.7	32	69.5 [63.0–80.0]	37.5	0.002	1.0	Poor
Barrett et al. (29)	2021	USA	Retro., long., single center study	0–28 days	Without critical illness	Critical illness defined as requiring mechanical ventilation or transfer to the ICU	NR~	NR	NR	NR~	NR	NR	NR	NR	Good
					Discharged	Death	NR	NR	NR	NR	NR	NR	NR	NR	
Bauer et al. (30)	2021	Germany	Pros., cohort, single center study	Admission	Non-ICU treatment	ICU admission	10	63.7 [52.5–71.0]	60	7	71.9 [57.5–76.8]	71	0.27	1.0	Fair
Comer et al. (31)	2021	Ireland	Retro., cohort, single center study	Day 0 and day 7 of hospital admission or transfer to ICU	Non-ICU admission	ICU admission	20	69.25 ± 17.7	35	34	59.4 ± 10.5	38	NR	NR	Poor

(Continued)

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TABLE 1 (Continued)

Study					Clinical outcome		Non-severe/survivor			Severe/non-survivor			Comparison of the two groups (*P*-value)		Quality rating
References	year	Country	Study design	Time of blood test	Non-severe/ survivor	Severe disease/ non-survivor	No.	Age	% female	No.	Age	% female	Age	% female	
de la Rica et al. (38)	2020	Spain	Retro., cohort, single center study	<24 h of admission	Non-ICU according to Son Latzer University Hospital for COVID-19 management	ICU according to Son Latzer University Hospital for COVID-19 management	27	66.30 ± 14.90	33	21	65.57 ± 12.87	33	0.856	1	Fair
Ding et al. (43)	2020	China	Retro., cohort, single center study	Admission	Non-severe defined according to the coronavirus pneumonia diagnosis and treatment program, and the criteria of clinical classification	Severe defined according to the coronavirus pneumonia diagnosis and treatment program, and the criteria of clinical classification	57	46 [35–60]	57.9	15	67 [55–76]	40	NR	NR	Poor
Dogan et al. (32)	2021	Turkey	Retro., cohort,single center study	Admission	Non-ICU defined according to the Turkish Ministry of Health COVID-19 guidelines	ICU defined according to the Turkish Ministry of Health COVID-19 guidelines	131	NR	NR	20	NR	NR	NR	NR	Fair
Eraybar et al. (41)	2021	Turkey	Retro., cohort, single center study	First admission	Survivor	Non-survivor defined as 28-day mortality	905	NR	53.7	33	NR	36.4	NR	0.05	Poor
Giusti et al. (23)	2020	Italy	Retro., cohort, single center study (letter)	Admission	Discharged	Non-discharged	117	59.9 ± 14.0	36.8	92	73.6 ± 11.6	35.9	< 0.001	0.895	Poor
					Survivor	Non-survivor	178	63.5 ± 14.0	37.1	31	63.5 ± 14.0	32.3	< 0.001	0.607	
Guclu et al. (44)	2020	Turkey	Retro.,cohort, single center study	Day of admission	Patients with room air oxygen saturation ≥90%	Patients with room air oxygen saturation < 90%	81	56.52 ± 15.95	45.7	134	69.04 ± 14.26	43.3	< 0.001	0.732	Fair
					Survivor	Non-survivor	159	61.15 ± 16	45.3	56	73.34 ± 12.58	41.1	< 0.001	0.697	

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10.3389/fcvm.2022.1031092

TABLE 1 (Continued)

Study					Clinical outcome		Non-severe/survivor			Severe/non-survivor			Comparison of the two groups (*P*-value)		Quality rating
References	Year	Country	Study design	Time of blood test	Non-severe/ survivor	Severe disease/ non-survivor	No.	Age	% female	No.	Age	% female	Age	% female	
Higuera-De-La-Tijera et al. (33)	2021	Mexico	Pros., cohort, single center study	Admission	Patients with COVID-19 pneumonia without severity criteria for ICU admission (evaluated by intensive care medical staff)	ICU admission (evaluated by intensive care medical staff)	139	49.1 ± 12.8	32.4	27	58.6 ± 12.7	25.9	0.001	0.51	Fair
Incir et al. (34)	2021	Turkey	Pros., cohort, single center study	First day of admission	Non-severe defined as patients not requiring oxygen support	Severe defined as the mechanical ventilation requirement, admission to the intensive care unit (ICU), and death	110	45 [18–89]$^{\Psi}$	50	44	63 [20–91] $^{\Psi}$	17	0.003	0.607	Good
Jamshidi et al. (20)	2021	Iran	Retro., cohort,multi-center study	1–3 days of ICU admission	Survivor	Non-survivor	105	58.0 [47.0–73.0]	46.7	158	72.5 [64.0–80.75]	53.3	<0.001	NR	Poor
Karaasla-n et al. (45)	2021	Turkey	Retro., cohort, multi center study	Admission	Survivor	Non-survivor	182	50.6 ± 15.4	50	24	69.7 ± 16.0	50	<0.001	NR	Poor
Kilercik et al. (24)	2021	Turkey	Retro., long., single center study	0–30 days	Non-critical and critical survivors. Followed the COVID-19 directory of the Ministry of Health of the Republic of Turkey for listed criteria.	Critical non-survivors. Followed the COVID-19 directory of the Ministry of Health of the Republic of Turkey for listed criteria.	82	49.2 ± 15.1 and 58.6 ± 16.4	37.8	15	69.2 ± 10.6	20	<0.001*	NR	Good

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TABLE 1 (Continued)

Study					Clinical outcome		Non-severe/survivor			Severe/non-survivor			Comparison of the two groups (*P*-value)		Quality rating
References	**Year**	**Country**	**Study design**	**Time of blood test**	**Non-severe/ survivor**	**Severe disease/ non-survivor**	**No.**	**Age**	**% female**	**No.**	**Age**	**% female**	**Age**	**% female**	
Ko et al. (39)	2020	China	Retro., cross., multi center study	<24 h of admission	Survivor	Non-survivor	195	50.39 ± 15.00	52.8	212	68.90 ± 11.93	38.3	<0.001[$]	<0.001[#]	Poor
Lanini et al. (46)	2020	Italy	Pros., long., single center study	0–21 days	Survivor. Patients who recovered and were discharged from hospital or who were still hospitalized within 30 days after symptoms onset	Non-survivor. Died within 30 days after onset of symptoms.	338	<60 yrs 95.73% >60 yrs 84.19%	87.7	41	<60 yrs 4.27% >60 yrs 15.81%	12.3	NR	NR	Fair
Mao et al. (35)	2021	China	Retro., long., single center study	0–>25 days	Moderate disease according to the Chinese management guidelines for COVID-19 (7th edition) released by the NHCC	Severe or critically ill according to the Chinese management guidelines for COVID-19 (7th edition) released by the NHCC	82	62 (53–68)	45	45	68 (61–73)	23	0.003	0.684	Fair
Mertoglu et al. (47)	2021	Turkey	Retro., long., single center study	Day of admission	Non-ICU admission	ICU admission	532	47.5 [32–64.75]	42.5	23	59.0 [41.0–75.0]	43.5	0.011	0.925	Fair
Ouyang et al. (48)	2020	China	Retro., long., single center study	First test after incharge	Survivor COVID-19 patients discharged, transferred for further treatment or due to the presence of improved symptoms or the closure of this emergency hospital	Non-survivor. COVID-19 patients who died by 18 March 2020	82	55.7[ϕ]	NR	25	63.5[ϕ]	NR	0.018	NR	Fair
Rolla et al. (22)	2021	Italy	Pros., cohort, single center study (letter)	Admission	Survivor	Non-survivor	152	62 [51–73]	43	31	80 [74–85]	19	<0.001	0.022	Poor

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10.3389/fcvm.2022.1031092

TABLE 1 (Continued)

Study					Clinical outcome		Non-severe/survivor			Severe/non-survivor			Comparison of the two groups (*P*-value)		Quality rating
References	Year	Country	Study design	Time of blood test	Non-severe/ survivor	Severe disease/ non-survivor	No.	Age	% female	No.	Age	% female	Age	% female	
San et al. (49)	2021	Turkey	Cross.; single center study	Admission	Non-severe based on clinical manifestation	Severe based on a slightly modified interim guidance of the World Health Organization was used.	344	42 ± 23	44.2	44	67.5 ± 13.75	38.6	<0.0001	0.59	Fair
Song et al. (19)	2020	China	Retro., cross., single center study	First inhospital results	Mild and moderate disease based on the New Coronavirus Pneumonia Prevention and Control Program (6th edition) by NHCC	Severe and critical disease based on the New Coronavirus Pneumonia Prevention and Control Program (6th edition) by NHCC	31	48.0 [37.0–59.0]	48.4	42	55.5 [48.0–64.3]	28.6	0.039	0.083	Poor
Tsui et al. (40)	2020	China	Retro., cross., multi center study	At admission and day 5	Satisfactory: progressing well and likely to be discharged soon; stable: with mild ILI symptoms;	Critical: require intubation, or ECMO or in shock; (2) serious: require oxygen supplement of 3 L or more per minute	502 and 485	35.6 ± 16.8 and 37.6 ± 17.5	41.4 and 52.2	50	60.6 ± 14.0	36	<0.0001*	0.0011*	Poor
Waris et al. (36)	2021	Pakistan	Retro., cross., single center study	2nd day of admission	Mild and moderate disease according to the NHCC guidelines	Severe and critical disease according to the NHCC guidelines	76	43.24 and 49.1	30.2	25	56.6 and 62.1	32	NR	NR	Fair
Wu et al. (18)	2020	China	Retro., cohort, single center study	Obtained from medical records when the patients were admitted for the first time	If none of the "severe disease" criteria were met during the whole hospitalization process	Severe disease if one of the listed criteria were met, including respiratory distress or failure, ICU admission or death	217	42.0 [33.0–59.0]	58.5	82	62.0 [53.0–71.8]	42.7	<0.001	0.019	Poor

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10.3389/fcvm.2022.1031092

TABLE 1 (Continued)

References	Year	Country	Study design	Time of blood test	Clinical outcome		Non-severe/survivor			Severe/non-survivor			Comparison of the two groups (*P*-value)		Quality rating
					Non-severe/survivor	Severe disease/non-survivor	No.	Age	% female	No.	Age	% female	Age	% female	
Xiong et al. (42)	2020	China	Retro., long., single center study	Day 1–14	Mild/non-severe. Clinical classification at admission based on APACHE II, SOFA and CURB-65 criteria	Severe and critical. Clinical classification at admission based on APACHE II, SOFA and CURB-65 criteria	355	47 [34–61] and	65	166 and 139	62 [52–70] And 67 [54–76]	53 and 33	<0.001*	<0.001*	Fair
Zhong and Peng (37)	2021	China	Retro., cohort, dual center study	Admission	MPR < 7.44	MPR > 7.44 Severe pneumonia of COVID-19 defined by clinical manifestation	59	41.0 [34.0–58.0]	57.6	26	51.0 [35.5–68.3]	26.9	0.064	0.009	Good

$ calculated from the raw data using a two-tailed Welch's unpaired test. # calculated from the raw data using a two-tailed Fisher's exact test. *Compares three patient groups. Data presented as mean ± standard deviation, median [range] (marked with a $^{\Psi}$), average (marked with a ϕ) or median [interquartile range]. ~ Study reported a total cohort of 3,915 COVID-19 patients. APACHE, Acute Physiology and Chronic Health Evaluation; Cross., Cross-sectional study; ECMO, extracorporeal membrane oxygenation; ICU, Intensive care unit; ILI, influenza-like illness; long., longitudinal; MPR, Mean platelet volume/platelet count ratio; NHCC, National Health Commission of China; NR, not reported; Pros., prospective; Retro., Retrospective; SOFA, Sequential Organ Failure Assessment.

did not fully describe the methods used to measure the platelet parameters. Fourteen studies provided the number of eligible patients and the total number included in the study (25, 27, 28, 33–35, 37–42, 45, 46), of which three had a participation rate < 50% (33, 41, 45). Possible bias related to participation rate could not be ascertained in the remaining 18 studies. Moreover, only two studies (26, 33) included a justification for sample size, hence, most authors were unable to make a valid inference about the population being studied.

Pre-analytical and analytical variables, such as the anticoagulant used, the time between blood collection, storage temperature and instrument type are known to significantly affect MPV measurements (50). For example, platelets collected into ethylenediaminetetraacetic acid (EDTA) anticoagulant undergo time-dependent platelet swelling and activation. Only eight (25%) of included studies reported the anticoagulant used (EDTA for all of them) (21, 24, 26, 27, 30, 34, 36, 37) and four (13%) reported the time to analysis (24, 28, 34, 37). A larger proportion of studies (41%) reported the analyzer used to measure PVI (21, 22, 24, 27–30, 32, 34, 36, 37, 43, 47), with the Sysmex XN series reported most frequently.

PVI may be influenced by various demographic factors including age, although no conclusive data are available on this topic (51). Of the 32 included studies, 12 considered age as a confounding variable and adjusted statistically for its impact on the relationship between PVI and disease severity or mortality (21, 23, 24, 28, 29, 33, 37, 38, 42, 44, 46, 47).

PVI as biomarkers of severity in COVID-19 patients

Baseline PVI measurements

Nineteen studies compared the baseline levels of MPV in COVID-19 patients at hospital admission (18, 21, 23, 25, 27, 28, 30–36, 38, 40, 42–44, 47, 49), of which nine reported a significantly higher MPV in the severe COVID-19 patients compared with non-severe patients (18, 21, 23, 31, 32, 34, 42, 43, 49). Of nine studies that assessed PDW in COVID-19 patients (18, 19, 27, 34–36, 38, 42, 44), one study reported a significantly higher PDW in the severe group compared to the non-severe group at admission (34). Of seven studies assessing P-LCR in COVID-19 patients (18, 27, 34–36, 42, 47), three reported significantly higher P-LCR in the severe patient group at admission (34, 42, 47).

Two studies measured PVI on different days of hospital admission. A retrospective cohort study (44) reported a non-significant mean MPV at day 0 in COVID-19 patients with room air oxygen saturation < 90%, but significantly higher MPV at day 3, though this significance was marginal ($p = 0.043$). No significantly different PDW was observed for both days. Comer et al. (31) observed higher median MPV in ICU patients

10.3389/fcvm.2022.1031092

TABLE 2 Values of platelet indices in the COVID-19 patient severe and non-severe groups at admission.

First author	Year	Platelet indices	Non-severe patients	Severe patients	Comparison of the two groups (*p*-value)
Ak et al. (25)	2021	PLT	203 ± 77.52	179 ± 81.46	NR
		MPV	9.6 ± 1.08	10 ± 1.16	NR
Alnor et al. (27)	2021	PLT	188 [161–271]	174 [127–203]	**0.037**
		MPV	10.5 [9.6–10.9]	10.9 [10.1–11.8]	0.126
		PDW	12.1 [10.5–13.2]	13.0 [11.6–14.5]	0.097
		P-LCR	28.5 [22.9–32.7]	31.1 [25.9–39.4]	0.093
Asan et al. (28)	2021	PLT	221 ± 60	198 ± 61	0.081
		MPV	9.9 ± 4.2	10.1 ± 1.6	0.241
		MPR	0.044 ± 0.018	0.053 ± 0.028	**0.049**
Bauer et al. (30)	2021	PLT	187.0 [161.0–233.0]	208.0 [167.0–233.0]	1.0
		MPV	10.2 [9.9–10.6]	10.5 [10.1–10.6]	0.49
Comer et al. (31)	2021	PLT	242 [105–488] [ω]	213 [40–550] [ω]	0.6
		MPV	9.8 [8.7–13] [ω]	10.8 [9–13] [ω]	**0.015**
de la Rica et al. (38)	2020	PLT	228.02 ± 108.82	209.56 ± 77.97	0.678
		MPV	7.92 ± 1.25	8.18 ± 1.19	0.596
		PDW	16.81 ± 1.24	16.99 ± 0.97	0.333
Ding et al. (43)	2020	PLT	180 [149–227]	160 [134–216]	0.515
		MPV	9.1 [7.1–10.0]	9.7 [9.2–11.6]	**0.017**
Dogan et al. (32)	2021	PLT	234 ± 74	235 ± 94	0.933
		MPV	10.30 [9.70–10.90]	11.05 [10.35–11.75]	**0.0042**
Giusti et al. (23)	2020	PLT	191 [156–235]	183 [141–217]	0.088
		MPV	10.4 [9.9–11.1]	10.9 [10.5–11.9]	**0.001**
Guclu et al. (44)	2020	PLT	187.4 ± 59.82	208.63 ± 135.72	0.573
		MPV	9.18 ± 1.24	9.61 ± 1.76	0.129
		PDW	17.37 ± 2.32	17.72 ±2.52	0.142
Higuera-De-La-Tijera et al. (33)	2021	PLT	226.4 ± 86.2	219.7 ± 73.1	0.77
		MPV	8.4 ± 0.9	8.9 ± 0.9	0.11
Incir et al. (34)	2021	PLT	244 [155–460] [ω]	196 [150–636] [ω]	0.065
		MPV	10.8 ± 1.10	12.1 ± 1.22	**<0.001**
		PDW	12.4 [7.50–24.5] [ω]	15.6 [10.3–21.9] [ω]	**0.001**
		P-LCR	31.4 ± 8.84	40.5 ± 9.30	**0.001**
Mertoglu et al. (47)	2021	PLT	233.0 [193.0–278.0]	219.0 [176.0–312.0]	0.872
		MPV	10.1 [9.5–10.7]	10.8 [9.6–11.7]	0.052
		P-LCR	25.60 [21.0–30.61]	32.70 [22.8–38.0]	**0.033**
San et al. (49)	2021	PLT	210 ± 96	199.5 ± 127	0.95
		MPV	8.2 ± 1.2	8.6 ± 1.75	**0.003**
		MPR	0.04 ± 0.02	0.04 ± 0.03	0.453
		LMR	6.42 ± 4.01	8.84 ± 5.5	**<0.0001**
Song et al. (19)	2020	PLT	178.0 [127.0–239.0]	189.0 [154.0–231.0]	0.321
		PDW	12.8 [10.7–13.9]	12.3 [11.0–13.5]	0.643
Tsui et al. (40)	2020	PLT	Stable: 0.60 ± 0.19 Satisfactory: 0.58 ± 0.20	Critical: 0.54 ± 0.19	**0.0094** *
		MPV	Stable: 0.88 ± 0.12 Satisfactory: 0.87 ± 0.15	Critical: 0.88 ± 0.10	0.9139
Waris et al. (36)	2021	PLT	Mild: 217.03 [191.52–240.55]	Severe: 205.55 [120.38–290.72]	0.16*

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10.3389/fcvm.2022.1031092

TABLE 2 (Continued)

First author	Year	Platelet indices	Non-severe patients	Severe patients	Comparison of the two groups (*p*-value)
			Moderate: 223.73 [185.93–261.54]	Critical: 165.06 [123.44–206.67]	
		MPV	Mild: 9.02 [8.60–9.43]	Severe: 9.34 [8.62–10.6]	0.31*
			Moderate: 12.60 [5.93–19.26]	Critical: 9.74 [9.15–10.33]	
		PDW	Mild: 12.18 [11.81–12.56]	Severe: 12.53 [11.31–13.75]	0.31*
			Moderate: 13.70 [10.92–16.48]	Critical: 12.21 [12.05–14.37]	
		P-LCR	Mild: 23.08 [21.28–24.87]	Severe: 23.52 [18.36–28.68]	0.39*
			Moderate: 25.40 [21.12–29.68]	Critical: 26.49 [22.29–30.69]	
Wu et al. (18)	2020	PLT	174.0 [140.8–214.5]	149.0 [116.5–188.8]	**<0.001**
		MPV	9.8 [9.2–10.6]	10.2 [9.4–10.09]	0.016
		PDW	12.9 [10.7–16.3]	12.4 [10.8–15.8]	0.371
		P-LCR	24.0 [19.7–30.1]	26.8 [21.1–32.3]	0.028
Xiong et al. (42)	2020	PLT	Mild and non-severe: 187.0 [153.0–235.0]	Severe: 193.0 [154.0–248.0] Critical: 122.0 [91.0–168.0]	**<0.001***
		MPV	Mild and non-severe: 9.70 [9.00–10.40]	Severe: 9.60 [8.93–10.30] Critical: 10.15 [9.30–11.0]	**<0.001***
		PDW	Mild and non-severe: 15.70 [10.80–16.33]	Severe: 15.90 [10.62–16.50] Critical: 13.40 [10.70–16.40]	0.9*
		P-LCR	Mild and non-severe: 23.30 [18.93–29.20]	Severe: 22.95 [18.22–28.78] Critical: 26.20 [21.08–34.30]	**<0.001***

Data presented as mean ± standard deviation, median [min-max range] (marked with a ω) or median [interquartile range]. Units for platelet indices are PLT: 10^9/L; MPV: fL; PDW: %; P = LCR: %. LMR, lymphocyte/mean platelet volume ratio; MPR, mean platelet volume/platelet count ratio; MPV, mean platelet volume; NS, not significant; P-LCR, platelet-large cell ratio; PLT, platelet count; PDW, platelet distribution width. *Comparison of three or more patient groups. Significant *p*-values (≤0.05) in bold.

compared with non-ICU patients at day 7 vs. day 0 ($p = 0.0014$ and $p = 0.015$, respectively). It should be noted, however, that this difference was due to the MPV decreasing in the non-ICU patients at day 7, rather than the MPV increasing in the ICU patients.

Longitudinal analysis

Three retrospective longitudinal studies evaluating PVI in non-severe and severe COVID-19 patients reported conflicting results. A Chinese study of non-severe/mild, moderate and critical patients (42), identified significantly higher median MPV and P-LCR in the more severe groups ($p \leq 0.001$) from day 1 to day 14 of hospitalization. PDW significantly increased from day 3 onwards. In contrast, a Turkish study (47) observed higher median MPV and P-LCR in ICU patients compared to non-ICU patients except > 7–8 days. Severe or critically ill COVID-19 patients without existing hematologic disease, were reported to have significantly higher mean MPV and P-LCR at ≥25 days after hospitalization ($p \leq 0.01$) compared to moderate patients, with no significant difference prior to this (35). However, they reported no significant difference

10.3389/fcvm.2022.1031092

TABLE 3 Values of platelet indices in the COVID-19 patient survivor and non-survivor groups at admission.

First author	Year	Platelet indices	Survivors	Non-survivors	Comparison of the two groups (*p*-value)
Ak et al. (25)	2021	PLT	179 ± 81.46	191.5 ± 100.3	NR
		MPV	10 ± 1.16	9.4 ± 1.64	NR
Al-Nimer et al. (26)	2021	MPV	8.9 ± 0.1	9.1 ± 0.1	0.310
		PDW	41.6 ± 1.2	41.4 ± 1.2	0.921
Barrett et al. (21)$^{\omega}$	2020	PLT	205.0 [164.8–253.8]	187.5 [147.5–257.5]	0.385
		MPV	10.55 [10.1–11.2]	11.00 [10.5–11.9]	**0.022**
Eraybar et al. (41)	2021	MPV	9.60 [9.00–10.30]	9.70 [9.20–11.10]	>0.05
		LMR	0.21 [0.15–0.29]	0.12 [0.07–0.20]	**<0.001**
Giusti et al. (23)	2020	PLT	189 [149–233]	174 [111–204]	0.127
		MPV	10.5 [10.0–11.3]	11.4 [10.6–12.4]	**<0.001**
Guclu et al. (44)	2020	PLT	207.69 ± 123.06	180.59 ± 78.14	0.094
		MPV	9.34 ± 1.37	9.77 ± 2.11	0.189
		PDW	17.44 ± 2.35	18.02 ±2.69	**0.040**
Jamshidi et al. (20)	2021	PLT	196.0 [151.5–260.0]	179.0 [125.0–255.0]	**0.04**
		MPV	9.7 [9.175–10.5]	10.0 [9.3–10.7]	0.3
		PDW	12.8 [11.5–14.0]	13.2 [11.4–14.7]	0.32
		P-LCR	24.4 [19.85–29.3]	26.7 [21.05–30.825]	0.07
Karaaslan et al. (45)	2021	PLT	199.93 ± 79.6	199.42 ± 124.9	0.97
		MPV	9.60 ± 1.4	9.72 ± 1.0	0.57
Ko et al. (39)	2020	PLT	212.48 ± 82.00	159.45 ± 86.54	**<0.0001***
		MPV	10.63 ± 0.87	11.16 ± 0.94	**<0.0001***
		PDW	12.29 ± 2.02	13.59 ± 2.64	**<0.0001***
		P-LCR	29.60 ± 7.08	33.88 ± 7.42	**<0.0001***
Lanini et al. (46)	2020	PLT	n/a	n/a	**0.019$^{\%}$**
		MPV	n/a	n/a	**0.001$^{\%}$**
Ouyang et al. (48)	2020	PLT	214.66 ± 91.61	178.77 ± 93.70	0.1
		MPV	9.23 ± 1.05	10.01 ± 1.15	**0.003**
		PDW	16.18 ± 0.42	16.63 ± 0.49	**<0.001**
		P-LCR	21.60 ± 7.00	26.75 ± 7.69	**0.003**
Rolla et al. (22)	2021	PLT	186.50 [152.25–226.50]	148.00 [125.00–197.00]	**0.005**
		MPV	10.50 [9.90–11.00]	11.40 [10.70–12.10]	**<0.001**
		P-LCR	28.5 [23.35–33.20]	35.90 [31.20–42.40]	**<0.001**

Data presented as mean ± standard deviation or median [interquartile range]. Units for platelet indices are PLT: 10^9/L; MPV: fL; PDW: %; *P* = LCR: %. LMR, Lymphocyte/mean platelet volume ratio; MPV, mean platelet volume; P-LCR, platelet-large cell ratio; PLT, platelet count; PDW, platelet distribution width. *Author calculated from raw data using Welch's unpaired *t*-test for continuous data. $^{\omega}$Study outcomes were death and/or thrombosis event. Significant *p*-values (≤0.05) in bold. $^{\%}$Temporal difference between the patient groups.

between patient groups for PDW for the entire course of the disease.

Measures of association

After adjustment for variables including age, comorbidities and prior medication, Barrett et al. (29) reported that patients with the highest MPV tertile at baseline had higher odds of requiring mechanical ventilation or transfer to the ICU (OR 1.5, 95% CI 1.3–1.8). Importantly, they show that this association remains significant in patients without thrombocytopenia, demonstrating that the high MPV was independent of low PLT. A prospective cohort study of 14 patients who experienced a thrombotic event, reported that MPV was not associated with thrombosis after adjustment for multiple variables including age, sex, anti-platelet therapy and PLT (21). Multivariate logistic regression to assess the effect of age and gender, found that P-LCR was not significant in determining admission to the ICU (47). Likewise, univariant logistic regression identified no association with MPV and ICU requirement in COVID-19 patients (32).

Other PVI

Zhong and Peng (37) investigated the relationship between mean platelet volume/platelet count ratio (MPR) and the

prognosis of COVID-19. They found that high baseline MPR (>7.44 fL) was significantly associated with severe pneumonia in COVID-19 patients. Contrary to this, MPR was not found to be a significant hematological marker for severity in a cross-sectional study (49) and retrospective cohort study (28). San et al. reported a significantly higher median lymphocyte/mean platelet volume ratio (LMR) in severe patients ($p \leq 0.0001$) (49), though multivariate logistic regression revealed that it was not an independent risk factor for severe disease.

PVI as biomarkers of mortality in COVID-19 patients

Baseline PVI measurements

Eleven studies assessed baseline MPV in COVID-19 patients who survived and died (20–23, 25, 26, 39, 41, 44, 45, 48). Of which, six studies reported a significantly higher MPV in non-survivors at hospital admission (21–23, 39, 44, 48). Five studies assessed baseline PDW in COVID-19 mortality (20, 26, 39, 44, 48), and three reported higher PDW in non-survivors at admission (39, 44, 48). P-LCR at hospital admission was assessed in only four included studies (20, 22, 39, 48), and three reported significantly higher P-LCR in non-survivors (22, 39, 48).

Guclu et al. (44) reported that deceased COVID-19 patients had non-significant MPV when compared to survivors on the first day of hospital admission ($p = 0.005$), but significantly higher MPV on the third day. Conversely, they found significantly higher PDW in the non-survivor group at both admission and the third day (day 0: $p = 0.040$; day 3: $p = 0.006$).

Longitudinal analysis

Varying trends were observed for the longitudinal studies. A retrospective study of 98 patients identified a significantly higher MPV, PDW and P-LCR in non-survivors for the first test of the hospital stay (48). MPV and P-LCR increased over the course of the disease, with non-survivors showing an average MPV that was above the normal reference range for the last test. In contrast, the temporal trends analysis appears to show PDW decreasing in the survivors over time.

A comprehensive study of 379 COVID-19 patients over 21 days reported patterns of temporal variations for MPV (46). Tests showed significantly higher MPV in survivors compared with non-survivors at the beginning of the disease ($p < 0.001$). While the opposite was observed at the end of the follow-up, with MPV significantly higher in non-survivors than in survivors ($p < 0.001$). Over time, MPV tended to normalize in survivors and steadily increase in non-survivors, exceeding the upper normal limit value (11 fL) by the seventh day after symptoms' onset. Paradoxically, age was not significantly associated with higher MPV, which contrasted with other included studies.

Lastly, a retrospective study that observed 97 patients over 30 days of hospitalization (24), reported a significantly higher MPV, PDW and P-LCR in critical non-survivors compared with non-critical and critical survivors ($p = 0.014$, $p = 0.011$, and $p = 0.006$, respectively). As the disease progressed, all PVI gradually increased, with the differences among the groups remaining significant over time ($p < 0.05$). Moreover, trends analysis of MPV showed a distinctive divergence for all three groups after 10 days.

Measures of association

A prospective cohort study of 183 COVID-19 patients reported that MPV and P-LCR were shown not to be independent predictors of in-hospital mortality in multivariate analysis (22). In contrast, a prospective cohort study of 100 patients, reported MPV was significantly associated with all-cause mortality after adjustment for multiple variables such as age, sex, anti-platelet therapy and PLT (OR 2.33, 95% CI 1.27–4.67) (21). Guclu et al. showed that an increase of 1-unit MPV difference (between 1st and 3rd day), significantly increases the probability of death within 28 days (OR 1.762, 95% CI 1.272–2.440) (44).

Discussion

For patients with severe COVID-19 infection, early decision making is critical for successful clinical management to prevent the development of acute respiratory distress syndrome and possible death. To the best of our knowledge, this is the first comprehensive systematic review to evaluate the usefulness of PVI as early predictors for severity and mortality in COVID-19 patients based on the current literature.

The quality assessment demonstrated that currently most studies published in this field are poor, fair at best. As such, we decided that the premises of meta-analysis were not met, and that the data used to generate the composite outcome would not be reliable. For this reason, we chose to perform a narrative analysis, summarizing the study results and exploring the limitations of the current research and recommendations for future work in this field.

While our systematic review showed a general trend for PVI to be higher in severe patients or non-survivors, it is evident that there are differing baseline results for the individual studies with 14 studies having reported significantly higher PVI ($p \leq 0.05$). Furthermore, longitudinal analysis showed both increasing and decreasing trends during disease progression. PVI taken at the emergency room could be useful to guide decisions on immediate treatment. Additionally, monitoring PVI trends over time could be used to guide other aspects of therapy and to determine disease outcome. Nonetheless, we cannot markedly conclude that COVID-19 patients with elevated PVI are more

likely to develop severe illness or are at higher risk of dying due to the variation in results.

Interestingly, some studies reported non-significant levels of PDW in severe patients but significantly higher MPV or P-LCR, either at admission or during the course of the disease (35, 42, 44). Studies evaluating the effect of storage time on platelet volume due to platelet swelling, identified a decrease in PDW and an increase in MPV (52, 53), indicating that PDW is a better indicator of platelet activation than MPV, since it was not elevated during single platelet distention caused by platelet swelling. It is possible that platelet swelling has occurred in these studies, though we can only surmise this has occurred due to absence of details on time to analysis and anticoagulant used.

Studies reporting that significantly higher PVI in severe or non-surviving patients was present at admission, also demonstrated that the platelet parameters were not independent predictors of disease progression (22, 47, 49), with age being the variable most likely to predict ICU requirement and mortality (23, 28). Patients with COVID-19 who enter a critical condition or die are mostly elderly, male and have comorbidities, with hypertension, diabetes and coronary artery disease being the most common (1, 54). PVI have been shown to be influenced by age, sex, hypertension, diabetes and coronary heart disease in some studies (55–60). Few included studies adjusted for confounding variables when assessing the association of PVI with COVID-19 disease, even though many reported significantly different ages and sex proportions when comparing the groups. Future studies should be designed to measure PVI in COVID-19 cohorts matching for age and gender. Giusti et al. (23) proposed a model, in which age and three routine coagulation parameters, including MPV, are measured to predict prognosis of hospitalized patients. Conceivably PVI could be used alongside other clinical and/or laboratory parameters to predict prognosis of COVID-19 disease rather than acting as a stand-alone biomarker.

A published letter has reported a significant association between MPV and combined severe illness and mortality in COVID-19 patients using pooled analysis of 18 studies (61). Pre-analytical and analytical variables were not considered however, and the statistical heterogeneity was extremely high (91%). Our review has identified many caveats in the current research which likely accounts for this heterogeneity including differences in participant characteristics, hematology analyzer, timing of the blood test, and clinical endpoints. As such we infer that the lack of robust standardization, along with the retrospective design and low patient numbers, renders the current research on PVI in COVID-19 patients inconclusive.

There is an inverse relationship between MPV and PLT in healthy adults (62). Increased MPV denotes an increase in circulating young platelets as a response to thrombocytopenia, possibly because of platelet consumption due to micro-thrombotic events in small vessels (63). Research has demonstrated that COVID-19 patients with thrombocytopenia have a significantly higher MPV and P-LCR, compared with COVID-19 patients with retained PLT (15). The general trend reported in our included studies showed a reduction in PLT at hospital admission, combined with increased PVI. Other studies have reported that patients with severe COVID-19 disease have a PLT only 23×10^9/L to 31×10^9/L lower than those with non-severe disease (64, 65). Bearing in mind that severely ill patients with systemic immune and coagulation activation maintain reasonable PLT, a compensatory platelet production response by the bone marrow at the early stage of the disease is probable. Notwithstanding, Barrett et al. (29) demonstrated that high MPV was independent of low PLT in COVID-19 patients. Therapies used early in the COVID-19 pandemic that cause thrombocytopenia, such as hydroxychloroquine and azithromycin, are given to COVID-19 patients at onset of disease (66, 67), possibly contributing to lower PLT in the later stages of the disease. Prospective studies that adjust for treatment could enable researchers to evaluate if thrombocytopenia is caused by disease progression and/or therapies.

It is important to note that research has shown platelet heterogeneity to be present from formation, and size may not necessarily reflect platelet age or activity. Platelet characteristics, including RNA content, may be dynamic over time (68–70). Thus, it is possible that platelet size heterogeneity predates COVID-19 infection. It also could reflect an inflammatory state due to other infection or co-morbidities. Therefore, the direction of effect cannot be inferred from the retrospective studies we review here, and the nature of this association must be elucidated by further prospective cause–effect analysis.

Research has shown that the median time for COVID-19 disease deterioration is 11 days to developing severe illness and entering a critical stage of severe pneumonia and organ damage (71). This corresponds with trends analysis by Kilercik et al. (24) who showed a distinctive divergence in MPV after 10 days when comparing non-survivors and survivors. Therefore, we propose that future work should comprise appropriately powered, prospective studies that analyze PVI trends over time, with consideration for confounders such as age, sex, co-morbidities and therapies. Notably, the majority of the included studies are from cohorts early in the pandemic before specific therapy was available, such as steroids, antivirals and vaccination, and with earlier COVID variants. Thus, the role of PVI biomarkers from early in the pandemic requires re-evaluation in the context of these factors with COVID-19 pneumonia now less common.

Conclusion

While there is a trend toward higher PVI in severe COVID-19 patients and non-survivors, the contradictory findings of this systematic review suggest that further work is required to evaluate the potential usefulness of PVI for early prognosis of

COVID-19. Most importantly, the technical concerns need to be addressed to fully demonstrate its use in clinical practice. Although some studies reported significant associations between clinical outcome and PVI, a causal relationship could not be inferred. Future studies should be prospective in design so that researchers can assess multiple outcomes at different time frames. They should give comprehensive methodology which includes careful study design, controlled measurement of platelet parameters, full reporting of how the data were acquired, and appropriate statistical considerations for confounding factors.

Data availability statement

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

Author contributions

SD made substantial contributions to the design of the study, with support from HW, DD, and MT. SD and HW carried out the full search and screened the literature for inclusion and exclusion and performed the data extraction and quality assessment. SD performed the analysis and interpretation of the data and drafted the first version. Substantial revisions were provided by HW, DD, and MT. All authors read and approved the final 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1031092/full#supplementary-material

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Vaccine-induced immune thrombotic thrombocytopenia: Updates in pathobiology and diagnosis

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Coronavirus disease 2019 (COVID-19) is a viral respiratory infection caused by the severe acute respiratory syndrome virus (SARS-CoV-2). Vaccines that protect against SARS-CoV-2 infection have been widely employed to reduce the incidence of symptomatic and severe disease. However, adenovirus-based SARS-CoV-2 vaccines can cause a rare, thrombotic disorder termed vaccine-induced immune thrombotic thrombocytopenia (VITT). VITT often develops in the first 5 to 30 days following vaccination and is characterized by thrombocytopenia and thrombosis in unusual locations (e.g., cerebral venous sinus thrombosis). The diagnosis is confirmed by testing for anti-PF4 antibodies, as these antibodies are capable of platelet activation without any cofactor. It can be clinically challenging to differentiate VITT from a similar disorder called heparin-induced thrombocytopenia (HIT), since heparin is commonly used in hospitalized patients. VITT and HIT have similar pathobiology and clinical manifestations but important differences in testing including the need for PF4-enhanced functional assays and the poor reliability of rapid immunoassays for the detection of anti-platelet factor 4 (PF4) antibodies. In this review we summarize the epidemiology of VITT; highlight similarities and differences between HIT and VITT; and provide an update on the clinical diagnosis of VITT.

KEYWORDS

COVID-19, platelet, antigen-antibody complex, immune complex, thrombosis, thrombocytopenia, heparin, vaccine

Introduction

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a recently-described hematological disorder that emerged during the mass coronavirus disease 2019 (COVID-19) vaccination programs (1). The incidence of VITT ranges from 1 in 26,500 to 263,000 people after the administration of an adenoviral vector vaccine, with an overall incidence of ∼1 in 100,000 (2). VITT is similar to heparin-induced thrombocytopenia (HIT), which is a side effect of heparin exposure, that is also characterized by thrombocytopenia and thrombosis (3, 4). VITT often presents with a severe clinical illness, yet the diagnosis can be missed since it is rare and clinical manifestations are non-specific. Laboratory testing is essential for VITT diagnosis and

is based on similar concepts as HIT testing but with important differences. These differences include the type of screening immunoassay used and the need for specialized functional assays.

In this review we provide a pragmatic overview of VITT. We review the differences in clinical presentation between HIT and VITT; VITT pathophysiology; and unique aspects of laboratory investigations.

Clinical characteristics of VITT

VITT is characterized by new-onset thrombocytopenia and thrombosis following vaccination with an adenoviral COVID-19 vaccine (1, 3). Examples of these include the ChAdOx1 nCoV-19 [AstraZeneca-Oxford] and Ad21.COV2.S [Johnson & Johnson/Janssen] vaccines. VITT patients have a medium time to symptom onset of 15 days post-vaccination (ranging from 7 to 61 days) based on a Canadian cohort of 43 patients (5). The median age of this cohort was 52 years old (ranging from 29 to 73 years old) with no apparent sex-related differences (5). Patients present with thrombocytopenia and the majority have concurrent thrombosis (85.7%) (5). Rarely, patients can present without thrombocytopenia, as occurred in 2/35 patients in the Canadian cohort. Cerebral venous sinus thrombosis (CVST) has been reported as the most common site of thrombosis (36.8%); other locations include deep-vein thrombosis of the lower extremity (23.7%); pulmonary embolism (31.6%); and splanchnic vein thrombosis (19%) (1, 3, 5, 6). Appropriate imaging based on symptom presentation (e.g., MRI venogram for severe headache) should be completed to confirm thrombosis.

A complete blood count, D-dimer, coagulation tests [international normalized ratio (INR), partial thromboplastin time (PTT)], and liver enzymes should be ordered in suspected VITT patients. The median platelet count in VITT ranges from 25,000 to 47,000 based on several reports (3, 7, 8). D-dimer levels are often markedly elevated and may worsen with disease progression (9). If the D-dimer level is <2,000 fibrinogen equivalent units (or four times the upper limit of normal), VITT is unlikely and alternate diagnoses should be considered (10). Coagulation testing (INR, aPTT, fibrinogen) are used to monitor for consumptive coagulopathy that can occur with severe or late presentations. Elevated liver enzymes (aspartate transaminase, alanine transaminase, and alkaline phosphatase) or liver function abnormalities may suggest underlying splanchnic vein thrombosis. While these initial investigations can support the diagnosis, specialized testing is ultimately required for confirmation (see Diagnostic Testing below).

A recent report described eleven VITT patients who presented to hospital with no thrombosis on initial assessment. All eleven patients had thrombocytopenia and severe headache following adenoviral vaccination (4). They also had biochemical features suggestive of VITT (thrombocytopenia, elevated D-dimer) with high anti-PF4/heparin IgG levels confirming the diagnosis. Two patients had CVST and seven did not develop thrombosis. This highlights the fact that VITT may present with isolated thrombocytopenia and, rarely, thrombosis with platelet count levels that are in the normal range. Treatment should not be delayed if clinical suspicion is high (e.g., strongly positive anti-PF4 testing with a high optical density).

Pathobiology of VITT and HIT

A review of VITT pathobiology is essential to understand the clinical application of specialized screening and diagnostic tests. VITT is a prothrombotic, autoimmune disorder that is a result of pathologic anti-PF4 antibodies (11–13). These antibodies target the cationic protein, PF4, that is normally released from platelets during coagulation (14, 15). Anti-PF4 antibodies bind to PF4 on platelet surfaces to form immune complexes that subsequently activate platelets through the FcγRIIa receptor (16, 17). This results in significant release of intracellular molecules, including serotonin (see Serotonin Release Assay below), with resulting thrombocytopenia and thrombosis. It is postulated that monocytes and neutrophils are also activated, in a similar manner to HIT, but this has yet to be confirmed (18–20).

Similarly, HIT is mediated through anti-PF4 antibodies, but the antibody characteristics and mechanism of platelet activation are different than VITT. HIT occurs following heparin exposure and the immune complex is dependent on this hapten. Heparin, a negatively charged molecule, serves as a scaffold to bridge PF4 molecules. This facilitates anti-PF4/heparin antibody immune complex formation. Heparin binds to a unique site on the PF4 protein that has been well characterized through alanine scanning mutagenesis studies (21, 22). Anti-PF4 antibodies (VITT) bind directly to this heparin-binding site on PF4 and thus do not require heparin as a hapten. Indeed, heparin appears to inhibit VITT anti-PF4 antibody binding to PF4 *in vitro* since they both bind to the same region (22, 23). The trigger for anti-PF4 antibody production is unclear but several reports have speculated that the negatively charged surface of the adenovirus vector or other vaccine components bind PF4 to stimulate antibody production and activation, similar to heparin (13, 24). These distinct molecular differences, including heparin-dependence and anti-PF4 antibody specificity, have important implications for VITT testing.

Diagnostic testing

Screening tests-rapid immunoassays and enzyme immunoassays

Screening tests for VITT were initially adapted from HIT assays due to the similarities in pathobiology and

antibody specificity. The two broad categories of testing include enzyme immunoassays (EIAs) and rapid assay (25). These immunoassays rely on antibody recognition of PF4, either alone or in complex with a negatively charged scaffold such as heparin (PF4/heparin) or polyvinylsulfate (PVS, PF4/PVS) (26–29). Rapid assays, as the name implies, offer faster turnaround time compared to EIAs and do not require sample batching. For HIT, both the EIA and rapid assays have a high sensitivity (90–99%) but low specificity (∼50–70%) for "pathogenic" anti-PF4/heparin antibodies (25, 30–36). Reduced specificity is due to false positive results from patients with "non-pathogenic" anti-PF4/heparin antibodies. These "non-pathogenic" forms are unable to bind the appropriate site on PF4 required for immune complex formation and subsequent platelet activation and thus do not manifest with HIT (21, 35). Although rapid assays have similar efficacy in HIT testing, this does not hold true in the context of VITT, as will be discussed below.

EIAs quantify the optical density emitted from anti-PF4/heparin antibodies in patient serum when mixed with a secondary antibody. They consist of microtiter plates containing antigen complexes (PF4/heparin) bound to the well surface. Patient sera is added to the well and anti-PF4/heparin antibodies bind to the antigen. A secondary antibody (anti-human IgG) that can emit light is added and the degree of transmission is quantified. Commercially available EIAs for HIT detect either all immunoglobulin classes (polyspecific, i.e., IgG/A/M) or IgG-only antibodies. When used for VITT testing, commercial EIA has been reported to have a sensitivity of 100% and the specificity of commercial, in-house, and anti-P4F/heparin ranging between 95.6 and 97.4% (5). Various rapid assays are commercially available for the diagnosis of HIT including the LIA, chemiluminescence immunoassay (CLIA), particle gel immunoassay (PaGIA), and lateral flow assay (LFA). The LIA measures light transmission of a sample containing PF4/PVS-coated latex nanoparticles mixed with a HIT-mimicking antibody (30, 31). This antibody agglutinates the nanoparticles, thus resulting in low light transmission through the sample. When patient plasma containing anti-PF4/heparin antibodies is added, there is competition for binding to the nanoparticles. This disrupts the agglutination and results in higher light transmission. Therefore, the amount of patient anti-PF4/heparin antibodies is directly related to the degree of light transmission. The CLIA also utilizes PF4/PVS coated particles to capture anti-PF4/heparin antibodies and then measures the emitted light of an isoluminol-labeled anti-human IgG secondary antibody (25). Similarly, the ID-PaGIA measures the ability of patient sera to agglutinate beads coated with PF4/heparin complexes (32). Lastly, LFAs capture antibodies bound to PF4/PVS when as patient samples migrate with buffer across a test strip, appearing as a visible colored line when positive (37). Rapid assays thus represent an attractive option for HIT testing because of their ease of use, rapid turnaround time (<1 h), and superior performance compared to standard EIAs (slightly lower sensitivity, but greater specificity) (30, 33). However, rapid assays should not be used for VITT testing due to their inferior sensitivity (high false-positive rate) compared to EIA (12, 37).

Platton et al. (11) tested 23 suspected or probable VITT patients using six commercially available EIAs and four rapid assays. IgG-specific EIAs had comparable diagnostic performances to the polyspecific EIAs, with 5/6 tests showing >90% sensitivity. On the other hand, the rapid tests performed poorly in VITT: three of the four rapid assays had diagnostic sensitivities between 0 and 5.9% while the fourth had a sensitivity of 45.5% (13). Another study also found similarly poor detection of anti-PF4 antibodies in VITT patients with the following assays: ID-PaGIA (3/12, 25% detected), CLIA/HemosIL AcuStar HIT-IgG (8%), and LFA/Milenia QuickLine HIT-Test (0%) (37). Using the same samples, two commercial EIAs (ZYMUTEST HIA IgG-EIA and Immucor PF4 IgG-EIA) were shown to better identify anti-PF4 antibodies in 11/12 (92%) and 12/12 (100%) VITT patients. The superiority of EIAs was confirmed in another study of 9 VITT patients that showed reduced accuracy of rapid assays for VITT antibodies (12). All rapid assays tested in this study were negative for anti-PF4 antibodies whereas the three EIAs used showed variable results depending on the antigen target. Only the PF4-PVS EIA (Lifecodes PF4 IgG, Immucor) successfully identified high titres of anti-PF4 antibodies in 7/9 patients. The PF4/heparin EIA (Asserachrom HPIA, Stago) identified anti-PF4 antibodies in 5/9 suspected patients while the platelet-lysate/heparin EIA (Zymutest HIA IgG, Hyphen) only identified antibodies in 4/9 suspected patients. Finally, a multi-center UK study evaluating anti-PF4 EIAs and rapid assays showed rapid assays to have inferior performance compared to EIAs for identifying anti-PF4 VITT antibodies (38).

Therefore, while rapid assays are often used to rule-out the presence of anti-PF4/heparin antibodies in HIT, they perform poorly in VITT. EIAs are the preferred screening assays as they can accurately identify anti-PF4 VITT antibodies. The Lifecodes Immucor PF4-EIA has demonstrated the best performance in this regard. Reference laboratories should be able to perform more than one type of EIA, given that no single assay detects all VITT sera. Future work should look to evaluate the diagnostic capabilities of EIAs to further substantiate their usefulness as a diagnostic test for VITT due to the limited sample size of these studies. However, functional platelet activating assays remain the gold-standard test for laboratory confirmation of VITT.

Functional assays (HIPA/PEA/SRA)

Functional platelet activation assays are required to confirm the diagnosis of HIT after a positive screen for anti-PF4/heparin antibodies with the EIA or rapid assays (39). Functional assays thus reduce false positive results due to non-pathogenic

antibodies (34, 40). Examples of HIT functional assays include the heparin-induced platelet aggregation assay (HIPA), heparin-induced multiple electrode aggregometry (HIMEA), PF4-dependent P-selectin expression assay (PEA), and the serotonin release assay (SRA) (39, 41–43). The HIPA, PEA and SRA rely on the use of fresh donor platelets that are mixed with patient serum, while the HIMEA is a whole blood-based assay (see Table 1). In the case of the HIPA, HIEMA and SRA, varying exogenous heparin doses are used to confirm heparin-dependence or independence. The assays mostly differ in their measured endpoints of platelet activation: HIPA measures platelet aggregation *via* turbidity change (assessed visually), while the HIMEA measures aggregation by electrical impedance; the PEA measures P-selectin expression on the platelet surface; and the SRA measures radioactive serotonin release from platelet granules (34, 39, 41–43, 47). These assays can also be used to identify HIT patients who may safely receive heparin products following treatment. For example, in a HIT patient treated with intravenous immunoglobulin, the SRA (functional) test will be negative despite a persistently positive EIA (48).

Functional HIT assays were quickly implemented for VITT testing once it was discovered that patients had anti-PF4 antibodies (45). However, standard functional assays often produced false-negative results in VITT. Therefore, a modification of the functional assay is needed by adding exogenous PF4 instead of heparin (1, 22, 44). In classic HIT, exogenous heparin serves as a scaffold to complex PF4 and induce a conformational change such that it can be recognized by anti-PF4/heparin HIT antibodies (40). This results in a "heparin-dependent" platelet activation, as evidenced in the SRA (39, 44). In contrast, heparin was found to *reduce* the sensitivity of platelet activation assays in VITT and produced a false-negative SRA. However, the addition of exogenous PF4 significantly increased the sensitivity of HIT assays and produced an activation pattern similar to HIT. The heparin-mediated inhibition (at pharmacological concentrations, i.e., 0.1 to 0.5 IU/mL heparin) of platelet activation in VITT can be explained by the recent finding that the majority of anti-PF4 VITT antibodies bind to the heparin-binding site on PF4 (22). Therefore, heparin should be avoided in VITT functional platelet testing and exogenous PF4 should be added (49). In one study, the addition of heparin to the PF4-enhanced HIPA assay reduced the sensitivity from 100 to 20% of clinically diagnosed VITT samples (5). In another study, sensitivity was reduced from 100 to 46.2% when heparin was added to the PF4-enhanced SRA (1). Interestingly, both the PEA and the PF4-induced flow cytometry–based platelet activation assay (PIFPA) already require PF4 and do not use heparin in their reactions (46). Both assays measure P-selectin expression *via* flow cytometry for their activation endpoints; the main differences between the PFIPA and PEA is the use of a whole blood-based assay compared to washed platelet-based assay, respectively.

TABLE 1 Summary of platelet functional assays in HIT and VITT.

HIT		VITT analog		
Assay	**Methodology**	**Assay**	**Difference in methodology**	**Reported results for clinical VITT**
Serotonin release assay (SRA)	• Measures release of radioactive serotonin from activated platelets • Washed platelets treated with heparin and patient sera	PF4-SRA	Modified SRA with no heparin and washed platelets preincubated with PF4	3/3 (44) 5/5 (22)
Heparin-induced platelet activation assay (HIPA)	• Visual detection of platelet aggregation • Washed platelets treated with heparin and patient sera	PIPA	Modified HIPA with no heparin and washed platelets preincubated with PF4	28/28 (1)
Heparin-induced multiple electrode Aggregometry (HIMEA)	• Platelet aggregation measured by changes in electrical impedance. • Whole blood mixed with heparin and patient sera	HEMA	Modified HEMA with no heparin	4/5 *(45) 50/52 **(8)
PF4-dependent P-selectin expression assay (PEA)	• Expression of P-selectin *via* flow cytometry • Washed platelets treated with patient sera and PF4	PIFPA***	Expression of P-selectin *via* flow cytometry Whole blood assay with PF4 preincubation before sera addition	16/16 (46)

*Negative sample was ruled inconclusive. **Negative samples were patients undergoing IVIG. ***Closest analogue. All samples tested were positive for anti-PF4 antibodies.

PF4-enhanced functional assays can confirm whether anti-PF4 antibodies are capable of platelet activation and whether persistent anti-PF4 antibodies continue to be pathogenic. This was highlighted in a recent study of 69 patients with thrombocytopenia and thrombosis syndrome (TTS, an epidemiologic classification that includes VITT) (8). The sensitivity and specificity of functional platelet assays in this cohort were 96 and 78%, respectively. The two patients who yielded false negative platelet activation had receive intravenous immunoglobulin prior to blood collection, which is known to interfere with these assays (44). This suggests that functional assays may even approach 100% sensitivity if appropriate clinical context and pre-analytical variables are used, and with optimal assay reactivity. Rarely, PF4 supplementation may be required as demonstrated by "SRA-negative HIT" patient sera (47, 50). In one study, four of eight VITT patients (50%) with thrombocytopenia but without thrombosis also tested strongly positive for anti-PF4, platelet-activating antibodies. Functional assays can thus confirm the diagnosis in patients with atypical VITT presentation (e.g., thrombocytopenia alone on initial presentation). However, given that the excellent operating characteristics of the EIAs, functional assays are not required to confirm the diagnosis of VITT. This contrasts with HIT where the screening test has a low specificity and confirmatory functional assays are needed to confirm the diagnosis.

Functional assays can also potentially identify disease resolution in the context of persistent anti-PF4 antibodies following VITT. In a longitudinal study of 35 VITT patients, 32 (91%) continued to test positive on anti-PF4/heparin IgG EIA, albeit with significantly reduced optical density results (51). In those who had follow-up >12 weeks, the majority (14/15, 93%) became negative in the platelet-activation assays. The remaining patient exhibited recurrent thrombocytopenia with persistently high-titre anti-PF4/heparin IgG and positive platelet-activation. Therefore, platelet functional assays may be used to monitor disease activity longitudinally in patients with ongoing symptoms or evidence of disease recurrence. It is still unclear whether persistent EIA positivity indicates potential for recurrent disease.

VITT functional assays are thus a useful adjunct and diagnostic tool for patient cases that are atypical or require longitudinal monitoring. When testing well clinically defined VITT patient samples, VITT functional assays have reliable and accurate detection. Neither the PF4 serotonin release assay (PF4- SRA), the PF4-induced platelet activation assay (PIPA), or flow-cytometry based assay (PIFPA) missed any of the clinically diagnosed VITT samples, whereas the HEMA missed three samples in two different studies. Of these three samples, one was inconclusive while the other two were patients undergoing IVIG treatment (Table 1) (1, 8, 44–46). Given the relative novelty of VITT and rapidity at which these assays were developed, the majority of studies featured small, well-selected patient populations. No significant large-scale comparative studies have been done to compare the functional assays to each other as of this publication. It is because of this small sample population that the sensitivity and specificity of these tests has not been well defined; however, by all initial indications the assays appear to be strong diagnostic tools for VITT and should be considered when EIA testing is not available or additional supportive testing is required.

Discussion

VITT is a prothrombotic disorder that has been characterized through application of existing knowledge related to HIT. Although these disorders share many similarities, clinicians should be aware of several important differences: First, HIT antibodies and VITT antibodies have different binding sites on PF4. Second, anti-PF4/heparin EIAs have high sensitivity and high specificity for the VITT diagnosis, whereas the specificity is low for HIT. Third, rapid diagnostic assays developed for HIT often yield false-negative results for VITT and should not be used as a diagnostic test for VITT.

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Conflict of interest

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SARS-CoV-2 Spike protein activates TMEM16F-mediated platelet procoagulant activity

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Thrombosis of the lung microvasculature is a characteristic of COVID-19 disease, which is observed in large excess compared to other forms of acute respiratory distress syndrome and thus suggests a trigger for thrombosis that is endogenous to the lung. Our recent work has shown that the SARS-CoV-2 Spike protein activates the cellular TMEM16F chloride channel and scramblase. Through a screening on >3,000 FDA/EMA approved drugs, we identified Niclosamide and Clofazimine as the most effective molecules at inhibiting Spike-induced TMEM16 activation. As TMEM16F plays an important role in stimulating the procoagulant activity of platelets, we investigated whether Spike directly affects platelet activation and pro-thrombotic function and tested the effect of Niclosamide and Clofazimine on these processes. Here we show that Spike, present either on the virion envelope or on the cell plasma membrane, promotes platelet activation, adhesion and spreading. Spike was active as a sole agonist or, even more effectively, by enhancing the function of known platelet activators. In particular, Spike-induced a marked procoagulant phenotype in platelets, by enhancing Ca^{2+} flux, phosphatidylserine externalization on the platelet outer cell membrane, and thrombin generation. Eventually, this increased thrombin-induced clot formation and retraction. Both Niclosamide and Clofazimine blocked this Spike-induced procoagulant response. These findings provide a pathogenic mechanism to explain lung thrombosis-associated with severe COVID-19 infection. We propose that Spike, present in SARS-CoV-2 virions or exposed on the surface of infected cells in the lungs, enhances the effects of inflammation and leads to local platelet stimulation and subsequent activation

of the coagulation cascade. As platelet TMEM16F is central in this process, these findings reinforce the rationale of repurposing Niclosamide for COVID-19 therapy.

KEYWORDS

platelets, SARS-CoV-2, TMEM16F, coagulation, Niclosamide, Clofazimine, COVID-19, Spike

1 Introduction

Thrombosis is a defining characteristic of advanced COVID-19 lung disease. Recent meta-analyses reveal that elevated D-dimers, fibrinogen and thrombosis-associated biomarkers are present in over 20% of patients with COVID-19 and in at least 50% of patients requiring intensive care (1–5). At the histological level, our own post-mortem analysis of 41 patients with COVID-19 revealed the presence of microthrombosis in the lungs of 83% of patients requiring intensive care and 75% of all the other patients (6). This high prevalence resonates with that of several other recent pathology investigations (7–11). While a thrombotic response is common in other causes of acute respiratory distress syndrome (ARDS), the magnitude of this response appears a hallmark of COVID-19. A post-mortem study reported that severe vascular injury, including alveolar microthrombi, was nine times more prevalent in COVID-19 lungs than in patients with influenza (7).

At least three observations suggest that lung thrombosis in COVID-19 is triggered by local events. First, thrombi and fibrin deposition in the lungs are asynchronous, by which recent thrombi infiltrated by inflammatory cells are close to older thrombi, in advanced stage of fibrotic organization (6). Second, the presence of macro- or micro-vascular thrombosis is only sporadically detected in other organs (6–11). Third, patients with severe COVID-19 have high levels of D-dimer and fibrinogen, but do not show an increase in prothrombin time or a decrease in antithrombin levels and rarely develop disseminated intravascular coagulation (12); this argues against thrombosis as a consequence of a systemic consumptive coagulopathy.

This pulmonary intravascular coagulopathy (13) has variously been attributed to endothelial dysfunction (6, 14), or the hyperinflammatory state that accompanies SARS-CoV-2 infection (15–19), or immunothrombosis, by which neutrophils and monocytes activate the coagulation cascade as a host immune defense against infection (20–22), including the release of neutrophil extracellular traps (NETs) (23, 24). While these mechanisms most likely contribute to the local pro-thrombotic state, they still do not explain why lung thrombosis is particularly frequent in COVID-19 compared to other causes of ARDS.

Several observations point to a specific involvement of platelets in the pathogenesis of COVID-19 thrombosis. A common characteristic of patients with advanced COVID-19 disease is thrombocytopenia (5, 25–27); COVID-19 lungs show increased number of pulmonary megakaryocytes, which could be indicative of increased local megakaryopoiesis in response to platelet consumption (28); SARS-CoV-2 infection is associated with platelet hyperreactivity, including increased P-selectin expression, platelet activation and spreading on both fibrinogen and collagen, and elevated levels of circulating platelet-neutrophil, -monocyte, and -T-cell aggregates (29–32). The actual molecular mechanisms leading to this platelet hyperactivity during SARS-CoV-2 infection, coupled with local thrombosis, remain largely elusive and is likely multifactorial.

In the course of SARS-CoV-2 infection, the viral envelope Spike protein exerts a fundamental function by mediating virus entry into the cells. In particular, the Spike surface unit (S1) binds the ACE2 cellular receptor (33), which facilitates docking of the virion onto the surface of the target cells. A subsequent activation step carried out by a cellular protease cleaves Spike at the S1/S2 boundary and thus exposes the S2 portion, which promotes membrane fusion and virion content internalization. In the case of SARS-CoV-2, the protease priming event and subsequent fusion can occur at the plasma membrane thanks to the involvement of the TMPRSS2 (33) or furin (34, 35) proteases, hence the capacity of virus-infected cells, which expose Spike on their surface, to form large syncytia with other infected or non-infected cells that express the ACE2 receptor.

While studying the molecular properties of SARS-CoV-2 Spike, we discovered a novel mechanism that regulates Spike-mediated cellular syncytia formation. We screened two libraries of EMA/FDA-approved small molecules to search for drugs that inhibit syncytia formation (36). As the most effective drugs, this screening identified Niclosamide, a synthetic salicylanilide developed in the 1950s as a molluscicide against snails (37) and later approved for tapeworm infection, and Clofazimine, an antibiotic used for the combination treatment of leprosy (38) and, more recently, for drug-resistant tuberculosis (39). Of relevance for platelets, we discovered that Niclosamide blocks the formation of syncytia by inhibiting the cellular Ca^{2+}-dependent chloride channel and scramblase TMEM16F, thus preventing the externalization of phosphatidylserine (PS) onto the outer leaflet of the cell plasma membrane (36). TMEM16F is essential for lipid scrambling in platelets during blood coagulation (40, 41), as externalized PS serves as an anchoring site for the assembly of the tenase and

prothrombinase complex, which jointly enhance the rate of thrombin generation by several orders of magnitude (42).

Based on these observations, it was tempting for us to speculate that Spike-driven activation of platelets in SARS-CoV-2 infected lungs could be causally involved in the thrombotic process. Here we show that Spike, exposed on either the envelope of virions or the surface of cells, induces a procoagulant phenotype in platelets, which is blocked by both Niclosamide and Clofazimine.

2 Materials and methods

2.1 Human blood collection and isolation of platelets

All investigations in this study conform to the principles outlined in the Declaration of Helsinki. All human subjects provided their informed written consent.

Studies using human platelets were approved by St Thomas's Hospital, London, UK Research Ethics Committee (ref. 07/Q0702/24). Blood was collected by venepuncture into tri-sodium citrate (3.2%; Sigma-Aldrich, Burlington, MA, United States) from healthy volunteers (aged 25–40), who had abstained from non-steroidal anti-inflammatory drug consumption for the preceding 14 days. Platelet rich plasma (PRP) was obtained by centrifugation of whole blood (175 × *g*, 15 min, 25°C). Further purification of platelets was achieved by centrifugation of PRP at 1,000 × *g* for 10 min at 25°C in the presence of apyrase (0.02 U/mL, Sigma-Aldrich, Burlington, MA, United States) and prostacyclin (PGI_2; 2 μM, Tocris Bioscience, Bristol, United Kingdom) followed by resuspension in modified Tyrode's HEPES buffer (134 mmol/L NaCl, 2.9 mM KCl, 0.34 mmol/L Na_2HPO_4, 12 mmol/L $NaHCO_3$, 20 mmol/L HEPES, and 1 mmol/L $MgCl_2$; pH 7.4; Sigma-Aldrich, Burlington, MA, United States) with glucose (0.1% w/v; Sigma-Aldrich, Burlington, MA, United States) and bovine serum albumin (Sigma-Aldrich, Burlington, MA, United States). Washed platelets were adjusted to a concentration of 3×10^8/ml, allowed to rest of 30 min and then supplemented with 2 mM $CaCl_2$ (Sigma-Aldrich, Burlington, MA, United States).

2.2 Patients

Patients' lung samples were obtained from post-mortem analysis of a cohort of patients who died of COVID-19 at the University Hospital in Trieste, Italy, after intensive care support (6). All patients scored positive for SARS-CoV-2 by RT-PCR tests on nasopharyngeal swab and presented symptoms (fever, cough, and dyspnea) as well as imaging data indicative of interstitial pneumonia related to COVID-19 disease. All patients eventually died of clinical acute respiratory distress syndrome related to COVID-19 infection. Further clinical details on these patients are reported in Bussani et al. (6). The use of these samples was approved by the Joint Ethical Committee of the Regione Friuli Venezia Giulia, Italy (re. 0019072/P/GEN/ARCS).

2.3 Cell culture

HEK-293T and Vero cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Burlington, MA, United States, 4.5 g/L) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Burlington, MA, United States).

2.4 Pseudotyped viral particles

For Spike pseudotyped lentiviral particle production, we generated the expression plasmid pEC120-S-D19-V5, in which the 19aa at the Spike C terminus, acting as an ER-retention signal (43), are replaced by a V5 epitope tag. The modified DNA segment was obtained by recombinant PCR and cloned into the pEC117-Spike-V5 vector (36). Pseudotyped viral particles were produced by transfecting HEK293T cell (2.5×10^6) in a 100 mm-dish with 10 μg pLVTHM/GFP (Addgene #12247), 7.5 μg psPAX2 (Addgene #12260), and 6 μg pMD2.G (Addgene #12259) or pEC120-S-D19-V5. The culture medium was collected after 48 h and concentrated three times using Vivaspin columns with 100 kDa cutoff (GE HealthCare, Chicago, IL, United States, #28932363). Aliquots were stored at −80°C.

For titration, RNA from pseudoparticle preparations was isolated using the Viral RNA isolation Kit (Takara, Tokyo, Japan). Viral RNA genome content was quantified using the Lenti-X qRT-PCR Titration Kit (Takara, Tokyo, Japan) and the Quant-X One-Step qRT-PCR TB Green Kit (Takara, Tokyo, Japan).

To assess pseudoparticle infectivity, HEK293T cells were bulk transfected with an ACE2-expressing plasmid and then seeded in a 96-well plate (3×10^3 cells per well). The subsequent day, 5 μL viral particles carrying the G-protein of the Vesicular Stomatitis Virus (VSV-G) or 10 μL particles carrying SARS-CoV-2 Spike were added to each well [the difference in volume was meant to partially compensate for the different transduction efficiency of the two viral preparations (44)]. The plate was fixed at 48 h, stained with Hoechst (Invitrogen, Carlsbad, CA, United States) and imaged.

2.5 Platelet aggregation with pseudotyped viral particles and cells

Platelet aggregation was performed as previously described (45) with minor modifications. Briefly, washed platelets were incubated with VSV-G or Spike pseudoparticles (1:10) for 10 min followed by stimulation with collagen related peptide

(CRP; 0.3 μg/mL), collagen (0.3 μg/mL, Takeda, Tokyo, Japan) or vehicle. Plates were shaken for 5 min (1,200 rpm, 37°C; BioShake IQ, QInstruments, Jena, Germany), and absorbance was measured at 595 nm using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland). Aggregation was calculated as percentage change in absorbance.

For platelet aggregation on Spike-expressing cells, Vero cells were kept in culture in DMEM supplemented with 10% FBS and transfected with a plasmid coding for either GFP (pZac-GFP) or V5-tagged Spike (pSARS-COV-2-S). After detachment, 20,000 cells were co-incubated with 5×10^5 pre-labeled platelets (1:2,000 Cell Tracker Deep Red Dye, Thermo Fisher Scientific, Waltham, MA, United States) in 96-well plates, and shaken at 200 rpm for 10 min at 37°C. The plate was centrifuged at 300 *g* for 10 min and fixed by 4% final paraformaldehyde (PFA, VWR, Radnor, PA, United States). The plate was permeabilized with 0.5% Triton (Sigma-Aldrich, Burlington, MA, United States) for 10 min, blocked with 1% BSA for 1 h at room temperature (RT) and stained with anti-GFP (1:1,000) or anti-V5 tag (1:500) antibodies for 2 h at RT. After washing three times with 1xPBS, anti-rabbit 488 or anti-mouse 488 secondary antibodies (1:500) were incubated for 1 h at room temperature. Then, plates were washed three times with 1xPBS and incubated with Cell Mask (1:2,000; Thermo Fisher Scientific, Waltham, MA, United States) for 30 min at RT. After further washing with 1xPBS, Hoechst (1:5,000, Thermo Fisher Scientific, Waltham, MA, United States) was added. Images (20 × magnification) were acquired using a Perkin Elmer Operetta CLS High Content Fluorescent microscope. Analysis was performed using the ImageJ software (Fiji).

2.6 Platelet adhesion and spreading

Washed platelets (3×10^8/mL) were allowed to adhere to type I Horm collagen (100 μg/mL; Takeda, Tokyo, Japan) coated plates, for 90 min at 37°C. Samples were fixed with 0.2% PFA for 10 min followed by permeabilization with 0.2% Triton (Sigma-Aldrich, Burlington, MA, United States) for 5 min. Samples were washed with 1xPBS and stained with AlexaFluor 488 Phalloidin (1:2,000). Images (63 × magnification) were acquired using a Perkin Elmer Operetta CLS High Content Fluorescent microscope. Analysis was performed using the ImageJ software (Fiji).

2.7 Flow cytometry

For externalized phosphatidylserine (PS) analysis, washed platelets were incubated with VSV-G or Spike pseudoparticles (1:10) for 10 min followed by activation with collagen (30 μg/ml) and thrombin (0.5 units, Sigma-Aldrich, Burlington, MA, United States) or vehicle for 15 min (350 rpm, 37°C; BioShake IQ). Platelets were stained with Annexin V-Pacific Blue (1:50; BioLegend, San Diego, CA, United States) and CD61-APC (1:100; BioLegend, San Diego, CA, United States) in annexin binding buffer for 20 min (350 rpm, 37°C; BioShake IQ). For $Ca2^+$ levels analysis, washed platelets were stained with Fluo-4 AM (5 μM, Thermo Fisher Scientific, Waltham, MA, United States) for 30 min at 37°C followed by incubation with pseudoparticles (1:10) for 10 min. Platelet samples were activated with vehicle or collagen (30 μg/ml) for 15 min.

In both cases, samples were diluted with modified Tyrode's HEPES buffer and analyzed on the ACEA Novocyte 3,000 (ACEA Biosciences, San Diego, CA, United States). Analysis was performed using the FlowJo software v.10 (TreeStar, Ashland, OR, United States).

2.8 Thrombin measurement

Thrombin activity was measured using a Thrombin Activity Assay Kit (Abcam, Cambridge, United Kingdom). Briefly, PRP was incubated with VSV-G or Spike pseudoparticles (1:10) for 10 min at 37°C, followed by stimulation with collagen (30 μg/ml) for 20 min (350 rpm, 37°C). PRP was diluted 1:10 with thrombin assay buffer, and 50 μl of thrombin substrate was added to each well. Thrombin activity was assessed by measuring the conversion of thrombin substrate into its fluorogenic state using a CLARIOstar fluorescent plate reader (BMG Labtech, Ortenberg, Germany) with 350/450 nm excitation and emission filter. Analysis was performed using MARS analysis software.

2.9 Clot retraction

Platelet rich plasma (PRP) was diluted 1:1 with modified Tyrode's HEPES buffer, supplemented with $CaCl_2$ (2 mM) and 10 μl of whole blood. PRP was incubated with VSV-G (1:10), Spike pseudoparticles (1:10), His-tag recombinant SARS-CoV-2 Spike Protein S1/S2 (S-ECD) (1 ng/ml; Thermo Fisher Scientific, Waltham, MA, United States; aa11-1208; RP-87680) or Flag-tag recombinant SARS-CoV-2 Spike RBD (1 ng/ml; Bio-Techne, Minneapolis, MN, United States, 10689-CV-100) for 10 min at 37°C, followed by stimulation with thrombin (0.5 units). Clot retraction was measured over 90 min, taking an image every 15 min. Analysis was performed using ImageJ (NIH).

2.10 Effect of drugs on Spike-induced platelet function

The effect of drugs was tested in the presence of 1 μM Niclosamide (Sigma-Aldrich, Burlington, MA, United States) or 5 μM Clofazimine (Sigma-Aldrich, Burlington, MA, United States) for 10 min. Niclosamide (Sigma-Aldrich, Burlington, MA, United States, N0560000) and Clofazimine

(Sigma-Aldrich, Burlington, MA, United States, Y0000313) stocks were prepared at 10 mM by resuspending in DMSO. Fresh dilutions were prepared for each experiment. In all the experiments, DMSO (vehicle) was used in controls at the same concentration as in the drug treated samples.

2.11 Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0. Unless stated, data followed a Gaussian distribution. Statistical significance between pairs was determined using unpaired Student's *t*-test, two tails; statistical significance among three groups of more was determined with one-way ANOVA, two tails, with Dunnett's *post-hoc* analysis for multiple comparisons.

2.12 Antibodies

Immunofluorescence analysis was performed for actin (AlexaFluor 488 Phalloidin, #A12379, Thermo Fisher Scientific, Waltham, MA, United States), GFP (#ab6556, Abcam, Cambridge, United Kingdom) V5 tag (#37-7,500, Thermo Fisher Scientific, Waltham, MA, United States), TMEM16F (ab256302, Abcam, Cambridge, United Kingdom), and ACE2 (ab87436 and ab15348, both from Abcam, Cambridge, United Kingdom). Fluorescent secondary antibodies were obtained from Sigma-Aldrich, Burlington, MA, United States. Immunohistochemistry for platelets was performed with antibody 760–4,249 (Roche, Basel, Switzerland) against CD61.

Immunoblots were performed with primary antibodies against Spike (#GTX632604, Genetex, San Antonio, TX, United States), TMEM16F (#HPA038958, Sigma-Aldrich, Burlington, MA, United States) and tubulin (Cell Signaling, #3873S). Anti-rabbit and anti-mouse HRP-conjugated antibody were obtained from Abcam, Cambridge, United Kingdom.

2.13 Immunohistochemistry

Post-mortem samples from patients were fixed in 10% formalin and embedded in paraffin. Antigen retrieval and antibody pre-treatment were performed according to the manufacturer's instructions. Immunohistochemistry for platelets (CD61 antigen) was performed on one-micron sections. Tissue staining was with Bluing Reagent (Ventana 760–2,037).

2.14 Immunoblotting

To detect Spike in pseudoparticles, concentrated Spike and VSV-G pseudoparticles (40 μL) were diluted in 4× protein loading dye. For the detection of TMEM16F by immunoblotting, platelets (9×10^8/mL) were lysed in 2% SDS and quantified by using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States). Samples (15–20 μg) were resolved by electrophoresis in 4–20% gradient polyacrylamide gels (Mini-PROTEAN, Bio-Rad, Hercules, CA, United States) and transferred to Trans-blot (Bio-Rad, Hercules, CA, United States). Membranes were blocked in TBST (PBS + 0.1% Tween-20) with 5% skim milk (#9999, Cell Signaling) for 1 h at room temperature. Blots were then incubated (4°C, overnight) with primary antibodies against Spike (1:1,000), TMEM16F (1:1,000) or tubulin (1:10,000). Blots were then washed three times with TBST. Membranes were incubated for 1 h at room temperature with anti-rabbit HRP-conjugated antibody (1:5,000) or anti-mouse HRP-conjugated antibody (1:10,000). After washing three times with TBST (10 min each), blots were developed with ECL (Amersham).

2.15 Real-time PCR

RNA was extracted from washed platelets lysed with Trizol and purified using the RNeasy kit from Qiagen, Hilden, Germany (ID: 74004), following the manufacturer's instruction. cDNA was obtained by retrotranscribing 0.5 μg of RNA. ACE2 expression was evaluated using primer pair: Forward primer CAAGAGCAAACGGTTGAACAC; Reverse primer CCAGAGCCTCTCATTGTAGTCT. The results shown refer to the sequences published in Manne et al. (31).

2.16 Data sharing

For original data, please contact Professor MG (mauro.giacca@kcl.ac.uk) or Professor TW (t.d.warner@qmul.ac.uk).

3 Results

3.1 SARS-CoV2 Spike potentiates platelet aggregation and adhesion

COVID-19 lung pathology is characterized by extensive local thrombosis. Our own analysis of post-mortem samples from patients who died of COVID-19 indicated that thrombosis of the lung micro- and macro- vasculature was present in 29/41 (71%) of all patients and in 83% of patients in intensive care units (6). Staining of lung sections with an antibody recognizing the platelet CD61 glycoprotein revealed that these thrombi are massively infiltrated by aggregated platelets (representative images for five patients in **Figure 1A**).

To understand whether platelet activation could be induced by SARS-CoV-2 Spike on the plasma membrane of infected cells,

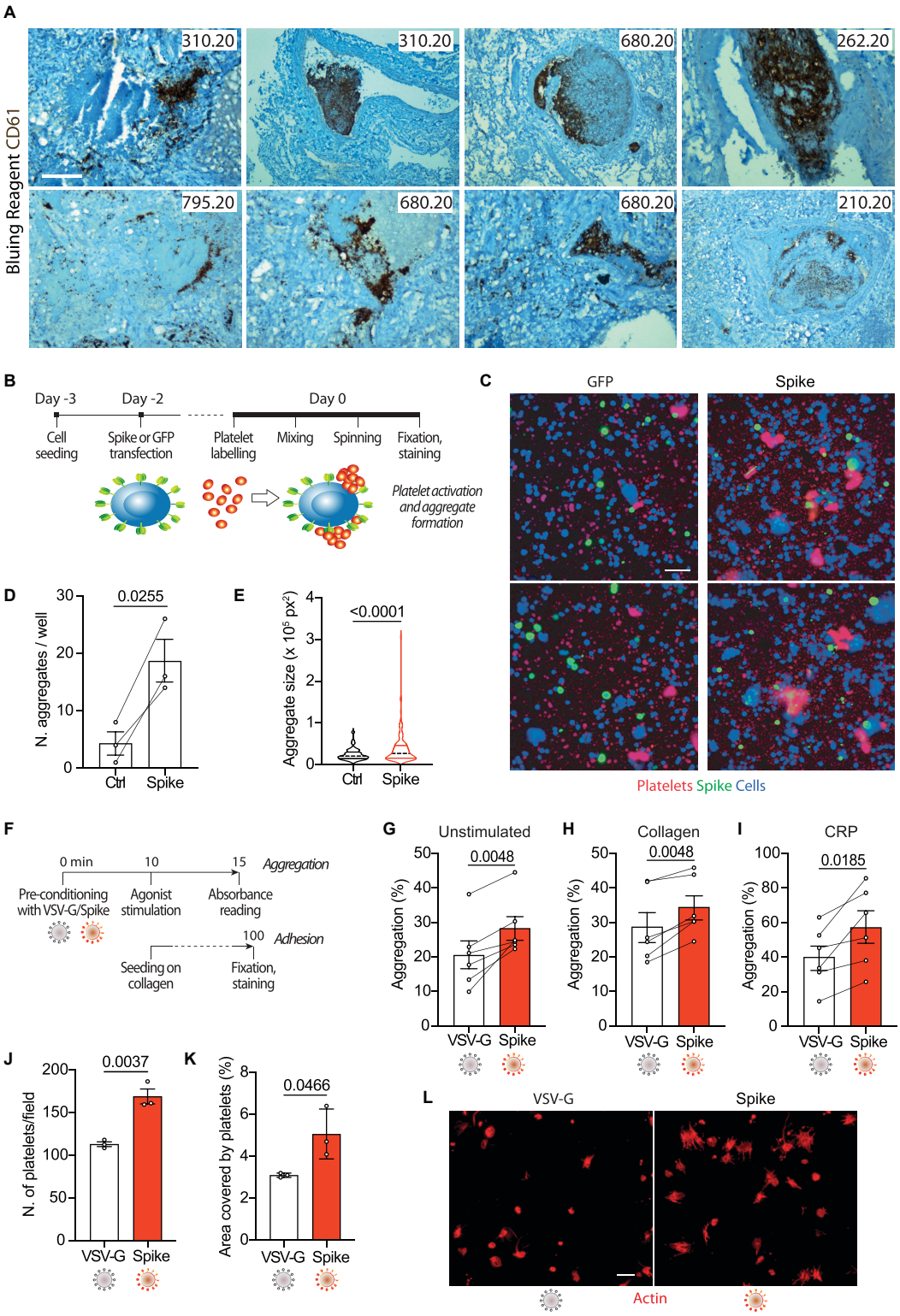

FIGURE 1
Spike enhances platelet activation. **(A)** Histopathological evidence of platelet aggregates in the thrombotic microvasculature of SARS-COV-2-infected lungs from four COVID-19 patients. Numeric codes identify patients. Platelets were stained using an anti-CD61 glycoprotein antibody. Scale bar: 100 μm. **(B)** Experimental scheme to study platelet activation and aggregate formation. Vero cells transfected to express either Green Fluorescent Protein (GFP) or SARS-CoV-2 Spike were incubated with pre-labeled washed platelets and shaken at

(Continued)

FIGURE 1 (Continued)
200 rpm for 10 min at 37°C. The plate was centrifuged, fixed, and stained with Cell Mask and antibodies recognizing either GFP or Spike. **(C)** Representative images showing platelet aggregates. Vero cells stained with Cell Mask are in blue; labeled platelets are in red; GFP or Spike are in green. Scale bar, 20 μm. **(D)** Number of aggregates larger than 40,000 px^2. Results are from $n = 3$ independent experiments. Data are mean ± SEM, statistical significance is indicated (paired Student's *t*-test). **(E)** Violin plot showing the size of the aggregates found in all the experiments performed. Statistical significance is indicated (unpaired Student's *t*-test). **(F)** Experimental scheme for platelet activation in suspension. **(G–I)** Percentage of platelet aggregation when incubated with vehicle **(G)**, stimulated with collagen **(H)** or CRP **(I)**. Results are from $N = 6$ independent experiments. Data are mean ± SEM statistical significance is indicated (paired Student's *t*-test). **(J)** Number of adherent platelets per field. Results are from $n = 3$ independent experiments each performed in duplicate. Each dot represents the mean of six images quantified. Data are mean ± SEM. Statistical significance is indicated (paired Student's *t*-test). **(K)** Percentage of the area covered by adherent platelets. Results are from $n = 3$ independent experiments performed in duplicate; each dot represents the mean of six images quantified. **(L)** Representative images of platelets adhering on collagen. Images were acquired using a high content fluorescent microscope followed by analysis using the ImageJ software (Fiji). Platelets were stained with F-actin (in red). Scale bar, 5 μm.

we incubated Vero cells transfected to express Spike (Wuhan strain) or GFP control with washed human platelets (1×10^5), which had previously been stained with the lipophilic dye CellTracker (scheme in **Figure 1B**). Transfection efficiency was verified by immunostaining for the transgenes (>40% efficiency in both cases). We observed that the cells expressing Spike significantly increased both the number of platelet aggregates and their overall aggregate area (**Figures 1D, E**, respectively; $P < 0.001$ in both cases; representative images in **Figure 1C**).

As far as the receptor that mediates these Spike activities in platelets, we could detect the ACE2 mRNA in platelets of three normal donors that we analyzed, albeit at levels that were lower than those of respiratory Calu-3 cells (**Supplementary Figure 1A**). When platelets of two of these donors were assessed by immunofluorescence, both ACE2 (visualized with two different antibodies) and TMEM16F were readily detectable (**Supplementary Figure 1B**).

Next, we wanted to assess whether Spike could also activate platelets in a cell-free context. We generated GFP-expressing lentiviral vectors pseudotyped with either SARS-CoV-2 Spike (Δ19) or the vascular stomatitis virus (VSV) G protein as a control. The vector preparations had comparable genome titres (**Supplementary Figure 1C**) and Spike was detected in the vector lysates by immunoblotting (**Supplementary Figure 1D**). Both pseudotyped preparations were effective at transducing HEK293/ACE2 reporter cells (quantification and representative images in **Supplementary Figures 1E, F**). As indicators for activation, we measured platelet aggregation and adhesion (scheme in **Figure 1F**). The Spike pseudoparticles significantly enhanced platelet aggregation (from 21 ± 4% in VSV-G controls to 28 ± 3% in Spike-treated platelets; $n = 6$, $P < 0.01$; **Figure 1G**). There was no effect of VSV-G particle-treated compared to untreated platelets (**Supplementary Figures 1G, H**). Spike pseudotyped virions also increased aggregation induced by either collagen (29 ± 4 vs. 34 ± 3%; $n = 6$, $P < 0.01$ in VSV-G vs. Spike; $n = 6$, $P < 0.01$) or collagen-related peptide (CRP; 40 ± 7 vs. 57 ± 9%; $n = 6$, $P < 0.01$; **Figures 1H, I**, respectively).

In addition to potentiating platelet aggregation, Spike also increased platelet adhesion and spreading. The Spike viral particles augmented both the number of platelets adhering on collagen (113 ± 3 vs. 169 ± 9 platelets per field in VSV-G vs. Spike; $n = 3$, $P < 0.01$) and the area covered by the adherent platelets (3% vs. 5 ± 1% in VSV-G vs. Spike; $n = 3$, $P < 0.05$); **Figures 1J, K**, respectively; representative images in **Figure 1L**. Also in this case, there was no significant difference between VSV-G particle-treated and untreated platelets (**Supplementary Figure 1I**).

Taken together, these observations indicate that Spike on either the virion envelope or the cell plasma membrane enhances platelet aggregation and adhesion.

3.2 SARS-CoV2 Spike induces a procoagulant response in platelets

As Spike activates TMEM16F (36) and this scramblase stimulates the procoagulant activity of platelets (40–42), we wanted to study platelet PS externalization upon treatment with Spike (**Figure 2A**). We observed that the dual stimulation of platelets with collagen and thrombin in the presence of Spike virions increased both the percentage of annexin V-positive platelets (7.0 vs. 10 ± 1% in VSV-G vs. Spike; $n = 4$, $P < 0.05$) and the amount of platelet-bound annexin V [1,524 ± 98 vs. 1,755 ± 82 arbitrary units (AU) in VSV-G vs. Spike; $n = 4$, $P < 0.01$; **Figures 2B, C**, respectively; representative flow cytometry profiles are in **Figure 2D**].

Given the relevance of Ca^{2+} signaling in platelet activation and procoagulant activity, next we investigated the effect of Spike on Ca^{2+} flux (**Figure 2E**). We observed that, in platelets activated with collagen, Spike increased the levels of the Fluo-4 indicator, which is sensitive to cytosolic Ca^{2+} (16,756 ± 2,115 AU vs. 19,281 ± 1,952 AU in VSV-G vs. Spike; $n = 4$, $P < 0.05$; **Figure 2F**; representative flow cytometry plots are in **Figure 2G**). This increase was only observed in the presence of extracellular Ca^{2+} (**Supplementary Figure 2**). This indicates that Spike-mediated platelet activation is not related to Ca^{2+} release from the platelet intracellular stores or, should release occur, this is not amplified and sustained by store-operated Ca^{2+} entry.

Considering these results, we wanted to assess whether Spike also affected clot formation and retraction, which are the last

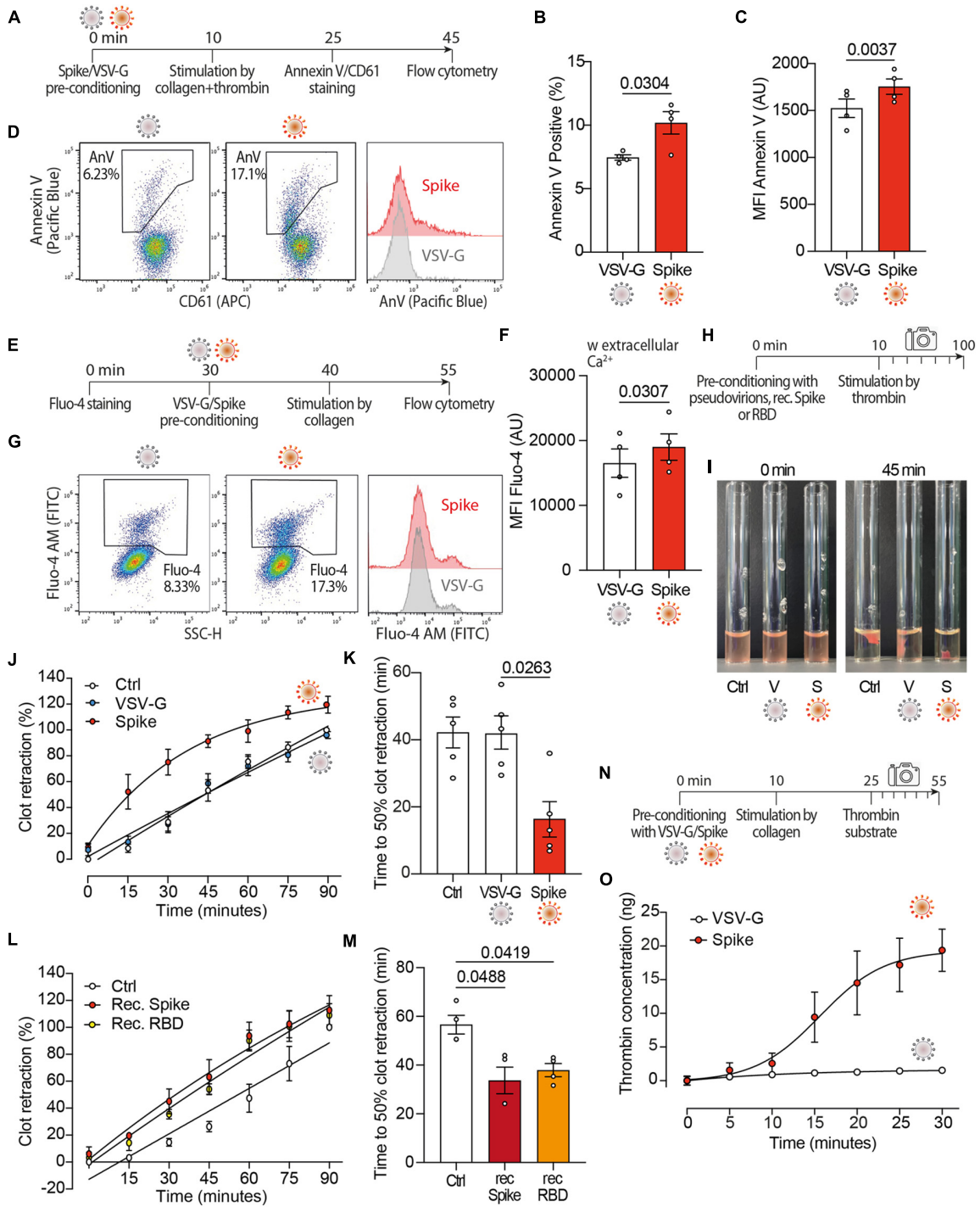

FIGURE 2

Spikes activates the procoagulant activity of platelets. **(A)** Experimental scheme to study platelet activation by pseudovirions. Washed platelets were incubated with 1:10 diluted VSV-G or Spike for 10 min, followed by incubation with collagen (30 μg/ml) and thrombin (0.5 units) for 15 min. Platelets were stained with Annexin V-Pacific Blue and CD61-APC for 20 min at 37°C and then analyzed by flow cytometry. **(B,C)** Percentage and mean fluorescence intensity (MFI) of annexin V positive platelets upon activation with Spike or VSV-G pseudovirions. Results are from $n = 4$ independent experiments. Data are mean ± SEM. Statistical significance is indicated (paired Student's t-test). AU, arbitrary units. **(D)** Representative flow cytometry plots. The boxed area shows the percentage of platelets positive for annexin V (AnV) binding following incubation with VSV-G or Spike. The histogram on the right shows the distribution of Annexin V positive platelets after the two treatments. For each flow cytometry plot, the first gating was based on CD61/FSC to define the platelet population. Thus, the Aneexin V plot excludes any

(Continued)

FIGURE 2 (Continued)
microvesicles which may have been present within the sample. **(E)** Experimental scheme to study Ca^{2+} flux in platelets. Washed platelets were stained with Fluo-4 for 30 min, followed by incubation with VSV-G or Spike pseudovirions diluted 1:10 for 10 min. Platelet samples were then incubated with collagen (30 μg/ml) for 15 min and then analyzed by flow cytometry. **(F)** Mean fluorescence intensity (MFI) of Fluo-4 (AU, arbitrary units) in platelets stimulated with Spike or VSV-G pseudovirions. Results are from $n = 4$ independent experiments. Data are mean ± SEM. Statistical significance is indicated (paired Student's *t*-test). **(G)** Representative flow cytometry plots. The boxed area shows the percentage of platelets positive for Fluo-4 fluorescence following incubation with VSV-G or Spike. The histogram on the right shows the distribution of Fluo-4 fluorescence after the two treatments. **(H)** Experimental scheme for the clot retraction assay following stimulation of platelets with Spike. PRP was supplemented with $CaCl_2$ and 10 μl of whole blood, then incubated with 1:10 diluted VSV-G or Spike pseudoparticles, recombinant Spike or recombinant RBD (1 ng/mL) for 10 min at 37°C, followed by incubation with thrombin. Clot retraction was measured over 90 min, taking an image every 15 min. **(I,K)** Clot retraction of PRP pre-incubated with PBS, VSV-G or Spike pseudoparticles. Representative images immediately after the addition of thrombin and after 45 min are in panel **(I)**, the percentage of clot retraction over a 90 min observation period is in panel **(J)**. The graph in **(K)** shows the time to 50% clot retraction. All data are mean ± SEM from $n = 5$ independent experiments. Statistical significance is indicated in panel **(K)** (one-way ANOVA with Dunnett's *post-hoc* correction for multiple comparisons). **(L,M)** Clot retraction of PRP pre-incubated with PBS, recombinant Spike (1 ng/mL) or recombinant receptor binding domain (RBD, 1 ng/mL). The percentage of clot retraction over the 90 min observation period is in panel **(L)**. The graph in panel **(M)** shows the time to 50% clot retraction in the three experimental conditions. All data are mean ± SEM from $n = 3$ independent experiments. Statistical significance is indicated in panel **(K)** (one-way ANOVA with Dunnett's *post-hoc* correction for multiple comparisons). **(N)** Experimental scheme to study thrombin generation upon platelet treatment with Spike pseudovirions. PRP was incubated with 1:10 diluted VSV-G or Spike pseudovirions for 10 min at 37°C, followed by stimulation with collagen (30 μg/mL) for 15 min (350 rpm, 37°C) and the addition of a fluorogenic thrombin substrate. Thrombin activity was assessed by measuring the conversion of thrombin substrate into its fluorogenic state using a CLARIOstar fluorescent plate reader with 350/450 nm excitation and emission filter. Analysis was performed using MARS analysis software.
(O) Concentration of thrombin formed during a 30 min-time period. Results are from $n = 5$ independent experiments. Data are mean ± SEM.

steps in the coagulation cascade (**Figure 2H**). We observed that Spike markedly enhanced thrombin-induced clot retraction (representative images in **Figure 2I**; complete time point set in **Supplementary Figure 3**). The kinetics of clot formation in the presence of Spike was faster compared to both VSV-G-treated or control platelets (**Figure 2J**; difference in Area Under the Curve, AUC: $P < 0.001$ for both controls), with a time to 50% clot retraction of 16 ± 5 min compared to 42 ± 5 min after treatment with VSV-G; $n = 4$, $P < 0.05$ (**Figure 2K**). The same result was also obtained by measuring the clot retraction after incubating platelets with recombinant proteins corresponding to full-length Spike or to the Spike receptor binding domain (RBD) (**Figures 2L, M** for kinetic analysis and time to 50% clot retraction, respectively; $P < 0.05$ in both cases).

We also observed that the kinetics of thrombin generation following stimulation with collagen (scheme in **Figure 2N**) was markedly increased by incubation with the Spike virions (1.5 ± 0.4 ng vs. 19.4 ± 3.1 ng thrombin concentration in VSV-G vs. Spike at 30 min; $n = 5$, AUC difference: $P < 0.01$; **Figure 2O**). There was no significant difference between untreated and VSV-G-treated platelets at any time point (not shown).

Collectively, these results indicate that Spike increases platelet Ca^{2+} flux, PS exposure and thrombin-induced clot retraction, all of which are markers of the procoagulant function in platelets.

3.3 Drugs inhibiting Spike-induced syncytia formation also block the effect of Spike on platelets

Our previous screening work has identified Niclosamide and Clofazimine as the two most potent drugs that inhibit Spike-induced cell fusion by targeting TMEM16F (36). We confirmed that this scramblase is well expressed in platelets (immunoblotting from six normal donors in **Supplementary Figure 4A**) and thus hypothesized that Niclosamide and Clofazimine could inhibit Spike-induced platelet activation (scheme in **Figure 3A**).

Pre-treatment of platelets with either drug, followed by incubation with VSV-G or Spike pseudotyped particles, reduced platelet aggregation upon stimulation with CRP (shown in **Figure 3B** for Niclosamide—from 42 ± 2 to 33%; $P < 0.05$, and in **Figure 3C** for Clofazimine; from 34 ± 3 to 23 ± 2%; $P < 0.01$). Clofazimine also inhibited aggregation in the absence of CRP treatment (from 31 ± 1 to 18 ± 3%; $P < 0.01$). There was no effect when the drugs were used in the presence of the VSV-G pseudotypes (**Supplementary Figures 4B, C**).

We next sought to understand whether Niclosamide and Clofazimine affected Spike-induced platelet adhesion on collagen. Pre-treatment with either drug significantly reduced the total number of adherent platelets (representative images and quantification in **Figures 3D, E**; 169 ± 9 platelets/field in vehicle-treated samples vs. 118 ± 7 and 88 ± 2 platelets/field in Niclosamide and Clofazimine-treated platelets, respectively; $n = 3$, $P < 0.01$ in both cases). Pre-treatment with neither Niclosamide nor Clofazimine affected platelet adhesion in VSV-G controls (**Supplementary Figure 4D**).

Finally, we assessed the spreading stage of adherent platelets as an indicator of drug efficacy. In VSV-G-treated samples, most platelets showed a rounded shape with no or single filopodia (filopodia patterns are in **Figure 3F**). Platelet activation by Spike increased the percentage of platelets with multiple filopodia and of platelets showing a fully spread phenotype (**Figure 3G**). Treatment with either Niclosamide and Clofazimine reversed this Spike-induced phenotype ($n = 3$ per condition; $P < 0.05$ for

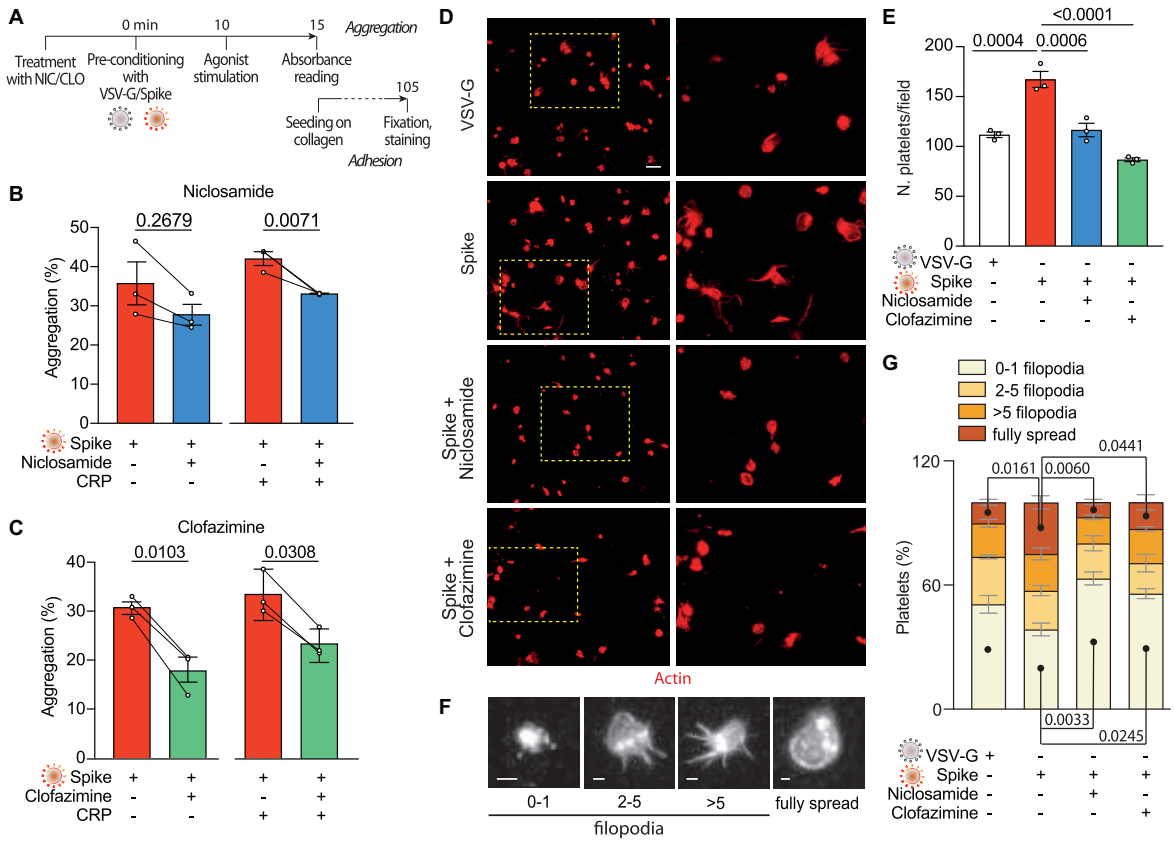

FIGURE 3
Niclosamide and Clofazimine inhibits Spike-induced activation of platelets. **(A)** Experimental scheme to assess the effect of drugs on platelet activation. Washed platelets were incubated with Niclosamide, Clofazimine **(C)** or vehicle for 10 min, then incubated with 1:10 diluted Spike or VSV-G pseudoparticles for further 10 min. Aggregation and adhesion were evaluated as described in **Figure 1**. **(B,C)** Percentage of platelet aggregation upon treatment with Spike in the presence of either Niclosamide **(B)** or Clofazimine **(C)**, and respective DMSO controls, with or without CRP. Results are from $n = 3$ independent experiments. Data are mean ± SEM. Statistical significance is indicated (paired Student's *t*-test). **(D)** Representative images of platelets adhering on collagen (magnification in the right panels). Platelets are stained in red for F-actin. Scale bar, 5 μm. **(E)** Number of adherent platelets per field. Results are from $n = 3$ independent experiments performed in duplicate. Each dot represents the mean of six quantified images. Data are mean ± SEM. Statistical significance is indicated (unpaired Student's *t*-test). **(F)** Representative images of platelet morphological changes. Adherent platelets were classified into four morphological categories representing different stages of platelet adhesion and activation: platelets with 0–1, 3–5, more than five protrusions or fully spread platelets, as indicated by the representative images under the graph. Scale: 1 μm. **(G)** Platelet morphological changes upon Spike and drug treatment. Results are from $n = 3$ independent experiments performed in duplicate. Data are mean ± SEM. Statistical significance is shown for the indicated morphological categories (one-way ANOVA with Dunnett's *post-hoc* correction for multiple comparisons).

both platelet phenotype distribution between VSV-G and Spike and for the effect of either drug vs. Spike).

3.4 Niclosamide and Clofazimine block Spike-induced platelet procoagulant activity

Given the reduction in aggregation and adhesion, we next investigated whether Niclosamide and Clofazimine also inhibited the procoagulant response to Spike (scheme in **Figure 4A**). Pre-treatment of platelets with either drug reduced PS exposure (2,467 ± 125 Annexin V AU vs. 1,627 ± 55 AU in Spike vs. Niclosamide for mean fluorescent intensity; 2,064 ± 343 AU vs. 1,341 ± 113 AU in Spike vs. Clofazimine; $n = 4$, $P < 0.05$ in both cases; representative flow cytometry plots and quantification in **Figures 4B, C**; the difference in the percentages of fluorescent cells was also statistically different for both drugs–not shown). The levels of Spike-induced intracellular Ca^{2+} were reduced by both drugs (scheme in **Figure 4D**; Fluo-4 fluorescence: 20,014 ± 2,627 AU vs. 16,607 ± 2,278 AU in Spike vs. Niclosamide for mean fluorescent intensity; 15,605 ± 2,083 AU vs. 13,804 ± 1,816 AU in Spike vs. Clofazimine; $n = 4$, $P < 0.05$ in both cases; **Figures 4E, F**). In a consistent manner and for both drugs, the percentages of fluorescent cells also showed statistically significant differences (not shown).

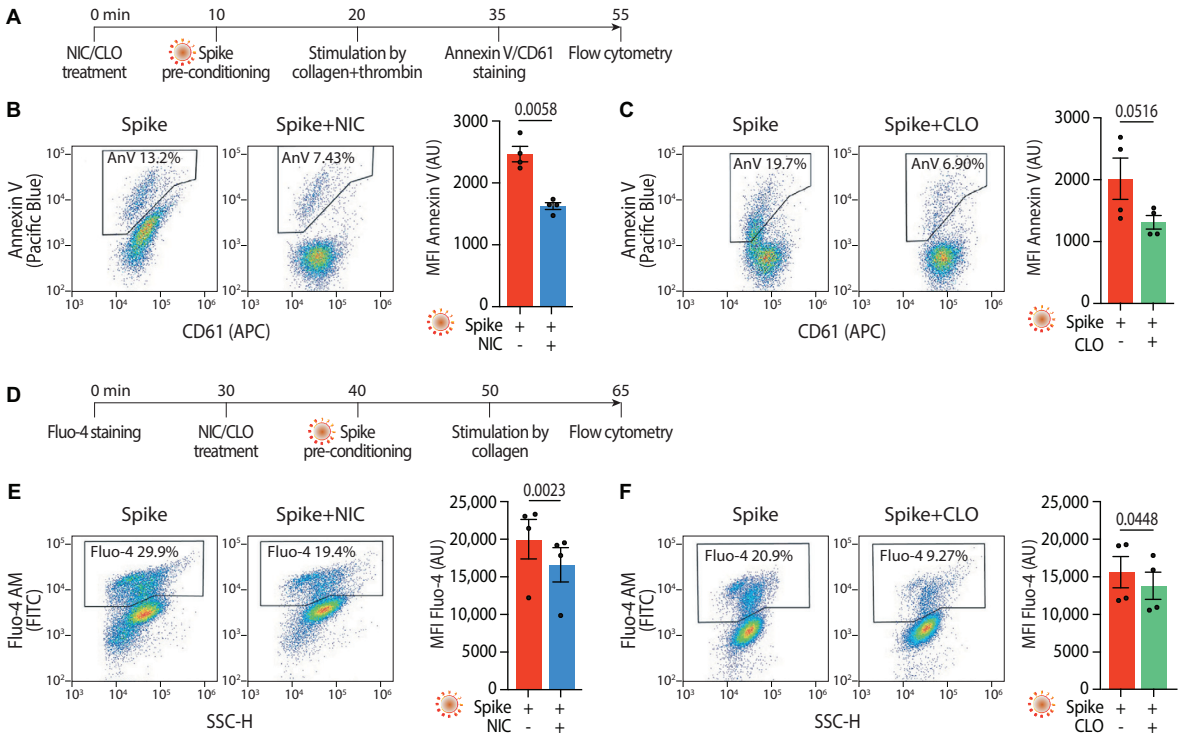

FIGURE 4
Niclosamide and Clofazimine reduce annexin V reactivity and intracellular calcium in platelets. **(A)** Experimental scheme to assess annexin V reactivity upon Spike stimulation and drug treatment. Platelets were pre-incubated with Niclosamide (NIC, 1 μM) or Clofazimine (CLO, 5 μM) for 10 min, followed by incubation with collagen (30 μg/ml) and thrombin (0.5 units) for 15 min. Platelets were then stained with Annexin V-Pacific Blue and CD61-APC and analyzed by flow cytometry. **(B)** Effect of Niclosamide on annexin V reactivity. Representative flow cytometry plots are on the left, quantification on the right. The boxed areas show the percentage of washed platelets positive for annexin V upon treatment with the drug or vehicle (DMSO at the same concentration and for the drug) and incubation with VSV-G or Spike pseudoparticles, followed by stimulation with collagen and thrombin. The graph shows the Mean fluorescence intensity (MFI) of annexin V positive platelets (AU, arbitrary units). Results are from $n = 4$ independent experiments. Data are mean ± SEM. Statistical significance is indicated (paired Student's t-test). **(C)** Same as panel **(B)** upon treatment with Clofazimine. **(D)** Experimental scheme to assess Ca^{2+} influx upon Spike stimulation and drug treatment. Platelets were stained with Fluo-4 for 30 min and then pre-incubated with Niclosamide (NIC, 1 μM) or Clofazimine (CLO, 5 μM) for 10 min, followed by incubation with collagen (30 μg/ml) and thrombin (0.5 units) for 15 min. Platelets were then assessed for fluorescence by flow cytometry. **(E)** Representative flow cytometry plots are on the left, quantification on the right. The boxed areas show the percentage of washed platelets positive for Fluo-4 upon treatment with the drug or vehicle (DMSO at the same concentration and for the drug) and incubation with Spike pseudoparticles, followed by stimulation with collagen and thrombin. The graph shows the Mean fluorescence intensity (MFI) of Fluo-4 (AU, arbitrary units). Results are from $n = 4$ independent experiments and are expressed as mean ± SEM. Statistical significance is indicated (paired Student's t-test). **(F)** Same as in panel **(E)** upon treatment with Clofazimine.

Both Niclosamide and Clofazimine reduced the rate of thrombin-stimulated clot retraction (experimental scheme in **Figure 5A**, representative images in **Figures 5B, C**, respectively; complete time courses in **Supplementary Figures 5A, D**). In the presence of either drug, the kinetics of Spike-induced clot formation was reduced (**Figures 5D, E**, respectively; AUC difference: $P < 0.001$ and $P < 0.05$ for the two drugs, respectively). The time to 50% clot retraction increased from 27 ± 4 min to 76 ± 6 min in the presence of Niclosamide and from 27 ± 7 min vs. 72 ± 17 min in the presence of Clofazimine ($n = 3$, $P < 0.05$ in both cases; **Figures 5F, G**). Of interest, Niclosamide had no significant effect on the rate of thrombin-stimulated clot retraction in the absence of Spike, namely upon addition of PBS or treatment with VSV-G pseudovirions (**Supplementary Figures 5B, C**, respectively). This is consistent with the conclusion that Niclosamide specifically blocks Spike-induced platelet activation through TMEM16F, in agreement with its role as a specific TMEM16F inhibitor. In the case of Clofazimine, partial inhibition of thrombin-stimulated clot retraction was also observed in the absence of Spike (**Supplementary Figures 5E, F**), albeit at a lower extent than upon stimulation with Spike. This could indicate that Clofazimine also acts through additional platelet activation pathways.

Finally, we assessed the effect of the two drugs on the kinetics of thrombin generation following stimulation with collagen in the presence of virions with Spike (scheme in **Figure 5H**). Both drugs markedly decreased thrombin formation (62%

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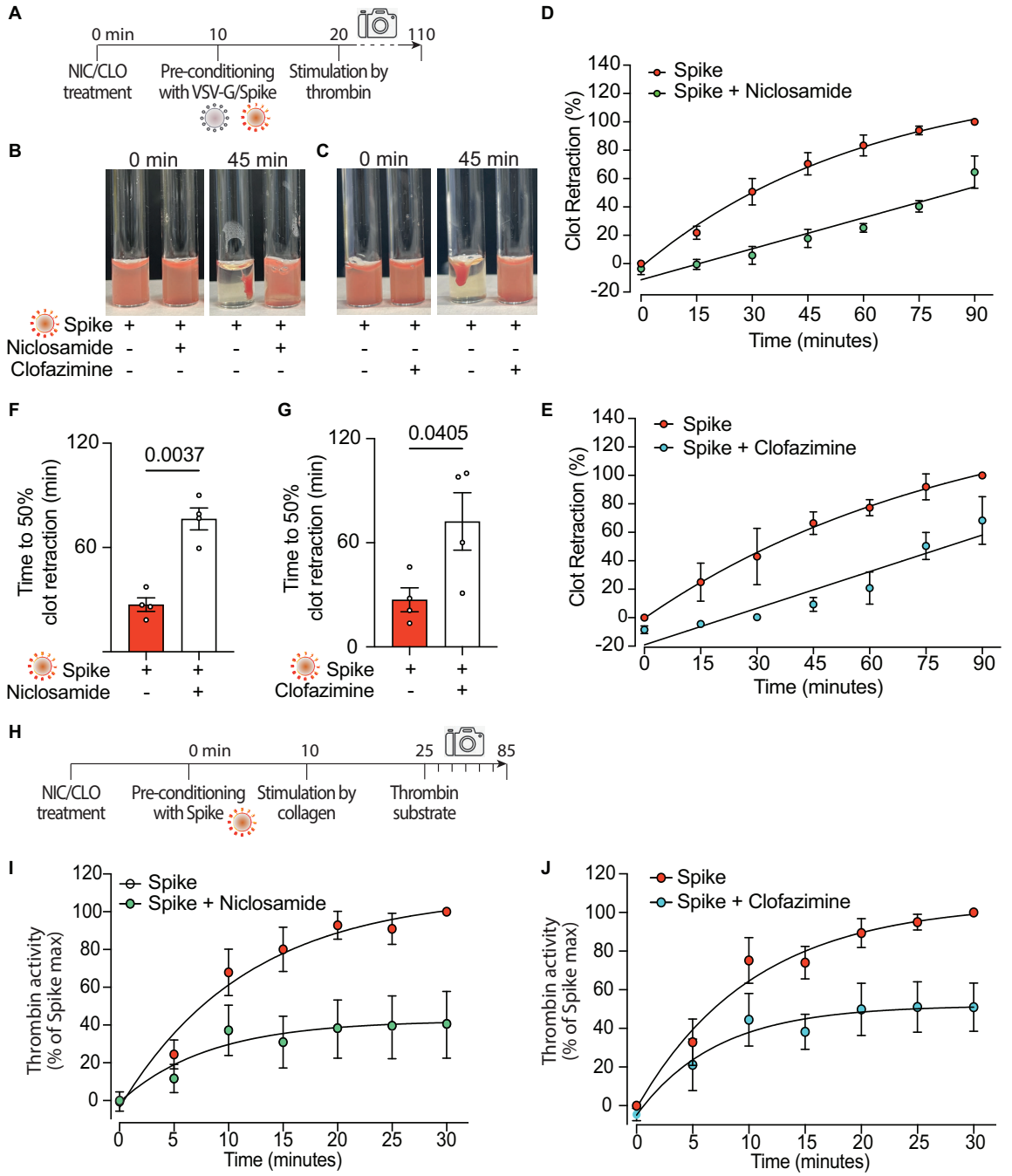

FIGURE 5

Niclosamide and Clofazimine block procoagulant activation of platelets. **(A)** Experimental scheme to study the effect of drugs on the clot retraction assay following stimulation of platelets with Spike. PRP, supplemented with $CaCl_2$ and 10 μl of whole blood, were incubated with Niclosamide or Clofazimine for 10 min, then treated with 1:10 diluted VSV-G or Spike pseudoparticles for additional 10 min. Clot retraction was measured over 90 min from the addition of thrombin, taking an image every 15 min. **(B,C)** Representative images of clot retraction when platelets were treated with Spike and either Niclosamide **(B)** or Clofazimine **(C)**, immediately after the addition of thrombin and after 45 min. **(D,E)** Percentage of clot retraction over 90 min, when platelets were treated with Spike and either Niclosamide **(J)** or Clofazimine **(K)**. Results are from $n = 4$ independent experiments. Data are expressed as mean ± SEM. **(F,G)** Time to 50% clot retraction when platelets were treated with Spike and either Niclosamide **(L)** or Clofazimine **(M)**. Results are from $n = 4$ independent experiments. Data are mean ± SEM. Statistical significance is shown (paired Student's *t*-test). **(H)** Experimental scheme to study the effect of drugs on thrombin generation upon platelet treatment with Spike pseudovirions. PRP was incubated with 1:10 diluted VSV-G or Spike for 10 min at 37°C, followed by incubation with collagen (30 μg/mL) for 15 min and the addition of a fluorogenic thrombin substrate. Thrombin activity was assessed by the measurement of the conversion of the thrombin substrate into its fluorogenic state. **(I,J)** Concentration of thrombin formed during a 30 min-time period in PRP treated with Spike and pre-conditioned or not with Niclosamide or Clofazimine. Data are expressed as mean ± SEM.

decrease vs. Spike at 30 min for Niclosamide, 55% decrease for Clofazimine; $n = 5$, $P < 0.01$ at all time points from 10 to 30 min; **Figures 5I, J**; AUC difference: $P < 0.01$ in both cases).

4 Discussion

There is broad evidence of platelet dysregulation during SARS-CoV-2 infection, by which platelets are hyperreactive in several of their phenotypes. Manne et al. reported that platelets from COVID-19 patients aggregate faster, showing increased spreading over fibrinogen/collagen–coated surfaces and increased P-selectin expression, despite integrin activation was reduced (31). Zaid et al. observed that platelets from COVID-19-positive patients showed increased aggregation, adhesion, and microvesiculation to subthreshold concentration of thrombin (32). Nicolai et al. reported that cases of intermediate severity showed an exhausted platelet phenotype, while patients severely affected with COVID-19 were characterized by excessive platelet activation in comparison with healthy controls and non-COVID-19 pneumonia (46). Finally, Zhang et al. observed platelet hyperreactivity based on increased integrin αIIbβ3 activation and P-selectin expression (47).

Here we show that exposure of platelets to SARS-CoV-2 Spike promotes platelet activation and adhesion, and enhances Ca^{2+} release and PS exposure to drive increased thrombin generation. Externalized, negatively charged PS can act as a platform for assembly of the tenase and prothrombinase complex, which massively amplify thrombin generation (42). In addition, increased PS exposure could contribute to inflammation and immune reactivity. These phenotypes in platelets, and in particular the procoagulant activity of Spike, were blocked by Niclosamide, a TMEM16A and TMEM16F inhibitor (48). The relevance of TMEM16F for the procoagulant phenotype of platelets is underscored by the phenotypes of Scott patients [reviewed in Nurden et al. (49)] and PF4-Cre-conditioned TMEM16F-null mice (41), both of which have a normal platelet count and do not show obvious defects in platelet activation but indeed display a reduced procoagulant phenotype in association with reduced thrombin generation.

The effects of Spike were present when we used this protein as a sole agonist, but was markedly enhanced in the presence of known platelet activators. This observation suggests that Spike, in the lungs of severely infected individuals, could contribute to the pro-thrombotic state, by cooperating with hyperinflammation (15–19), endothelial dysfunction (6, 14) and NETosis (23, 24) in determining thrombosis. This could explain the prevalence of thrombosis in the lungs of COVID-19 patients with severe disease, which is significantly higher than in other forms of ARDS.

In our experiments, SARS-CoV-2 Spike stimulated platelets both when present on the virion envelope or upon expression on the cell plasma membrane. When and in what compartment would platelet become in direct contact with the SARS-CoV-2 Spike protein in patients? There are at least three possibilities. First, through direct viral infection of endothelial cells. There is evidence of endothelial cell infection by SARS-CoV-2 by viral RNA analysis (7, 14), while we and others have detected Spike by immunostaining on endothelial cells in SARS-CoV-2 lungs of COVID-19 patients at post-mortem analysis (6, 50, 51). Second, SARS-CoV-2 can be found in the blood (52) and viral production is particularly robust in the lung and lower tract respiratory epithelium of SARS-CoV-2 infected patients, leading to the continuous production of infectious particles (53). Our own work has shown that SARS-CoV-2 RNA is detectable in plasma or serum of COVID-19 ICU patients when neutralizing antibody response is low. RNAemia was associated with higher 28-day ICU mortality (54). Third, and probably most relevant, the hyperinflammatory environment in the infected lungs can promote the disruption of the endothelial barrier, which would allow platelets to enter the infected lung tissue and get in contact with infected pneumocytes. These infected cells (36) persist for prolonged periods during infection while expressing viral antigens, including Spike. Cell surface expression of Spike leads to fusion of the infected cells with neighboring cells expressing the ACE2 receptor, as SARS-CoV-2 Spike, in contrast to the homologous protein from SARS-CoV, contains a furin cleavage site that allows protein activation while the protein is produced during the ER-Golgi transit or when at the cell surface (55–57). The syncytia formed because of these characteristics can be found in over 90% of patients with severe infection and express detectable amounts of Spike on their surface (36). These infected cells and syncytia represent a platform for platelet activation and the consequent induction of thrombosis.

In our experiments, Spike was also active when administered as a recombinant protein, which raises the question as to what the mechanism is for Spike-mediated platelet activation. Based on our previous observations on the activation of TMEM16F by Spike (36), we can envisage at least two mechanistic possibilities. Activation could occur directly upon binding of Spike to its receptor, leading to the direct activation of TMEM16F on the platelet membrane. Alternatively, activation of TMEM16F could be triggered by the increase on Ca^{2+} we observe after stimulation with Spike. In other cell types, Spike-mediated TMEM16 activation increases the amplitude of spontaneous Ca^{2+} signals (36), which is in line with previous reports showing that both TMEM16A and TMEM16F augment intracellular Ca^{2+} by increasing the filling of ER stores and augmenting IP3R-induced Ca^{2+} release (58). Two of the experiments that we report here, however, favor the former possibility, namely that Spike-induced platelet activation is consequent to a molecular event occurring at the membrane level. First,

activation of platelets did not occur when the medium was depleted of extracellular Ca^{2+}, likely indicating that intracellular Ca^{2+} stores are not required for activation. Second, and most important, platelet activation also occurred upon treatment with the isolated spike RBD domain. These observations are in favor of a direct TMEM16F activation event occurring upon binding of Spike at the plasma membrane level.

As far as the platelet Spike-binding receptor is concerned, this remains still controversial. A couple of reports failed to detect ACE2 in platelets from both COVID-19 patients and healthy individuals (31, 32). Other investigators, however, have reported expression of ACE2 on platelets from both healthy individuals and mice by RT-PCR (47), and also expression of the ACE2 protein by immunodetection (59). In our own experiments, we could readily detect expression of ACE2 in the healthy donors we analyzed, from whom platelets were collected for our experiments. Irrespective of the actual expression of ACE2, however, all the studies so far conducted, including those reporting against ACE2 expression, are concordant in showing platelet activation in SARS-CoV-2 infection, and several of these studies have also confirmed that the viral genome can be found within platelets (31, 32, 59). Should ACE2 not be the relevant receptor, there may be ACE2-independent mechanisms whereby SARS-CoV-2 directly interacts with, and possibly enters, platelets [reviewed in ref: (60)].

Both Niclosamide and Clofazimine were remarkably effective at inhibiting Spike-induced platelet activation. In the case of Niclosamide, this in line with the inhibition of TMEM16F and the known role that this protein plays in amplifying the rate of platelet procoagulant activity (42). Niclosamide acted at concentrations in the low hundred nanomolar range, which are even lower than those needed to inhibit syncytia (36). Niclosamide is a synthetic salicylanilide developed in the 1950s as a molluscicide against snails (37) and subsequently approved for use in humans, where it has been employed for over 50 years to treat tapeworm infections (61). Solubility of the currently available oral formulation is relatively low, but there is anyhow evidence of significant systemic absorption, with plasma levels that can reach 1–20 μM (62). Besides inhibiting TMEM16 proteins, multiple evidence indicates that this drug can exert pleiotropic effects in mammalian cells, which include modulation of the Wnt/β-catenin and Notch signaling pathways, repression of mTORC1 and inhibition of STAT3 and NF-κB transcriptional regulators [reviewed in Chen et al. (63)]. Some of these various functions could explain the effects of Niclosamide on platelet aggregation, adhesion and spreading, which are not directly relatable to TMEM16F activation. In this respect, however, it should be noted that the magnitude of these effects, albeit their statistical significance, is markedly lower than that of the inhibition of platelet pro-coagulant activity.

Finally, an interesting open question is whether the mechanism for Spike-induced platelet activation could be causally linked to the thrombocytopenia in the course of COVID-19 (5, 25–27). Decreased platelet levels due to clearance in the spleen are reported in other conditions of PS overexposure (64), including mice with reduced levels of Bcl-xL (64). Recent evidence links this feature specifically to TMEM16F and extracellular Ca^{2+} influx, as mice with sphingomyelin synthase one deficiency show marked thrombocytopenia due to increased PS exposure consequent to excessive TMEM16F activation (65). Based on these observations, we speculate that thrombocytopenia during severe COVID-19 may be consequent to abnormal platelet activation through Spike-mediated TMEM16F stimulation.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Joint Ethical Committee of the Regione Friuli Venezia Giulia, Italy (re. 0019072/P/GEN/ARCS). The patients/participants provided their written informed consent to participate in this study.

Author contributions

AC, ES, HA, and IS performed the molecular and cellular biology experiments and generated pseudovirions. HEA and MC performed experiments with human platelets. SV, RS, and LM participated in experiments on Spike-expressing cells. SZ contributed to conceiving the project. TW and MG conceived the project, coordinated the work, and wrote the manuscript. All authors read and edited the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1013262/full#supplementary-material

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Microvascular significance of TGF-β axis activation in COVID-19

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As 2023 approaches, the COVID-19 pandemic has killed millions. While vaccines have been a crucial intervention, only a few effective medications exist for prevention and treatment of COVID-19 in breakthrough cases or in unvaccinated or immunocompromised patients. SARS-CoV-2 displays early and unusual features of micro-thrombosis and immune dysregulation that target endothelial beds of the lungs, skin, and other organs. Notably, anticoagulation improves outcomes in some COVID-19 patients. The protein transforming growth factor-beta (TGF-β1) has constitutive roles in maintaining a healthy microvasculature through its roles in regulating inflammation, clotting, and wound healing. However, after infection (including viral infection) TGF-β1 activation may augment coagulation, cause immune dysregulation, and direct a path toward tissue fibrosis. Dysregulation of TGF-β signaling in immune cells and its localization in areas of microvascular injury are now well-described in COVID-19, and such events may contribute to the acute respiratory distress syndrome and skin micro-thrombosis outcomes frequently seen in severe COVID-19. The high concentration of TGF-β in platelets and in other cells within microvascular thrombi, its ability to activate the clotting cascade and dysregulate immune pathways, and its pro-fibrotic properties all contribute to a unique milieu in the COVID-19 microvasculature. This unique environment allows for propagation of microvascular clotting and immune dysregulation. In this review we summarize the physiological functions of TGF-β and detail the evidence for its effects on the microvasculature in COVID-19. In addition, we explore the potential role of existing TGF-β inhibitors for the prevention and treatment of COVID-19 associated microvascular thrombosis and immune dysregulation.

KEYWORDS

fibrosis-pulmonary-histopathology-diagnosis, SARS-CoV-2, acute respiratory disease syndrome (ARDS), thrombosis, TGF-β1, TSP1/THBS1, COVID-19

Introduction

The transforming growth factor-beta (TGF-β) superfamily consists of 33 known mammalian proteins classified into subfamilies that include bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins, inhibins, and three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) (1). TGF-β1 is a ubiquitous pleiotropic cytokine expressed by numerous cell types and is well-known for its regulation of inflammation and wound healing. TGF-β1 is the dominant protein expressed in most tissues (including immune cells) whereas expression of TGF-β2 and TGF-β3 are more restricted. In this review "TGF-β" will refer to TGF-β1 unless otherwise specified. The amino acid sequence of TGF-β1 is markedly conserved between species, with 100% sequence homology between human, simian, bovine, porcine, murine, and chicken proteins. TGF-β1 shares moderate sequence homology with TGF-β2 and TGF-β3, with some overlapping functions. The phenotypes of null mice for each of these isoforms are strikingly different, suggesting key functions are distinct for each isoform. TGF-β1 null mice have the most severe phenotype, dying at 2 weeks of life from widespread inflammation, while TGF-β2 and TGF-β3 null mice each succumb to developmental abnormalities soon after birth (2–4).

In its most common form, TGF-β is inactive, or "latent" (L-TGF-β), being covalently bound to the latency-associated peptide (LAP) derived from the 5' end of its gene transcript, which shields its receptor binding sites. This forms the small latent complex (SLC). TGF-β is secreted by most cells in this SLC form. LAP can also form a disulfide bond with the latent TGF-β binding protein (LTBP, from different genes for each TGF-β isoform) forming the large latent complex (LLC) (**Figure 1**). Interestingly, mice null for LTBP also suffer from widespread inflammation (5). Latency of TGF-β is essential to sequester its pluripotent effects while also maintaining a state where it can be rapidly activated (such as during tissue injury and acute inflammation). These inactive complexes can be manipulated through a variety of pathways that lead to release of active TGF-β, including non-covalent interactions with thrombospondin-1 (TSP1) or integrins, or proteolytic degradation of the LAP by enzymes such as metalloproteinases (5–9). TSP1 is able to conformationally alter LAP through its "KRFK" motif (lysine arginine phenylalanine lysine), one of the few molecular known to activate the latent form of TGF-β (10). Other such "keys" are contained in motifs of αvβ6 and αvβ8 integrins, which typically require membrane or matrix anchoring to bind to RGD amino acid motifs (arginine glycine aspartic acid) in LAP and deform its shape (11). This post-translational regulation of all TGF-β isoforms is the main determinant of their availability, rather than from changes in gene expression. The lung is one organ with high latent TGF-β expression and protein levels, both within structural and immune cells and intercalated into the extracellular matrix (after cell secretion) (**Figure 1**; 12). Thus in most tissues but particularly in the lungs, inflammatory and other stimuli can rapidly release active TGF-β from its latent stores (13). TGF-β release in this scenario is context-dependent, but often is a key event leading to a post-inflammatory tissue healing phase of illness (1). In some contexts TGF-β has anti-inflammatory effects, whereas in other contexts persistent stimulation by TGF-β may lead to tissue fibrosis (14).

Abbreviations: ACE2, angiotensin converting enzyme 2; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BMP, bone morphogenetic proteins; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; ENaC, apical sodium channel; FN1, fibronectin; GARP, glycoprotein-A repetitions predominant (cell surface molecule); FOXP3, forehead box P3 protein (transcription factor); GDF, growth differentiation factors; HIF-1α, hypoxia inducible factor alpha; IFN, interferon; IL, interleukin; IPF, idiopathic pulmonary fibrosis; KRFK, lysine arginine phenylalanine lysine; HPL, human peripheral lung; ICU, intensive care unit; LAP, latency-associated peptide; LLC, large latent complex; LTBP, latent TGF-β binding protein; L-TGF-β, latent transforming growth factor-beta; M2, macrophage phenotype, anti-inflammatory; MDSC, myeloid-derived suppressor cells; MIS-C, multisystem inflammatory syndrome of children; NET, neutrophil extracellular traps; NK, natural killer; NOX4, NADPH oxidase 4; ORF8, protein encoded by open reading frame 8 of SARS-CoV-2 genome; PAI-1, plasminogen activator inhibitor-1; PASC, post-acute syndrome of COVID; PHD2, prolyl hydroxylase 2; RGD, arginine glycine aspartate amino acid motif; SARS, severe acute respiratory syndrome coronavirus of 2004 (2003–5); SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SIN3A, SIN3 transcription regulator family member A; SLC, small latent complex; SMAD, suppressor of mothers against decapentaplegic; TF, tissue factor; TGF-β, transforming growth factor-beta; TGF-βI, transforming growth factor-beta inhibitors; TGFBIp, TGF beta induced protein; TGFBRI, transforming growth factor-beta receptor I, aka ALK5; TGFBRII, transforming growth factor-beta receptor II; Th2, type 2 immune response; TLR, toll-like receptors; Tregs, T regulatory cells; TSP1, thrombospondin-1; WXXW, tryptophan, any amino acid, any amino acid, tryptophan motif.

TGF-β is a mediator of thrombosis

The initial physiological response in mammals to vascular injury is hemostasis which begins with platelet aggregation at the wound site. Platelets are the most abundant source of human TGF-β1 (40–100 times the level of TGF-β1 compared with other cells), and this protein is stored in its latent form in platelet alpha granules (15). Coagulation factors V, XI, and XIII, and the TGF-β activator protein TSP1 are also stored in the alpha granules (15, 16). Therefore, measuring TGF-β levels in blood samples is complex due to much higher TGF-β levels in serum (coagulated blood) compared to that in plasma (anticoagulated blood) (17). Thus, comparison of serum TGF-β levels between populations or during illnesses may be confounded by platelet counts, intrinsic platelet aggregation properties, and/or use of anti-platelet agents (17, 18). Both latent forms of TGF-β are released from platelets with the LLC and SLC conformations comprising 65 and 35%, respectively (19, 20). These latent TGF-β forms are then likely activated by co-secreted platelet enzymes (like furin), co-secreted proteins (TSP1), shear stress (which can double in

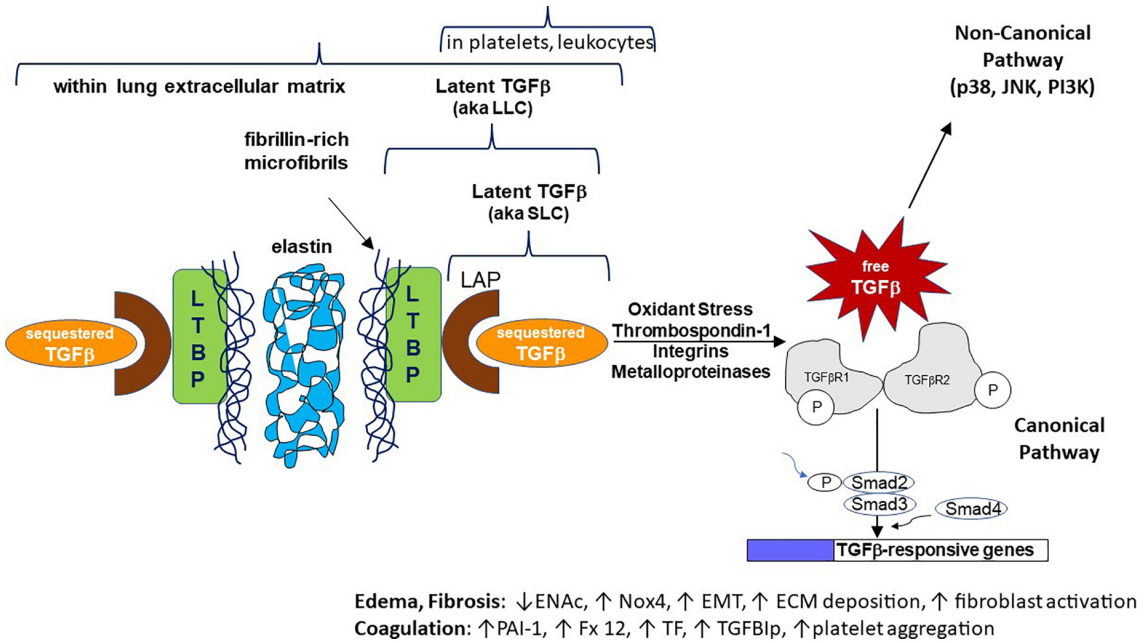

FIGURE 1
Cartoon schematic representing normal human transforming growth factor-beta (TGF-β) expression, latency, activation mechanisms, intracellular signaling, and effects on specific genes/pathways. The non-canonical signaling pathway is not discussed in this manuscript. For space purposes the binding of active TGF-β as a dimer to its tetrameric receptor complexes is not shown. ENaC, epithelial sodium channel; Nox4, NADPH oxidase 4; EMT, epithelial to mesenchymal transition; ECM, extracellular matrix; PAI-1, plasminogen activator inhibitor 1; Fx 12, coagulation factor 12; TF, tissue factor; TGFBIp, TGF-beta induced protein.

a thrombosing vasculature), or inflammation-induced enzymes like metalloproteinases (20, 21). Altered release of TGF-β from platelets has been described in several inflammatory diseases (17, 22). Notably, some cancers appear to "hijack" platelets and use TGF-β and other proteins to promote tumor growth and provide a permissive tumor environment (23).

Platelets express TGF-β receptors, so they can respond to TGF-β in an autocrine or paracrine manner (24). In normal platelets, exogenous TGF-β1 induces platelet aggregation (25). Exogenous TGF-β1 may also induce a procoagulant state *via* release of vascular or platelet stores of Factor XII, tissue factor, and plasminogen activator inhibitor-I (PAI-I) (26–28). In SARS-CoV-2 infected lung epithelial cells, the PAI-I gene (SERPINE1) is strongly upregulated (29). TGF-β also indirectly augments platelet activation *via* induction of TGF-β induced protein (TGFBIp) in platelets, respiratory epithelial cells, endothelial cells, keratinocytes, fibroblasts, and monocytes. TGFBIp is secreted and is also stored in the extracellular matrix, accumulating at sites of inflammation. TGFBIp has RGD sequences and fasciclin 1 domains that appear to bind to platelet surface integrins and activate platelets. Transgenic mice that overexpress TGFBIp are more prone to pulmonary embolism (30). Interestingly, platelets also maintain a small amount of L-TGF-β on their external surface bound to the glycoprotein-A repetitions predominant (GARP) protein. However, GARP-bound TGF-β seems more important for immune interactions rather than for coagulation (31).

Transgenic mouse models have given substantial insight into the role of platelet-derived TGF-β1 in coagulation. TSP1 null mice lose the major non-integrin mechanism by which L-TGF-β is activated. These mice have less active TGF-β in both platelets and in healing wounds, defects in platelet aggregation and wound healing, and markedly less platelet TGF-β release during aggregation (32). TGF-β1 null mice are difficult to study due to their demise *in utero* or shortly after birth, but conditional knockout of TGF-β1 in megakaryocytes (platelet precursors) reveals lower blood TGF-β1 levels and a bleeding diathesis with decreased platelet aggregation from fibrinogen receptor binding defects (25). In summation, existing evidence places TGF-β as an important orchestrator of coagulation whose role may loom larger during wound healing following inflammation. The key studies supporting this conclusion are summarized in **Table 1**.

TGF-β has critical roles in inflammation and immune regulation

Transforming growth factor-beta (TGF-β1) is the major TGF-β isoform involved in the regulation of immunity (12).

TABLE 1 Key evidence for transforming growth factor-beta (TGF-β) as a mediator of thrombosis.

References	Key findings
Assoian et al. (15)	Identification of TGFβ; description of high expression in human platelets
Huber et al. (23)	Human platelet TGFβ is principally in the latent form and is resistant to protease digestion
Grainger et al. (19)	Human platelet TGFβ exists as both the LLC and SLC forms; plasmin releases additional TGFβ from clots
Crawford et al. (16)	TGFβ in platelets is bound to thrombospondin-1 (an key activator of latent TGFβ)
Hoying et al. (25)	TGFβ enhances platelet aggregation *via* effects on the fibrinogen receptor
Dong et al. (28)	TGFβ activity correlates with PAI-I expression in vascular endothelium; TGFβ increases PAI-I expression in vascular cells
Lev et al. (24)	Platelets have TGFβ receptors; TGFβ1 does not cause direct platelet aggregation but modulates ADP-induced aggregation
Ahamed et al. (21)	Shear stress causes activation of circulating latent TGFβ (present in the LLC form), *in vivo* clotting releases TGFβ
Kim et al. (30)	TGFβ indirectly activates platelets through platelet TGFβ induced protein, which potently increases *in vivo* clotting
Jablonska et al. (26)	TGFβ induces coagulation factor XII *via* a SMAD binding element in its gene promoter
Meyer et al. (18)	Targeted genetic ablation of platelet TGFβ decreases cardiac fibrosis after injury and decreases circulating TGFβ
Maloney et al. (17)	Platelet TGFβ release is decreased in chronic inflammatory lung disease (cystic fibrosis)
Saito et al. (27)	Platelet TGFβ induces tissue factor expression

All listed studies are cited in the references section. TGF-β, transforming growth factor beta; LLC, large latent complex; PAI-I, plasminogen activator inhibitor; SLC, small latent complex; SMAD, small mothers against decapentaplegic (transcription factor named as an anagram after homologies with fly and worm genes).

Augmented TGF-β1 signaling drives a local suppression of the host immune system *via* direct inhibition of effector immune cells such as T cells, natural killer (NK) cells, and dendritic cells. This effect is amplified by activation of immune suppressor T cells known as T regulatory cells (Tregs) (33). Tregs further suppress T effector cell, dendritic cell, and NK cell function–thus limiting their responses to invading pathogens and blunting inflammation (34–36). Much of this occurs due to direct effects of SMAD transcription factors. TGF-β1 binding to its receptors leads to intracellular phosphorylation of SMAD3 and SMAD4 transcription factors, which then enter the nucleus and affect expression of multiple genes that leads to an inhibition of inflammatory responses (37). Notably, stimulated Tregs also secrete TGF-β1 with paracrine effects on neighboring cells, and keep a small amount of L-TGF-β1 on their surface bound to the GARP protein (31). On balance, these TGF-β1 driven pathways dampen inflammation on route to wound healing. The importance of these predominantly anti-inflammatory pathways is illustrated by the inflammatory phenotype of the TGF-β1 null mouse, which dies several weeks after birth from widespread inflammation in multiple organs (2).

However, TGF-β1 is pleiotropic and can also stimulate immune responses and drive inflammation through the Th17 pathway, particularly in the context of high IL-6 activity (38). TGF-β1 and IL-6 cooperatively drive induction of Th17 cells and secretion of proinflammatory IL-17 (39). Th17 pathway activation may also contribute to tissue fibrosis (40). Furthermore, IL-17 is a potent neutrophil chemokine and increased absolute neutrophil number, percentage of neutrophils, and neutrophil: lymphocyte ratio in the blood of COVID-19 patients are predictive of progression to severe disease (41).

TGF-β dysregulation in ARDS and pulmonary fibrosis

Prior to the COVID-19 pandemic, there was strong evidence that TGF-β1 is a key driver in the pathophysiology of the acute respiratory distress syndrome (ARDS) and in chronic fibrotic lung diseases such as idiopathic pulmonary fibrosis (IPF). In the early phases of ARDS, inflammatory signals likely release active TGF-β1 from latent stores in the lung extracellular. Released TGF-β1 can immediately inhibit alveolar fluid reabsorption through rapid endocytosis of the apical sodium channel (ENAc) at the airway surface of alveolar type II cells and drive oxidant stress through NADPH oxidase 4 (NOX4) induction (42–44). TGF-β1 also downregulates prolyl hydroxylase 2 (PHD2), and thereby stabilizes the transcription factor hypoxia inducible factor alpha (HIF-1α) leading to further upregulation of hypoxia-inducible genes such as mediators of endothelial permeability (45). TGF-β1 null mice die of multi-organ inflammation, including lung inflammation with features of ARDS. Thus, the complete absence of TGF-β1 and its related immune suppressive functions also favors lung inflammation, suggesting that TGF-β1 dysregulation in either direction has effects on inflammation. Interestingly, TSP1 null mice display milder chronic lung inflammation as their principal phenotype and can be rescued to a near-normal state by administration of short peptides of the L-TGF-β1 activating motif from TSP1, suggesting that TGF-β activation may be the most important constitutive function of TSP1 (16).

Transforming growth factor-beta (TGF-β1) staining is prominent in human ARDS lung, and TGF-β1 concentrations are elevated in ARDS bronchoalveolar lavage (BAL) specimens, but are minimal in BAL of healthy controls (42, 46–49).

Continued TGF-β signaling akin to what occurs in IPF may drive a small subgroup of patients to develop fibroproliferative ARDS and permanent fibrosis. To date, no TGF-β1 targeted clinical trials have been published in non-COVID ARDS.

Transforming growth factor-beta (TGF-β1) has a clear pathologic role in the chronic disease pathophysiology of IPF. TGF-β1 staining is prominent in the IPF lung, and active TGF-β1 concentrations are elevated in IPF BAL specimens, far exceeding TGF-β2 and TGF-β3 levels (whereas free TGF-β3 levels are highest in healthy lung BAL) (50). Transient adenoviral over-expression of TGF-β1 in mouse lungs causes progressive pulmonary fibrosis that becomes severe by day 60, whereas TGF-β3 overexpression displays mild histologic fibrosis that mostly resolves by day 60 (51). Of the TGF-1β isoforms, TGF-β1 has the most robust effects on differentiation of fibroblasts into activated myofibroblasts by epithelial to mesenchymal transition, upregulating TGF-β receptors, increasing connective tissue growth factor, driving collagen synthesis, and inhibiting degradation of collagenous intercellular matrix (1, 51). To date, two clinical trials of anti-TGF-β drugs in IPF have failed to show a significant clinical benefit (an anti-TGF-β antibody, and an oral integrin blocker; unpublished), while one oral agent (epigallocatechin-3-gallate) showed benefit in a single center study (52).

Viral evasion of host immune recognition is aided by "TGF-β hijacking"

The principal fitness advantage of SARS-CoV-2 occurs through gain-of-function mutations of its spike protein that enhance infectivity *via* stronger binding to the angiotensin converting enzyme 2 (ACE2) cell entry receptor. While the main immune alteration in response to severe SARS-CoV-2 infection is the tendency toward a pro-inflammatory state, in part triggered by the spike protein, it is less clear how SARS-CoV-2 can escape immune detection during early infection. This leads to its unique asymptomatic carriage in many hosts, only to cause inflammatory illness much later post-exposure than other respiratory viruses.

Curiously, mechanisms to increase TGF-β tissue levels have been exploited by microbes for eons as a path to evade host immune detection, to increase infectivity, and to achieve latency/chronic infection. All of these paths augment a microbe's reproductive fitness. Even an animal as primitive as a sea anemone employs TSP1 motifs to regulate the activation of L-TGF-β that permits its primitive immune system to tolerate symbiotic algae colonization. Mutation of these motifs leads to rejection of algae and death of the anemone (35, 53). L-TGF-β1 is highly expressed in airway and alveolar epithelium, alveolar macrophages, and in the lung intercellular matrix where inhaled microbes encounter host defenses during their pulmonary phase (12). Augmented TGF-β1 signaling initially drives a local suppression of the host immune system *via* direct inhibition of effector immune cells like T cells and dendritic cells, an effect amplified by activation of immune suppressor Tregs. Tregs further suppress the function of T effector cells, dendritic cells, and NK cells–thus limiting their responses to the invading pathogen (34–36). These effects typically occur due to direct binding of SMAD transcription factors, activated by TGF-β receptors, to gene promoters of IL-2, IL-10, forkhead box P3 protein (FOXP3) and other key immune cytokines and transcription factors (37). TGF-β1 also drives class switching of plasma cells from IgM and IgG to IgA antibody formation even outside mucosal barriers. Since IgA does not fix complement, it is less effective at directing immune responses to an invading pathogen (54).

Augmentation and exploitation of host TGF-β activity (known as "TGF-β hijacking") by microbes occurs by various mechanisms. Since no known pathogen synthesizes TGF-β, host TGF-β must be hijacked from the host. The hepatitis C virus increases host TGF-β and TSP1 gene expression, increasing activation of latent TGF-β (55). *Trypanosoma cruzi* also increases host TSP1 (56). The influenza virus produces a protease that degrades LAP and thereby releases active TGF-β (57). The SARS 2004 (aka SARS-CoV) virus nucleocapsid protein associates with the TGF-β intracellular signaling transcription factors SMAD2 and SMAD3 and augments TGF-β induced gene transcription (58). Malaria and cryptosporidium parasites produce a TSP-like protein that activates latent TGF-β through TSP1-like WXXW domains (tryptophan, any amino acid, any amino acid, tryptophan) that inhibit the LAP-TGF-β interaction (59, 60). Schistosoma induces host monocyte TSP1 to activate pulmonary TGF-β as a contributor to the pulmonary vascular disease typical with this chronic infection (61, 62). Mitigating the TGF-β exploitation by these microbes consistently leads to decreased infectivity. For example, transgenic mice specifically lacking bronchial epithelial cell TGF-β1 display robust protection from influenza-induced weight loss, airway inflammation, and tissue injury; these airway null mice also have a heightened antiviral immune response (63). There is emerging evidence that SARS-CoV-2 has also evolved to exploit TGF-β and increase its infectivity. Parallel immunoprecipitation studies of SARS-CoV-2 or SARS-CoV-infected human respiratory epithelial cells (A549) reveal a strong and exclusive interaction of the open reading frame 8 (ORF8) protein of SARS-CoV-2 with TGF-β1, TGF-β2, TGFBR2, LTBP1 (and thus latent TGF-β1) that was not seen with SARS-CoV infection. The mechanism for these interactions is unclear, as the small ORF8 protein has no known amino acid sequences for activating LAP, LTBP, or for TGF-β isoform binding such as KRFK, RGD, or WXXW motifs. Whether the ORF8 interaction leads to TGF-β1 activation is unknown (29). Moreover, the TGF-β induced genes fibronectin (FN1) and plasminogen activator inhibitor (PAI-I, SERPINE1 gene) were prominently

upregulated, but this was not seen with SARS-CoV infected cells. Another study found that phosphorylation and activation of the TGF-β pathway is prominent in SARS-CoV-2 infection (64). These findings suggest that exploitation of host TGF-β is a key mechanism of SARS-CoV-2 infection, and that inhibition of this pathway is a promising treatment for SARS-CoV-2 infection.

Well-characterized immune responses that occur during SARS-CoV-2 infection include aberrant activity of both the innate and adaptive immune systems (65). Cell-mediated innate immunity functions by activating toll like receptors (TLRs) and triggering interferon (IFN) production at sites of infection (66). Cytokines released by pro-inflammatory macrophages also play a key role in antiviral defense, activating T cells which then produce IFN-gamma and cytokines that activate more macrophages, driving a loop of anti-viral inflammation (67, 68). One hypothesis is that interference with IFN production is a key perturbation that allows SARS-CoV-2 to replicate freely during early infection. In later stages of severe disease, a rebound IFN response can be exaggerated–leading to cytokine storm and inflammation. Complement is also activated in SARS-CoV-2 and animal studies have shown that inhibition of complement may be beneficial during infection (69). The formation of neutrophil extracellular traps (NETs) is critical to the disruption of the microvasculature, with an imbalance in formation and degradation driving inflammation and occlusive NET-derived immunothrombosis (70). Adaptive immunity also plays a key role, with B cells producing multiple classes of antibodies. The production of these antibodies does not occur until at least day 7–8 of infection, and delays in antibody production have been associated with more severe COVID-19 illness (54). T cells are also activated with a predominance of CD4+ and CD8+ cells. Novel T cell responses are seen in SARS-CoV-2 infection, with increased T cell death by apoptosis and increased syncytia activity, presumably induced by SARS-CoV-2, which when internalized can kill T cells. It has also been observed that highly active CD16+ T cells are linked to microvascular endothelial cell injury and these cells can persist after acute infection which may contribute to long COVID. Other key players include pro-fibrotic CD163-expressing monocyte derived macrophages, and NK cells that exhibit impaired anti-fibrotic function (65). This review will focus on the multi-faceted TGF-β response seen with SARS-CoV-2 infection.

Linking TGF-β activation with thrombo-inflammatory responses in COVID-19

SARS-CoV was a much less prevalent coronavirus that also demonstrated TGF-β dysregulation, with frequent ARDS and pulmonary fibrosis, but it caused less than a thousand deaths (71). Using human peripheral lung epithelial (HPL) cell models infected with SARS-CoV, Zhao et al. found high levels of TGF-β expression, leading to increased plasminogen activator inhibitor-1 (PAI-1) related tissue fibrosis (58). This mechanism of TGF-β induced expression of PAI-1 has been well-described in prior models of lung fibrosis (72). In this same model of HPL cells, low levels of SMAD3/4 TGF-β induced apoptosis were found, leading to persistence of virus infected cells. The SARS-CoV papain-like protease was also shown to induce TGF-β signaling including fibrotic pathways in lung epithelial cells and blood monocytes (73, 74). Elevated levels of TGF-β1 in blood and high expression in autopsied lung were also reported in SARS 2003 patients (75, 76). Therefore, it is thought that TGF-β played an important role in the pathogenesis of SARS-CoV by promoting replication and proliferation of the virus and contributing to coagulation dysregulation and lung fibrosis.

SARS-CoV-2 infection has also been associated with dysregulation of the TGF-β pathway, resulting in increased TGF-β levels and pathway activity. Signatures of increased TGF-β activity in COVID-19 patients include increased plasma and serum TGF-β1 levels (77, 78), increased RNA levels of TGF β-induced protein in the blood of ARDS patients (79), plasmablast and monocyte TGF-β signatures in blood (80), increased IgA class switching pathognomonic of a TGF-β effect (81), and TGF-β mediated suppression of circulating NK cells (78). Furthermore, increased blood TGF-β levels were characteristically correlated with increased disease severity (80, 82). Strikingly, myeloid-derived suppressor cells (MDSC) from COVID-19 patients suppress T effector cells but this effect can be blocked with a neutralizing antibody to TGF-β, and this TGF-β antibody also rescued interferon responses in MDSC to SARS-CoV-2 antigens, where the number of MDSC also correlated with plasma TGF-β1 levels (83).

Another function of TGF-β is to dampen excessive immune responses and maintain homeostasis. This effect is unfortunately aberrant in SARS-CoV-2 infection due to the timing of its expression. In the sera of patients with severe COVID-19 infections, TGF-β gene expression was found to peak during the first 2 weeks of infection (78). At this time, NK cells, a key part of the innate immune system with activity against RNA viruses, were found to be inhibited in a TGF-β-dependent fashion. After pretreatment with TGF-β blocking antibodies, NK cell function recovered (78). This shows that it is not only the expression, but also the timing of TGF-β responses that allows the virus to proliferate, undetected and undisturbed by the immune system. In parallel, TGF-β1 could also contribute to inflammation through Th17 pathway activation in an IL-6 rich environment such as in the pulmonary phase of COVID-19 (67, 84–86). Interestingly, a subset of SARS-CoV-2 infections leads to a multisystem inflammatory syndrome in children (MIS-C; can also occur in adults) similar to Kawasaki's disease–a disease associated with common genetic variation in the TGF-β pathway (87, 88).

Evidence of TGF-β as a mediator of lung microvascular injury and thrombosis during COVID-19

Most COVID-19 deaths occur from ARDS. While ARDS was also the major cause of death in SARS 2004, our understanding of lung pathophysiology in the SARS 2004 pandemic was limited by its brief duration and limited mortality (89). Due to its longer pandemic duration and an extensive number of investigations, substantial insights into COVID-19 ARDS have been made. Many of these insights resulted from its comparison to data from investigations of ARDS due to influenza, bacterial, and other etiologies (designated here as non-COVID ARDS). Compared to non-COVID ARDS, COVID-19 ARDS has evidence of more extensive microvascular thrombosis (in skin and lung) and a higher propensity to cause lung fibrosis (90, 91).

Microvascular clotting and venous thromboembolism are more common in COVID ARDS (90). Empiric treatment dose anticoagulation has been shown to benefit hospitalized COVID-19 patients outside of intensive care units (ICU), but not in severely-ill patients–suggesting that full dose anticoagulation may come too late in these patients to be of benefit (this subgroup still benefits from prophylactic anticoagulation) (92). The severity of microvascular clotting in COVID-19 ARDS has been surprising, with extensive clotting in lung and skin far surpassing that seen in non-COVID ARDS, with no clear explanations (93, 94).

In one study, postmortem lung samples and antemortem plasma samples were collected from a cohort of COVID-19 ARDS patients. In these samples, TGF-β expression and blood TGF-β1 levels paralleled PAI-1 expression in fibrotic areas of lung and in the endothelium of alveolar septal capillaries and blood vessels (95). PAI-1, a potent inhibitor of fibrinolysis, would be expected to promote clotting in these regions (28). This correlation of TGF-β with PAI-1 highlights an imbalance between prothrombotic and antifibrinolytic pathways in COVID-19. In autopsy studies of patients who died from COVID-19 versus those who died of causes without ARDS, there was a significant increase in expression of TGF-β1 in COVID-19 lungs (96). This TGF-β increase was associated with activation of the Th2 pathway, since increased immune expression of sphingosine-1 (M2 macrophages) and IL-4 were found. M2 macrophages are known to stimulate TGF-β which, in turn, potentiates tissue fibrosis and ineffective pathways for viral clearance. Most COVID-19 autopsy studies have not reported TGF-β staining.

Elevated blood TGF-β1 levels are described in multiple reports of COVID-19 cohorts and may also be a biomarker of COVID ARDS severity. Colarusso et al. described higher plasma TGF-β1 levels and higher stimulated release of TGF-β1 from blood monocytes in patients with signs of lung fibrosis on imaging (97, 98). Ghazavi et al. found increased serum TGF-β1 levels in COVID-19 patients that correlated positively with disease severity and serum IL-17 (82). Serum TGF-β1 levels have also been show to correlate with circulating monocyte phenotype in severe COVID-19 (99). In another study, blood levels of TGF-β-induced protein (TGF-βIp) were markedly elevated in severe COVID-19 (79). This protein is of particular interest as its transgenic overexpression worsens pulmonary emboli in a murine model of venous thromboembolism (30). The cause of increased blood TGF-β1 and activity in COVID-19 is likely in large part related to elevated pro-inflammatory cytokines and oxidant stress. IL-1 and IL-17 are proinflammatory cytokines that are typically elevated in blood of severe COVID-19 patients. IL-6 increases TGF-β1 expression, and IL-17 increases TGF-β receptor II expression (100, 101). TGF-β-induced epithelial-to-mesenchymal transition (EMT) *via* upregulation of NOX4 expression and activity that is augmented by intracellular oxidant stress (102). Thus, inhibition of TGF-β production by IL-6 may be one mechanism underlying the survival benefit seen with IL-6 inhibitor administration during severe COVID-19 (84). Likewise, corticosteroids improve survival in hypoxic COVID-19 patients, and steroid effects include suppression of TGF-β1 gene expression (103, 104).

Assessment of airspace levels of TGF-β or airway immune cell TGF-β expression during COVID-19 ARDS have been limited to a few reports based on understandable desire to limit aerosolization of sputum from COVID-19 patients to research or clinical personnel. While multiple groups have reported on RNA expression or inflammatory cell distributions within BAL fluid from mechanically ventilated COVID-19 patients, to our knowledge none have reported an assessment of TGF-β protein levels in BAL fluid or other respiratory specimens. Grant et al. collected BAL from 88 COVID-19 patients and reported RNA sequencing results on 10 specimens and cell subset analyses by flow cytometry; in this study TGF-β was not reported as a dysregulated gene nor was a TGF-β driven gene signature reported among findings of T cell and macrophage drive alveolar inflammation (68). Ronit et al. studied lymphocyte subsets and a limited cytokine panel from BAL of four COVID-19 patients and found an inflammatory pattern, but TGF-β was not a target of the assays (105). Xiong et al. reported that RNA sequencing results from two COVID-19 BAL specimens demonstrated significantly increased TGF-β2 gene expression in BAL cells compared to three healthy controls (106). Zhou et al. reported RNA sequencing results from cells of eight COVID-19 BAL specimens compared to healthy control and non-COVID pneumonia patients, but TGF-β genes were not reported as dysregulated. Ferreira-Gomes et al. reported day 31 and day 46 BAL cell analysis from one mechanically-ventilated COVID-19 ARDS patient and found persistently high TGF-β protein in T cells (80). Since TGF-β availability is regulated at the post-transcriptional level, measurement of active TGF-β levels in lung secretions and lung tissue will be important to assess.

Cell-based models of respiratory epithelial cell infection with SARS-CoV-2 have provided additional insights into the role of TGF-β in epithelial cell injury. In an interactome study, human A549 lung epithelial cells transfected with individual SARS-CoV-2 proteins showed a prominent and exclusive binding of the viral ORF8 protein with TGF-β1-2 isoforms, TGF-β receptor 2 (TGFβR2), and L-TGF-β (29). This finding was unexpected, as the small ORF8 protein has no known protein sequences for LAP, LTBP, or TGF-β isoform binding such as KRFK, RGD, or WXXW motifs (107, 108). Moreover, known TGF-β1 induced genes fibronectin and PAI-1 were upregulated.

A recent animal model of COVID-19 used the MA10 mouse strain to evaluate longitudinal changes in lung pathology with a non-lethal SARS-CoV-2 dosing approach. This model revealed a post-transcriptional pattern of increased alveolar TGF-β1 pathway signaling (such as fibronectin and type I collagen upregulation) at days 15 and 30 post-infection. Moreover, increased TGF-β1 gene transcription was seen in areas of residual fibrosis at 15–120 days after clearance of viral infection (109).

Evidence of TGF-β as a mediator of skin microvascular injury and thrombosis during COVID-19

COVID-19 is associated with variable dermatologic presentations with vasculopathies being common. The rich capillary supply network of skin and its safe and feasible biopsy have given important insights into the microthrombotic and inflammatory features of COVID-19. Of particular interest is the ability of skin biopsies to give information on living COVID-19 patients. In comparison, study of tissues such as lung or heart microvascular injury during COVID-19 are mostly limited to animal or human autopsy studies.

Prevalence estimates for COVID-19 skin disease are confounded by the predominance of case reports, nevertheless, data from reviews suggest that dermatologic microvascular damage may be present in up to 20–25% of cases, and in almost all severe cases (110). Of these, the most frequently reported conditions are chilblain-like vasculitic acral lesions (pernio-like lesions, "COVID toe") and reticular lesions such as livedo reticularis and retiform purpura, typically associated with microvascular clotting (110). Several pathophysiologic mechanisms have been implicated in the development of these cutaneous microvascular lesions. In one study, microthrombosis was seen in 87% of premortem skin biopsies of severe COVID-19 cases with ARDS, but no microthrombi were seen in the premortem skin biopsies of mild COVID-19 cases or in skin from ARDS patients with non-COVID etiologies (111). As to inflammatory mediators in COVID-19 affected skin, terminal complement C5b-9 and MASP2 (lectin complement pathway) deposition are prominent in skin biopsies of severely ill COVID-19 patients with livedoid or purpuric cutaneous reactions that clinically suggest vasculitis. Strikingly, deposition of C5b-9 has also been demonstrated in biopsies of apparently normal skin–where its deposition tracks COVID-19 severity (highest in COVID-19 ARDS), while such C5b-9 deposition is absent from the skin of patients with non-COVID ARDS (69, 111). This complement deposition damages the endothelium resulting in further clotting cascade activation and a pro-coagulative state, likely explaining the tight correlation of complement deposition with micro-thrombosis. Complement activation has also been demonstrated in acro-ischemic chilblain-like lesions frequently seen in milder COVID-19 cases and in younger patients (112).

Prominent expression of the transcription factor SIN3A has been demonstrated in premortem skin biopsies of severe COVID-19 patients, where it also correlates with disease severity (111). The potential significance of this finding in the context of TGF-β is that SIN3A is known to co-localize with SMAD transcription factors on TGF-β driven gene promoters, suggesting it may have a cooperative effect with TGF-β on the genesis of microvascular injury during COVID-19 (113). To our knowledge, no biopsy studies have directly evaluated levels of skin TGF-β protein or RNA expression in COVID-19. At autopsy, skin is among the highest ACE2 receptor expressing organs in COVID-19 patients (second only to the lungs) (69). Binding of spike protein ("pseudovirus") to dermal microvessel ACE2 also correlates strongly as a signal for subsequent complement deposition and inflammation (110, 114). These reports demonstrate unique pathophysiologic features linked with cutaneous microvascular damage, overall disease severity, and patient outcomes. This evidence suggests an interplay of several inflammatory pathways in the pathogenesis of microvascular skin conditions in COVID-19. Further high-quality clinical studies are needed to fully elucidate the pathophysiology of these microvascular manifestations as well as their clinical significance in relation to overall COVID-19 severity and treatment options. Importantly, skin biopsies in living subjects remain a feasible and minimally invasive technique to better understand the longitudinal microvascular pathophysiology of COVID-19, particularly related to investigation of TGF-β pathways. Importantly, anti-TGF-β therapies have been shown to have dramatic effects on TGF-β driven genes in analyses of skin biopsy from clinical trial subjects with scleroderma (115). Analysis of skin biopsies from COVID-19 patients treated with anti-TGF-β therapies also promise to be a useful, safe, and feasible window in understanding the effects of these therapies on microvascular injury due to inflammation and clotting.

These data show that TGF-β is a key mediator in the pathogenicity of COVID-19 and likely vital to the virus's asymptomatic carriage that contributes to high viral loads and increased viral spread, delayed progression to severe illness, and transition in a subset of patients into a pro-inflammatory, pro-fibrotic final phase of illness with prominent microvascular clotting. A summary of the key evidence for this paradigm during COVID-19 illness is listed in **Table 2** and illustrated by **Figure 2**.

TABLE 2 Key evidence for transforming growth factor-beta (TGF-β) as a mediator of COVID-19 immune dysregulation and thrombosis.

References	Key findings
Park et al. (79)	TGFβIp acetylated at Lysine 676 is a diagnostic marker of severity in SARS-CoV-2 pneumonia
Sacchi et al. (83)	Polymorphonuclear myeloid-derived suppressor cells inhibit T-cell IFN-λ production during SARS-CoV-2 peptide stimulation through TGFβ mechanisms
Ghazavi et al. (82)	Serum TGFβ levels increase with increasing disease severity in patients with COVID-19
Kvedaraite et al. (99)	Serum TGFβ levels correlate with circulating monocyte phenotype in severe COVID-19
Ferreira-Gomes et al. (80)	SARS-CoV-2 triggers an immune response instructed by TGFβ, including IgA class switching
D'Agnillo et al. (95)	TGFβ1 staining co-localizes with PAI-1 in pulmonary blood vessels and developing fibrotic lesions in COVID-19 lung tissue
Witkowski et al. (78)	Serum levels of TGFβ peak during the first 2 weeks of severe COVID-19 infection and inhibit NK cell function
Colarusso et al. (97)	Higher TGFβ levels predict an increased relative risk of lung fibrosis-like changes in post-COVID patients
Vaz de Paula et al. (96)	Immuno-expression of TGFβ1 is increased in lung tissue of patients who died of COVID-19
Dinnon et al. (109)	Transcriptomic analysis of diseased alveolar regions shows up-regulated signaling by the TGFβ receptor complex in mouse-adapted SARS-CoV-2
Colarusso et al. (98)	TGFβ has higher stimulated release in post-COVID patients with signs of lung fibrosis on chest computed tomography scans

All listed studies are cited in the references section. TGF-β, transforming growth factor beta; LLC, large latent complex; NK, natural killer; PAI-I, plasminogen activator inhibitor; TGFβIp, TGFβ induced protein.

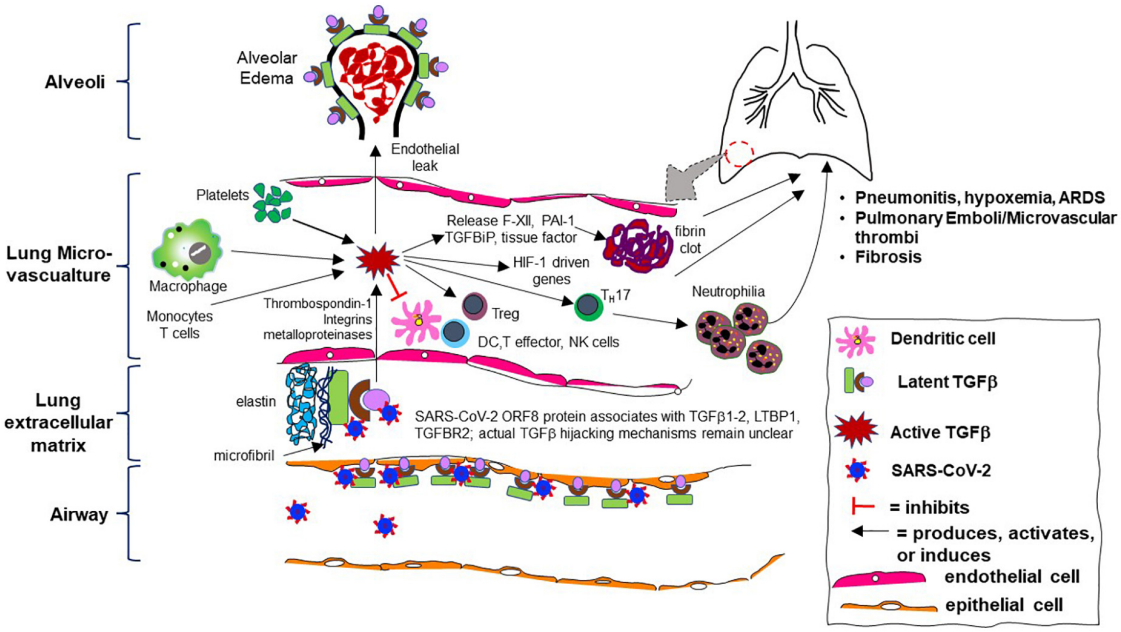

FIGURE 2
Cartoon schematic of aberrant transforming growth factor-beta (TGF-β) pathway activity during SARS-CoV-2 infection in the human lung microvasculature and its consequences. Skin, kidney, and other organ involvement are not depicted but similar mechanisms likely occur.

TGF-β pathway inhibitors may ameliorate TGF-β driven thrombosis and immune dysregulation in COVID-19

Given the substantial evidence that SARS-CoV-2 stimulates host TGF-β pathways, TGF-β antagonism arises as a potential therapeutic target for improving outcomes during COVID-19 illness. Such an approach was suggested by multiple investigators early in the COVID-19 pandemic (80, 108, 116, 117). Over a dozen TGF-β inhibitors (TGF-βi) have been evaluated in human clinical trials of cancer and inflammatory diseases. Thus given the evidence presented above, the TGF-βi space is ripe for immediate repurposing for animal "proof of principle" studies and subsequent clinical trials of COVID-19 (**Table 3**; 118). A number of robust animal models exist for

COVID-19, allowing rapid assessment of candidate TGF-βi for efficacy and safety to identify agents worth promoting to human clinical trials (119–122).

To date, the majority of TGF-βi have failed to benefit primary outcomes in phase I-II clinical trials of various cancers and inflammatory diseases (123–127). For some agents, recognition of concerning adverse events limited further development (127, 128). None of these studies evaluated thrombosis as an outcome. These existing TGF-βi target multiple points in the TGF-β pathway (**Table 3**). TGF-β receptor inhibitors are typically small molecule oral drugs that inhibit intracellular TGF-β receptor I (TGFBRI, also known as ALK5) or TGF-β receptor II (TGFBRII) kinase activity, including catechin polyphenol derivatives of green tea extracts (52, 126). Antibodies to TGF-β ligand (both to individual and to all TGF-β isoforms) also exist, as do ligand traps (124, 129–131). Agents targeted at preventing L-TGF-β activation such as integrin inhibitors have already entered clinical trials of inflammatory diseases (125), and antibodies and small molecules blocking L-TGF-β are in preclinical development (132, 133). Angiotensin converting enzyme inhibitors and angiotensin-II receptor blockers such as losartan has been evaluated in clinical trials of COVID-19 (NCT04312009 and NCT04312009) given their low cost, worldwide availability as approved drugs for hypertension and heart failure, and their potential modulation of surface expression of the ACE2 receptor where the SARS-CoV-2 spike protein binds for its cellular uptake route. These agents are also known to have off-target effects *via* decreased TGF-β signaling through inhibiting synthesis of TSP1 and other mediators that increase active TGF-β (134). They have been used to slow TGF-β mediated thoracic aortic aneurysm progression in Marfan's disease with evidence for improved outcomes (135). To date, losartan has not been shown to improve outcomes in COVID-19 (136). As these agents are not specifically directed at TGF-β, they are not a subject of this review.

Antisense RNA agents targeting TGF-β1 or TGF-β2 are also in development or in clinical trials (137). Notably, while only one of these agents is FDA-approved, in most cases these agents could undergo a rapid production phase as most have already completed phase I trials. These would be reasonable agents to test in animal models of COVID-19, selecting promising agents for further evaluation in human trials. One TGF-βi, luspatercept, is an FDA-approved TGF-β "trap" that is already marketed worldwide in a subcutaneous formulation to decrease red cell transfusion needs in thalassemia. In thalassemia, marrow production of red blood cells is inhibited by TGF-β (129). Pirfenidone and nintedanib are an antifibrotic agents that have anti-TGF-β properties and are approved in many countries for the treatment of idiopathic pulmonary fibrosis. Pirfenidone works in part by inhibiting furin, an enzyme that activates TGF-β from its precursor form. Furin is also necessary for SARS-CoV-2 internalization (138). A phase 2 trial of pirfenidone in 146 inpatients with acute COVID-19 (NCT04282902) did not meet its primary endpoint for change in pulmonary-related quality of life scoring at week 4, but improvements in secondary outcomes such as decreased proinflammatory cytokines, decreased coagulation biomarkers, and decreased hospital days were reported (139). Pirfenidone is currently in a phase II clinical trial for post-COVID-19 pulmonary fibrosis (NCT04607928). Nintedanib is also being evaluated in post-COVID fibrosis in several phase III-IV trials (NCT04541680 and NCT04619680). To date, only one clinical trial of a specific TGF-βi for use during COVID-19 illness has begun and is ongoing. This trial tests the effect of trabedersen, an antisense RNA for TGF-β2 (137). Trabedersen has been repurposed for evaluation in COVID-19, a decade after an initial clinical trial in brain tumors did not show improved outcomes (140). The ongoing phase 2 double blind placebo-controlled trial in South America of hospitalized severe COVID-19 patients involves a 7-day intravenous infusion of trabedersen or placebo in combination with the oral anti-malarial drug artemisinin (which binds to TGF-βR2) (141); results have not been published (NCT04801017).

Transforming growth factor-beta (TGF-β) inhibition will also have direct or indirect effects on other cytokines that should on balance be beneficial during COVID-19 illness. TGF-β1 drives proinflammatory Th17 cell production (38), and Th17 cell number and activity (including IL-17 and IL-23 secretion) are increased during COVID-19 illness (86, 142). TGF-β1 also indirectly upregulates HIF-1α induced genes *via* decreased expression of PHD2, which leads to an increase in intracellular HIF-1α levels (since PHD2 normally leads to hydroxylation and ubiquitination/destruction of HIF-1α) (45). Thus, HIF-1α driven genes such as vascular endothelial growth factor, erythropoietin, heme oxygenase-1, and glucose transporter 1 would be expected to decrease with TGF-β i.

Scope and limitations of using TGF-β inhibitors and antisense RNA agents

The best timing of administration of TGF-βi in this paradigm remains unclear. Existing data suggests that SARS-CoV-2 exploitation of TGF-β and related immune evasion most likely occurs during early infection (week 1) when a milder illness is present. During this early stage, an immune reconstitution driven by TGF-βi administration would strengthen anti-viral immunity and be less likely to have adverse effects. However, even after the onset of fever and a proinflammatory state or cytokine storm in more severe COVID-19 cases (correlating with immune recognition of infection, typically week 2 post-infection), evidence of ongoing TGF-β activity persists–such as IgA antibody class switching, high TGF-β blood levels, Th17 pathway activation, and the onset of lung fibrosis (54, 80, 86, 97, 105, 143). Thus, TGF-β pathway activity may remain high over most of

TABLE 3 Transforming growth factor-beta (TGF-β) pathway inhibitors (approved, currently in clinical trials, or preclinical) potentially relevant to COVID-19 prevention or treatment.

Drug	Manufacturer	Route	Mechanism	Status diseases	Clinical trials	Clinical trial stage/Findings (as of 8/2022)
GSK3008348	Glaxo	Inhaled	αvβ6 integrin inhibitor	Investigational lung fibrosis	NCT02612051 NCT03069989 NCT02612051	Phase I Ongoing or completed/ Unpublished
PLN-74809	Pliant Therapeutics	Oral	Small molecule αvβ6/αvβ1 integrin inhibitor	Investigational lung fibrosis cholangitis	NCT04072315 NCT04396756 NCT04480840	Phase IIa Ongoing or completed/ Unpublished
MORF-720	Morphic (AbbVie)	Oral	Small molecule αvβ6, 4β7 integrin inhibitors	Pre-clinical fibrosis cancer	NA	NA
LY2157299 (Galunisertib)	Eli Lilly	Oral	Small molecule, TGFβRIi	Investigational cancer	Of 25 trials: NCT02304419 NCT02008318	Phase 1–3 Well-tolerated, benefit seen in phase 2 studies
LY573636 (Tasisulam)	Eli Lilly	IV	Small molecule, TGFβRIi; long half-life	Investigational cancer	Of 13 studies: NCT00992225	Phase 2 Hepatic and marrow toxicity
LY3200882	Eli Lilly	Oral	Small molecule, TGFβRIi	Investigational cancer	Of 4 trials: NCT04031872 NCT02937272	Phase 1–2 Well-tolerated
Vactosertib (TEW7197)	Bristol Myers Squibb (Forbius)	Oral	Small molecule, TGFβRIi	Investigational cancer	Of 17 trials: NCT03074006 NCT04515979	Phase 2 Ongoing or completed/ Unpublished
SRI-35241	Southern Research	IV	Small molecule Inhibits activation of latent TGFβ	Preclinical fibrosis	Preclinical	Preclinical
Epigallocatechin Gallate (ECGC)	Multiple producers	Oral	Green tree extract Catechin Kinase inhibitor	Investigational lung fibrosis many diseases	Of 104 trials: NCT03928847 NCT03928847	Marketed as over-the-counter supplement (FDA approval not required)
AVID200	Bristol Myers Squibb (Forbius)	IV	TGFβ 1/3 trap	Investigational cancer skin fibrosis myelofibrosis	NCT03831438 NCT03834662 NCT03895112	Phase 2 Well-tolerated, biologic effects demonstrated *in vivo*
Luspatercept	Acceleron (Merck)	SQ	TGFβ trap (ACtRIIb)	FDA-approved myelofibrosis thalassemia	Of 25 trials: NCT02604433 NCT04717414	Marketed after phase 3 trials Improves hemoglobin levels Other indications being tested in clinical trials
Sotatercept (ACE-001)	Acceleron (Merck)	SQ	TGFβ trap (ACtRIIa)	Investigational pulmonary hypertension	Of 21 trials: NCT03496207 NCT04896008	Phase 2
Bintrafusp-α (M7824)	EMD Serrano (Merck-GSK)	IV	Bifunctional Ab: TGFβ-trap, aPDL1	Investigational cancer	Of 46 trials: NCT02517398 NCT04727541	Phase 1–2; ongoing or completed Tolerable (rash, pruritis)
Dalutrafusp-α (GS-1423)	Gilead	IV	Bifunctional Ab: aCD73, TGFβ-Trap	Investigational cancer	NCT03954704	Phase 1, terminated

(Continued)

TABLE 3 (Continued)

Drug	Manufacturer	Route	Mechanism	Status diseases	Clinical trials	Clinical trial stage/Findings (as of 8/2022)
Trabedersen AP12009 OT-101	Autotelic (Isarna)	IV topical	Antisense RNA to TGFβ2	Investigational cancer COVID-19 myopia	NCT00844064 NCT00431561 NCT00761280 NCT04801017	Phase 2–3 Ongoing or completed/ Unpublished
ISTH0036	Isarna Therapeutics	Eye	Antisense RNA to TGFβ1	Investigational glaucoma AMD	NCT02406833	Phase 2, Ongoing
TRK-250	Toray Industries	Inhaled	Antisense RNA to TGFβ1	Investigational Lung fibrosis	NCT03727802	Phase 1, Ongoing
XOMA089	Novartis	IV/SQ	TGFβ 1-3 Ab (all isoforms)	Preclinical	NA	NA
LY2382770	Eli Lilly	SQ	TGFβ1 Ab	Investigational kidney disease	NCT01113801	Phase 2, completed No clinical benefit to date Well-tolerated
Fresolimumab (GC1008)	Sanofi	IV	TGFβ 1-3 Ab (to all isoforms)	Investigational cancer fibrosis	Of 12 trials: NCT00125385 NCT01665391	Phase 2; completed No clinical benefit to date Skin toxicity
SRK-181	Gilead (Scholar-Rock)	IV	Latent-TGFβ1 Ab	Investigational cancer	NCT04291079	Phase 1, Ongoing
NA	Merck (Tilos)	IV	Anti-LAP integrin Ab	Pre-clinical cancer	NA	NA
BG00011 (STX100)	Biogen	SQ	anti-αvβ6 integrin Ab	Investigational Lung fibrosis	NCT01371305	Phase 2, unpublished Trials stopped early 2017
VTX-001	Venn Therapeutics	IV/SQ	anti-αvβ6 integrin Ab	Pre-clinical fibrosis	None to date	NA

Some pre-clinical agents and other agents with halted development are not shown. Only one drug is FDA-approved (luspatercept), and only one drug is currently in COVID-19 clinical trials (trabedersen). Ab, antibody; ACtRIIa, activin receptor IIa; ACtRIIb, activin receptor IIb; AMD, adult macular degeneration; aPDL1, anti-programmed death ligand 1; IV, intravenous; LAP, latency activating peptide; NA, not applicable; SQ, subcutaneous; TGF-β, transforming growth factor beta; TGFβIR, TGF-β receptor I, also known as ALK5; TGFβR2 trap, Fc-based compound containing copies of TGF-β receptor II; TGFβRIi, TGF-β receptor I inhibitor.

the illness course, particularly in severe cases and during lung fibrosis. During these later inflammatory stages, an immune reconstitution driven by TGF-βi administration could trigger adverse inflammatory effects. For example, in one study of cancer patients given an antibody to TGF-βRII, a cytokine release syndrome developed that limited further drug development and suggested that immune stimulation occurred with this agent (144). However, such adverse events have been rare with TGF-βi and may be ameliorated in COVID-19 by concomitant therapies such as steroids and anti-inflammatory agents that are already given in hospitalized patients with severe illness requiring oxygen therapy (84, 103). Current studies are insufficient to implicate TGF-β in the "long covid" syndrome, also known as the post-acute syndrome of COVID (PASC). However, Colarusso et al. have demonstrated elevated blood levels of TGF-β1 and stimulated blood monocyte release of excessive TGF-β1 in patients with persistent lung abnormalities on computed tomography scans months after clearance of SARS-CoV-2 (97, 98, 145). If clinical trials of TGF-βi are shown to be effective in acute COVID-19, it will be important to assess their effect on PASC.

Any salutary effects of TGF-βi on COVID-19 induced thrombosis would have to be additional to those already proven for the standard therapeutic anticoagulant heparin (92). Moreover, due to TGF-β's pluripotent effects on inflammatory and immune mediators, separating out specific TGF-βi effects on thrombosis will be difficult in the greater context of its more direct effects on immune function and inflammation. Moreover, unexpected side effects could occur with TGF-βi given the panoply of genes and processes that TGF-β regulates.

Summary

Thrombosis, inflammation, and immune dysregulation are all key events that contribute to microvascular injury and adverse outcomes during SARS-CoV-2 infection. Increasing evidence suggests that activation of the TGF-β pathway

is a prominent feature of the microvascular clotting and inflammatory injury characteristic of SARS-CoV-2 infection. Given the substantial evolution seen in other viruses and pathogens where enhancement of their fitness is achieved by TGF-β hijacking, a paradigm exists where SARS-CoV-2 elicits initial immune evasion by increasing activation of host TGF-β. Ongoing TGF-β activation then promotes collateral downstream adverse effects during the inflammatory phase of COVID-19 such as continued immune dysregulation, inflammatory injury including Th17 pathway activation, and fibrosis. These reports suggest that TGF-β pathway inhibitors may be a useful approach to improve outcomes in SARS-CoV-2 infection. One such inhibitor is already being tested in a clinical trial of COVID-19, one inhibitor is FDA-approved for another illness, and over a dozen inhibitors are available for repurposing after unsuccessful or ongoing clinical trials in cancer and inflammatory diseases. Evaluation of these inhibitors in animal models of COVID-19 is highly feasible and will be an important first step for "proof of concept" before clinical trials in humans with SARS-CoV-2 infection.

Author contributions

EC and JM created the original figures. All authors wrote the manuscript, edited and contributed to tables and figures, and approved the final version of the manuscript.

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Conflict of interest

JM was the inventor of a pending United States intellectual property patent owned by the University of Colorado entitled "TGF-β1 inhibitors for preventing and treating SARS-COV-2".

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

Publisher's note

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The intersection of obesity and (long) COVID-19: Hypoxia, thrombotic inflammation, and vascular endothelial injury

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The role of hypoxia, vascular endothelial injury, and thrombotic inflammation in worsening COVID-19 symptoms has been generally recognized. Damaged vascular endothelium plays a crucial role in forming *in situ* thrombosis, pulmonary dysfunction, and hypoxemia. Thrombotic inflammation can further aggravate local vascular endothelial injury and affect ventilation and blood flow ratio. According to the results of many studies, obesity is an independent risk factor for a variety of severe respiratory diseases and contributes to high mechanical ventilation rate, high mortality, and slow recovery in COVID-19 patients. This review will explore the mechanisms by which obesity may aggravate the acute phase of COVID-19 and delay long COVID recovery by affecting hypoxia, vascular endothelial injury, and thrombotic inflammation. A systematic search of PubMed database was conducted for papers published since January 2020, using the medical subject headings of "COVID-19" and "long COVID" combined with the following keywords: "obesity," "thrombosis," "endothelial injury," "inflammation," "hypoxia," "treatment," and "anticoagulation." In patients with obesity, the accumulation of central fat restricts the expansion of alveoli, exacerbating the pulmonary dysfunction caused by SARS-CoV-2 invasion, inflammatory damage, and lung edema. Abnormal fat secretion and immune impairment further aggravate the original tissue damage and inflammation diffusion. Obesity weakens baseline vascular endothelium function leading to an early injury and pre-thrombotic state after infection. Enhanced procoagulant activity and microthrombi promote early obstruction of the vascular. Obesity also prolongs the duration of symptoms and increases the risk of sequelae after hospital discharge. Persistent viral presence, long-term inflammation, microclots, and hypoxia may contribute to the development of persistent symptoms, suggesting that patients with obesity are uniquely susceptible to long COVID. Early interventions, including supplemental oxygen, comprehensive antithrombotic therapy, and anti-inflammatory drugs, show effectiveness in many studies in the prevention of serious hypoxia, thromboembolic events, and systemic inflammation, and are therefore recommended to reduce intensive care unit admission, mortality, and sequelae.

KEYWORDS

COVID-19, long COVID, hypoxia, thrombotic inflammation, vascular endothelial injury

Introduction

As we all know, obesity is an independent risk factor for severe or lethal complications of many diseases. Features such as weight load, low-grade inflammation, neuroendocrine factors, metabolic abnormalities, and nursing difficulties all play a role (1–4). The mechanical action of central fat affects the compliance of the respiratory system. Patients with obesity have higher levels of proinflammatory cytokines and inflammatory cells infiltrating adipose tissue, along with leptin resistance and low levels of anti-inflammatory adiponectin, which can modulate immune responses, affecting tissues and organs throughout the body. Additionally, obesity is often accompanied by diseases such as insulin resistance, abnormal lipid metabolism, high blood pressure, fatty liver, and coronary heart disease. These are independent risk factors in many diseases and may have additive effects. In the influenza A (H1N1) pandemic, the delayed antiviral response in obese patients exacerbated disease and increased mortality, while prolonged influenza A shedding and chronic inflammation contributed to poor recovery (3, 4). Despite recent worldwide efforts to study the cross-population of coronavirus disease 2019 (COVID-19) and obesity, little is known about how obesity adversely affects COVID-19 symptoms and long COVID sequelae (5–13).

A large number of cohort and case-control studies have shown that high body mass index (BMI) is a risk factor for increased disease severity and mortality in COVID-19 patients. This is primarily measured as increased prevalence of severe and critical illness, hospitalization rate, mechanical ventilation rate, intensive care unit (ICU) hospitalization rate, and in-hospital mortality (**Table 1**). In our pooled study, compared with normal-weight individuals, patients with a high BMI had a 1.35-fold increased risk of severe illness and a 2.35-fold increased risk of critical illness (14, 15). Data published by the American Heart Association, which included clinical information of 7,606 confirmed patients, showed that 61% of hospitalized patients were overweight while only 28% were normal weight (9). Individuals who were overweight or obese had a higher risk of invasive mechanical ventilation (IMV), with an adjusted risk ratio (aRR) of 1.12 [95% confidence interval (CI) 1.05–1.19] and 2.08 (95% CI 1.89–2.29), respectively. There was also a strong correlation between increased BMI and death (16). The risk of ICU admission in individuals with obesity ranged from 1.06 to 1.89 (6, 14, 16). In long COVID, higher BMI was associated with longer symptom duration and delayed recovery. Palaiodimos et al. showed that the in-hospital mortality rate of patients with BMI $\geq$ 35 kg/m^2 was approximately twice that of patients with BMI between 25 and 34 kg/m^2 (34.8 vs. 17.2%) (17). In another study, aRR for patients with a BMI of 30–34.9 kg/m^2 was 1.08 (95% CI 1.02–1.14) and 1.61 (95% CI 1.47–1.76) for patients with BMI $\geq$ 45 kg/m^2 compared to patients of normal weight (16). The obesity rate of non-survivors and survivors was 27.1 and 13.5%, respectively (18). The adjusted hazard ratio (aHR) of prolonged symptoms in patients with BMI 25–30 kg/m^2 and those with BMI > 30 kg/m^2 were 1.07 (95% CI 1.04–1.10) and 1.10 (95% CI 1.07–1.14), respectively (11). Individuals with a BMI of >30 kg/m^2 were less likely to recover within 1 year after discharge (12, 13).

In addition, in acute COVID-19, higher BMI is associated with deep vein thrombosis and pulmonary embolism events. A study showed that after multivariate adjustment analysis, patients with Class II obesity (BMI 35.0–39.9 kg/m^2) had a higher risk of thromboembolism than participants with normal BMI [hazard ratio (HR) 2.01, 95% CI 1.30–3.12] (9). Hypoxia, vascular endothelial injury, and thrombotic inflammation play a well-recognized role in exacerbating COVID-19 symptoms. The combination of insufficient cavity ventilation and poor pulmonary perfusion leads to severe respiratory distress symptoms (shortness of breath with the respiratory rate $\geq$ 30 times/min) or respiratory failure (19). Even with prompt mechanical ventilation, there can be a failure to reverse pulmonary conditions due to ventilation-perfusion mismatch (20) whereas maintaining normal oxygen saturation improves survival (21, 22). Biomarkers of vascular endothelial injury and platelet activation [such as von Willebrand factor (vWF) antigen, soluble E-selectin, soluble P-selectin, angiopoietin (Ang) 2, and soluble intercellular adhesion molecule-1] were maintained at high levels in hospitalized patients, and to a greater extent in ICU patients (23, 24). During the acute phase of COVID-19, pulmonary interstitial inflammatory infiltration and elevated levels of inflammatory markers (such as cytokines, chemokines, lactate dehydrogenase, C-reactive protein, ferritin, and procalcitonin) have also been reported in multiple imaging studies (25–27). Thromboembolic events accelerate the progression to severe disease in COVID-19, and even in recovering patients, microthrombi and pulmonary blood flow restriction have been reported (28, 29). Patients with obesity are more likely to exhibit vascular injury and develop hypoxemia and thrombotic inflammation during the acute phase of COVID-19. Therefore, obesity can aggravate of COVID-19 by affecting endothelial cells, inflammatory response, hypercoagulation, and thrombosis (30, 31). This review focuses on the relationship between hypoxemia, vascular endothelial injury, and thrombotic inflammation, the synergistic effect of obesity and acute COVID-19, the influence of obesity on long COVID, and suggestions for treatment.

Hypoxia, vascular endothelial injury, and thrombotic inflammation in COVID-19

In one study of more than 900,000 COVID-19 patients followed for 90 days, the cumulative incidence of venous thromboembolism ranged from 0.2 to 0.8% and was as high as 4.5% in hospitalized cases (32). Another observational study involving more than 1 million COVID-19 cases showed that SARS-CoV-2 infection significantly increased the risk of thromboembolic events, with a 3-fold and 7-fold increase in the risk of deep vein thrombosis and pulmonary embolism, respectively, even in mild cases, although the risk was greater in severe cases. The heightened risk of venous thrombosis and pulmonary embolism lasted as long as 3–6 months (33). Damaged vascular endothelium is a crucial cofactor in forming *in situ* thrombosis. Biomarkers of endothelial activation and the formation of endothelial-derived extracellular vesicles were consistently observed in both acute and long COVID (23, 34, 35). Injured vascular endothelium exposes collagen, which binds to glycoprotein Ib/IX/V complex on the platelet membrane through the bridging molecule vWF to enhance platelet adhesion. The levels of protective factors (prostacyclin, nitric oxide, and NTPDase-1) decreased, weakening the inhibition of platelet activation, aggregation and expansion, thus forming a pro-thrombotic environment. Activated platelet phenotypes have

TABLE 1 Studies reporting on the outcomes of obese patients with COVID-19.

References	Study population	BMI (kg/m^2)	Outcomes
Simonnet et al. (140)	124 patients admitted to ICU	30 < BMI ≤ 35 (47.6%) BMI > 35 (28.2%)	The proportion of patients requiring mechanical ventilation (p < 0.01): BMI > 35 (85.7%) 30 < BMI ≤ 35 (75%) 25 < BMI ≤ 30 (60.4%) BMI < 25 (47.1%)
Lighter et al. (141)	3,615 SARS-CoV-2 positive patients	30 ≤ BMI < 35 (21%) BMI ≥ 35 (16%)	Critical disease: BMI of 30–34 is 1.8 times more than normal. BMI > 35 is 3.6 times more than normal.
Cai et al. (142)	383 confirmed inpatients	18.5 ≤ BMI ≤ 23.9 (53.1%) 24.0 ≤ BMI ≤ 27.9 (32.0%) BMI ≥ 28 (10.7%)	Probability of developing severe cases: BMI: 18.5–23.9 (19.2%) BMI: 24.0–27.9 (29.3%) BMI ≥ 28 (39.0%) (p = 0.001)
Hamer et al. (7)	334,329 cases of samples 640 confirmed inpatients	BMI > 25 (66.6%)	Possibility of hospitalization compared to normal weight: BMI: 25–30 (OR 1.39) BMI: 30–35 (OR 1.70) BMI > 35 (OR 3.3)
Giacomelli et al. (18)	233 confirmed inpatients	BMI > 30 (16.3%)	Obesity rate among survivors (13.5%) Obesity rate among non-survivors (27.1%) Mortality of BMI > 30 (aHR 3.04)
Huang et al. (6)	A summary of 33 articles	Not reported	Univariate analysis of COVID-19 patients with obesity: Risk of hospitalization (OR 1.76, p = 0.003) Risk of ICU admission (OR 1.67, p < 0.001) Risk of death (OR 1.37, p = 0.014) Risk of IMV (OR 2.19, p < 0.001)
Palaiodimos et al. (17)	200 confirmed inpatients	BMI < 25 (19%) BMI: 25–34 (58%) BMI ≥ 35 (23%)	In-hospital mortality rate: BMI < 25 (31.6%) BMI: 25–34 (17.2%) BMI ≥ 35 (34.8%) Intubation rate: BMI < 25 (18.4%) BMI: 25–34 (16.4%) BMI ≥ 35 (34.8%)
Kass et al. (143)	265 patients admitted to ICU	BMI < 26 (25%) BMI > 34.7 (25%)	Younger individuals admitted to hospital were more likely to be obese.
Bhatraju et al. (144)	24 patients admitted to ICU	BMI: 18–25 (3) BMI: 25–30 (7) BMI > 30 (13)	BMI > 30 (85% required mechanical ventilation and 62% died) BMI < 30 (64% required mechanical ventilation and 36% died)
Petrilli et al. (145)	4,103 confirmed patients	BMI > 30 (26.8%)	Rate of obesity among hospitalized patients: 39.8% BMI > 40 kg/m^2 is the biggest risk factor for hospitalization (OR 6.2)
Goyal et al. (146)	393 confirmed inpatients	BMI > 30 (35.8%)	Obesity accounted for 43.3% of patients requiring invasive ventilation. Obesity accounted for 31.9% of non-invasive ventilation patients.
Caussy et al. (14)	340 patients with severe condition	BMI > 30 (25%)	After standardization of age and sex, compared to the average French person: The incidence of obesity in severe COVID-19 is 1.35 times higher (p = 0.0034). The prevalence of obesity in the ICU is 1.89 times higher (p = 0.0011).
Du et al. (15)	109,881 patients with COVID-19 in the meta-analysis	Not reported	The observational studies showed that patients with a BMI ≥ 30 kg/m^2 were 2.35 times more likely to develop critical COVID-19 and had a 2.68-fold risk for mortality, compared with patients with a BMI < 30 kg/m^2. Random-effects dose-response meta-analysis showed that the incidence of critical cases and mortality augmented by 9 and 6% for each 1 kg/m^2 increase in BMI, respectively.
Tartof et al. (147)	6,916 patients with COVID-19	BMI: 18.5–24 (n = 1,240) BMI: 25–29 (n = 2,207) BMI: 30–39 (n = 2,537) BMI: 40–44 (n = 372) BMI ≥ 45 (n = 262)	Compared with patients with 18.5 ≤ BMI<24 kg/m^2, those with BMI of 40–44 kg/m^2 and greater than 45 kg/m^2 had relative risks of 2.68 and 4.18, respectively.

(Continued)

TABLE 1 (Continued)

References	Study population	BMI (kg/m^2)	Outcomes
Hendren et al. (9)	7,606 patients hospitalized with COVID-19	Underweight, BMI < 18.5 (n = 194) Normal, BMI: 18.5–24.9 (n = 1,793) Overweight, BMI: 25.0–29.9 (n = 2,308) Class I obesity, BMI: 30.0–34.9 (n = 1,623) Class II obesity, BMI: 35.0–39.9 (n = 846) Class III obesity, BMI ≥ 40.0 (n = 842)	Higher risks of in-hospital death or mechanical ventilation than normal weight group (18.5–24.9 kg/m^2): Class I obesity, BMI 30.0–34.9 kg/m^2 (OR 1.28) Class II obesity, BMI 35.0–39.9 kg/m^2 (OR 1.57) Class III obesity, BMI ≥ 40.0 kg/m^2 (OR 1.80)
Kompaniyets et al. (16)	148,494 patients with COVID-19	Underweight, BMI < 18.5 (n = 79,988, 2.5%) Healthy weight, BMI: 18.5–24.9 (n = 829,474, 25.6%) Overweight, BMI: 25–29.9 (n = 936,132, 28.9%) Obesity, BMI ≥ 30 (n = 1,397,055, 43.1%)	aRRs for hospitalization for patients with different BMI compared with healthy-weight cohort: Hospitalization: BMI 30–34.9 kg/m^2: 1.07 (95% CI 1.05–1.09) BMI ≥ 45 kg/m^2: 1.33 (95% CI 1.30–1.37) Death: BMI 30–34.9 kg/m^2: 1.08 (95% CI 1.02–1.14) BMI ≥ 45 kg/m^2: 1.61 (95% CI 1.47–1.76) ICU admission BMI 40–44.9 kg/m^2: 1.06 (95% CI 1.03–1.10) BMI ≥ 45 kg/m^2: 1.16 (95% CI 1.11–1.20) IMV: BMI 25–29.9 kg/m^2: 1.12 (95% CI 1.05–1.19) BMI ≥ 45 kg/m2: 2.08 (95% CI 1.89–2.29)
Yamashita et al. (10)	1,236 patients with COVID-19	Mean body: 67.6 kg Mean BMI: 24.0 kg/m^2	COVID-19 patients with VTE showed a higher body weight (81.6 vs. 64.0 kg, p = 0.005) and BMI (26.9 vs. 23.2 kg/m^2, P = 0.04) compared with those without.
Thompson et al. (89)	1.1 million individuals with COVID-19 diagnostic codes in electronic healthcare records	Acute COVID-19 (n = 1,064,491) Not obese (n = 800,439) Obese I, BMI (30–34.9) (n = 151,782) Obese II (35–39.9) (n = 67,470) Obese III (40+) (n = 44,800) Long COVID (n = 4,189) Not obese (n = 2,694) Obese I (30–34.9) (n = 787) Obese II (35–39.9) (n = 411) Obese III (40+) (n = 297)	Overweight/obesity was associated with increased odds of symptoms lasting for 4+ weeks (OR 1.24, 95% CI 1.01–1.53) but not with symptoms lasting 12+ weeks specifically (OR 0.95, 95% CI 0.70–1.28).
Subramanian et al. (11)	486,149 adults with confirmed SARS-CoV-2 infection	BMI < 18.5 (n = 13,261, 2.7%) BMI: 18.5–25 (n = 148,295, 30.5%) BMI: 25–30 (n = 138,771, 28.5%) BMI > 30 (n = 121,943, 25.1%)	Compared with patients with normal BMI, patients with a BMI of 25–30 kg/m^2 reported an aHR of 1.07 (95% CI 1.04–1.10) for prolonged symptoms and those with a BMI of >30 kg/m^2 reported an aHR of 1.10 (95% CI 1.07–1.14).
PHOSP-COVID Collaborative Group (12)	924 post-COVID participants who had a 1-year visit	BMI < 30 kg/m^2 (n = 349, 40.3%) BMI ≥ 30 kg/m^2 (n = 517, 59.7%)	In multivariable analysis, BMI ≥ 30 kg/m^2 (OR 0.50, 95% CI 0.34–0.74, p = 0.0007) was an independent factor associated with being less likely to recover at 1 year.
Wynberg et al. (13)	342 COVID-19 patients during the first 12 months after illness onset	Underweight or normal weight, BMI < 25 (140, 41%) Overweight, BMI: 25–30 (108, 32%) Obese, BMI > 30 (82, 24%)	In the 1-year post-COVID recovery study, the obese patients recovered 38% more slowly than participants with normal BMI (aHR 0.62, 95% CI = 0.39–0.97). Recovery was slower in those with a BMI ≥ 30 kg/m^2 compared to BMI < 25 kg/m^2 (HR 0.62, 95% CI = 0.39–0.97).
Xie et al. (99)	18,818 outpatients with COVID-19	Mean BMI: 27.64	In patients with COVID-19, obesity was independently associated with higher risk, with aHR of 1.83 (95% CI, 1.28–2.61).
Lacavalerie et al. (104)	51 chronic post-COVID-19 patients	Non-obese 18, Mean BMI: 25 Obese 33, Mean BMI: 34	Obese patients with chronic COVID-19 develop exaggerated ventilatory drive and impaired oxygenation at peak exercise, lower lung volumes, reduced ventilatory reserve (25 vs. 40, p = 0.011) and lower peripheral capillary oxygen saturation (96 vs. 98, p = 0.036).

COVID-19, coronavirus disease 2019; BMI, body mass index; OR, odds ratio; aHR, adjusted hazard ratio; aRRs, adjusted risk ratio; VTE, venous thrombus embolism; CI, confidence interval; HR, hazard ratio.

been observed in convalescent patients after mild SARS-CoV-2 infection (36). Disruption of homeostasis leads to high intracellular Ca^{2+} concentration and the subsequent activation of Ca^{2+}-dependent scramblase on the cell membrane. The resulting increased phosphatidylserine (PS) exposure on the outer membrane of injured vascular endothelial cells promotes activation of the intrinsic tenase complex and the formation of the prothrombinase complex (37, 38). The number of PS^+ peripheral blood mononuclear cells in patients at the initial stage of COVID-19 diagnosis was higher than that of healthy controls (39). Moreover, tissue factor (TF) is also decrypted by PS, promoting the activation of the exogenous tenase complex. Anti-TF cannot completely inhibit the coagulation cascade, but lactadherin can inhibit coagulation in multiple ways by competing for PS binding sites (40). In the early stages of

COVID-19, immune cells recognize and clear a small amount of virus without inducing severe inflammatory reactions, and laboratory tests show no significant changes or only decreases in peripheral blood white blood cell counts (41). In more severe cases, drastic replication and release of SARS-CoV-2 result in accumulation of immune cells in the lung tissue. Cytokines initiate the associated transduction pathways and trigger a cascade of inflammation that leads to cytokine storms (42–44). In addition, when immune cells remove pathogens, they release a large number of PS^+ microvesicles. Thrombus and inflammation interact to further damage the vascular endothelium. Fogarty et al. recently reported that significantly elevated intermediate monocytes and activated $CD4^+$ and $CD8^+$ T cells were associated with sustained EC activation and poor hemostatic function in long COVID (45).

Vascular endothelial dysfunction and structural destruction also contribute to the development of hypoxemia. SARS-CoV-2 enters the alveolar interstitium and infects the capillary endothelial cells at the thin part of the air-blood barrier. The injured capillary endotheliocytes begin to contract and narrow the microcirculation. Moreover, due to the binding of SARS-CoV-2 and angiotensin converting enzyme 2 (ACE2), the available ACE2 is reduced, and the conversion of Ang II to Ang 1-7 is suppressed, causing vasoconstriction, inflammation promotion, enhanced vascular permeability, and pulmonary edema (46–48). ACE2 knockout mouse models exhibit more severe acute respiratory distress syndrome (49), and ACE2/Ang-(1-7) inhibition has been implicated in endothelial dysfunction or endotheliitis in COVID-19 stroke patients (50). The combination of damaged endothelial cells and vasoconstriction results in stenosis or obstruction in the tiny alveolar capillaries, interfering with normal gas exchange and promoting hypoxemia. Ackermann et al. found extensive alveolar capillary microthrombi, microangiopathy, and perivascular T-cell infiltration in the lungs of patients who died from COVID-19. The incidence of microvascular thrombosis in COVID-19 patients was 9 times higher than that in H1N1 patients ($p < 0.001$) and the number of new blood vessels was 2.7 times that of H1N1 patients ($p < 0.001$) (51). Neovascularization, capillary remodeling, microvascular sclerosis, and uneven vessel wall lead to the formation of local eddy currents, which promote platelet activation and PS exposure. Several studies have detected microclots in blood samples from long COVID patients and observed manifestations of poor blood flow in the vessels downstream of microthrombus-blocked capillaries (28, 29). At the same time, hypoxia can further damage the vascular endothelium through the release of free radicals, reactive oxygen species, and lipid hydroperoxide. The resulting decrease in adenosine triphosphate (ATP)-dependent translocation enzyme function influences membrane phospholipid stability and perturbs the normal function of endothelial cells.

Hypoxia and thrombosis can also be mutually aggravating, in addition to having common promoters. Hypoxia reduces ATP production, inhibits ATP-dependent translocase (flippase and floppase), and prevents the reversion of PS into the inner cell membrane. In addition to the vascular endothelial cells, various types of blood cells (such as red blood cells, platelets, neutrophils, lymphocytes, and monocytes) also exhibit abundant PS exposure, upregulating the coagulation cascade and accelerating thrombogenesis. PS is also exposed on the surface of the microvesicles released by these cells during apoptosis. The presence of pulmonary microcirculation thrombosis leads to pulmonary capillary hypertension, which increases the pressure difference between the two sides of the air-blood barrier. Driven by the pressure difference and the damaged alveolar structure, water molecules, albumin, and platelets enter the alveolar cavity, inducing increased blood viscosity and aggravated vascular stasis. In severe cases, macromolecules such as globulins and red blood cells also appear in the alveolar lumen. The increased fluid causes a decrease in the effective alveolar volume and exacerbates dyspnea and hypoxemia.

Obesity and acute COVID-19

Limited alveolar dilatation

In individuals with obesity (especially central obesity), adipose accumulation in the chest wall and abdomen restricts the lungs' expansion and impedes diaphragm movement, resulting in a decrease in lung volume (52, 53). Visceral fat also increases airway resistance. Additionally, since alveolar ventilation and pulmonary blood flow progress from the apex to the bottom of the lung, compression of the lower part has more significant effects on lung function. Studies have shown that obesity itself can lead to respiratory impairment, with decreases in expiratory reserve capacity, functional capacity, forced vital capacity, functional residual capacity, expiratory reserve capacity, and total lung volume (2, 54).

Chronic low-grade inflammation aggravates tissue damage and inflammation diffusion

Wherever SARS-CoV-2 goes, it recruits a variety of immune cells, and later inducing the release of cytokines, including monocyte chemoattractant protein 1, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and IL-6 (55–60). As adipocytes swell to store excess energy, close interactions between adipocytes and host immune cells enhance lipolysis, resulting in abnormal adipocyte secretion (more leptin and less adiponectin), insulin resistance, and persistent low-level inflammation (61–63). Mitochondrial dysfunction and reactive oxygen species production induced by hyperglycemia promote vigorous generation of cytokines (such as TNF-α, IL-1, IL-6, IL-18, and interferon γ) (64, 65). In addition to the increase in pro-inflammatory mediators, anti-inflammatory regulatory substances (such as adiponectin, IL-4, IL-10, IL-33, and Tregs) are reduced. In terms of host defense, obesity suppresses the adaptive immune system against the influenza virus, suggesting that this could also be true for COVID-19 (**Figure 1A**; 1, 66). Although the immune system actively clears the pathogens, it does not produce completely specific anti-viral immune response, increasing the likelihood of viral escape. The abnormal obese state throughout the body promotes the inflammatory cascade, endothelial dysfunction and thrombosis, which worsens local tissue damage and facilitates the distant spread of SARS-CoV-2 (62, 67–69). Some scholars have proposed that the imbalance of the intestinal microenvironment and host immune system may mediate infection susceptibility in obese individuals (70).

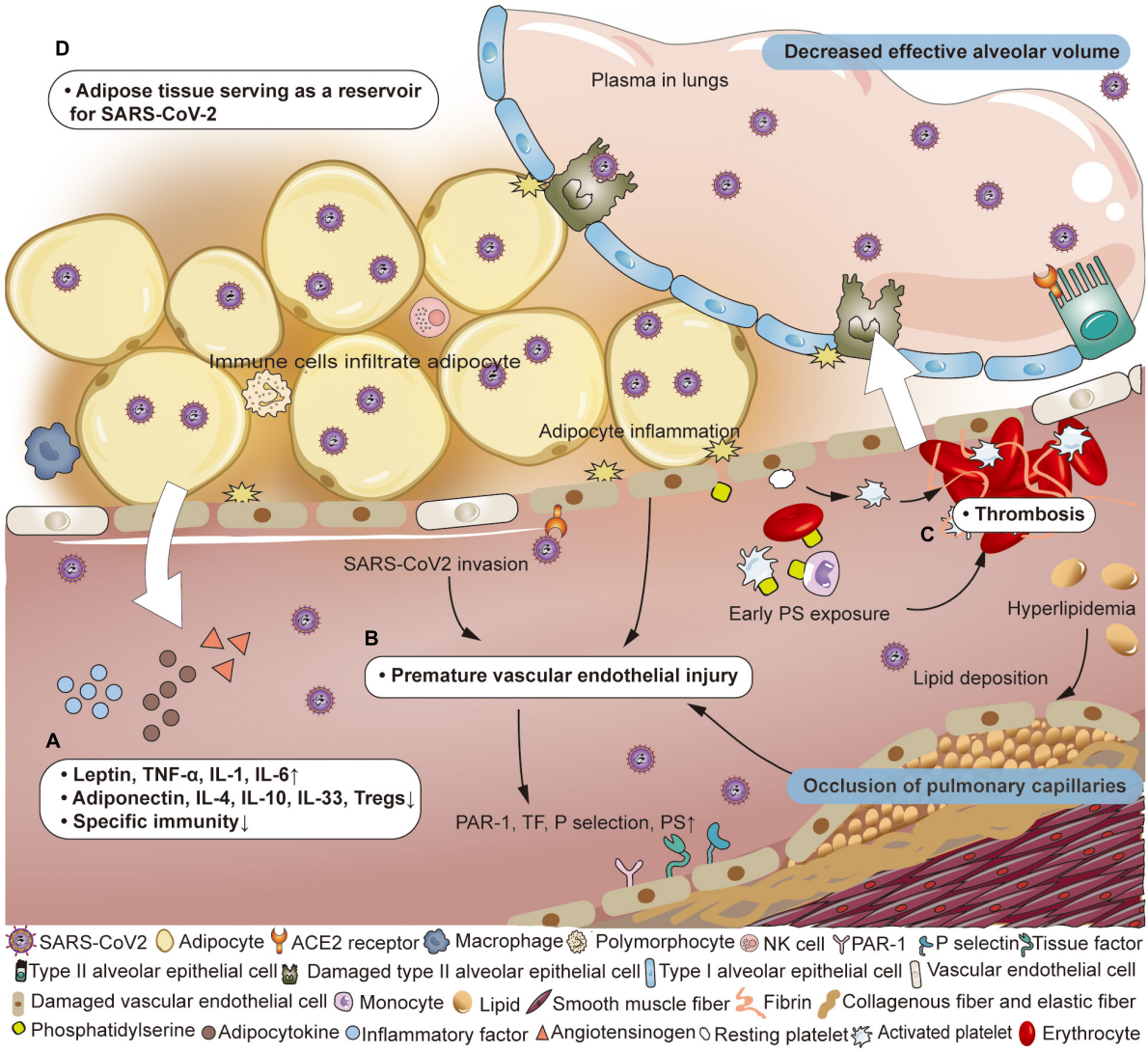

FIGURE 1
Pathophysiological changes of air-blood barrier in obese patients with COVID-19. **(A)** Adipose tissue has the potential to serve as a reservoir for SARS-CoV-2. **(B)** Immunocytes infiltrate adipose tissue, producing inflammatory mediators and adipokines, accompanied by a weakened specific immune response. **(C)** Under the combined effects of virus invasion, inflammation of adipocytes and lipid deposition, vascular endothelial destruction occurs prematurely, and the vascular wall structure is destroyed. The expression of protease activated receptor 1 (PAR-1), tissue factor (TF), P selectin, and phosphatidylserine (PS) on endothelial cells is up-regulated. **(D)** PS exposure on vascular endotheliocyte, erythrocyte, platelet, neutrophil, and lymphocyte appears earlier and participates in thrombosis. Adapted from "Adipocyte (white)," by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

Endothelial dysfunction induces a pre-existing damaged and prothrombotic state

The primary function of vascular endothelial cells is to ensure unobstructed blood flow and maintain a barrier between the circulatory system and surrounding tissues. However, in patients with obesity, the level of adiponectin and nitric oxide is too low to effectively maintain this ordinary protective function. Because of obesity-related chronic inflammation, immunocytes infiltrate adipose tissue and release inflammatory factors, exerting adverse effects on peripheral vascular endothelial cells (5). Additionally, the high incidence of hyperlipidemia (increased low-density lipoprotein, triglyceride, and cholesterol) results in atherosclerotic plaque formation that renders the underlying vascular endothelium more susceptible to damage (46). Overall, although individuals with obesity have relatively mild endothelial dysfunction which rarely causes severe adverse effects, this impaired protective effect leaves them more susceptible to developing severe complications from other diseases. Under the dual impact of obesity and SARS-CoV-2, viruses can more easily invade vascular endotheliocytes due to early phase weakening of the air-blood barrier. As the disease progresses to the middle stages, endothelial dysfunction becomes prominent, leading to more vulnerable and rigid pulmonary vessels (**Figure 1B**). Hypoxemia is further exacerbated by pulmonary edema caused by the enhanced permeability of the alveolar membrane. Although endothelial change is not specific, endothelium-induced thrombosis plays a significant role in COVID-19 (71). Vascular endothelium expresses more protease activated receptor 1 (PAR-1), TF, P selectin, and membrane PS, and releases microvesicles, vWF,

and clotting factor VIII (72). This alteration, together with increased soluble thrombomodulin and the surface chemokines, causes platelet overactivation and thrombosis (73).

Enhanced procoagulant activity blocks vascular perfusion

After adjustment for age, sex, and race/ethnicity, class II obesity (BMI 35.0–39.9 kg/m^2) was associated with a higher risk of thromboembolism compared with normal BMI (HR 2.01, 95% CI 1.30–3.12) (9). A retrospective multicenter cohort study showed that the BMI of COVID-19 patients with venous thrombus embolism (VTE) (26.9 vs. 23.2 kg/m^2, p = 0.04) was higher than that of patients without VTE (10). Autopsy results showed fibrin deposition and thrombosis in both macro and micro pulmonary vessels. In patients with obesity, increased levels of fibrinogen, vWF and plasminogen activator inhibitor-1 causes hypercoagulability (66, 74). Endothelial cell dysfunction appears prematurely under the effect of high inflammation promoted by both obesity and SARS-CoV-2, further inducing PS exposure on the outer layer of endotheliocytes and forming microthrombi (**Figure 1C**). It has been reported that most of the PS$^+$ microvesicles in COVID-19 patients are from the endothelium and platelets (75). Althaus et al. found higher levels of PS exposure on platelets from ICU patients with SARS-CoV-2 infection than in the non-severe group, and that PS exposure was associated with organ failure and elevated D-dimer (76). Because of the high viscosity and slow blood flow in patients with obesity, platelets are more likely to adhere to vascular endothelial cells and participate in coagulation function. In addition, the surrounding inflammatory state caused by the infiltration of immune cells into adipocytes damages the vascular endothelium, increasing the prevalence of procoagulant platelets, leading to thrombin formation. More importantly, these microthrombi can contribute to extrapulmonary thrombosis, leading to ischemia and necrosis of the corresponding organs (77). In addition, excess fat tissue produces high levels of angiotensin, which is rapidly converted into Ang II. Ang II accumulation changes local hemodynamics primarily through pulmonary vasoconstriction, constituting a pre-thrombus environment (78). In the later stage, fibrinolysis is inhibited by the depletion of fibrinolytic factors, decreasing the clearance of cross-linked fibrin and thrombi (79, 80). As a result of early blocked pulmonary blood perfusion, ineffective luminal ventilation occurs even while the alveolar structure is undamaged. Later, after diffuse alveolar injury, there is a lower proportion of air-blood exchange.

Obesity and long COVID

Viral persistence

Adipose tissue has the potential to serve as a reservoir for viruses (**Figure 1D**). Damouche et al. detected replicative human immunodeficiency virus (HIV) in adipose CD4$^+$ T cells in six patients with antiretroviral therapy-controlled HIV, which contributing to viral persistence and long-term immune activation (81, 82). Another study in mice infected with H5N1 has found high virus titers in adipose tissue, including tissue attached to thymus, spleen, kidney, and heart (83). Evidence of SARS-CoV-2 infecting adipose tissue has also been found in patients with COVID-19. Martínez-Colón et al. have detected SARS-CoV-2 in adipose tissue around the heart and intestines of patients who died from COVID-19. *In vitro* experiments in which adipose tissue was cultured with SARS-CoV-2-containing solution showed that the virus infected and replicated within adipocytes (84). It is controversial whether adipose tissue mediates SARS-CoV-2 infection through the high expression of ACE2 (85, 86). Some studies have proposed that ACE2 RNA can occasionally be detected in fresh mature adipocytes, although no ACE2 protein is detected, suggesting that there may be other ways to mediate viral invasion (84, 87, 88). However, the detrimental effects of obesity on recovery from COVID are widely recognized. Overweight/obesity was associated with increased odds of symptoms lasting for 4+ weeks in longitudinal studies [odds ratio (OR) 1.24, 95% CI 1.01–1.53], and long COVID code in electronic health records (OR 1.31, 95% CI 1.21–1.42) (89). A retrospective matched-cohort study showed that patients with BMI > 30 kg/m^2 had a 10% relative increased risk (aHR 1.10, 95% CI 1.07–1.14) of reporting prolonged symptoms compared to patients with a normal BMI (11). The PHOSP-COVID Collaborative Group found that obesity (patients with BMI > 30 kg/m^2 vs. BMI < 30 kg/m^2) is an independent factor associated with not feeling fully recovered 1 year after hospital discharge in both severe (70.8 vs. 29.2%) and very severe recovery clusters (64.0 vs. 36.0%) (12). In the 1-year post-COVID recovery study, the patients with obesity recovered 38% more slowly than participants with normal BMI, when controlling for the effects of age, sex, and comorbidities (aHR 0.62, 95% CI 0.39–0.97) (13). These finding raise the possibilities of exploring the specificity of long COVID symptoms in patients with obesity. More research is needed into whether adipose tissue provides a reservoir for the virus to re-emerge from during long COVID.

Long-term inflammation

The cytokine storm induced during the acute phase by SARS-CoV-2 can develop into long-term systemic inflammation (65, 90, 91). At autopsy, infection-driving inflammation was found in almost all SARS-CoV-2-infected adipose tissue samples (84). The virus infects the immune cells in the adipose tissue and recruits a large number of inflammatory mediators, affecting the surrounding normal cells and causing inflammation to spread. Studies of high-resolution computed tomography lung scans of individuals recovering from COVID 6–12 months after discharge commonly showed ground-glass opacity (GGO) associated with pulmonary inflammatory exudation (92, 93). A prospective observational study demonstrated that higher C-reactive protein concentration was related to the more severe post-hospital cohort, and IL-6 was significantly increased in the moderate disease cluster compared with the mild cohort. Systemic inflammatory characteristics (e.g., serum C-reactive protein concentration > 5 mg/L) have no overall change between 5 months and 1 year (81.1 vs. 79.7%) (12) post-infection. In HIV infection, adipose tissue is thought to be a contributing factor to chronic immune activation/inflammation, with macrophages and CD4$^+$ and CD8$^+$ T cells in adipose tissue showing intense activation characteristics (81). Adipocytes and immune cells, acting as inflammatory partners, are also likely to promote and perpetuate persistent inflammation in long COVID (94–96). Moreover, the vascular endothelium of patients with obesity has a high risk of injury

due to lipid deposition, and the presence of microthrombi stimulates the vascular wall, facilitating the formation of aseptic inflammation.

Microclots

Although difficult to detect, the formation of microthombi can have a severe detrimental effect on microcirculation and can contribute to organ dysfunction such as impairment of respiratory function and renal function injury. Pretorius et al. detected fibrin amyloid microclots and activated platelets in blood samples from all 80 enrolled long COVID individuals by fluorescence microscopy (28). A prospective cohort study that analyzed hematological data from patients discharged from hospital with COVID-19 showed a decrease in mean D-dimer at 60 days of discharge compared with admission, but still elevated above baseline (900.71 vs. 1,350) (97). Townsend et al. evaluated coagulation markers in patients 4 months after initial COVID-19 diagnosis and statistically showed that 25.3% of patients had elevated D-dimer levels (>500 ng/ml), although prothrombin time and activated partial thromboplastin time returned to normal in >90% of patients (98). However, in many cases, microthrombus cannot be ruled out even if coagulation and thrombus-related indicators are normal. It has been reported that, while the results of blood tests and lung X-rays were basically normal, SPECT/CT of discharged patients indicated poor pulmonary blood flow caused by microthrombi blocking the microcirculation (29). Microclots are not easy to be detected. Often, when laboratory and imaging results are abnormal, microclots have affected organ function and need to be removed quickly. Therefore, it is necessary to focus on the indicators that may be related to the prothrombotic state to prevent the formation of microthrombi. Several studies have shown increased levels of vascular endothelial activation markers in long COVID (such as vWF antigen, vWF propeptide, and soluble thrombomodulin). *In vitro* studies have shown that convalescent plasma can damage vascular endothelial cells suggesting that the vascular endothelial cells can be continuously activated and in a pro-thrombotic state (34, 35). McCafferty et al. consistently observed that in samples from patients both in the acute and long COVID contained platelets with an activated phenotype (expressing activation markers CD62P and PAC1) (36). High PS level is also associated with poor prognosis in the convalescent period. It is reported that the levels of PS^{+} $microvesicles^{+}$ peripheral blood mononuclear cells in the blood of patients with long COVID-19 are higher than those in the healthy cohort (39). Higher BMI was associated with deep vein thrombosis and pulmonary embolism, and Xie et al. showed that individuals with obesity were at higher clinical risk for post-COVID-19 VTE events than non-obese patients (aHR 1.83, 95% CI 1.28–2.61) (99).

Hypoxia

Cardiopulmonary response to exercise may remain limited after hospital discharge in COVID-19 patients with obesity. Under the influence of airflow, fluid in the alveoli evaporates, leaving plasma proteins to form hyaline membranes, which can eventually develop into lung fibrosis and consolidation (20, 100). Studies have shown that convalescent patients still have symptoms of dyspnea, radiographic findings of interstitial lung infiltration and GGO, and impaired lung function at 1 year after discharge. In these individuals, the proportion of the diffusing capacity of the lung for carbon monoxide < 80% is associated with disease severity (92, 93, 101–103). Patients admitted with nasal catheters or mechanical ventilation were more likely to have diffusion disorders after discharge than patients who did not require supplemental oxygen (OR 4.60) (93). Meanwhile, obesity can have additional effects on the pulmonary sequelae in long COVID. Analysis of an observational study found that patients with obesity and chronic COVID-19 displayed exaggerated ventilatory drive and impaired oxygenation at peak exercise, accompanied by lower lung volume, decreased ventilation reserve (25 vs. 40, $p = 0.011$), and lower peripheral capillary oxygen saturation values (96 vs. 98, $p = 0.036$) 6 months after hospital discharge (104). The PHOSP-COVID Collaborative Group compared patient-reported outcomes between 5 months and 1 year and demonstrated that FEV1% < 80% predicted only minimal change, while cognitive impairment significantly improved (12). Compared with non-COVID-19 participants with obesity, post-COVID patients with obesity had significantly reduced oxygen pulse (66 vs. 76, $p = 0.003$), indicating poor cardiac function in convalescence (104). Both hypoxia and thrombotic inflammation can affect myocardial metabolism, resulting in loss of normal systolic and diastolic functions of the damaged myocardium, pulmonary congestion, and insufficient systemic circulation blood volume. Furthermore, due to the reduced effective lung volume, the blood in the pulmonary microcirculation cannot adequately carry oxygen, inducing reduced oxygen saturation. These effects may persist into the recovery period.

Therapy

Respiratory support

Hendren et al. showed that COVID-19 participants who were overweight and class I to III obese had a higher risk of requiring mechanical ventilation after multivariate analysis (OR 1.28, 1.54, 1.88, and 2.08, respectively) (9). In a Seattle study of 105 hospitalized patients with COVID-19, the survival rate was 98.9% in patients with oxygen saturation greater than 90%, while only 35 in 51 (68.63%) patients with arterial oxygen saturation < 90% survived. Ensuring sufficient blood oxygen saturation can effectively improve the survival rate of patients (21, 22). However, ventilation does not remove the etiological factors or produce lung healing; it merely keeps patients alive until their biological mechanisms can overcome SARS-CoV-2 (101). Many COVID-19 patients have almost no difficulty breathing until arterial oxygen partial pressure drops below 60 mmHg. The resulting dyspnea and shortness of breath are common symptoms which prompt patients' initial visit, and thus the patient's condition is often remarkably advanced (105–107). With further progression, pulmonary hyaline membrane or lung consolidation complicate gas exchange even with ventilation support (108). Therefore, even patients with mild symptoms should receive oxygen inhalation through a nasal catheter at 5 L/min upon admission, to maintain at least 95% peripheral oxygen saturation (21, 109, 110). In more serious cases, oxygen storage mask (initial flow 8–15 L/min) should be utilized and if there is no improvement after 1–2 h, progressive treatments [such as High-flow nasal cannula oxygen therapy, non-invasive ventilation, invasive ventilation, and extracorporeal membrane oxygenation

(ECMO)] should be performed (111). In patients with obesity, particular attention should be paid to suitable methods (ECMO is contraindicated in patients with BMI > 45 kg/m^2) and the risk of iatrogenic infection due to mechanical ventilation. Without the moisture and temperature regulation effects of nasal mucosa, inhaled gas can exert intense irritation to the airway, making it prone to infection. Nursing difficulties and persistent inflammatory state can both contribute to increased susceptibility to associated infection.

Comprehensive antithrombotic therapy

A study involving 176,137 hospitalized COVID-19 cases found a difference in case fatality between patients with and without pulmonary embolism (28.7 vs. 17.7%) (112). In addition to directly affecting microcirculation, thrombosis can also lead to increased local intravascular pressure, aggravated pulmonary exudation, and pulmonary dysfunction. Severe hypoxemia and pulmonary hypertension can even lead to myocardial dysfunction. Therefore, it is possible to prevent disease progression if comprehensive treatment can be taken early in the disease to relieve the hypercoagulable state (including antiplatelet and anticoagulation), and if necessary to dissolve (micro) thrombi (113, 114). Early comprehensive antithrombotic therapies aim to maintain unobstructed blood flow and ensure adequate alveolar blood perfusion, without resulting in pulmonary arterial hypertension. It also seeks to delay the onset of pulmonary edema, thereby reducing the incidence of respiratory distress and respiratory failure. Unobstructed blood circulation also promotes the clearance of viruses and damaged blood cells, thus inhibiting the spread of inflammatory reactions and preventing disease progression. When the damaged vascular endothelium results in the exposure of basement membrane collagen, platelets react to the endothelium injury, interweave with fibrin, and become the starting point of thrombus formation. Inhibition of platelet adhesion, aggregation, and release can also be used as therapeutic targets, through the use of aspirin (75–100 mg/d), clopidogrel (75 mg, qd), and dipyridamole (100 mg, tid), thereby inhibiting the formation of intrinsic and extrinsic tenase and prothrombinase complexed caused by PS exposure on outer cell membranes (115). Santoro et al. found that in-hospital use of antiplatelet drugs was associated with lower mortality after multivariate adjustment [relative risk (RR) 0.39, 95% CI 0.32–0.48, $p < 0.01$] (116). Anticoagulant drugs, which are the most commonly used and studied drugs in clinical practice, can prevent thrombosis by inhibiting coagulation factors and activating antithrombin III. Results from multiple randomized controlled trials have shown that the use of therapeutic heparin in non-critically hospitalized COVID-19 patients reduces the number of days requiring organ support, the incidence of VTE and 28-day mortality events, and the proportion requiring respiratory support or IMV, compared with standard-dose thromboprophylaxis cohorts (117–120). However, a similar meta-analysis of non-critically ill patients with COVID-19 found that therapeutic thromboprophylaxis had a higher incidence of bleeding than standard-dose anticoagulation, including major bleeding (HR 1.86, 95% CI 1.04–3.33) and minor bleeding (HR 5.23, 95% CI 1.54–17.77) (121). Using thrombotic events (arterial and/or venous) as the primary endpoint, Spyropoulos et al. found a benefit of therapeutic anticoagulation in non-critically ill patients with elevated D-dimer (RR 0.46, 95% CI 0.27–0.81) (119). High D-dimer levels can predict poor prognosis of COVID-19. D-dimer levels have been found to be generally higher in critically ill patients than in mild patients (2.4 vs. 0.5 mg/L) (122). Standard doses of thromboprophylaxis are recommended for adults who are critically ill during hospitalization (123). Although guidelines for clinical inpatients recommend only standard or therapeutic doses, intermediate doses (defined as low molecular weight heparin bid or increased weight-based dosing that is less than the recommended therapeutic dose) are often used in clinical trials (123, 124). Drug distribution and metabolic clearance in patients with obesity may necessitate adjustments to dosing. The clinical effect of moderately increasing anticoagulant dose still needs to be investigated by high-quality trials (78, 125). For patients with body weight > 90 kg or BMI ≥ 30 kg/m^2, enoxaparin 30–40 mg bid or UFH 7500 IU bid/tid can be used as a prophylactic dose, and enoxaparin can be used at a therapeutic dose of 1mg/kg bid. With regard to treatment of outpatients with COVID-19, there is currently no evidence to support the routine use of antithrombotic agents such as aspirin, factor Xa inhibitors, or low molecular weight heparin for the prevention of arterial/venous thrombosis or COVID-19 progression, and several studies were stopped early due to lower-than-expected primary event rates (126). Currently, the omicron variant is milder in pathogenicity, with a lower hospitalization rate, lower mortality rate, and shorter duration of acute symptoms than the delta variant, but with rapid bronchial replication and high transmissibility (127, 128). In the new round of COVID-19 transmission led by omicron variant, in addition to high-risk groups such as the elderly and people with underlying diseases or immune deficiencies, individuals with obesity (BMI ≥ 30) are also more likely to develop critical illness than the general population. For those without anticoagulant contraindications, the time of anticoagulation should be seized. Timely and sufficient anticoagulant treatment can effectively relieve hypercoagulability, prevent the occurrence of symptomatic thrombotic events, and improve the prognosis of patients with obesity.

Other treatments

Systemic corticosteroids are recommended for patients with severe and critical illness but should be used with caution in patients with diabetes or underlying immune deficiency (129). In a randomized, controlled clinical study, dexamethasone (6 mg per day for 10 days) reduced mortality in patients requiring oxygen support, both on IMV (29.3 vs. 41.4%; RR 0.64, 95% CI 0.51–0.81) and non-IMV (23.3 vs. 26.2%, RR 0.82, 95% CI 0.72–0.94) (130). Intravenous dexamethasone plus standard care increased the number of ventilator-free days within 28 days in patients with COVID-19 related acute respiratory distress syndrome (6.6 vs. 4.0, $p = 0.04$) (131). Tocilizumab (4–8 mg/kg IV, single dose) is also recommended as an anti-inflammatory drug in many guidelines and is usually recommended in patients with peripheral capillary oxygen saturation ≤ 94% on room air and CRP ≥ 75 mg/L. It inhibits IL-6 signaling by reducing the binding of soluble and membrane-bound receptors (sIL-6R and mIL-6R) of IL-6 to block T cell activation, plasmocyte immunoglobulin secretion, and macrophage activity (132–134). Results of a meta-analysis showed a 12% reduction in mortality in the tocilizumab group compared with a control group that did not receive tocilizumab (RR 0.27, 95% CI 0.12–0.59) (135). JAK inhibitors (such as baricitinib), which blocks the signaling of inflammatory and immune responses, is also used in severely ill patients with pneumonia and hypoxia (136). Other medications may also be considered. Remdisivir, an adenosine analog, binds to

new strands of viral RNA and leads to premature termination of virus replication, improving recovery and reducing adverse events (137). Statins, which improve immune system function and fight inflammation and oxidative stress, could also be an option in the treatment of COVID-19 (138). Cytokine storm is associated with a higher risk of multiple organ failure and death. During severe and critical episodes, anti-granulocyte-macrophage colony-stimulating factor and IL-6 inhibitors can reduce inflammation severity, clear cytokines, and reduce disease risk (139). While it is possible to target cytokine storms in theory, it does not play a significant role in clinical practice and can be used as a complementary therapy (64).

Precaution

Patients with obesity are more likely to experience hypertension, hyperlipidemia and diabetes before admission, and the incidence of various comorbidities is high during their hospitalization. It is unlikely that even prompt treatment after hospital admission can normalize the rate of mechanical ventilation and the ICU occupancy in patients with obesity to the level of normal-weight patients. Therefore, for patients with obesity, daily weight management before COVID-19 diagnosis is critical in preventing COVID-19 and mitigating risk. A healthy diet and proper exercise help support immune health. As with everyone else, use of personal protective equipment and avoiding crowds are also important risk reducing strategies.

Conclusion

The ongoing worldwide epidemic of COVID-19 is a problem that every country faces. Preventing the progression of COVID-19 to severe disease and reducing the incidence of sequelae are two major priorities for disease management. COVID-19 patients with obesity exhibit increased thrombotic inflammation and hypoxia, which are associated with mechanical compression, persistent inflammation, vascular endothelial damage, and hypercoagulable state. Obesity also contributes to the development and persistence of sequelae in long COVID, and may be involved in persistent viral presence, chronic inflammation, microclots, and hypoxemia, although reliable evidence from larger, high-quality studies is still needed. Although vaccines are important in preventing severe disease, effective treatment of COVID-19 is still critical, given continued viral mutation and the limited effectiveness of vaccines. Early intervention, including timely oxygen supplementation, prevention of microthrombi, and relief of the spread and persistent effects of inflammation can prevent or reverse disease progression and reduce the occurrence of sequelae.

Author contributions

MX wrote the manuscript and drew the figure and table. XW and HJ searched the manuscript and provided comments. JS proposed the project, designed the study, and revised the structure. VN reviewed the text and polished the language. All authors read and approved the final 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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Use of fondaparinux in patients with heparin-induced thrombocytopenia on veno-venous extracorporeal membrane oxygenation: A three-patient case series report

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Heparin-induced thrombocytopenia is a life-threatening immune-mediated complication of unfractionated heparin therapy. Fondaparinux is a therapeutic alternative, but it has limited evidence for its use in patients on extracorporeal membrane oxygenation (ECMO). We present a series of three adult patients with COVID-19 on ECMO who were diagnosed with heparin-induced thrombocytopenia after 7–12 days of unfractionated heparin treatment and were switched to fondaparinux. Fondaparinux was initiated with an intravenous loading dose of 5 mg, followed by a dose of 2.5 mg subcutaneously every 8–12 h. Dosage was adjusted according to daily measured anti-Xa concentration with a target range of 0.4–0.7 mg/L. The total duration of treatment with fondaparinux and ECMO ranged from 13 to 26 days. One major bleeding episode unrelated to fondaparinux therapy was observed, and the transfusions requirement was also low in all patients. The ECMO circuit was changed once in each patient. This series provides a deep insight into the use of fondaparinux over an extended period of time in patients on ECMO. Based on the presented data, fondaparinux can be considered a reasonable and affordable anticoagulant in patients without a high risk of bleeding.

KEYWORDS

fondaparinux, pharmacokinetics, heparin-induced thrombocytopenia, extracorporeal membrane oxygenation, pharmacoeconomics

1. Introduction

Extracorporeal membrane oxygenation (ECMO) is a life-saving therapy for severe acute respiratory distress syndrome (ARDS) in COVID-19 patients. The use of an extracorporeal circuit requires contact of blood with artificial materials (cannulas, tubing, pump, and oxygenator membrane). This leads to the activation of platelets and the coagulation cascade. Anticoagulation is necessary for most ECMO patients to suppress this undesirable phenomenon and prevent circuit clotting. Unfractionated heparin (UFH) is the most frequently used drug because it is cheap, has a short half-life and an antidote, and is relatively easy to monitor. Although the optimal target for UFH anticoagulation is not clearly defined, recent Extracorporeal Life Support Organization (ELSO) guidelines recommend a target

anti-Xa range of 0.3–0.7 IU/ml (1). Concordantly, data from COVID-19 patients on ECMO show that the most frequent (two-thirds of patients) therapeutic target is 0.3–0.6 or 0.3–0.7 IU/ml (2). This was associated with the following clinical outcomes: 29% of patients had only bleeding events, 16% had only thrombotic events, and 20% had both bleeding and thrombosis.

A decrease in platelet counts is common in ECMO patient group—mainly due to the consumption in the extracorporeal circuit or sepsis. Moreover, heparin-induced thrombocytopenia (HIT) is diagnosed in 0.8–22.2% (mean 3.7%) patients on ECMO (3). Platelet-activating immune complexes have been described in patients with COVID-19 (4); however, the incidence of HIT in critically ill patients with COVID-19 is comparable (5). HIT is a complication influencing morbidity and mortality in critically ill patients (6) and patients with COVID-19 (7). HIT management is based on the immediate discontinuation of heparin and its replacement with non-heparin alternatives—argatroban, bivalirudin, or fondaparinux among parenteral anticoagulants. In the Czech Republic, only fondaparinux is an available and economically acceptable alternative for longer-term administration, although its use in HIT is off-label (8).

To our knowledge, only a few papers describing the administration of fondaparinux in adult patients on ECMO have been published. A cohort of 8 patients on ECMO treated with fondaparinux 2.5 mg per day due to HIT was reported by Loforte et al. (9). Two case reports describe a young female after mitral valve replacement and having fondaparinux 2.5 mg subcutaneously (SC) daily to provide anticoagulation for 5 days on veno-arterial ECMO (10) and a patient with ARDS due to SARS-CoV-2 infection receiving fondaparinux 7.5 mg SC daily during 5 days of veno-venous ECMO (11). Nevertheless, none of these papers provide a more detailed view on achieved plasma concentrations and longer-term administration of fondaparinux in patients on ECMO, as presented in the following description of three patients.

1.1. Fondaparinux and its potential interference with ECMO

Fondaparinux is a synthetic anti-Xa inhibitor with a molecular weight of 1,728, highly selective, and with a high affinity to antithrombin. Fondaparinux binding to antithrombin leads to irreversible conformational changes, which considerably increase the factor Xa inhibition rate (12). The high protein binding of fondaparinux determines the low distribution volume (V_D). In healthy volunteers, the V_D ranges from 7.4 to 10.9 L over the dose range of 2–20 mg of intravenous (IV) fondaparinux resp. 10–10.8 L over the dose range of 2–8 mg of SC fondaparinux and 8.2 L for a single fondaparinux SC 2.5 mg dose (12, 13). The partition coefficient logP of fondaparinux is 0.4 (14). Based on these properties, the effect of ECMO on plasma concentrations of fondaparinux/risk of sequestration can be tentatively predicted using the criteria proposed by Ha et al. (15). Given the small V_D (<1 L/kg), ECMO may theoretically increase V_D; however, when comparing the volume of the ECMO circuit in adults (~600 mL) and the average blood volume (~5 L), only a mild increase in V_D can be expected without the need for a higher loading dose. There is also a moderate risk of sequestration of fondaparinux on the ECMO circuit, which may translate into the necessity of higher maintenance dosage.

The anticoagulant effect of fondaparinux can be measured using an anti-Xa assay calibrated with fondaparinux (in mg/L). Anti-Xa assay calibrated with LMWH can also be used, however, a dose-dependent bias must be taken into account. Within the therapeutic range, the anti-Xa assay calibrated with LMWH overestimates fondaparinux concentration by ~20% (16). The test validated with LMWH calibrators (in IU/mL) serves only as a rough guide and may be helpful especially for monitoring of accumulation.

1.2. Target plasma concentrations and fondaparinux dosing

Anticoagulation therapy is always a balance between thrombosis (i.e., ECMO circuit dysfunction and decreased circuit lifespan) and bleeding complications. Target anti-Xa levels of fondaparinux for therapeutic, prophylactic, and ECMO indications are not established. Mean peak and trough plasma concentrations observed after 2.5 mg per day were 0.39–0.50 mg/L and 0.14–0.19 mg/L, respectively; in therapeutic dosing reflecting body weight, observed mean peaks and troughs were 1.20–1.26 mg/L, and 0.46–0.62 mg/L, respectively (8, 17, 18). Wahby et al. report very similar peak and trough anti-Xa levels as targets for prophylaxis in critically ill patients (0.47 ± 0.2 mg/L and 0.18 ± 0.1 mg/L, respectively) (19). On the other hand, few data are available confirming similar plasma levels of fondaparinux to be effective in venous thromboembolism prophylaxis (20). The correlation between plasma concentration and clinical outcomes is not proven in larger and/or specific populations (17, 21). The above-mentioned values may thus serve only as an approximate guide for dose titration.

In estimating the dosage of fondaparinux in our ECMO patients, we aimed at plasma concentrations of ~0.4–0.7 mg/L as a boundary between prophylactic and therapeutic levels or the lower part of the therapeutic range.

Our calculation of the individual dose was based on the pharmacokinetic data from healthy volunteers because data from the critically ill are generally unavailable. A loading dose (LD) was used to quickly achieve the target plasma concentration (c_{target}) set at 0.6 mg/L in a drug with a long biological half-life. We entered the values of 7–11 L for the V_D and 17–21 h for the biological half-life ($T_{1/2}$; taking into account its prolongation in impaired kidney function) (18). The maintenance dose was then estimated based on clearance and adjusted according to repeatedly measured anti-Xa levels taken prior to the next dose. We chose to divide the total daily dose into multiple single doses to minimize fluctuations in plasma levels and to prevent potential bleeding around peak concentrations. We did not expect any significant contribution of ECMO to V_D or clearance in our adult patients.

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TABLE 1 Basic demographics and time course.

Patient	Age (years)	Sex	Weight (kg)	Height (cm)	ABW (kg)	BMI (kg/m^2)	ECMO duration = ICU stay (days)	HIT diagnosis (days after UFH started)	Fondaparinux therapy duration (days)	PRBC units during fondaparinux therapy—total (per day)	ECMO circuit exchange (days after HIT diagnosis)
A	63	F	90	165	70	33.1	25	7	18	6 (0.3)	1
B	61	M	90	180	81	27.8	20	7	13	4 (0.3)	8
C	52	M	118	189	97	33.0	37	12	26	5 (0.2)	2

M, male; F, female; ABW, Adjusted Body Weight; BMI, Body Mass Index; ECMO, Extracorporeal Membrane Oxygenation; PRBC, Packed Red Blood Cells; UFH, Unfractionated Heparin; HIT, Heparin-Induced Thrombocytopenia.

The following formulas were used for calculations:

$$LD = c_{target} \times V_{D.}$$
$$MD = c_{target} \times clearance$$
$$clearance = \frac{\ln 2 \times V_D}{T_{1/2}}$$

A single dose was then calculated from the daily maintenance dose, considering the available size of fondaparinux injections (2.5 mg) and the interval at which this dose should be administered. Generally, the loading dose was 5 mg, followed by the maintenance dose of 2.5 mg every 8–12 h for a patient with more than 60 kg of body weight and normal renal functions. Subsequently, the anti-Xa value was checked 0.5–2 h prior to the next dose almost daily. For dose adjustment, the measured anti-Xa level, renal function, platelet count, and clinical signs of bleeding and thrombosis were considered.

Clotting in the extracorporeal circuit is usually challenging to diagnose. Visual inspection of tubing and membrane oxygenator may detect fibrin deposits or thrombi formation. Significant oxygenator thrombosis leads to an increase in the pressure gradient across the oxygenator (ΔP) and is also associated with impaired gas exchange in the oxygenator. Thrombosis in cannulas, tubing, or especially in the blood pump leads to intravascular hemolysis. Free plasma hemoglobin is a standard marker of its severity, with normal values below 0.5 g/L. Since we record ΔP hourly and measure free plasma hemoglobin daily, we used these parameters to assess anticoagulation efficacy. The most common complication of anticoagulation therapy is bleeding.

2. Case descriptions

Fondaparinux was used to provide anticoagulation in three critically ill COVID-19 patients with severe ARDS who developed HIT while on veno-venous ECMO. One patient had positive both HIT antibodies and the aggregation test (patient A), while two other patients had negative HIT antibodies and positive aggregation test (patients B and C). HIT diagnosis was confirmed in the National Reference Laboratory (Institute of Hematology and Blood Transfusion, Prague, Czech Republic) for all three patients. Demographic data and time course of HIT diagnosis and fondaparinux and ECMO therapy are summarized in Table 1. If not stated otherwise, day 0 is the date of HIT diagnosis and commencement of fondaparinux therapy. The glomerular filtration rate was calculated according to the CKD Epidemiology Collaboration Group (GFR CKD-EPI) equation based on serum creatinine; all values are calculated for the actual body surface and stated as median [range].

2.1. Patient A

Patient A was a 63-year-old woman with normal renal function during the entire ICU stay—GFR CKD-EPI was 113 (94–118) ml/min. Fondaparinux was started as a loading dose of 5 mg IV followed by a 2.5 mg IV dose 12-hourly. The determination of anti-Xa by the fondaparinux-calibrated method

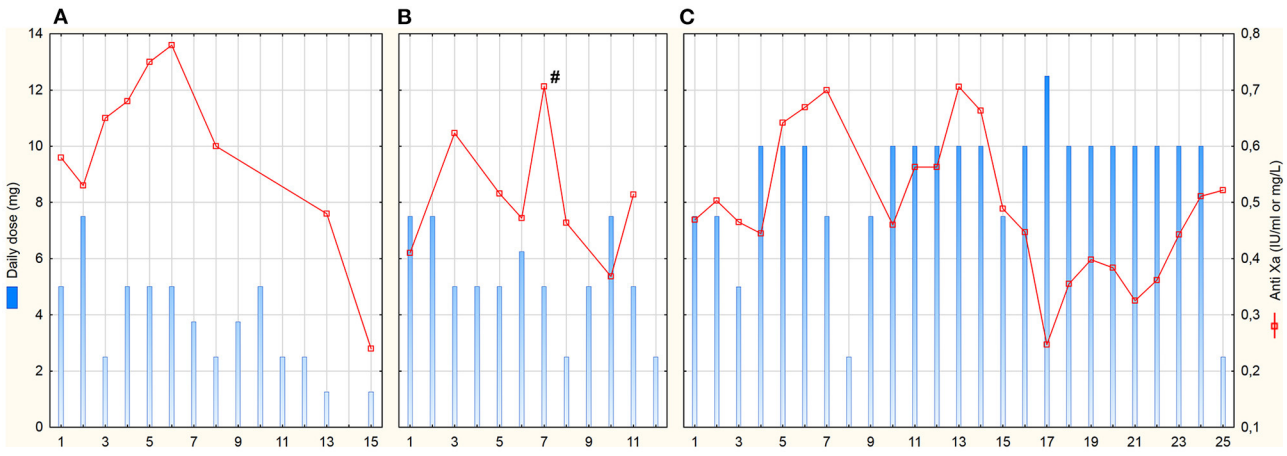

FIGURE 1
Daily fondaparinux doses and anti-Xa trough levels. Anti-Xa in IU/mL for patient **(A)** (assay calibrated for low molecular-weight heparins), in mg/L for patients **(B, C)**; # means peak anti-Xa 3 h after administration (trough levels were taken max. 2 h before the next dose); x-axis represents days after HIT diagnosis and commencement of fondaparinux treatment.

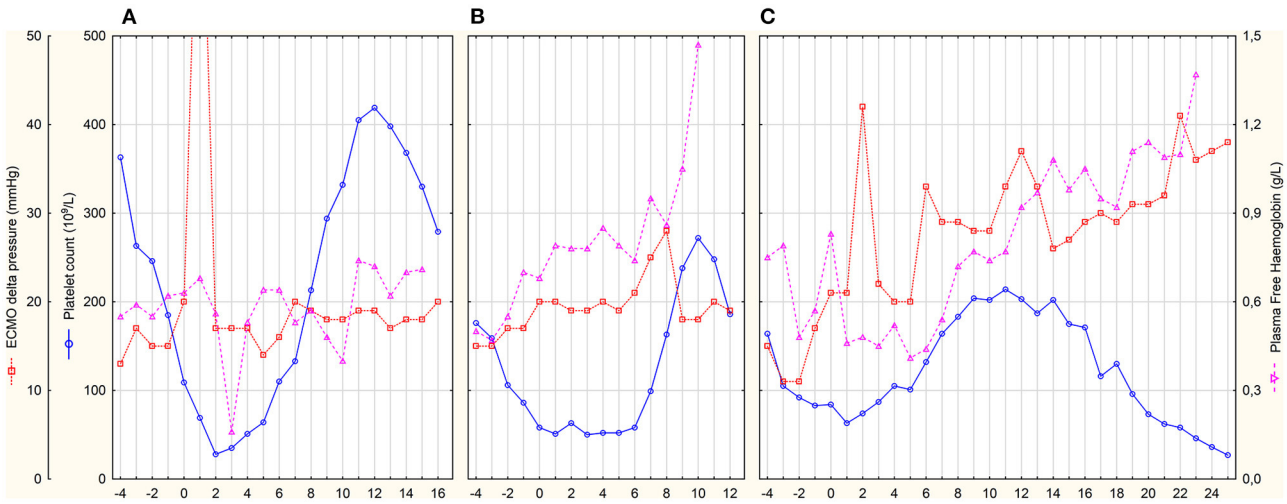

FIGURE 2
Daily platelet count and markers of ECMO circuit thrombosis. x-axis represents days after HIT diagnosis and commencement of fondaparinux treatment. **(A–C)** represent individual patients.

was not available at that time; therefore, anti-Xa was determined by the LMWH-calibrated assay. Only one anti-Xa value was determined by both methods in parallel (0.48 IU/ml vs. 0.43 mg/L). Fondaparinux doses and anti-Xa levels are shown in Figure 1A. There were several episodes of minor bleeding from the cannulation sites, upper and lower airways, and urinary tract. Fondaparinux doses were intermittently reduced due to bleeding episodes and relatively high anti-Xa levels. No patient-related thrombotic complications were observed. ECMO circuit exchange was necessary 1 day after HIT diagnosis due to significantly elevated ΔP (Figure 2A). Plasma free hemoglobin was moderately elevated throughout the whole course of ECMO therapy (Figure 2A). She underwent a surgical tracheostomy on day 14 of fondaparinux therapy; fondaparinux was withheld for 24 before the procedure. She died after therapy limitation on day 33 of her hospital stay due to persistent severe lung failure.

2.2. Patient B

Patient B was a 61-year-old man with initially normal renal function (GFR CKD-EPI 125 (122–135) ml/min until day 9) who developed acute kidney injury on day 9. Continuous veno-venous hemodialysis (CVVHD) was used for the last 3 days of his ICU stay. Fondaparinux loading dose of 5 mg IV was followed by 2.5 mg SC 8-hourly. Trough anti-Xa levels were determined by fondaparinux-calibrated assay (Figure 1B). From day 6, fibrin deposits were visible in the ECMO oxygenator. The ECMO circuit had to be exchanged on day 8 due to impaired ability to

eliminate CO_2. Fondaparinux was reduced on day 8 before surgical tracheostomy on day 9. Subsequently, the patient was complicated with pneumonia with empyema. Besides several episodes of minor bleeding from the cannulation sites and lower respiratory tract, this patient experienced a major bleeding according to the International Society on Thrombosis and Haemostasis definition on day 10 (22). Electrocauterization of the bleeding artery in a pressure sore on the tongue and transfusion of 3 units of packed red blood cells (PRBC) was necessary. Since the source of bleeding was the artery injured by teeth and anti-Xa level was below 0.4 mg/L, we do not consider this bleeding related to the fondaparinux therapy. There were no patient-related thrombotic events. Plasma free hemoglobin levels were persistently elevated with further increase after ECMO set exchange (Figure 2B). The terminal peak in plasma free hemoglobin was not solved due to the decision not to exchange the ECMO circuit because of poor prognosis. The patient died due to septic shock with multiple organ failure after 20 days of ICU stay.

2.3. Patient C

Patient C was a 52-year-old man with normal renal function [GFR CKD-EPI 167 (123–185) ml/min] during the entire ICU stay. After HIT diagnosis, fondaparinux was started with a 5 mg IV loading dose followed by 2.5 mg SC 8-hourly. Trough anti-Xa levels were also determined by fondaparinux-calibrated assay (Figure 1C). From day 0 on, blood clots were detected in the oxygenator. Due to an increase in ΔP (Figure 2C) with concomitant deterioration of blood gases, the ECMO circuit was exchanged on day 2. Fondaparinux dose was decreased on day 3 due to the cannulation site and lower respiratory tract bleeding. A thrombotic complication—ischemia of fingers on both hands—was diagnosed on day 6. Although perfusion of the left hand improved later, necrosis of two fingers developed on the right hand. On day 8, fondaparinux was omitted for 24 h, and a surgical tracheostomy was performed on day 9. Subsequently, the inter-dose interval was resumed at 6 h until day 35. On day 15, barotrauma required the insertion of a chest drain with the subsequent complication of chest drain and lower respiratory tract bleeding. Fibrin deposits, detected in the oxygenator on day 22, were accompanied by a mild increase in ΔP and plasma free hemoglobin (Figure 2C). Sepsis with thrombocytopenia gradually progressed while lung function remained stationary poor. The patient died after 36 days of ECMO support and ICU stay.

One episode of major bleeding unrelated to fondaparinux therapy and minor bleedings are described in case descriptions. The administration of PRBC is summarized in Table 1. Neither fresh frozen plasma nor platelets were administered during fondaparinux therapy.

3. Discussion

This case series provides a detailed insight into fondaparinux anticoagulation in ECMO patients complicated with HIT, who are prone to develop both bleeding and thrombotic complications, and the anticoagulation therapy becomes exceptionally challenging. In a group of three patients, we proved the fondaparinux efficacy demonstrated by decent ECMO circuit lifespan during the prolonged treatment. One major bleeding episode was unrelated to fondaparinux therapy, and transfusion requirement was low and comparable with a large cohort of veno-venous ECMO patients (23). Daily monitoring and related dosage adjustments contributed to achieving the predefined target plasma concentrations. We believe that this tight control secured the efficacy and safety of fondaparinux therapy.

The majority of previously published cases on fondaparinux anticoagulation on ECMO stated only prophylactic dosage (2.5 mg daily) without an anti-Xa measurement (9, 10). This approach might be insufficient for patients without an increased risk of bleeding because the HIT guidelines recommend using full-dose anticoagulation once the HIT diagnosis has been established (24). ECMO circuits might be extremely prone to clotting due to a hypercoagulable state during HIT and reduced anticoagulation therapy, as demonstrated by the early ECMO circuit exchange in two of three patients. Only one published case report (11) used a therapeutic dosage (7.5 mg daily) with a target anti-Xa range of 0.8–1.2 IU/ml; however, no achieved anti-Xa were reported.

3.1. Disadvantages/risks

The major disadvantage of fondaparinux is its long biologic half-life in combination with the unavailability of an antidote. This combination presents a significant risk in case of bleeding because the anticoagulation effect would persist for many hours or days. Moreover, impaired renal functions—a common complication in critically ill patients—further prolong the elimination half-life with a need for dose adjustment and the risk of prolonged bleeding. In contrast to APTT, monitoring of anti-Xa calibrated with fondaparinux is not widely spread. The optimal therapeutic target is unknown; however, this is similar to the other alternative drugs. Generally, fondaparinux is not suitable for patients with a high risk of bleeding and with limited benefits in patients with renal impairment.

3.2. Pharmacoeconomics

Although fondaparinux is an off-label drug for patients with HIT, it is an easily available and most affordable option in the Czech Republic. The other options are argatroban and bivalirudin,

TABLE 2 Pharmacoeconomics—expenses per day of treatment, including monitoring.

Drug	Fondaparinux	Bivalirudin	Argatroban
Daily dose	7.5 mg	576 mg (0.3 mg/kg/h)	35 mg (0.3 μg/kg/min)
Price of treatment (USD)	16.8	967	308
Monitoring (*n* per day)	Anti-Xa (1)	APTT (3)	APTT (3)
Price of monitoring (USD)	24.8	12.3	12.3
Total price per day (USD)	41.6	979.3	320.3

which have limited availability in some countries. The daily doses of bivalirudin and argatroban were calculated using the previously published dose requirements in ECMO patients (25, 26), and a body weight of 80 kg (see Table 2). Actual prices in the Czech Republic were used except for bivalirudin, which is not available on the Czech market, and where the price from two studies from the USA was adopted (~420 USD per 250 mg vial) (27, 28). Another factor for pharmacoeconomic analysis is the size of the vial, which is usually higher than the required daily dose in the case of argatroban. Once opened, the unused part must be discarded within 24 h. For calculations in Table 2, we used the price of one 100 mg argatroban vial per day. Typical monitoring for argatroban and bivalirudin is APTT 3–4 times daily, while once daily anti-Xa determination seems a reasonable option for fondaparinux due to its long half-life.

4. Conclusion

In this case series report, all three patients on ECMO received fondaparinux as an alternative anticoagulation agent in HIT. The average time of fondaparinux administration was 18 days. A therapeutic dose given as a loading dose followed by several daily maintenance doses adjusted to reach the target trough anti-Xa of 0.4–0.7 mg/L provided effective, safe, and economically acceptable form of anticoagulation.

Data availability statement

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

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the next of kin to participate in this study. Written informed consent for the publication of this case series was obtained from the next of kin.

Author contributions

Conceptualization, drafting the manuscript, and collecting data: JR and PS. Critical revision of the manuscript: VŠ. All authors approved the submitted version.

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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Fibrin monomers evaluation during hospitalization for COVID-19 is a predictive marker of in-hospital mortality

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Background: Coagulopathy is one of the main triggers of severity and worsening of Coronavirus disease 2019 (COVID-19) particularly in critically ill patients. D-dimer has been widely used to detect COVID-19 coagulation disorders and has been correlated with outcomes such as disease severity and in-hospital mortality. Involvement of other fibrin degradation products, particularly fibrin monomers (FM), remains an ongoing question.
Methods: We performed a monocentric study of adult patients with COVID-19, who were admitted either in the medical ward (MW) or in the intensive care unit (ICU) and who had FM measurements performed on them during the first wave of COVID-19 outbreak. We analyzed the positivity of FM levels (FM > 7 μg/mL) to assess the ability of FM monitoring during the first days of hospitalization to predict COVID-19 outcomes.
Results: In our cohort, 935 FM measurements were performed in 246 patients during their first 9 days of hospitalization. During patient follow-up, the FM levels were higher in patients admitted directly to the ICU than in those admitted to the MW. Moreover, we observed significantly increased levels of FM in patients when the data were stratified for in-hospital mortality. At hospital admission, only 27 (11%) patients displayed a positive value for FM; this subgroup did not differ from other patients in terms of severity (indicated by ICU referral at admission) or in-hospital mortality. When analyzing FM positivity in the first 9 days of hospitalization, we found that 37% of patients had positive FM at least once during hospitalization and these patients had increased in-hospital mortality ($p = 0.001$). Thus, we used non-adjusted Kaplan–Meier curves for in-hospital mortality according to FM positivity during hospitalization and we observed a statistically significant difference for in-hospital mortality (hazard ratio = 1.48, 95% CI: 1.25–1.76, $p < 0.001$). However, we compared the AUC of FM positivity associated with a ratio of D-dimer >70% and found that this combined receiver operating characteristic (ROC) curve was superior to the FM positivity ROC curve alone.

Conclusion: Monitoring of FM positivity in hospitalized patients with COVID-19 could be a reliable and helpful tool to predict the worsening condition and mortality of COVID-19.

KEYWORDS

SARS-CoV-2, COVID-19, fibrin monomers, microthrombosis, coagulopathy, ICU, mortality, D-dimer

Introduction

Coagulopathy is one of the main triggers of disease severity and fatal outcome in Coronavirus disease 2019 (COVID-19), particularly in critically ill patients (1, 2). Inflammation and related endothelial lesions are probably at the origin of this coagulopathy associated with pulmonary vascular obstruction (2). Coagulation activation during COVID-19 is largely reflected by an increase in D-dimer levels (3). Extensive literature exists on the predictive value of D-dimer (4), mainly at patient admission in hospital, and D-dimer measurement in the first 48 h after admission has been proposed as a sensitive biomarker of initial severity and in-hospital mortality (5). In our previous multicenter study involving 24 French hospitals, 1,154 patients had D-dimer measurement performed on them upon admission in the medical ward (MW). We demonstrated that a D-dimer level above 1,128 ng/mL was a relevant predictive factor for in-hospital mortality among patients admitted in the MW for COVID-19. This held true regardless of the occurrence of venous thromboembolism (VTE) during hospitalization (6). More recently, we proposed a new algorithm with a specific D-dimer threshold in COVID-19 patients according to lung extension disease, to safely exclude pulmonary embolism (PE) (7) and to reduce the use of the computed tomography pulmonary angiogram. Finally, we demonstrated that daily monitoring of D-dimer was a hallmark of severe COVID-19 disease (8), since modified kinetics are associated with intensive care unit (ICU) referral and in-hospital mortality.

The coagulation process results in an insoluble clot of cross-linked fibrin. Subsequently, the fibrinolytic system activates to limit the clot size. Lastly, plasmin degrades cross-linked fibrin into different soluble fragments, including D-dimer. Disseminated intravascular coagulation (DIC) was first suspected in SARS-CoV-2 infection in early reports of COVID-19. DIC is characterized by the systemic activation of coagulation, which can lead either to thrombosis of the small and midsize vessels, contributing to organ failure, or to bleeding with platelet and coagulation factor consumption. DIC is secondary to other conditions such as severe infection, cancer, trauma, or obstetric complications. The International Society on Thrombosis and Haemostasis (ISTH) established a scoring algorithm for the diagnosis of DIC. The score relies on platelet count, prolonged prothrombin time, fibrinogen level (9), and the level of fibrin markers, including D-dimer. Nevertheless, D-dimer is not a specific biomarker; it also increases in pregnancy, inflammatory disease, and sepsis, for instance. The results must therefore be interpreted along with clinical features and other laboratory assays. The loss of coagulation factors or antithrombin and acquisition of a DIC-like phenotype was not typically seen in COVID-19 (10). However, this does not prevent the patient from developing DIC, although this is a rather rare event in COVID-19 until the later stages of severe disease (1). Another fibrin-related biomarker is fibrin monomers (FM) whose concentration has been largely described to reflect prothrombin activity. FM have been proposed as a diagnostic marker of DIC (9) and a predictor of thrombosis and/or a hypercoagulable state earlier than D-dimer (11). In healthy individuals, FM levels are very low in peripheral blood, generally below the limit of detection (12). In the early stages of the pandemic, some experts proposed evaluating FM levels in COVID-19 patients (13). Despite these expert recommendations, less data are available on the relevance of FM evaluation during COVID-19 (14–19) in contrast to D-dimer. None of the studies made a strong case for a clear-cut clinical use and a daily-life evaluation.

The aim of the present study was to determine, with a large retrospective study, the incidence of FM positivity among adult patients hospitalized for COVID-19 both at admission and daily during hospitalization. In addition, we examined the prognostic value of FM positivity when assessing in-hospital mortality.

Methods

Study design and population

We performed a monocentric study of adult patients (≥18-years old) with COVID-19, who were admitted in the European Georges Pompidou Hospital between 1 February and 30 June 2020. We retrospectively analyzed, for the study period, all patients with a confirmed diagnosis of COVID-19, using a reverse transcriptase–polymerase chain reaction as previously described (8, 20), and who had FM measurements performed on them. The patients were classified according to World Health Organization (WHO) guidelines as either non-critical (median oxygen requirement of 3 L/min and a WHO score range of 4–7) or critical (requiring mechanical ventilation, WHO score range 8–9). Patient characteristics included age, sex, and body mass index (BMI). Clinicians used the local protocol recommended during this period. This protocol was based on the international guidelines from the ISTH, advocating for the use of a prophylactic regimen of anticoagulation for both non-critical patients and critical patients admitted in the ICU. Few patients had therapeutic or intermediate prophylaxis dose in surgical intensive care. No patient received glucocorticoids or immunosuppressant treatments. Venous blood was collected from patients in 0.129 M trisodium citrate tubes (9NC BD Vacutainer, Plymouth, UK) and processed according to standard

laboratory techniques. Platelet-poor plasma was obtained after centrifugation twice at 2,500× *g* for 15 min at room temperature.

We studied only those patients who were hospitalized and whose FM levels had been measured at least twice during the first 9 days. We excluded all patients who did not meet these criteria. The flow chart of our study is presented in **Figure 1A**. The median [interquartile range (IQR)] number of FM tests was 3 [2–6], with a minimum of 2 and a maximum of 10 (Day 0 for day of admission; **Figure 1B**).

Fibrin monomer evaluation

We quantified the plasma FM level in platelet-poor plasma (STA-Liatest FM, Diagnostica Stago, France) using a STA-R Max analyzer (Diagnostica Stago, as recommended by the manufacturer). According to the manufacturer's recommendation and calibration curves, the limit of detection was 6 µg/mL. Furthermore, as part of a requirement of the COFRAC ISO 15189 label of our laboratory,

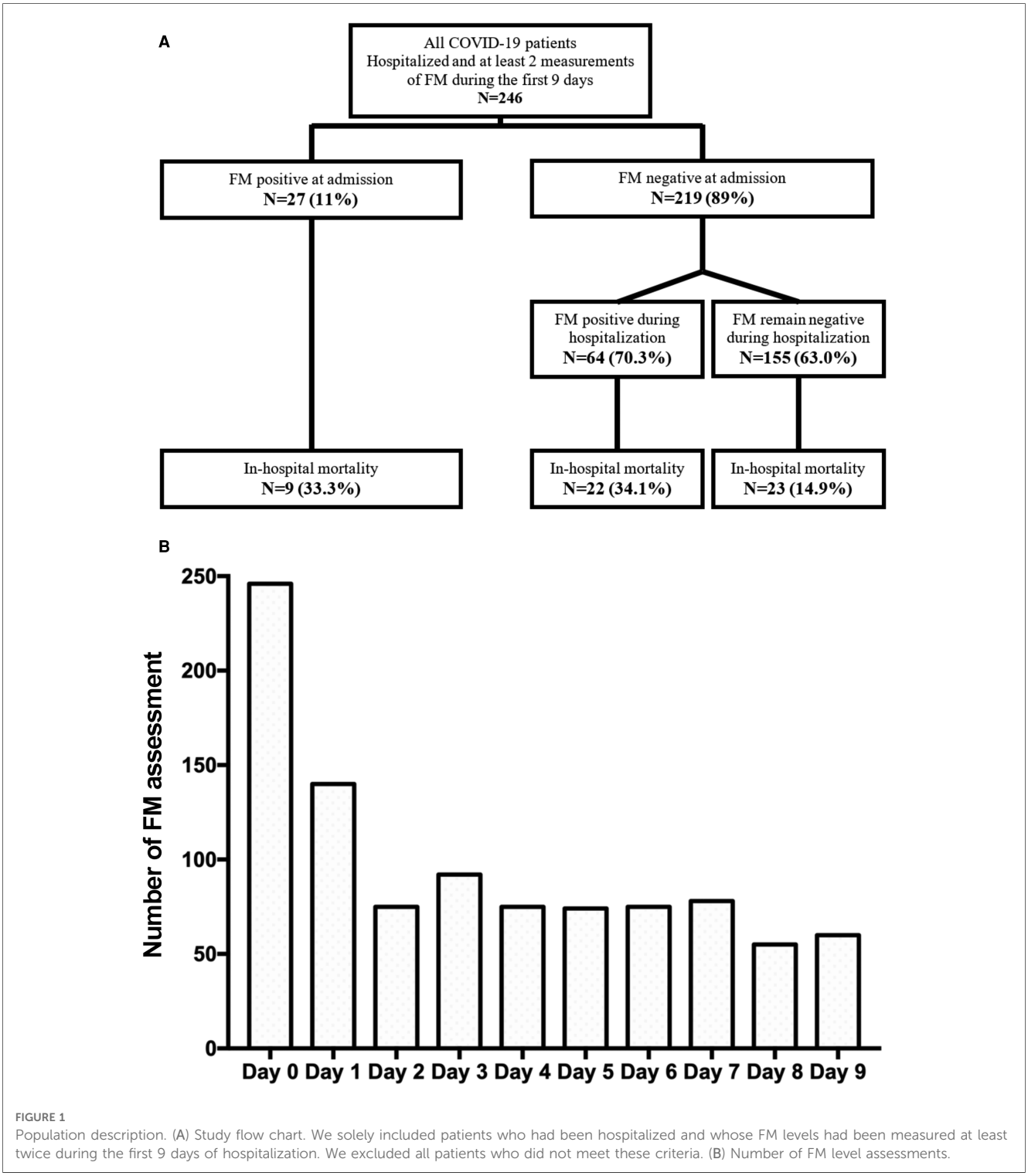

FIGURE 1
Population description. (A) Study flow chart. We solely included patients who had been hospitalized and whose FM levels had been measured at least twice during the first 9 days of hospitalization. We excluded all patients who did not meet these criteria. (B) Number of FM level assessments.

the limit of quantification was determined in our coagulometers and was identified as 7 μg/mL. Hence, positivity for FM was indicated by values above 7 μg/mL. The local protocol for COVID-19 management suggested the physician to measure FM in COVID-19 patients at admission and thereafter 72 h.

Statistical analysis

For the descriptive analysis, data were expressed as the median (IQR) for continuous data and as frequencies and percentages for categorical data. We used the Mann–Whitney test and Fisher's exact test to compare the continuous and categorical variables, respectively. In the survival analysis, we used the Kaplan–Meier curve to estimate the survival function from diagnosis to in-hospital death stratified to FM positivity. Survival curves were compared using the log rank test. We used the Cox proportional hazard (PH) model adjusted for age, BMI, and sex to investigate the association between in-hospital mortality and FM positivity. FM was a binary variable coded as follows: If the patient had at least one positive measurement above 7 μg/mL during the first 9 days, FM positivity was coded as 1; if the patient had any other positive measurement with FM ≤ 7 μg/mL, FM was coded as 0. To assess the PH model assumptions, we first estimated the regression coefficients using the functions *coxph* (*package survival*) and *crr* (*package cmprsk*). We tested for PH using the functions *prop.coxph* and *prop.crr* with default resampling methods implemented by the *goftte* package. We used two methods to examine the changes in the value of FM over time within the patient groups, which are defined as follows: (1) ICU at admission, (2) MW at admission and then transferred to the ICU during hospitalization, and (3) MW during the whole period of hospitalization. In the first method, during the first 9 days of hospitalization, we compared the mean value of FM in the group of patients who were admitted in the ICU with the mean value of patients admitted in an MW, using a Wilcoxon test with Bonferroni-corrected alpha level applied for each comparison. Second, to assess how the value of FM changed over time within each of the three patient groups, we used a linear mixed-effect model. In the model, time was considered a continuous measure, the patient group variable was an independent variable, and we added a random effect for the patient and an interaction term between the patient group and the period of time. To assess the prognostic ability of FM positivity and D-dimer value—at admission and during follow-up—we used receiver operating characteristic (ROC) curve analysis and compared the results with DeLong's test. The issue of missing data were addressed by imputation using a linear interpolation from observed values with an approximation function of the *stats* package of R software. Based on the imputed data, we created a graph using the *geom_smooth* function in the *ggplot2* visualization package of R. All analyses were two-sided, and statistical significance was set to $p < 0.05$. Statistical analyses were performed using R studio software, including R version 3.6.3 (RStudio Inc., Boston, MA, USA).

Results

FM levels during the first 9 days of hospitalization differed according to clinical outcomes in COVID-19 patients

Overall, 935 FM measurements were performed for 246 patients during their first 9 days of hospitalization (**Figure 1B**). Since the number of measurements of FM after 9 days of hospitalization was very low, we restricted our analysis because of low statistical power after more than 9 days. In our cohort, 169 (68.7%) patients were male, the median age was 66.0 years (IQR: 56.0–76.0), and 28 patients (11.4%) had a BMI above 30 kg/m^2. The cohort included 154 (62.6%) patients with COVID-19 who were initially admitted in an MW and 92 (37.4%) initially admitted in the ICU. The median duration of hospital stay was 16.0 days (IQR: 7.0–25.5) and the median delay from hospital admission to in-hospital death was 12.5 days (IQR: 6.0–24.0). The study period corresponded to the first wave of the pandemic, and clinicians used the local protocol recommended during this period. This protocol was based on the international guidelines from the ISTH and included the use of a prophylactic regimen of anticoagulation for non-critical patients and critical patients admitted in the ICU. A few patients underwent therapeutic or intermediate prophylactic dose in the surgical ICU (13, 21). None of the patients received glucocorticoids/immunosuppressants treatments. **Table 1** describes the range and positivity of FM observed during the first 9 days of hospitalization. In comparison with COVID-19 patients admitted in the MW, the mean FM levels of patients directly admitted in the ICU were not different at Day 0 and Day 1 (**Figure 2A**). In contrast, from Day 3 onwards in the MW, the mean FM levels were significantly higher in patients admitted initially in the ICU than in those admitted initially in the MW ($p < 0.05$, Wilcoxon test for all days following admission). Consistently, by considering the trend in FM levels throughout the period of the patients' follow-up, we found that the mean FM levels were significantly higher in patients admitted directly in the ICU than in those admitted to the MW (**Figure 2B**). Among the 154 COVID-19 patients directly admitted to the MW, 40 (26%) were referred to the ICU during their hospitalization, whereas 114 (74%) remained in the MW. Among patients initially admitted in the MW, starting from Day 4, a significant increase in FM levels was evidenced only for those referred to the ICU ($p < 0.001$, Wilcoxon test; **Figure 2C**). Finally, we observed significantly increased levels in the mean FM levels in patients (**Figure 2D**) when stratified on survival.

Iterative FM measurements during hospitalization, but not FM levels at admission, were predictive of worsening COVID-19 and in-hospital mortality

At hospital admission, only 27 (11%) patients displayed a positive value of FM (FM > 7 μg/mL, 17 of these patients were

admitted in the ICU and 10 in MW). We analyzed the positivity of FM levels (FM > 7 μg/mL) to assess the ability of FM monitoring during the first days of hospitalization to predict COVID-19 outcomes. We first analyzed FM positivity at hospital admission (Day 0). As demonstrated in **Table 2**, patients having positive FM at admission showed no differences in terms of age, sex, and

TABLE 1 Patients' value of fibrin monomers during hospitalization for COVID-19.

		All population		ICU at admission		MW at admission and then ICU		MD during the whole period of hospitalization	
		n = 246		*n* = 92		*n* = 40		*n* = 114	
Days	FM positivity	FM		FM		FM		FM	
	n (%)	Median [IQR]	Mean (SD)	Median [IQR]	Mean (SD)	Median [IQR]	Mean (SD)	Median [IQR]	Mean (SD)
0	27 (10.9%)	7.0 [7.0–7.0]	13.4 (27.2)	7.0 [7.0–7.0]	18.1 (36.4)	7.0 [7.0–7.0]	7.6 (3.2)	7.0 [7.0–7.0]	11.7 (22.5)
1	49 (19.9%)	7.0 [7.0–7.0]	25.4 (105.6)	7.0 [7.0–7.3]	31.9 (81.5)	7.0 [7.0–7.0]	14.3 (28.7)	7.0 [7.0–7.0]	24.1 (136.0)
2	48 (19.5%)	7.0 [7.0–7.0]	30.2 (104.5)	7.0 [7.0–8.8]	39.9 (122.0)	7.0 [7.0–7.7]	20.4 (56.8)	7.0 [7.0–7.0]	25.7 (102.2)
3	51 (20.7%)	7.0 [7.0–7.0]	52.9 (199.2)	7.0 [7.0–10.6]	80.7 (256.8)	7.0 [7.0–9.6]	33.1 (87.8)	7.0 [7.0–7.0]	37.3 (171.0)
4	57 (23.2%)	7.0 [7.0–7.0]	51.7 (182.6)	7.0 [7.0–14.9]	98.2 (277.6)	7.0 [7.0–19.0]	41.6 (117.1)	7.0 [7.0–7.0]	17.7 (50.4)
5	59 (24.9%)	7.0 [7.0–7.0]	67.9 (226.0)	7.0 [7.0–26.2]	134.3 (329.5)	7.0 [7.0–13.9]	64.5 (215.2)	7.0 [7.0–7.0]	15.5 (31.4)
6	61 (25.8%)	7.0 [7.0–7.8]	70.3 (222.2)	7.0 [7.0–51.1]	139.0 (325.8)	7.0 [7.0–9.1]	50.2 (158.4)	7.0 [7.0–7.0]	21.8 (80.6)
7	58 (24.6%)	7.0 [7.0–7.0]	53.2 (177.2)	7.0 [7.0–35.9]	102.4 (266.7)	7.0 [7.0–8.0]	29.2 (56.0)	7.0 [7.0–7.0]	21.9 (80.8)
8	54 (22.9%)	7.0 [7.0–7.0]	45.5 (159.5)	7.0 [7.0–13.2]	80.1 (237.7)	7.0 [7.0–7.1]	26.8 (49.6)	7.0 [7.0–7.0]	24.1 (85.2)
9	50 (20.3%)	7.0 [7.0–7.0]	48.1 (182.6)	7.0 [7.0–10.5]	75.8 (237.0)	7.0 [7.0–7.0]	23.5 (43.8)	7.0 [7.0–7.0]	34.4 (159.4)

ICU, intensive care unit; MW, medical ward; FM, fibrin monomers; IQR, interquartile range; SD, standard deviation.

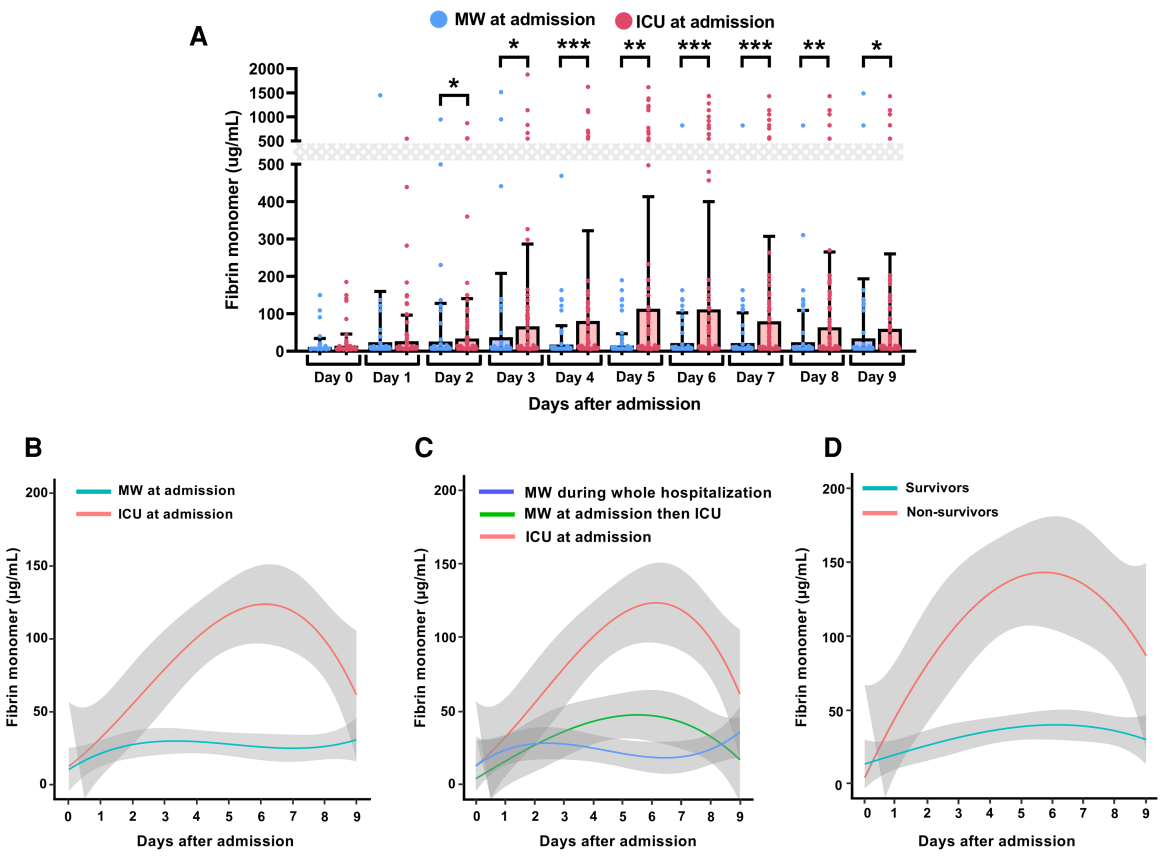

FIGURE 2
Daily monitoring of fibrin monomers levels and outcome predictions in hospitalized COVID-19 patients. For each group, the lines indicate the trendline of the mean FM levels measured daily during the first 9 days of hospitalization and the gray-colored area denotes the 95% confidence interval. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. (**A**) Comparison of FM levels during the first 9 days of hospitalization. (**B**) Temporal trends of daily FM levels during the first 9 days of hospitalization according to initial admission at hospital: medical ward (MW; blue Line) or intensive care unit (ICU; red line). (**C**) Temporal trends of daily FM levels during the first 9 days of hospitalization according to the care pathway: MW during the whole hospitalization (blue line); MW at admission then referred to the ICU (green line); ICU at admission (red line). (**D**) Temporal trends of daily FM levels during the first 9 days of hospitalization according to in-hospital mortality: survivors (blue line); non-survivors (red line).

TABLE 2 Patients' characteristics and outcomes according to fibrin monomer measurement during hospitalization for COVID-19.

		Negative	Positive	*p*-value
FM measured at admission in hospital ($n = 246$)				
Patients, *n* (%)		219 (89.0)	27 (11.0)	
Male, *n* (%)		151 (68.9)	18 (66.7)	0.98
Age, years [median (IQR)]		66.0 [55.5–76.0]	65.0 [59.5, 77.0]	0.97
BMI ≥ 30 kg/m^2, *n* (%)		26 (11.9)	2 (7.4)	0.71
Median [IQR]		7.0 [7.0–7.0]	29.0 [16.5–134.5]	<0.001
ICU admission (%)	ICU at admission	115 (52.5)	17 (63.0)	0.41
	Medical ward at admission	104 (47.5)	10 (37.0)	
In-hospital mortality, *n* (%)		45 (20.5)	9 (33.3)	0.21
		Negative	Positive	*p*-value
FM measured during the whole period of hospitalization ($n = 246$)				
Patients, *n* (%)		155 (63.0)	91 (37.0)	
Male, *n* (%)		105 (67.7)	64 (70.3)	0.78
Age, years, median [IQR]		66.0 [55.0–75.0]	66.0 [58.0–78.0]	0.53
BMI ≥ 30 kg/m^2, *n* (%)		22 (14.2)	6 (6.6)	0.11
Median [IQR]		7.0 [7.0–7.0]	11.0 [7.0–88.9]	<0.001
FM at admission in hospital	ICU at admission	46 (29.7)	46 (50.5)	<0.001
	Medical ward at admission	109 (70.3)	45 (49.5)	
In-hospital mortality, *n* (%)		23 (14.9)	31 (34.1)	0.001
		Positive during follow-up	Positive at admission only	
Positive FM measured at least one time during hospitalization ($n = 91$)				
Patients, *n* (%)		64 (70.3)	27 (29.7)	
Male (%)		46 (70.4)	18 (66.7)	0.83
Age, years [median (IQR)]		66.0 [58.0–78.0]	65.0 [59.5–77.0]	0.78
BMI ≥ 30 kg/m^2, *n* (%)		4 (6.6)	2 (7.4)	1.00
Median [IQR]		10.0 [7.0–85.0]	29.0 [16.5–134.5]	0.001
FM at admission in hospital	ICU at admission	29 (70.6)	17 (63.0)	0.52
	Medical ward at admission	35 (29.4)	10 (37.0)	
In-hospital mortality, *n* (%)		22 (34.1)	9 (33.3)	1

ICU, intensive care unit; MW, medical ward; FM, fibrin monomers; IQR, interquartile range; SD, standard deviation.

BMI and no difference was evident for severity (ICU referral at admission) or in-hospital mortality. Thus, we demonstrate that evaluating FM specifically at admission has no significance in terms of severity or in-hospital mortality prediction. In contrast, an analysis of FM positivity during the first 9 days of hospitalization indicated that 155 (63%) patients had negative FM during the whole period of hospitalization, whereas 91 (37%) patients had positive FM at least once during their stay in hospital. Patients having at least one positive FM during hospitalization did not show any differences in terms of age, sex, and BMI. However, they required more ICU referral at admission ($p < 0.001$) and had increased in-hospital mortality ($p = 0.001$). To confirm the association between FM positivity during the first 9 days of hospitalization and in-hospital mortality, we generated a non-adjusted Kaplan–Meier curve of in-hospital survival (**Figure 3**). The result demonstrated a statistical difference [hazard ratio (HR) 1.48, 95% CI: 1.25–1.76, $p < 0.001$]. Finally, a Cox regression model for in-hospital mortality adjusted for age, sex, and BMI confirmed a significant difference for in-hospital mortality of patients with positive FM during the first 9 days of hospitalization (adjusted HR: 1.47, 95% CI: 1.23–1.76, $p = 0.001$, data not given). To complete our analysis, we conducted a logistic regression test to determine whether the positivity of FM was associated with ICU referral. The results confirmed the link between FM positivity and ICU referral with an odds ratio of 2.27 (95% CI: 1.65–3.12, $p < 0.001$, data not shown). Finally, among patients with at least one positive FM during hospitalization, we analyzed the difference between those patients with positive FM at admission (Day 0) and those with positive FM solely during hospitalization (Day 1 in MW). We observed no significant differences in terms of demographics, COVID-19 severity, or in-hospital mortality.

Combination of FM positivity and the ratio of D-dimer assessed during the first 9 days of hospitalization improves in-hospital mortality prediction

We previously described a ratio of D-dimer (RoD) defined as either the D-dimer value on the day of outcome occurrence or the highest value during the first 9 days (if the outcome did not occur), divided by the D-dimer level at admission (8). We also previously identified different optimal thresholds for RoD: for patients with COVID-19 admitted directly to the MW, a threshold of 69% increase in RoD predicted in-hospital mortality; for patients admitted directly to the ICU, a threshold of 74% increase in RoD predicted in-hospital mortality (8). Thus, we

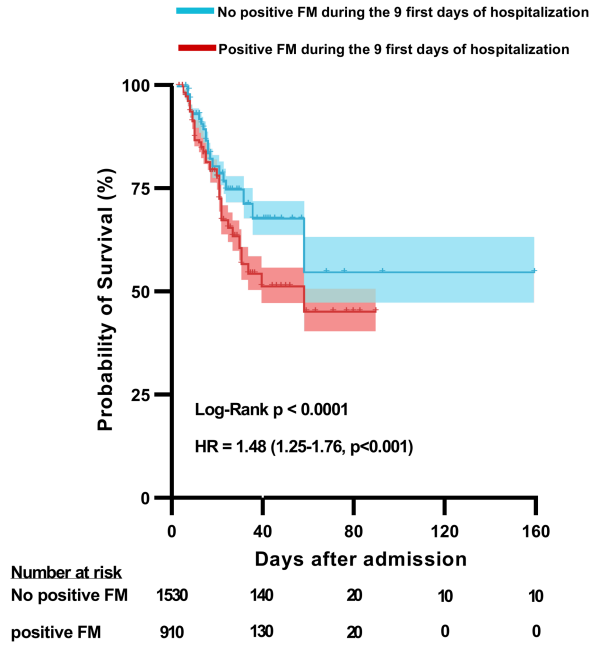

FIGURE 3
In-hospital mortality according to fibrin monomer positivity during the first 9 days of hospitalization for COVID-19. Unadjusted Kaplan–Meier survival curve for mortality among all hospitalized COVID-19 patients according to whether patients displayed at least one positive fibrin monomer (FM) level (Blue line) or not (Red line) during hospitalization. The *p*-value refers to the log rank test. Estimates of the hazard ratio (HR) and 95% confidence intervals derived from a single Cox-model model assessing the association between at least one positive FM level during hospitalization and in-hospital mortality.

TABLE 3 D-dimer and FM involvement during hospitalization for COVID-19: comparison of ROC curve analysis associating D-dimer or FM at admission or during the first 9 days of hospitalization.

Delong's test for two correlated ROC curves 2 by 2	ROC curve for FM positivity at admission	ROC curve for FM positivity and D-dimer at admission
ROC curve for FM positivity at admission	–	$p = 0.66$
ROC curve for D-dimer at admission	$p = 0.65$	$p = 0.63$
	ROC curve for FM positivity during hospitalization	ROC curve for FM positivity and RoD > 75% during hospitalization
ROC curve for FM positivity during hospitalization	–	$^{*}p = 0.0009$
ROC curve for RoD > 70% during hospitalization	$p = 0.09$	$p = 0.63$

compared the RoD predictive value with FM positivity and D-dimer at admission or FM positivity and RoD > 70% during hospitalization. An ROC curve analysis was created using D-dimer levels, FM positivity, and both parameters together at admission for the prediction of in-hospital mortality. In addition, we performed an ROC curve analysis for RoD > 70%, FM positivity, and both parameters together during hospitalization for the prediction of in-hospital mortality. We compare the predictability of three ROC curves in **Table 3** with DeLong's test for two correlated ROC curves 2 by 2 using the package *pROC*. No significant difference was found at admission regarding the ability to predict in-hospital mortality among positive FM, D-dimer, or both criteria. However, we compared the AUC of FM positivity associated with an RoD > 70% and found that this combined ROC curve was superior to the FM positivity ROC curve alone. This result demonstrates that FM is not superior to RoD > 70% during hospitalization, but that adding positivity to ROD allows us to more accurately predict in-hospital mortality.

Discussion

Coagulation activation is a hallmark of COVID-19 severity and probably reflects microthrombosis. Endotheliopathy associated with SARS-CoV-2 infection could be explained at least in part by coagulopathy, microthrombosis, and severity of symptoms (2). In this study, we demonstrated that FM positivity measured during the first 9 days of hospitalization was associated with COVID-19 severity and in-hospital mortality. Using a monocentric study of patients hospitalized for COVID-19, we observed that patients who displayed positive FM had a higher in-hospital mortality rate than those who never displayed a positive FM measurement during the first 9 days of hospitalization. Importantly, our study explored the usefulness of FM in a large cohort of patients with COVID-19 of varying degrees of clinical severity.

In terms of biomarkers, hemostasis parameters such as D-dimer have been highly "popular" and relevant biomarkers during the COVID-19 outbreak. Thrombus formation could be the origin of increased D-dimer observed in COVID-19; however, D-dimer can also originate from the extravascular space through the breakdown of alveolar fibrin deposits (22, 23). This extravascular origin could explain why FM plasma levels are generally within the normal range but D-dimer is high (16). Indeed, D-dimer is the most frequently observed abnormal coagulation parameter measured during SARS-CoV-2 infection. Before the COVID-19 outbreak, D-dimer was used in clinical practice to exclude a diagnosis of VTE, to estimate the risk of VTE recurrence, and to support the diagnosis of DIC (3, 9). The D-dimer levels at admission is an important indicator of COVID-19 severity and a relatively accurate good predictor of a worsening clinical state and in-hospital mortality, independent of VTE risk (6). Moreover, the course of D-dimer levels during hospitalization also seems relevant and may better predict outcomes (8). Thus, in contrast to early descriptions, it is now clear that increased D-dimer and COVID-19-induced coagulopathy is a clinical entity that differs from DIC, at least in part, at admission.

D-dimer is a specific marker of fibrin degradation (24), but it is not specific to VTE or DIC. Many conditions and diseases may increase D-dimer levels, including pregnancy and inflammatory diseases. The D-dimer/fibrinogen ratio has been proposed to increase the specificity of VTE diagnosis, but the results are inconsistent (25). With the high involvement of coagulopathy in COVID-19 for mortality prediction, it is important to identify other fibrin degradation biomarkers such as FM. FM is produced by thrombin cleavage of fibrinogen, releasing fibrinopeptide A and

B, and has been proposed as a marker of DIC or thrombosis (12). The presence of FM in circulation indicates an ongoing process of fibrinogen transitioning to fibrin after thrombin activation. In COVID-19, FM evaluation has been proposed by the French Society of Anesthesiology in the event of clinical worsening (13). However, little data on FM evaluation are available. Godon et al. studied a cohort of 164 COVID-19 patients and found that FM did not add any benefit, in contrast to D-dimer, to predict thrombotic events (14); analyzed events included VTE, arterial thrombosis, catheter-related thrombosis, and clotting related to dialysis filter and extracorporeal membranous oxygenation (14). Sridharan et al. demonstrated that only 23% of COVID-19 patients with increased D-dimer levels had elevated FM (15). This result is interesting in terms of specificity for DIC or COVID-19-associated coagulopathy diagnoses.

We previously described the clinical interest of FM monitoring for patients who are supported with ventricular assist device in order to assess appropriate anticoagulation (26, 27). Indeed, these patients had high levels of D-dimer, without any detectable thrombotic events, but interestingly, D-dimer levels were correlated to the amount of fibrin deposits on the surface of hemocompatible materials. In these patients, due to their high basal D-dimer level, DIC secondary to cessation of anticoagulant therapy is not easy to diagnose using D-dimer level alone, in contrast to FM (26, 27). In patients with COVID-19, FM levels are generally below the limit of detection and hence negative, whereas D-dimer is elevated in most of these patients and particularly those admitted in the ICU (8). Furthermore, the interpretation of D-dimer results in large cohorts may involve some challenges because of different reagents used in healthcare laboratories as stated by ISTH SSC during the COVID-19 outbreak (28, 29). Indeed, D-dimer assays can yield non-identical results. The reasons are differences in antibody specificity because of the heterogeneity in affinity for high- or low-molecular-weight fibrin degradation products and/or cross-linked and non-cross-linked fibrin derivatives.

Here, we demonstrate that evaluating FM specifically at admission has no relevance in terms of severity stratification or in-hospital mortality prediction. However, FM positivity during the follow-up period and its capacity to predict in-hospital mortality in any period are could be of clinical interest. D-dimer is associated with fibrin deposits, regardless of the location of fibrin: it can be extravascular deposits, which have been proposed as a marker of extravascular fibrinolysis correlated to the extent of lung injury (30). Fibrinolysis dysregulation in COVID-19 remains poorly understood. Several studies have shown that critically ill patients with COVID-19 display hypo-fibrinolysis (31, 32) or fibrinolysis shutdown associated with thrombosis, with the need for hemodialysis (33). Moreover, fibrinolytic shutdown should result in low D-dimer levels. Some studies have shown that COVID-19 patients have increased plasma thrombin levels and plasmin potential—in particular, high levels of plasmin-antiplasmin complex levels—compared with healthy donors and sepsis patients (34); nonetheless, *in vitro* analysis shows a hypofibrinolytic profile (31, 33) with an impaired response to r-tPA (32). Further studies are needed to better identify the role of fibrinolysis dysregulation in micro- and macrothrombosis and the origins of high D-dimer levels during COVID-19. Overall, it appears that FM detection could be a biomarker of worsening COVID-19 and could help in clinical characterization and management, regardless of the D-dimer level. Furthermore, FM quantification in plasma is now well standardized and is available on automated coagulometers with external quality controls.

Our study has some limitations: (i) the issue of missing data was addressed by imputation using a linear interpolation from observed values with an approximation function of the *stats* package of R software, (ii) the course of FM plasma levels could be influenced by DIC during ICU stay, and (iii) the course of FM plasma levels could be influenced by the different anticoagulation regimens.

All in all, the findings indicate that positive FM detected through iterative FM measurement during the first 9 days of hospitalization was associated with ICU referral and in-hospital mortality among COVID-19 patients. Hence, monitoring of FM during hospitalization could be an important tool for evaluating disease progression added to D-dimer. The predictive value should be confirmed in large multicentric studies that assess the association between routine measurement of FM levels and markers of thromboinflammation and endotheliopathy.

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 Comité de protection des personnes Sud Ouest et Outre Mer IV. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All the undersigned authors have substantially contributed to the paper. DS, NG, and RC designed the present study. DS wrote the manuscript. RC performed statistical analyses. All authors reviewed the paper. All authors declare that the submitted work is original and has not been published before (neither in English nor in any other language) and that the work is not under consideration for publication elsewhere. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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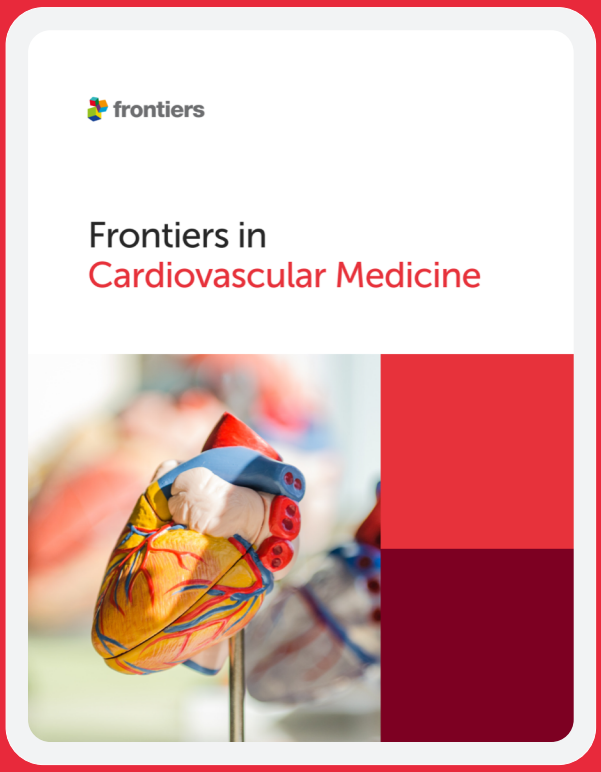

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