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The immunological role of platelet activation in the pathophysiology of COVID-19

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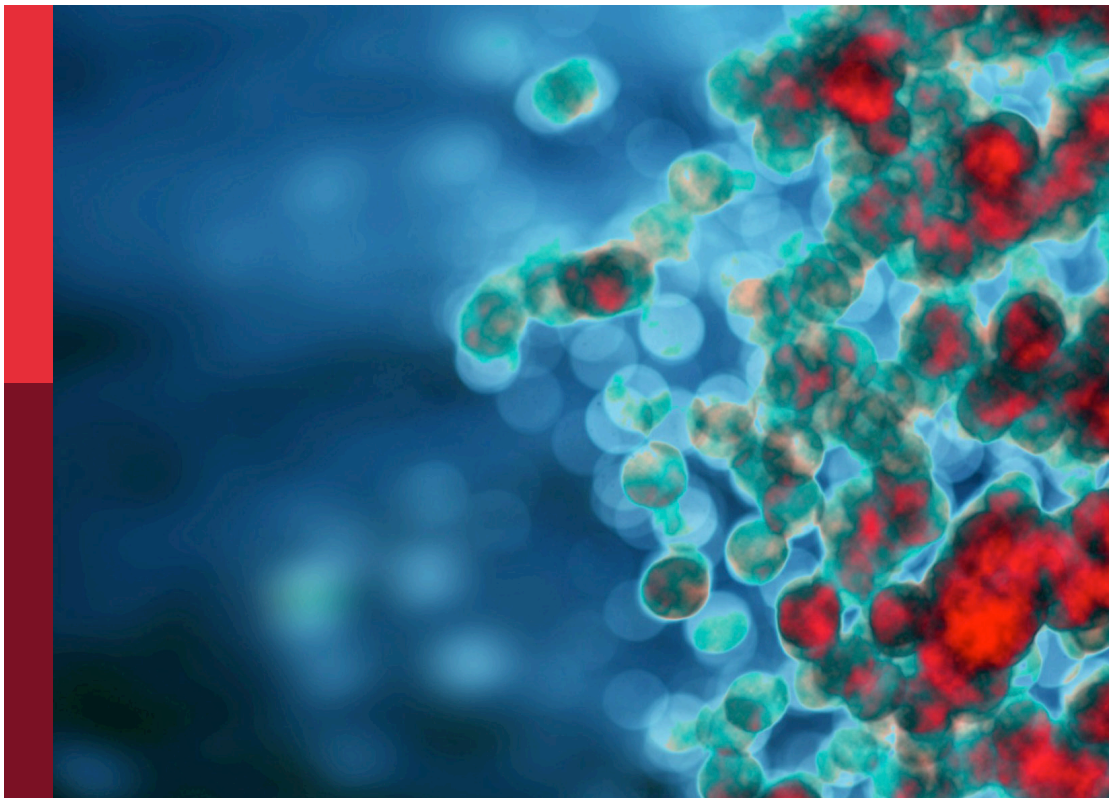

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The immunological role of platelet activation in the pathophysiology of COVID-19

Topic editors

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Editorial: The immunological role of platelet activation in the pathophysiology of COVID-19

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KEYWORDS

platelets, SARS-CoV-2, inflammation, immuno-thrombosis, vaccine-induced immune thrombocytopenia and thrombosis

Editorial on the Research Topic
The immunological role of platelet activation in the pathophysiology of COVID-19

Platelets are increasingly recognized for their role as mediators of immune response and inflammation. As major components of the hematological system, they form an important bridge between immunity and coagulation. When platelets become hyperactivated in response to an infection, patients can develop immuno-thrombosis and coagulopathy. These derangements of hemostasis are particularly relevant in the context of infection with the novel coronavirus, SARS-CoV-2, and the subsequent development of coronavirus disease, COVID-19, a disease in which thromboembolic events are an important cause of morbidity and mortality.

This Research Topic focuses on the immunological role of platelet activation in the pathophysiology of COVID-19 as well as Vaccine-induced Immune Thrombocytopenia and Thrombosis (VITT) in *in vitro* and *in vivo* environments.

Jevtic and Nazy provide an elegant review of platelet hyperactivation in COVID-19. They describe how the disease is associated with changes in platelet surface markers, secretion responses, and gene expression as well as increased circulating platelet-leukocyte aggregates. Immune complexes appear to contribute significantly to this platelet-activating effect; the antigen specificity and additional characteristics of these immune complexes remain to be determined.

Platelet activation *in vivo* in COVID-19 patients can lead to reduced responsiveness to additional *in vitro* stimuli, due to functional exhaustion of these anucleate corpuscles. Apostolidis et al. show that COVID-19 is associated with increased markers of basal platelet activation and reduced agonist responsiveness. Exposure to COVID-19 plasma increases donor platelet activation and, in a flow model, causes more platelet adhesion. The ability of patient plasma to activate donor platelets correlates with worsened patient outcomes. These effects occur through FcgRIIa-Syk and C5a-C5aR pathways; this identifies

Syk kinase inhibitors and C5 inhibitors as therapeutics with potential promise for COVID-19 thromboinflammation.

The role of complement activation is also explored by Perico et al. They describe a novel mechanism through which SARS-CoV-2-derived S1 protein sufficiently induces inflammatory and thrombogenic processes in the microvasculature. They demonstrate that the *in vitro* activation of endothelial cells with S1 protein, *via* ACE2, lead to leukocyte recruitment and C3 and C5b-9 deposition on endothelial cells, along with C3a and C5a generation that further magnify spike-induced complement activation. They propose that this process possibly recapitulates the systemic thromboembolic complications observed in severe COVID-19 cases, hence strengthening the argument for the use of complement inhibitors.

Capozzi et al. investigate patients with COVID-19 who experienced ischemic stroke. Immunoglobulin from such patients, particularly when containing antiphospholipid antibodies, is shown to activate donor platelets through phosphorylation of ERK and p38 as well as cause aggregation and ATP secretion.

Extracellular vesicles (EV) in biological fluids can be identified through light scatter properties and the presence of phosphatidylserine (PS) on their outer membrane leaflet. Annexin A5 binding is often used to identify PS positive particles. Jacob et al. report a significant increase in annexin A5 avid circulating EV (total plasma as well as endothelial- and platelet- derived EV) in COVID-19 ICU patients. Such microparticles are associated with increased thrombotic events.

Overall, these studies demonstrate the significant interplay of the humoral immune system and platelet activation in COVID-19, contributing to the high rate of thromboembolism seen in this disease.

The blood of people living with HIV (PLWH) are known to be hypercoagulable and they might therefore be particularly vulnerable to the thromboembolic complications of SARS-CoV-2. In an insightful study, van der Mescht et al., assess platelet and endothelium-associated cytokines, chemokines, and growth factors in 174 patients hospitalised with COVID-19. HIV co-infection was not associated with increased intensity of the inflammatory response. They do, however, report an interesting association between increased levels of the angiogenic factor, plasma vascular endothelial growth factor (VEGF), with SARS-CoV-2/HIV co-infection. Of note, PLWH with low CD4 counts or uncontrolled HIV replication, had lower levels of VEGF concentrations, possibly indicating an inappropriate immune response.

Rossouw et al. review the interaction of platelets with the vascular endothelium in the context of COVID-19-related myocardial injury. They describe the interaction of the viral spike protein with endothelial ACE2 together with alternative mechanisms that involve nucleocapsid and viroporin in the generation of a generalized endotheliitis. They further explain how platelet-derived calcium-binding proteins, SA100A8 and SA100A9, intensify endothelial activation and dysfunction. These events create a SARS-CoV-2–driven cycle of intravascular inflammation and coagulation, which contributes significantly to a poor clinical outcome in patients with severe disease.

Finally, three articles explore the immunological role of platelet activation in the pathophysiology VITT.

In an unprecedented head-to-head comparison between the Oxford/AstraZeneca [ChAdOx1] (AZ) and mRNA vaccines, Ostrowski et al. present original data about the diverse responses elicited by different vaccines. Their findings reveal enhanced inflammation, platelet activation, and thrombin generation following AZ vaccination and shed light on potential triggers and mechanisms underlying complications like VITT.

Pang et al. in a hypothesis and theory paper, venture into the uncharted territory of anionic substances binding to platelet factor 4 (PF4) in the context of VITT induced by the ChAdOx1-S vaccine. They identify five potential candidates within the vaccine that could trigger VITT, with negatively charged impurity proteins emerging as the most likely instigator. By unravelling the molecular interactions between these anionic substances and PF4, the authors shed light on the intricate web of events that give rise to VITT.

These hypotheses lay the groundwork for a deeper understanding of VITT's, offering a potential avenue for risk assessment and intervention strategies to ensure the safety of vaccine recipients.

In a succinct mini review, Hirsch et al. explore the interplay between platelets and neutrophils, both in the context of COVID-19 and VITT. The review underscores the central roles played by hyperactivated platelets and neutrophil extracellular traps in the coagulopathy associated with COVID-19. The authors highlight the emergence of a new paradigm in thrombophilia, catalysed by auto-antibody formation in response to adenoviral vector vaccines.

The collective insights from the papers, spanning original data to theoretical exploration and concise synthesis, featured in this Frontiers of Immunology Research Topic, underscore the need for multidisciplinary research and edge us forward towards unravelling the complexities of platelet activation in the context of infection.

Author contributions

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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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Inflammation and Platelet Activation After COVID-19 Vaccines - Possible Mechanisms Behind Vaccine-Induced Immune Thrombocytopenia and Thrombosis

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Introduction of vaccines against COVID-19 has provided the most promising chance to control the world-wide COVID-19 pandemic. However, the adenovirus-vector based Oxford/AstraZeneca [ChAdOx1] (AZ) and Johnson & Johnson [Ad26.CoV2.S] COVID-19 vaccines have been linked with serious thromboembolic events combined with thrombocytopenia, denominated Vaccine-induced Immune Thrombocytopenia and Thrombosis (VITT). The pathogenesis of COVID-19 VITT remain incompletely understood; especially the initial events that trigger platelet activation, platelet factor (PF)4 release, complex formation and PF4 antibody production are puzzling. This is a prospective study investigating the impact of different COVID-19 vaccines on inflammation (CRP, TNF-α, IL-1β, IL-6, IL-8, IL-10), vascular endothelial activation (syndecan-1, thrombomodulin, E-selectin, ICAM-1, ICAM-3, VCAM-1), platelet activation (P-selectin, TGF-β, sCD40L) and aggregation (Multiplate® impedance aggregometry), whole blood coagulation (ROTEM®), thrombin generation and PF4 antibodies to reveal potential differences between AZ and mRNA vaccines in individuals without VITT. The study included 80 (55 AZ and 55 mRNA) vaccinated individuals and 55 non-vaccinated age- and gender matched healthy controls. The main findings where that both vaccines enhanced inflammation and platelet activation, though AZ vaccination induced a more pronounced increase in several inflammatory and platelet activation markers compared to mRNA vaccination and that post-vaccination thrombin generation was higher following AZ vaccination compared to mRNA vaccination. No difference in neither the PF4 antibody level nor the proportion of individuals with positive PF4 antibodies were observed between the vaccine groups. This is the first study to report enhanced inflammation, platelet activation and thrombin generation following AZ vaccination compared to mRNA

vaccination in a head-to-head comparison. We speculate that specific components of the AZ adenovirus vector may serve as initial trigger(s) of (hyper)inflammation, platelet activation and thrombin generation, potentially lowering the threshold for a cascade of events that both trigger complications related to excessive inflammation, platelet and coagulation activation as observed in epidemiological studies and promote development of VITT when combined with high-titer functionally active PF4 antibodies.

Keywords: COVID-19, platelet factor 4, thrombocytopenia, thrombosis, vaccines, VITT, TTS

INTRODUCTION

Introduction of vaccines against COVID-19 has provided the most promising chance to control the world-wide COVID-19 pandemic. The chimp adenovirus vector-based Oxford/AstraZeneca [AZD1222/ChAdOx1] (AZ) COVID-19 vaccine was approved in the United Kingdom ultimo December 2020 and by the European Medicines Agency at the end of January 2021 and it is one of the most widely used COVID-19 vaccines. In March 2021, the first cases of serious thromboembolic events combined with thrombocytopenia following AZ vaccines were reported (1–3). These events lead to a pause in the use of the AZ vaccine in several European countries, and Denmark later chose to completely remove the AZ vaccine from the general vaccination program. In April 2021, vaccination with another adenovirus vector-based COVID-19 vaccine from Johnson & Johnson [Ad26.CoV2.S] was paused in the United States when similar clinical cases were reported (4–6). Subsequently, larger case series have been published delineating the occurrence and severity of this complication (7–9), now denominated Vaccine-induced Immune Thrombocytopenia and Thrombosis (VITT) (10).

VITT is characterized by multiple thromboses at unusual sites most often as cerebral venous sinus thrombosis (CVST) and splanchnic vein thromboses combined with thrombocytopenia, increased D-dimer and often decreased fibrinogen (1, 2, 7–9, 11). VITT is occasionally complicated by arterial thrombosis as ischemic stroke, myocardial infarction or limb ischemia (3, 12, 13).

The mechanisms behind the pathogenesis of VITT remain incompletely understood, but there is much to suggest that the platelet consumption and the progressive thrombogenic state is initiated by formation of antibodies against the protein platelet factor 4 (PF4). Unusual high serum levels of PF4 antibodies have been found in the vast majority of patients with VITT (1–4, 7, 8). PF4 is a tetrameric chemokine (CXCL4) stored mainly in megakaryocytes and in the α-granules of platelets (14) and released upon platelet activation. In VITT, patients form IgG antibodies against the PF4-polyanion complexes, which activate the platelets *via* the Fcγ receptor on the surface of the platelets. This platelet activation induces increased release of PF4, which contributes to complex formation with the newly formed PF4 autoantibodies leading to thrombocytopenia, increased platelet aggregation and thrombus formation. It is, however, not yet known what triggers the strong autoimmune response in VITT or which components of the vaccines that trigger antibody formation to PF4.

To investigate the impact of COVID-19 vaccines on inflammation and platelets, we analyzed markers of inflammation, vascular endothelial activation, platelet activation and aggregation, whole blood coagulation, thrombin generation and PF4 antibodies pre- and post-vaccination in a cohort of individuals without VITT receiving either AZ or mRNA [Pfizer/BioNTech BNT162b2 and Moderna mRNA-1273] vaccines. For further comparison, an age and gender matched control group comprising healthy non-vaccinated individuals was included.

MATERIALS AND METHODS

Study Participants

Vaccinated study participants were included in the Danish national vaccine trial ENFORCE (*National Cohort Study of Effectiveness and Safety of SARS-CoV-2/Covid-19 vaccines*, https://chip.dk/Research/Studies/ENFORCE/About-ENFORCE) EudraCT no (2020–006003–42). The ENFORCE trial is funded by The Danish Ministry of Health. The ENFORCE trial was approved by the National ethical committee (1-10-72-337-20) and the Danish data protection agency (1-16-02-289-21). Oral and written informed consent from all participants were obtained. The study was conducted in accordance with the declaration of Helsinki.

Individuals recently vaccinated with either AZ or mRNA vaccines against COVID-19 were invited to provide an early post-vaccination blood sample (see **Figure 1** for study design). The present study included eighty participants recently vaccinated (median of 11 days (range 8-16) post-vaccination) with either AZ (n=55, Oxford/AstraZenica [AZD1222/ChAdOx1]) or mRNA (n=25 in total: n=16 Pfizer/BioNTech [BNT162b2] and n=9 Moderna [mRNA-1273]) vaccines. Pre-vaccination samples were available from all participants, as they were participants in the ENFORCE study.

Since blood samples for platelet aggregation, whole blood coagulation and thrombin generation were not available at the pre-vaccination sample, non-vaccinated healthy individuals (controls) were invited to participate. The non-vaccinated controls were included in a separate study approved by the regional ethical committee (1-10-72-208-21) and the data protection agency (1-16-02-246-21). Oral and written informed consent from all participants were obtained. The study was conducted in accordance with the declaration of Helsinki. Eighty controls volunteered to participate and among these,

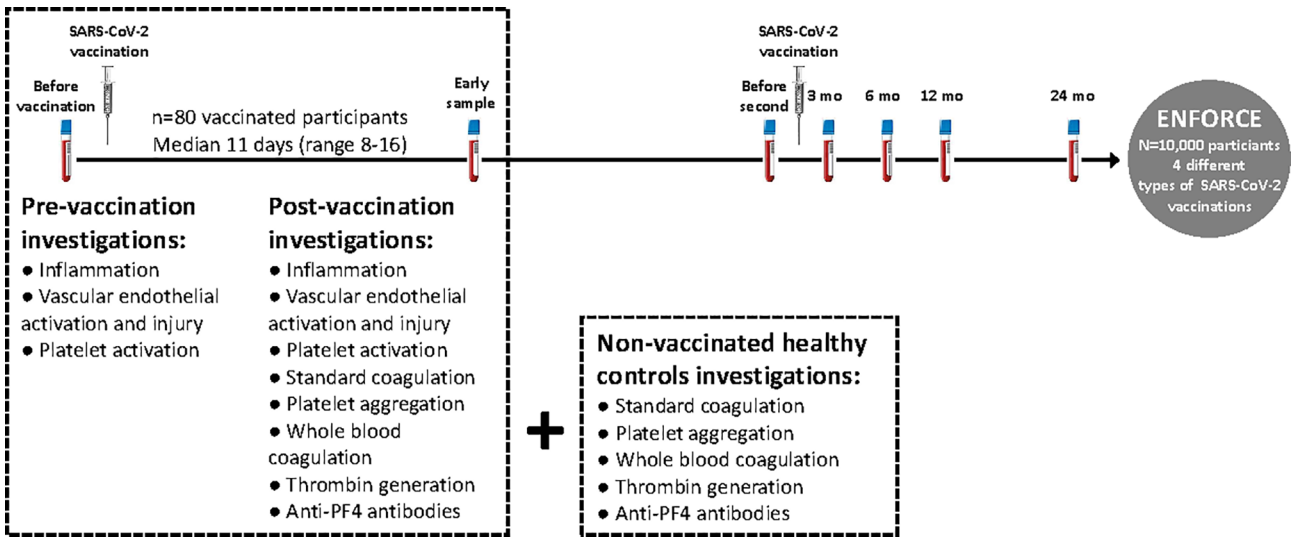

FIGURE 1 | Study design. Eighty participants (n=80) included in the Danish national vaccine trial ENFORCE, recently vaccinated with AZ or mRNA vaccines against COVID-19 were invited to provide an early blood sample post-vaccination (median of 11 days (range 8-16) post-vaccination). Pre-vaccination samples were available from all participants *via* the ENFORCE study. Since standard coagulation tests, platelet aggregation, whole blood coagulation (Thromboelastometry) and thrombin generation were not available at the pre-vaccination sample, non-vaccinated age and gender matched healthy individuals were invited to participate as controls.

we selected a subgroup that was age matched (age distribution matched from 20-70+ years for each 10-year interval with random and data blinded removal of individuals within age intervals with overrepresentation) and gender matched (similar approach as for the age matching) with the entire vaccination group (n=80). The age and gender matched controls comprised 55 individuals.

Participant Data

Age, gender and information about anticoagulant medication or platelet inhibitors were registered upon study participation and blood sampling.

Biomarkers of Inflammation, Vascular Endothelial Activation and Platelet Activation

Biomarker analysis was conducted pre- and post-vaccination. Markers reflecting inflammation (C-reactive protein (CRP), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10), vascular endothelial activation (syndecan-1, thrombomodulin, endothelial (E)-Selectin, intercellular adhesion molecule (ICAM)-1, ICAM-3, vascular cell adhesion molecule (VCAM)-1) and platelet activation (platelet (P)-Selectin, transforming growth factor (TGF)-β, soluble CD40 ligand (CD40L)) were measured in EDTA plasma by MesoScale Discovery (MSD) single or multiplex panels according to the manufacturer's recommendation by a MESO QuickPlex SQ 120 instrument (Rockville, Maryland, US). The following panels were applied: Proinflammatory panel I (TNF-α, IL-1β, IL-6, IL-8, IL-10). Human vascular injury kit I (E-Selectin, ICAM-3, P-Selectin, Thrombomodulin). Human vascular injury (CRP, ICAM-1, VCAM-1). Human syndecan-1 assay (syndecan-1). Human effector checkpoint assay (soluble CD40L). Human TGF-β1 assay (TGF-β). Results are displayed as concentrations (either pg/ml, ng/ml or μg/ml as appropriate).

Standard Coagulation, Platelet Aggregation (Multiplate), Whole Blood Coagulation (Thromboelastometry, ROTEM) and Thrombin Generation

All analyses were performed post-vaccination and in non-vaccinated healthy controls in an ISO 15189 accredited laboratory.

Standard Coagulation

In brief, platelet count was measured in EDTA plasma employing Sysmex XN-9000 (Sysmex, Kobe, Japan). Blood samples for the remaining coagulation tests were obtained using 3.2% sodium citrate. Fibrinogen (functional, Clauss methods), D-dimer, international normalized ratio (INR), and activated partial thrombin time (aPTT) were analyzed employing Sysmex C5100 (Sysmex, Kobe, Japan).

Platelet Aggregation

Blood was collected in tubes anticoagulated by hirudin. The analysis was performed within 2 hours employing impedance aggregometry (Multiplate® Analyzer, Roche, Germany). Platelets were activated with adenosine diphosphate (ADP) (Roche ADPtest), collagen (Bio/Data Corporation, BioNordika, Herlev, Denmark), and arachidonic acid (AA) (Roche ASPItest), according to the manufacturer's recommendations. The area under curve (AUC, aggregation units* min) was registered.

Whole Blood Coagulation

Whole blood for thromboelastometry (ROTEM, Instrumentation Laboratory, Bedford, USA) was collected using 3.2% sodium citrate tubes. The samples were left to rest for 30 min and analyzed within 2 hours. The standard assays EXTEM, INTEM, and FIBTEM were performed according to the manufacturer's recommendations. The following parameters were registered: clotting time (CT, seconds), maximum clot formation (MCF, mm) and lysis at 30 minutes (LI30, %).

Thrombin Generation

Blood was collected using sodium citrate 3.2% tubes, centrifuged at 3,000 g for 25 min within 1 hour after blood sampling to obtain platelet-poor plasma, aliquoted, and stored at -80°C until analysis. The thrombin generation assay (calibrated automated thrombogram®, Thrombinoscope® BV, Maastricht, the Netherlands) was performed as previously described, with final concentration of 5 pM tissue factor in wells (15). The following parameters were registered: Lag time (min), time to peak thrombin concentration (ttPeak, min), peak thrombin concentration (peak, nM), and endogenous thrombin potential (ETP, area under curve, nM*min).

Platelet Factor (PF)4 Antibodies

PF4 antibodies were investigated post-vaccination and in non-vaccinated healthy controls.

The immunoglobulin-G (IgG) specific ELISA-based assay Lifecodes PF4 IgG (Immucor, Solihull, United Kingdom) was employed to measure antibodies against PF4 (16). The blood samples were collected in tubes without anticoagulant, and serum was separated from red blood cells within 2 hours and stored at -80°C until analysis. The analysis was performed in duplicate and in case of more than 20% deviation the test was re-run. Based on 120 serum samples from healthy individuals, the manufacturer calculated a 95% reference interval with a 90% confidence and stated the upper end of the reference interval to be 0.352 O.D. units. According to the manufacturer, test results showing O.D. values equal to or greater than 0.400 are regarded as positive results.

Statistics

Data on demography, biomarkers, aggregation, whole blood coagulation, thrombin generation and PF4 antibodies are displayed as medians with interquartile ranges (IQR) or as n and proportions. AZ and mRNA vaccinated individuals (pre-, post-vaccination, delta values) and non-vaccinated controls were compared by Mann-Whitney U tests or Chi-square/Fishers exact tests, as appropriate. Delta values were calculated as post-vaccination values minus pre-vaccination values. Changes in variables over time (from pre- to post-vaccination) within sub-groups (AZ and mRNA vaccinated) were compared by Wilcoxon singed-rank test. Spearman correlations were conducted to investigate correlations between platelet count post-vaccination and platelet activation biomarkers and to investigate the intercorrelation between platelet activation biomarkers, results are displayed as rho and p-values. IBM SPSS statistics v. 25 were used for all analyses and figures. P-values <0.050 are considered significant.

RESULTS

Study Participants

Eighty vaccinated were included; 55 vaccinated with AZ and 25 vaccinated with mRNA COVID-19 vaccines. The median age of the entire group (AZ + mRNA) was 49 years (IQR 37-58), 73% were women. The AZ and mRNA groups had comparable age (median 48 (IQR 35-57) *vs* 49 (IQR 38-61) years), but the AZ group comprised a higher proportion of women (n=44, 80%) than the mRNA group (n=14, 56%) (p=0.026). It should be emphasized that based on the vaccination program in Denmark, the mRNA vaccinated individuals were more vulnerable and had a higher degree of co-morbidity compared to the AZ vaccinated individuals, as the latter mainly comprised healthcare professionals. This "vaccine group bias" may also explain the higher proportion of women in the AZ group compared to the mRNA group.

Among the vaccinated individuals, four subjects (two in each group) received aspirin (ASA). One individual in the mRNA group received a direct oral anticoagulant drug and one individual in the AZ group received a vitamin K antagonist.

Fifty-five non-vaccinated age and gender matched healthy individuals (controls) were included (median age 49 years (IQR 38-56), 73% women). We considered the matching successful, as median age and gender composition in the controls were comparable to both the entire cohort, the AZ and the mRNA groups (all p=NS).

Inflammation and Vascular Endothelial Activation

Pre-Vaccination

Several inflammation markers were higher in the mRNA group compared to the AZ group: CRP, TNF-α, IL-1β, IL-6 and IL-8 (**Figure 2**). Furthermore, pre-vaccination, syndecan-1, a vascular endothelial glycocalyx marker, was increased in the mRNA group whereas the remaining vascular endothelial markers were comparable between groups (**Figure 3**).

Post-Vaccination

CRP and IL-6 remained higher in the mRNA group whereas TNF-α, IL-1β and IL-8 changed in magnitude, so the AZ and mRNA groups had comparable levels (**Figure 2**). IL-10 neither differed between groups pre- nor post-vaccination. Post-vaccination, TNF-α and IL-8 only increased in the AZ group whereas IL-6 and IL-10 increased in both groups. CRP did not change in either group and IL-1β did not change in the AZ group whereas it declined in the mRNA group. Post-vaccination, no differences between groups were observed in any vascular endothelial markers and the increase in these (delta values) was comparable (**Figure 3**).

Delta Changes

The delta increases in TNF-α, IL-1β and IL-8 were higher in the AZ group whereas the increase in IL-6 was higher in the mRNA group (**Figure 2**). The delta change in CRP and IL-10 did not differ between groups.

Platelet Activation

Pre-Vaccination

P-selectin and CD40L were both higher in the mRNA group than in the AZ group whereas TGF-β did not differ between groups.

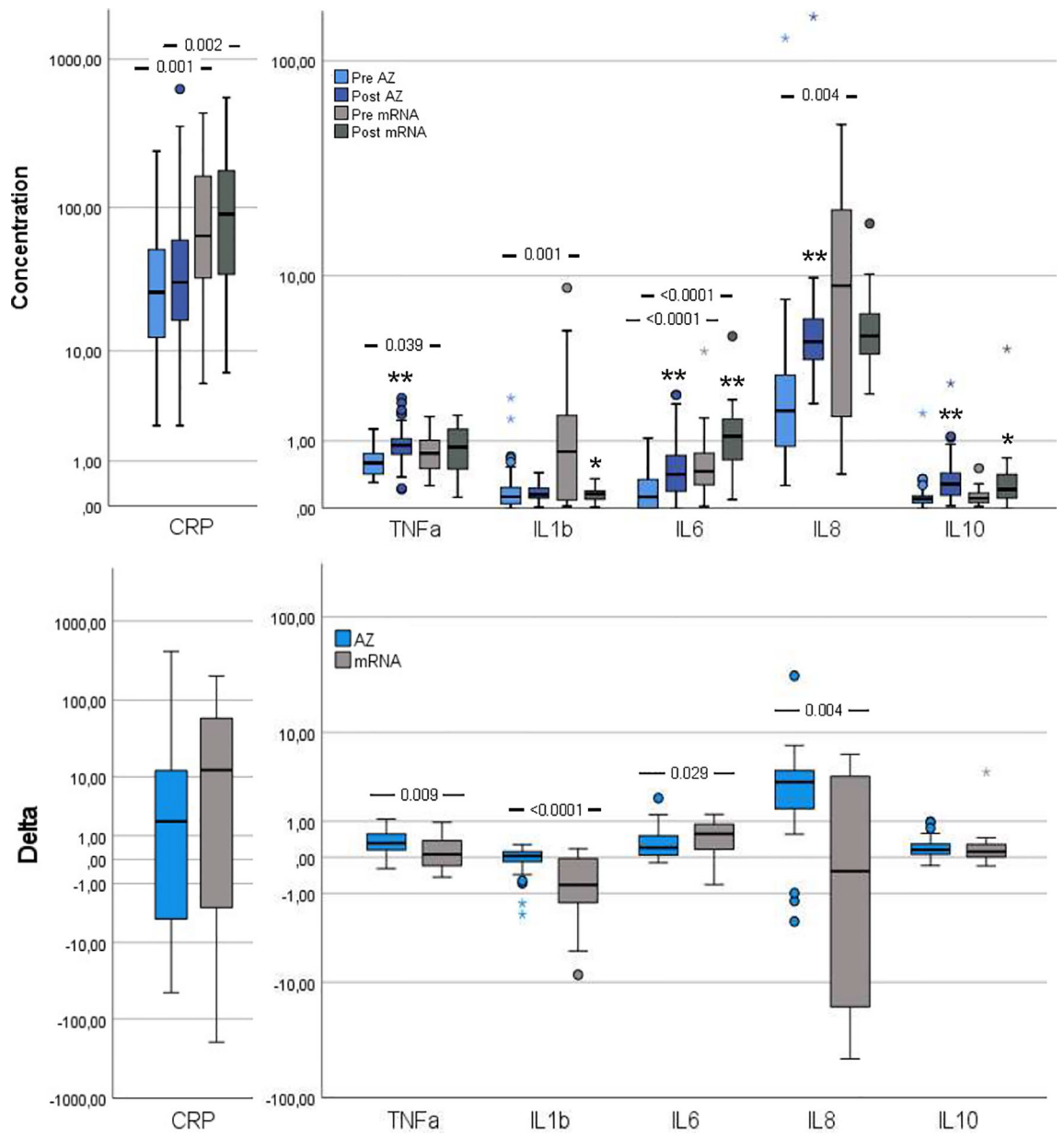

FIGURE 2 | Inflammation markers pre- and post-vaccination and delta changes with either AZ (n=55) or mRNA (n=25) vaccines. Levels of C-reactive protein (CRP, µg/ml), Tumor necrosis factor (TNF)-α (pg/ml), interleukin (IL)-1β (pg/ml), IL-6 (pg/ml), IL-8 (pg/ml) and IL-10 (pg/ml) are displayed on log scales. The top figure displays pre- and post-vaccination values, and the bottom figure displays delta changes. The box plots display median, first and third quartile (box mid-line, button and top, respectively) and the whiskers display maximum and minimum values with outliers (circles) and extremes (small asterisks) displayed. AZ vaccinated individuals are displayed by blue bars (top figure: pre=light blue, post=dark blue) and mRNA vaccinated individuals are displayed by gray bars (top figure: pre = light gray, post=dark gray). Differences between AZ and mRNA vaccinated individuals either pre- and post-vaccination and delta changes are investigated by Mann-Whitney U test, with p-values displayed. Changes over time within the AZ or mRNA groups are investigated by Wilcoxon signed-rank test, with **p < 0.0001 and *p < 0.010 (displayed over the post-vaccination bars).

Post-Vaccination

Though the three platelet activation markers increased from pre- to post-vaccination in both groups, compared to pre-vaccination, a different pattern was observed post-vaccination, with higher TGF-β in the AZ group and comparable levels of P-selectin and CD40L between the vaccination groups (**Figure 4**).

Delta Changes

The delta increase in TGF-β and CD40L were higher in the AZ group compared to the mRNA group (**Figure 4**).

Since the investigated platelet activation biomarkers, are also released to a lesser extend from other cells than platelets, we investigated the intercorrelations between the investigated

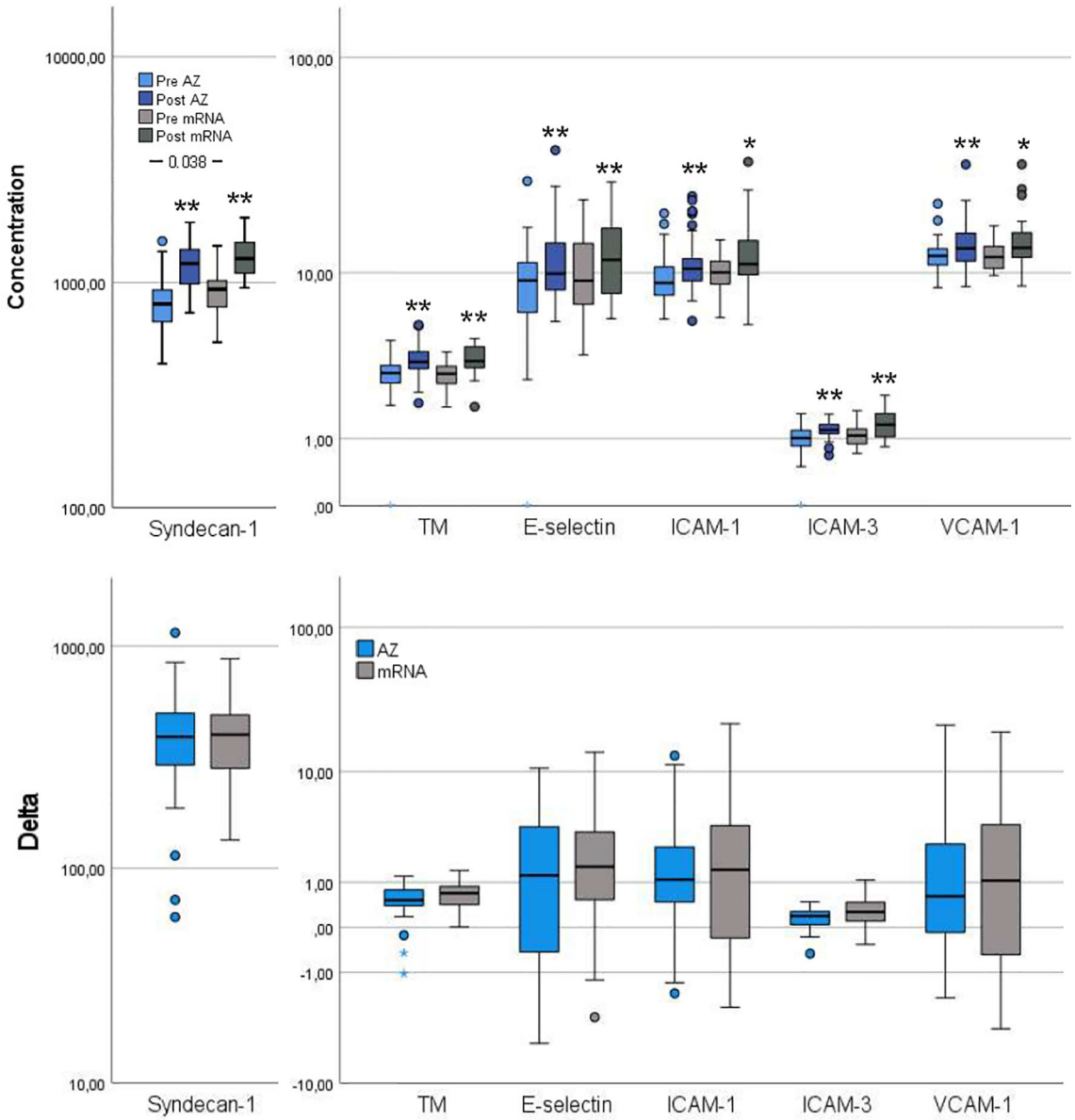

FIGURE 3 | Vascular endothelial activation markers pre- and post-vaccination and delta changes with either AZ (n=55) or mRNA (n=25) vaccines. Levels of Syndecan-1 (pg/ml), thrombomodulin (TM, ng/ml), endothelial (E)-selectin (ng/ml), intercellular adhesion molecule (ICAM)-1 (µg/ml), ICAM-3 (ng/ml) and vascular cell adhesion molecule (VCAM)-1 (µg/ml) are displayed on log scales. The top figure displays pre- and post-vaccination values, and the bottom figure displays delta changes. The box plots display median, first and third quartile (box mid-line, button and top, respectively) and the whiskers display maximum and minimum values with outliers (circles) and extremes (small asterisks) displayed. AZ vaccinated individuals are displayed by blue bars (top figure: pre=light blue, post=dark blue) and mRNA vaccinated individuals are displayed by gray bars (top figure: pre=light gray, post=dark gray). Differences between AZ and mRNA vaccinated individuals either pre- and post-vaccination and delta changes are investigated by Mann-Whitney U test, with p-values displayed. Changes over time within the AZ or mRNA groups are investigated by Wilcoxon signed-rank test, with **$p < 0.0001$ and *$p < 0.010$ (displayed over the post-vaccination bars).

platelet activation markers (**Table S1**) and between post-vaccination platelet count and platelet activation markers (**Figure S1**).

In brief, all platelet activation markers were intercorrelated, with the strongest being between TGF-β and CD40L (pre-vaccination: rho=0.93-0.94; post-vaccination and delta: rho=0.69-0.90). P-selectin generally correlated weaker with the other platelet activation markers. Despite the mRNA group being the smallest, some of the strongest intercorrelations were observed in this group post-vaccination and for delta values (**Table S1**).

Post-vaccination platelet count correlated generally with both pre- and post-vaccination platelet activation markers, with the strongest correlations observed between post-vaccination platelet

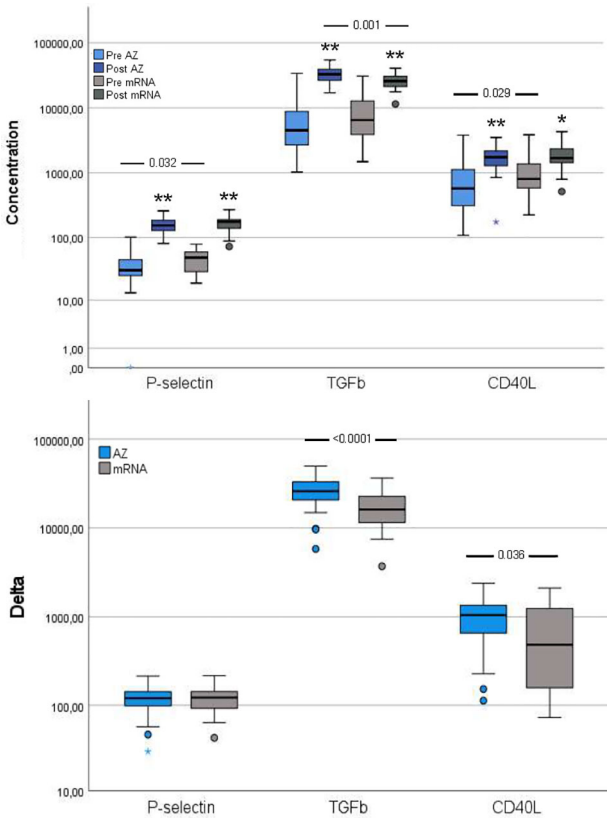

FIGURE 4 | Platelet activation markers pre- and post-vaccination and delta changes with either AZ (n=55) or mRNA (n=25) vaccines. Levels of platelet (P)-selectin (ng/ml), transforming growth factor (TGF)-β (pg/ml) and soluble CD40 ligand (CD40L, pg/ml) are displayed on a log scale. The top figure displays pre- and post-vaccination values, and the bottom figure displays delta changes. The box plots display median, first and third quartile (box mid-line, button and top, respectively) and the whiskers display maximum and minimum values with outliers (circles) and extremes (small asterisks) displayed. AZ vaccinated individuals are displayed by blue bars (top figure: pre=light blue, post=dark blue) and mRNA vaccinated individuals are displayed by gray bars (top figure: pre=light gray, post=dark gray). Differences between AZ and mRNA vaccinated individuals either pre- and post-vaccination and delta changes are investigated by Mann-Whitney U test, with p-values displayed. Changes over time within the AZ or mRNA groups are investigated by Wilcoxon signed-rank test, with **p < 0.0001 and *p < 0.010 (displayed over the post-vaccination bars).

count and post-vaccination TGF-β (rho=0.56-0.60) followed by CD40L (rho=0.37-0.42). P-selectin only correlated weakly with platelet count (**Figure S1**).

Standard Coagulation, Platelet Aggregation, Whole Blood Coagulation and Thrombin Generation

We only had data on standard coagulation, platelet aggregation, whole blood coagulation and thrombin generation post-vaccination due to the design of the study (**Figure 1**).

Post-vaccination, the AZ group had higher platelet count than the mRNA group whereas the mRNA group had higher INR, fibrinogen and higher INTEM LI30 indicating less fibrinolysis (**Table 1**). The AZ group had shorter lagtime and ttPeak and higher Peak and ETP than the mRNA group, all parameters indicating higher thrombin generation in the AZ group (**Table 1**).

Compared to non-vaccinated controls, the AZ group had higher platelet count and higher D-dimer post-vaccination whereas the mRNA group had higher fibrinogen and lower aPTT and INR (**Table 1**). Furthermore, both vaccination groups had higher post-vaccination platelet aggregation indicated by higher COLtest aggregation, and stronger clot formation indicated by higher EXTEM MCF and FIBTEM MCF than non-vaccinated controls (**Table 1**). Finally, the AZ group had longer post-vaccination INTEM CT, lower INTEM LI30 (indicating more fibrinolysis) and higher ETP (indicating more thrombin generation) than controls whereas, the mRNA group had longer lagtime and ttPeak than controls (**Table 1**).

Platelet Factor (PF)4 Antibodies

PF4 antibodies were analyzed post-vaccination and in controls. Post-vaccination, one individual in the mRNA group and two individuals in the control group had positive PF4 antibodies (>0.400 O.D.) whereas no individuals had O.D. >0.400 in the AZ group (p=NS). The post-vaccination median level of PF4 antibodies in the AZ and mRNA groups did not differ (0.11 O.D. (IQR 0.08-0.16) *vs.* 0.09 O.D. (IQR 0.07-0.11), p=NS).

TABLE 1 | Coagulation, primary and secondary hemostasis and thrombin generation post-vaccination either AZ (n=55) or mRNA (n=25) vaccines and in non-vaccinated age and gender matched controls (n=55).

Group	Unit	AZ Median (IQR)	mRNA Median (IQR)	AZ *vs* mRNA p-value	Controls Median (IQR)	Con *vs* AZ	Con *vs* mRNA
Standard coagulation							
Platelet count	10^9/L	285 (251-329)	265 (217-279)	**0.013**	245 (215-281)	**<0.0001**	NS
D-dimer	FEU/L	0.30 (0.25-0.43)	0.26 (0.25-0.38)	NS	0.25 (0.25-0.32)	**0.004**	NS
Fibrinogen	µmol/L	8.78 (7.39-9.92)	10.59 (8.45-12.54)	**0.004**	8.29 (7.08-9.71)	NS	**0.001**
aPTT	Sec	23.3 (21.8-25)	22.6 (21.7-24.4)	NS	23.7 (22.7-24.7)	NS	**0.032**
INR	IU	1.00 (1.00-1.06)	1.00 (1.00-1.00)	NS	1.00 (1.00-1.05)	NS	**0.042**
Platelet aggregation (Multiplate)							
COLtest	AUC	688 (582-770)	695 (645-766)	NS	464 (367-611)	**<0.0001**	**<0.0001**
ADPtest	AUC	809 (690-984)	808 (677-964)	NS	810 (754-1,007)	NS	NS
ASPItest	AUC	1,060 (896-1,194)	1,066 (791-1,164)	NS	1,074 (962-1,164)	NS	NS
ASPItest*	AUC	1,065 (946-1,206)	1,066 (800-1,175)	NS	1,074 (962-1,164)	NS	NS
Thromboelastometry (ROTEM)							
EXTEM CT	Sec	62 (60-67)	64 (58-66)	NS	63 (56-67)	NS	NS
EXTEM MCF	mm	65 (62-67)	66 (62-68)	NS	63 (60-66)	**0.007**	**0.023**
EXTEM LI30	%	100 (100-100)	100 (100-100)	NS	100 (100-100)	NS	NS
INTEM CT	Sec	187 (178-198)	184 (175-212)	NS	181 (173-190)	**0.015**	NS
INTEM MCF	mm	63 (62-66)	64 (61-67)	NS	63 (60-65)	NS	NS
INTEM LI30	%	99 (98-100)	100 (99-100)	**0.007**	100 (99-100)	**0.007**	NS
FIBTEM MCF	mm	16 (13-19)	16 (14-20)	NS	15 (11-17)	**0.007**	**0.014**
Thrombin generation							
Lagtime	min	2.74 (2.40-3.40)	3.33 (3.00-3.33)	**0.011**	2.83 (2.67-3.00)	**NS**	**<0.0001**
Peak	nM	241 (198-279)	198 (174-253)	**0.030**	221 (190-254)	NS	NS
ttPeak	min	6.24 (5.41-7.24)	7.00 (6.50-7.71)	**0.003**	6.33 (6.00-7.00)	NS	**<0.0001**
ETP	nM*min	1,430 (1,346-1,673)	1,318 (1,195-1,612)	**0.050**	1,360 (1,270-1,492)	**0.015**	NS

*Platelet count normal range: Females: 165-400; Males: 145-350. D-dimer normal range: <50 years <0.5 FEU/L; >50 years <0.6 FEU/L. *Two individuals in each group received ASA, median (IQR) values are displayed without these individuals included. Post-vaccination values in AZ and mRNA vaccinated individuals are compared by Mann-Whitney U test. P-values <0.050 are displayed in bold. P-values >0.10 are displayed as NS (not significant). aPTT, activate partial thrombin time; INR, international normalized ratio; CT, clotting time. MCF, maximum clot firmness; LI30, lysis after 30 min; ttPeak, time to peak; ETP, endogenous thrombin potential.*

The PF4 antibody level in controls was 0.09 O.D. (IQR 0.06-0.13), which was lower than the AZ group post-vaccination (p=0.021) but comparable to the mRNA group post-vaccination (p=NS).

DISCUSSION

The main finding in the present study was that AZ vaccination against COVID-19 induced a more pronounced increase in several inflammatory and platelet activation markers compared to mRNA vaccination. Also, post-vaccination thrombin generation was higher for all parameters in AZ vaccinated individuals than in mRNA vaccinated individuals. We found no difference in neither the PF4 antibody level nor the proportion of PF4 antibody positives between the vaccination groups.

The present study investigated individuals vaccinated against COVID-19 with either AZ or mRNA vaccines. None of the study participants developed VITT so our findings reflect normal vaccination responses to the investigated vaccines. The finding that AZ vaccination induced enhanced inflammation, platelet activation and thrombin generation compared to mRNA vaccination in a head-to-head comparison is notable and has to our knowledge not previously been reported. Importantly, our finding is in accordance with that of a recent epidemiologic study, reporting that AZ vaccination is associated with an increase in venous thromboembolic events, including CVST, and slightly higher rates of thrombocytopenia, coagulation disorders and bleeding (17). Together this indicates that even in a population with normal vaccination responses, higher levels of complications related to excessive inflammation, platelet and coagulation activation are observed following AZ vaccination.

Vaccines are designed and administered to inflict an immune response (18, 19), and some degree of inflammation and platelet activation is to be expected post-vaccination [the latter due to the close link between innate immunity and platelet activation (20)], in accordance with the findings in the present study that both vaccines resulted in enhanced inflammation and platelet activation one week after vaccination. Despite this common vaccination response, it is however notable, that the AZ vaccine induced higher increases in several key inflammatory mediators (TNF-α, IL-1β and IL-8) compared to mRNA vaccines, also given that the mRNA vaccinated individuals had a higher degree of low-grade inflammation pre-vaccination.

Though the mechanisms behind the pathogenesis of VITT remain incompletely understood, the initial events that trigger platelet activation, PF4 release, complex formation and PF4 antibody production (whichever comes first initially) are especially puzzling. It has been reported that PF4 antibodies are present at low levels in up to 7% of healthy individuals (21) and that healthy individuals have a reservoir of B cells specific for PF4 complexes, but that different immunoregulatory mechanisms hold these B cells under control (22, 23). It has also been reported that almost 7% of COVID-19 vaccinated individuals (both adenovirus

vector-based vaccines and mRNA vaccines) have low titers of PF4 antibodies, but these antibodies are not functionally active (24). Given that PF4 in healthy individuals is stored (hidden) in the megakaryocytes and in the α-granules of platelets (14), an early event post-vaccination leading to a breach of tolerance and generation of functionally active PF4 antibodies would be necessary to induce ensuing platelet activation and release of PF4 (25). Several studies have focused on vaccine components from the AZ adenovirus vector vaccine as a trigger of the cascade of early events (1, 25, 26). Though no specific component(s) have yet been identified as trigger of VITT, it has been suggested that one critical downstream event may be hyperinflammation induced by the AZ adenovirus vector vaccine (25). This notion is supported by our finding of higher increases in inflammation following the AZ vaccine compared to mRNA vaccines and the finding of higher increases in the chemokine IL-8/CXCL8, a potent neutrophil chemoattractant and activator (27, 28), is notable given that neutrophil activation and NETosis have been proposed to be involved in VITT pathophysiology (25). This study can only speculate on mechanisms contributing to the observed (hyper) inflammation observed following the AZ vaccine and further studies are needed to reveal potential structural components of the Adenovirus vector vaccines that may contribute to the (hyper) inflammation observed in this and other studies (1, 25, 26).

The AZ and mRNA vaccines both enhanced inflammation, platelet activation and vascular endothelial activation, a notable finding given that COVID-19 itself is also associated with extensive inflammation and platelet activation with e.g. high circulating proinflammatory cytokine levels, enhanced P-selectin expression and high soluble levels and extensive widespread vascular endothelial activation (29–33). Several studies of COVID-19 associated platelet activation or vascular endothelial activation have included advanced *OMICS* analyses, and despite the few biomarkers in common with our study, it is difficult to directly compare the COVID-19 vaccine and SARS-CoV-2 infection/COVID-19 induced changes. Though it is not possible from the design of the present study, to determine to which degree the observed platelet and vascular endothelial activation observed following COVID-19 vaccines is attributed to direct effects of SARS-CoV-2 antigens or indirect effects due to (hyper) inflammation, we envision that (hyper)inflammation is the main driver following vaccination whereas SARS-CoV-2 antigens (viremia) may be a greater contributor in COVID-19. Despite the common platelet activation following different COVID-19 vaccines, the present study also found higher increases in platelet activation and enhanced thrombin generation following the AZ vaccine compared to mRNA vaccines. This is to our knowledge the first study reporting this based on a head-to-head comparison. Given that platelet activation is a hallmark of VITT, the finding of higher increases in biomarkers suggestive of platelet degranulation combined with enhanced thrombin generation following the AZ vaccine is highly intriguing. Though VITT (34, 35) and CVST (9) have been reported following COVID-19 vaccination with mRNA vaccines, only CVST following AZ vaccination is associated with thrombocytopenia (9), emphasizing the critical involvement of platelets in VITT and potentially also the cascade of events that trigger VITT. We speculate that specific components of the AZ adenovirus vector may serve as initial trigger(s) of (hyper)inflammation, platelet activation and thrombin generation, potentially lowering the threshold for a cascade of events that both trigger complications related to excessive inflammation, platelet and coagulation activation as observed in epidemiologic studies (17) and promote development of VITT when combined with high-titer functionally active PF4 antibodies.

Finally, the present study found no evidence of enhanced PF4 antibody production following AZ vaccination compared to mRNA vaccination. This finding is not surprising given the previous finding from larger and better powered studies investigating PF4 antibodies in AZ vaccinated individuals (24, 36). The finding however emphasizes that the observed higher increase in platelet activation and thrombin generation in AZ vaccinated individuals compared to mRNA vaccinated individuals is not dependent only of PF4 antibodies. This points to specific components in the AZ adenovirus vector vaccine as initial trigger(s) of (hyper) inflammation, platelet activation and thrombin generation.

The study had several limitations. The study included a low number of participants and conducted many different investigations, together increasing the risk of both Type I and Type II errors. This should be taken into account, when interpreting the results (p-values just below 0.050 may be by chance). Furthermore, for the whole blood coagulation tests, we did not have pre-vaccination values for comparison but instead had to include an age and gender matched control group for these comparisons. Finally, we did not collect a full medical history on the vaccinated study participants, despite inferring that the vaccination program in Denmark introduced a "vaccine group bias" as mainly vulnerable individuals/patients were vaccinated with mRNA vaccines and healthcare professionals were vaccinated with AZ vaccines at the time of conduct of the study. The strengths of the study were however the quick response to the VITT focus with early blood sampling post-vaccination and the availability of pre-vaccination samples collected uniformly from the same individuals and participants in the ENFORCE vaccine trial. Furthermore, the head-to-head comparison of the AZ and mRNA vaccine response in the same study, minimizing variation is a clear strength. Finally, the inclusion of a broad range of both plasma and whole blood based analyses revealing inflammation, platelet activation and thrombin generation in the same study, is a clear strength.

In conclusion, the present study investigated the influence of different COVID-19 vaccines on inflammation, vascular endothelial activation, platelet activation and aggregation, whole blood coagulation, thrombin generation and PF4 antibodies to reveal potential differences between AZ and mRNA vaccines in individuals without VITT. The main findings were that the AZ vaccine induced a more pronounced increase in inflammation and platelet activation and higher thrombin generation compared to mRNA vaccines and that none of the vaccinated individuals developed PF4 antibodies. We speculate that specific components of the AZ adenovirus vector may serve as initial trigger(s) of (hyper) inflammation, platelet activation and thrombin generation,

potentially lowering the threshold for a cascade of events that both trigger complications related to excessive inflammation, platelet and coagulation activation as observed in epidemiologic studies and promote development of VITT when combined with high-titer functionally active PF4 antibodies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Ethical Committee (Denmark). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SO, OS, MT, NS, JL, LØ, and AH contributed to the design of the study. NS, OS, and AH participated in the enrolment of study participants and data collection. SO and AH led and supervised the laboratory analyses. SO and AH analyzed the data and drafted the manuscript. All authors contributed to the data interpretation and in revising the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.779453/full#supplementary-material

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HYPOTHESIS AND THEORY
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Potential Anionic Substances Binding to Platelet Factor 4 in Vaccine-Induced Thrombotic Thrombocytopenia of ChAdOx1-S Vaccine for SARS-CoV-2

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Recent reports of rare ChAdOx1-S vaccine-related venous thrombosis led to the suspension of its usage in several countries. Vaccine-induced thrombotic thrombocytopenia (VITT) is characterized by thrombocytopenia and thrombosis in association with anti-platelet factor 4 (PF4) antibodies. Herein, we propose five potential anionic substances of the ChAdOx1-S vaccine that can combine with PF4 and trigger VITT, including (1) the proteins on the surface of adenovirus, e.g., negative charged glycoprotein, (2) the adjuvant components of the vaccine, e.g., Tween 80, (3) the DNA of adenovirus, (4) the S protein antigen expressed by the vaccine, and (5) the negatively charged impurity proteins expressed by the vaccine, e.g., adenovirus skeleton proteins. After analysis of each case, we consider the most possible trigger to be the negatively charged impurity proteins expressed by the vaccine. Then, we display the possible extravascular route and intravascular route of the formation of PF4 autoantibodies triggered by the negatively charged impurity proteins, which is accordant with the clinical situation. Accordingly, the susceptible individuals of VITT after ChAdOx1-S vaccination may be people who express negatively charged impurity proteins and reach a certain high titer.

Keywords: vaccine-induced thrombotic thrombocytopenia (VITT), ChAdOx1-S vaccine, SARS-CoV-2, anionic substances, PF4

INTRODUCTION

Due to severe thrombotic adverse events named vaccine-induced thrombotic thrombocytopenia (VITT) (1, 2) reported in Denmark, Norway, Germany, Austria, and the United Kingdom, the usage of AstraZeneca recombinant adenoviral ChAdOx1-S was limited in several countries (3). VITT was more frequent in young people, therefore, the health authorities of several European countries and Canada modified their immunization strategies, reserving the ChAdOx1-S vaccine for older people (4). The

United States also reported similar events related to the Ad26.COV2-S Janssen vaccine, leading to a pause in its roll-out (4, 5). According to a recent report (6), as of July 2021, 342 patients had died in Taiwan after receiving the ChAdOx1-S vaccine which had been supplied with a total of 1.24 million doses since 15 June; the mortality was as high as 287 parts per million.

Even though patients with VITT had similar mortality after two vaccine doses, the VITT occurrence rate was higher in the ChAdOx1-S vaccine (7, 8). Greinacher et al. reported that people receiving ChAdOx1-S had one or more thrombotic complications beginning 5 to 16 days after vaccination (9). So far, most of the reported cases became symptomatic within 30 days of the first dose of the ChAdOx1-S vaccine, and VITT was more frequent in women and patients aged < 55 years (5, 9). VITT patients often showed laboratory signs of disseminated intravascular coagulation with severe thrombocytopenia (9), and most thrombotic complications occurred at unusual sites, particularly cerebral venous sinus thrombosis (CVT). On the basis of such a situation, healthcare authorities advised vaccine recipients who suffered symptoms such as shortness of breath, chest, abdominal, or extremities pain, severe headache, dizziness, visual disturbances, or other neurologic symptoms within 30 days of ChAdOx1-S vaccination should be urgently investigated for VITT by associated laboratory tests (10, 11).

Then, the serious question is, among the various vaccines approved worldwide, why has the ChAdOx1-S vaccine caused so many VITT cases?

THE KEY PLAYER: PF4 AND ANIONIC SUBSTANCES

The ChAdOx1-S vaccine utilizes chimpanzee adenovirus, which is considered safe, as its vaccine vector is not transmitted in humans, but it seems that this may not be the case.

According to a previous report (1), PF4-heparin antibodies were detected in the blood of patients with severe thrombosis, but these patients did not use heparin. So which component produced a similar effect to heparin after the injection of the ChAdOx1-S vaccine, forming the PF4-component complex, and then led to the formation of the PF4 autoantibody, triggering the thrombosis process just like PF4 immune activation in heparin-induced thrombocytopenia (HIT)?

From the perspective of biochemical properties, McGonagle et al. (12) pointed out that PF4 is easily combined with anionic substances, such as DNA, heparin, etc. Then, which anionic substances of the ChAdOx1-S vaccine may bind to PF4?

FIVE POTENTIAL ANIONIC SUBSTANCES

According to the related reports, we suggest five potential anionic substances of the ChAdOx1-S vaccine that can combine with PF4 as follows:

1. The proteins on the surface of adenovirus, for example, negatively charged glycoprotein
2. The adjuvant components of the vaccine, for example, Tween 80
3. The DNA of adenovirus
4. The S protein antigen expressed by the vaccine
5. The negatively charged impurity proteins expressed by the vaccine, for example, adenovirus skeleton proteins

For substance 1, although part of the adenovirus vaccine can enter the blood after intramuscular injection (13), this reason does not sound plausible, because this could not explain the rarity of the clinical observation of VITT. Moreover, even if some people have been infected with human adenovirus before, there are neutralizing antibodies against human adenovirus, when other adenoviruses enter again, the more possible result is the neutralization of adenovirus, not VITT.

For substance 2, the adjuvant components of the vaccine, such as Tween 80 (14), are also anionic, they may enter the blood and combine with PF4 to cause thrombosis theoretically. However, up to now, no relevant literature has been found to prove that they are related to VITT. More importantly, the adjuvant components are widely used in vaccines or other drugs, this also could not explain the rarity of the clinical observation of VITT (15).

As for substance 1 and substance 2, Gresele et al. (16) pointed out that VITT develops usually at least 1 week after vaccination, it is very unlikely that circulating Ad-vector or vaccine excipients would still be present in the blood, rendering more likely alternative explanations, and in particular an immunological reaction.

For substance 3, McGonagle et al. (12) pointed out that local tissue microtrauma, along with local microbleeding and immune cell activity, will bring adenoviral DNA in contact with PF4, which is then taken up by APCs and memory B cell engagement in the regional lymph nodes, leading to substantially increased PF4 autoantibody production. It is related to failed extravascular tissue tolerance mechanisms which are different from HIT. But how could the DNA enveloped in the Chadox1 adenovirus capsid be released to bind with PF4? Although Kircheis R et al. (17) pointed out that among the 50 billion virus particles in each dose, some may break apart and release their DNA, then the Ad26.COV2.S vaccine would also have released DNA and caused the same incidence and severity of VITT, which does not accord with the current situation (18).

For substance 4, in COVID-19 patients, anti-SARS-CoV-2 IgG-spike glycoprotein immune complexes can activate platelets through FcγRIIa. Kadkhoda et al. (13) figured out that adenoviral vectors leak into the circulation, travel to distant sites, and infect permissive cells. Once infected, copious amounts of soluble spike glycoproteins lead to a relatively high level of SARS-CoV-2 spike "antigenemia". In a person with a prior SARS-CoV-2 infection and/or with cross-reactive antibodies to common coronaviruses (CoVs), a high enough titer of aberrantly glycosylated antibodies would be induced. But if a person is pre-infected with SARS-CoV-2 and there are SARS-CoV-2 antibodies, the vaccine would be ineffective rather than the cause for VITT. As for the cross-reactive antibodies, there are no direct references supporting its relation to VITT. Greinacher et al. (15) found that antibodies tested against PF4 induced by vaccination do not cross-react with the SARS-CoV-2 spike protein, which indicates that the SARS-CoV-2 spike protein may not be a trigger of VITT.

From another perspective, the above four anionic substances are common in other adenovirus-based vaccines, for example, the Ad26.COV2.S vaccine, if they are plausible, which means that the Ad26.COV2.S vaccine would cause the same incidence and severity of VITT as the ChAdOx1-S vaccine, but that is apparently not the situation (18). Or at least for the ChAdOx1-S vaccine, the above four anionic substances cannot cover all substances that may bind to PF4, triggering VITT.

For substance 5, Almuqrin et al. (19) found that the genome of Chadox1 may express low-level adenovirus skeleton protein, although replication-defective in normal cells, 28 kbp of adenovirus genes is delivered to the cell nucleus alongside the SARS-CoV-2 S glycoprotein gene in some cell lines such as A549 and MRC5, and the expression of adenovirus cytoskeleton protein can then be detected. Besides, McGonagle et al. (12) also pointed out that a role for adenoviral proteins has been suggested as a potential factor in VITT in susceptible individuals. Susceptible individuals may be people who express the negatively charged impurity proteins—adenovirus skeleton protein—and reach a certain high titer. Just as the viral RNA can innate immune-driven immunothrombosis in severe COVID-19 pneumonia, the negatively charged proteins in these adenovirus skeleton proteins may play the same role as viral RNA and form adenovirus skeleton protein-PF4 complexes (12), with this adjuvanticity likely contributing to autoantibody development and ultimately thrombosis in VITT.

THE POSSIBLE ROUTES OF THE FORMATION OF PF4 AUTOANTIBODIES TRIGGERED BY NEGATIVELY CHARGED IMPURITY PROTEINS—ADENOVIRUS SKELETON PROTEINS

The two possible routes for the formation of PF4 autoantibodies include the following:

(1) The extravascular route (**Figure 1**): after the intramuscular injection of the ChAdOx1-S vaccine, the adenovirus enters into the muscle cell, mainly expressing the spike protein, but for some susceptible individuals as depicted above, the adenovirus gene is also expressed and there are adenovirus vector proteins. And if local microbleeding accidently happens, the adenovirus vector proteins have the chance to bind to PF4, which are then taken up by APC cells, memory B cells engage, and regional lymph nodes secret PF4 autoantibody which then enters the blood through lymphatic circulation and triggers the VITT process.

(2) The intravascular route (**Figure 1**): after the intramuscular injection of the ChAdOx1-S vaccine, the adenovirus leaks into the blood circulation, travels to distant and different tissues, and infects a range of permissive cells, which confer susceptibility, such as epithelial and endothelial cells, etc., that then secret the adenovirus vector proteins along with copious SARS-CoV-2 spike proteins, and the adenovirus vector proteins bind to PF4, and trigger the VITT process.

Route 1 is the extravascular immune response hypothesis which takes a relatively short amount of time while route 2 is the intravascular immune response hypothesis which takes a relatively long time, which is concordant with the clinical situation where there are urgent VITT cases occurring about 5 days after vaccination and chronic cases occurring about 24 days after vaccination (1, 12, 18). And young women have a more intense immune response than men and the elderly, so they are more prone to the above immune response routes.

DISCUSSION

Based on the biochemical properties, we suppose that five possible anionic substances in association with the ChAdOx1-S vaccine may bind to PF4. Although they all have potential, we consider the negatively charged impurity proteins expressed by

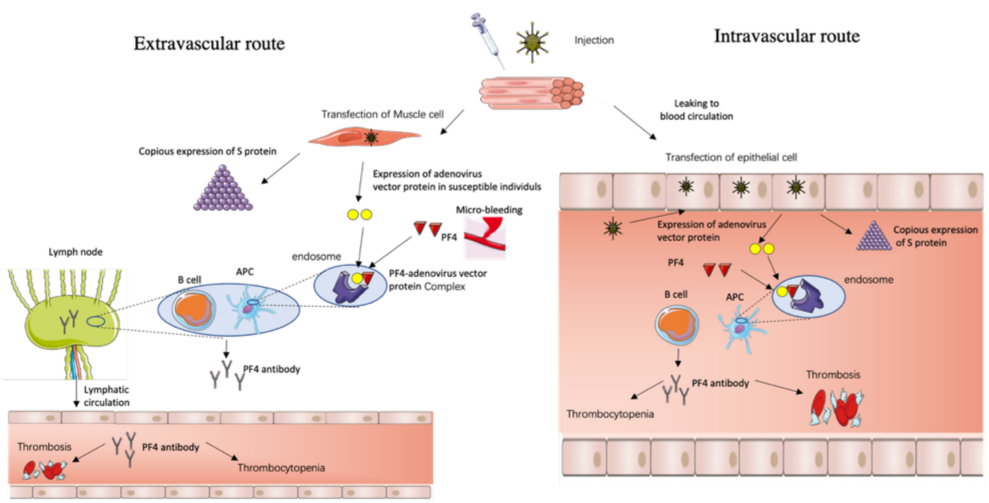

FIGURE 1 | The possible routes for the formation of PF4 autoantibodies triggered by negatively charged impurity proteins—adenovirus skeleton proteins.

the vaccine as the most possible trigger, but whether the extravascular and the intravascular models exist together or just one happens needs to be substantiated.

ChAdOx1-S-vaccinated individuals must undergo some concurrent events in order to develop VITT syndrome. (1) They are "predisposed" individuals who could express negatively charged impurity proteins—adenovirus skeleton protein and reach a certain high titer. That "prevalence" of the susceptible individuals determines the basic occurrence rate of VITT, for which the "prevalence" of VITT is different in different countries and regions. (2) For the extravascular route, local accident microbleeding needs happening in unison. (3) For the intravascular route, after the adenoviruses leak into the blood circulation, they need to travel to distant and different tissues, e.g., the cerebral sinus, and infect a range of permissive cells, which would induce lethal cerebral VITT and take a relatively long time in accordance with the cerebral venous thrombosis symptom feature in patients with VITT (20).

We propose one novel and data-supporting (19) potential anionic substance of the ChAdOx1-S vaccine that may combine with PF4 and trigger VITT, which could be verified by testing whether the negatively charged impurity proteins expressed by the vaccine could be detected in the sera from VITT patients through quantitative proteomics, or by separation and purification of the impurity proteins and testing their binding ability with PF4.

Although the mechanism of VITT is still unclear, there are many other hypotheses exploring the potential triggering causes of VITT, such as soluble shorter spiker protein variants binding to PF4, genetically determined enhanced expression of FcγRIIa, and the altered glycosylation state of IgG (16), etc., all of which need hypothetical condition support and verification.

The anionic substances discussed in this article that may bind to PF4 are mainly for the ChAdOx1-S vaccine, other adenovirus-based vaccines need further investigation.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XP, HL, YZ, and YC developed ideas and drafted the manuscript. XH and TJ acquired the data and revised the manuscript. All the authors participated in revising the manuscript and approved the final version. XP and HL contributed equally to this work.

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REVIEW
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Emerging Role of Platelet-Endothelium Interactions in the Pathogenesis of Severe SARS-CoV-2 Infection-Associated Myocardial Injury

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Cardiovascular dysfunction and disease are common and frequently fatal complications of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Indeed, from early on during the SARS-CoV-2 virus pandemic it was recognized that cardiac complications may occur, even in patients with no underlying cardiac disorders, as part of the acute infection, and that these were associated with more severe disease and increased morbidity and mortality. The most common cardiac complication is acute cardiac injury, defined by significant elevation of cardiac troponins. The potential mechanisms of cardiovascular complications include direct viral myocardial injury, systemic inflammation induced by the virus, sepsis, arrhythmia, myocardial oxygen supply-demand mismatch, electrolyte abnormalities, and hypercoagulability. This review is focused on the prevalence, risk factors and clinical course of COVID-19-related myocardial injury, as well as on current data with regard to disease pathogenesis, specifically the interaction of platelets with the vascular endothelium. The latter section includes consideration of the role of SARS-CoV-2 proteins in triggering development of a generalized endotheliitis that, in turn, drives intense activation of platelets. Most prominently, SARS-CoV-2–induced endotheliitis involves interaction of the viral spike protein with endothelial angiotensin-converting enzyme 2 (ACE2) together with alternative mechanisms that involve the nucleocapsid and viroporin. In addition, the mechanisms by which activated platelets intensify endothelial activation and dysfunction, seemingly driven by release of the platelet-derived calcium-binding proteins, SA100A8 and SA100A9, are described. These events create a SARS-CoV-2–driven cycle of intravascular inflammation and coagulation, which contributes significantly to a poor clinical outcome in patients with severe disease.

Keywords: ACE2 receptor, acute myocardial injury, cardiovascular disease, corona virus disease (COVID-19), endotheliitis, nucleocapsid (N) protein, platelet activation, spike protein

INTRODUCTION

Coronavirus disease-19 (COVID-19), caused by the novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a multi-system inflammatory disorder, which, in its severe form, is characterized by endothelial damage and hypercoagulability (1). Although the etiology of the arterial and venous thromboembolic events observed in 10-25% of patients admitted with SARS-CoV-2 infection (2) is still being debated, the role of the complex interactions between platelets, the endothelium and SARS-CoV-2 are increasingly recognized. COVID-19-associated thrombosis has been reported in many sites, such as the skin (3), respiratory tract (4, 5), brain (6), gastrointestinal tract (7) and extremities (8), but is particularly catastrophic when the heart is involved (9), especially in the context of a pro-atherosclerotic milieu.

From early on in our understanding of infection with this coronavirus, it has been recognized that underlying cardiac comorbid conditions are important risk factors for COVID-19 infection and that cardiac complications may occur, even in patients with no underlying cardiac disorders, as part of the acute infection. One of the early publications from Wuhan, describing 41 hospitalized cases with COVID-19 pneumonia, noted that 73% of the patients were men, with 32% having underlying co-morbidities, including hypertension in 15% and cardiovascular diseases in 15% (10). A number of complications were noted in the patients, including acute cardiac injury in 12%. Shortly thereafter, several reports confirmed that underlying cardiovascular co-morbidities, as well as cardiac complications due to acute COVID-19 infection, were associated with more severe disease and increased morbidity and mortality (11–16). From these collective publications, it was noted that the most common cardiac complication was acute cardiac injury, defined by significant elevation of cardiac troponins. The acute cardiac injury was secondary to a number of cardiac conditions, including acute myocardial infarction due to acute coronary syndrome (ACS), myocarditis, arrhythmias, and cardiomyopathy, while cardiogenic shock and cardiac arrest were also reported. Thromboembolic disease, both venous and arterial, was also described. The potential mechanisms of cardiovascular complications included direct viral myocardial injury, systemic inflammation induced by the virus, sepsis, arrhythmia, myocardial oxygen supply-demand mismatch, electrolyte abnormalities, and hypercoagulability (17–21). As was noted in 2020, despite many publications there remain many uncertainties regarding the origins of these cardiac complications (22).

The aim of the current manuscript is to review recent data on the clinical manifestations and mechanisms of acute cardiac involvement in patients with SARS-CoV-2 infection. We specifically address acute myocardial injury and ACS, and the potential mechanisms of these complications, especially with regard to the role of platelet-endothelium interactions, concentrating on publications that appeared mainly during 2020/2021.

THE CLINICAL ASPECTS OF COVID-19 AND CARDIOVASCULAR DISEASE

While underlying cardiovascular comorbidities are a risk factor for COVID-19 infection and severity, the infection itself has the potential to cause a significant impact on the cardiovascular health of millions of people worldwide. The virus can infect the heart, as well as vascular tissue, circulating cells, and various organs, through its interaction with the ACE2 receptor, which it uses as a host cell receptor through its spike protein. It is, therefore, not surprising that cardiovascular events are a common extra-pulmonary manifestation of COVID-19 infection (extensively reviewed in 17 and 18). The potential cardiac manifestations of COVID-19 infection are shown in **Table 1**, with the putative mechanisms depicted in **Table 2** (17–21).

The cardiac manifestations that may occur during COVID-19 infection include acute cardiac injury, myocarditis, acute myocardial infarction (AMI), heart failure, arrhythmia, cardiomyopathy, hypotension, shock, and cardiac arrest (18, 23, 24). An echocardiographic study of patients with COVID-19 documented pre-existing abnormalities, which prompted the authors to suggest caution with regard to attributing such abnormalities to acute COVID-19 infection (25). Among the vascular complications documented are venous and arterial thrombotic events, which are associated with greater need for respiratory support, vasopressors, and hemodialysis, and a longer hospital stay (24, 26). Autopsy studies from patients who died from COVID-19 infection have demonstrated a range of cardiac abnormalities including cardiac hypertrophy and/or enlargement, ventricular dilatation, infarction, and fibrosis (24).

A number of investigators have shown the potential value of a range of cardiac biomarkers, including high sensitivity troponins (Tn; TnT and TnI), creatinine kinase isoenzyme-MB (CK-MB) and N-terminal pro-brain-type natriuretic peptide (BNP; NT-proBNP), either as early indicators of cardiac involvement in COVID-19 infections and/or as markers of disease prognosis (27–34), while measurement of D-dimers is thought to be potentially important in preventing, diagnosing and managing vascular complications (24).

The troponins TnT and TnI are known as the cardiac troponins, and act on cardiac muscle contraction by regulating the calcium-dependent interactions of actin and myosin (35). The CK component of CK-MB is an enzyme that catalyzes the reversible transformation of creatinine and ATP to creatinine phosphate and ADP (35). Lastly, BNP is a hormone produced by

TABLE 1 | Potential cardiac complications in COVID-19 infections*.

- Acute myocardial injury – elevated troponin levels
- Acute coronary syndrome
  - Non-ST-elevation acute coronary syndrome – NSTEMI or unstable angina
  - ST elevation myocardial infarction (STEMI)
  - Acute myocardial infarction
    - *Type 1 – acute atherothrombotic coronary artery disease, mostly plaque rupture or thrombosis*
    - *Type 2 – oxygen supply-demand mismatch*
- Cardiomyopathy
  - Include stress cardiomyopathy – Takotsubo cardiomyopathy
- Myocarditis
- Arrhythmia
- Drug-induced cardiac effects
- Cardiac failure
- Hypotension and/or cardiogenic shock
- Cardiac arrest

**See References (17–21).*

TABLE 2 | Possible causes of cardiac events in COVID-19 infection*.

- Direct cardiac injury
  - Viral-induced myocardial injury
  - Tropism of the virus for the ACE-2 receptor
- Systemic inflammation—cytokine release syndrome and multisystem inflammatory syndrome
- Sepsis
- Plaque rupture or thrombosis
- Hematological changes—arterial thrombosis, hypercoagulability/coagulopathy and disseminated intravascular coagulation
- Hypoxia
- Myocardial oxygen supply-demand mismatch
- Dysfunctional endothelial response
- Arrhythmia
- Electrolyte disorders
- Direct drug toxicity and/or drug-drug interactions

*See References (17–21).

the heart, with both BNP and NT-proBNP being released in response to pressure changes within the heart (36). The former two biomarkers indicate myocardial injury, whereas the latter is a marker of hemodynamic stress (37).

In one systematic review and meta-analysis the pooled incidence of elevated levels of TnI, CK-MB and CK in COVID-19 cases were 15.16%, 10.92%, and 12.99%, respectively (38). Another detailed review indicated that troponin levels were elevated in 12% to 28% of cases with SARS-CoV-2 infection (18). While studies have indicated significant differences in biomarker levels in patients with differing COVID-19 infection severity, these levels also depended on whether the biomarkers were measured early on, or later, in the course of the infection (32, 38). Importantly, in COVID-19 cases, particularly those with underlying pre-existing cardiac disease, the prognostic cut-off values of these biomarkers may be lower than the reference standards (39). Furthermore, studies have shown that TnI, CKMB, and Pro-BNP are higher in non-survivors compared with survivors among critically ill patients with COVID-19 infection (28).

Lastly, it is important to be aware that cardiovascular sequelae can occur even in patients with mild COVID-19 infections, as well as in asymptomatic carriers of SARS-CoV-2 who may not even have been tested for this virus, and who may be at risk of developing cardiovascular disorders as bystander effects of the virus (40, 41). It remains essential to recognize and manage cardiovascular events during COVID-19 infection as soon as possible, as they are associated with worse outcomes (26, 42).

Acute Myocardial Injury

In studies of COVID-19 infection, myocardial injury has been defined as elevation of cardiac troponin I (TnI), or troponin T (TnT) to >99th percentile of the upper reference limit, or the presence of new electrocardiographic or echocardiographic abnormalities (18). Myocardial injury can occur *via* ischemic and non-ischemic mechanisms and the overall rate of acute cardiac injury in older studies has been reported to be between 7.2 and 36%, with rates as high as 59% in those who died (18, 43, 44). The rate of acute cardiac injury has been found to be higher in the case of infection with viruses, such as SARS-CoV-2, that bind the ACE2 receptor, than in those with viruses that do not (45). Cardiac injury can occur early or later in the course of COVID-19 infection and recurrent episodes of cardiac injury may occur, with the latter events being associated with worse outcome (46). Importantly, more severe cardiac injury is associated with higher levels of TnI, greater need for ICU admission, and higher mortality (18).

The potential etiologies of COVID-related acute myocardial injury include i) ACS due to plaque rupture or thrombosis (Type I myocardial infarction), or supply-demand mismatch (Type II myocardial infarction), or ii) injury due to disseminated intravascular coagulation or non-ischemic injury, associated with myocarditis, stress-induced cardiomyopathy, cytokine release or acute pulmonary embolism (47). Cardiac injury is associated with more severe disease and a worse prognosis (43).

Interestingly, one hypothesis-generating study from the US, which mirrored similar studies from other countries, such as European countries, as well as Australia and New Zealand, indicated that most metropolitan cities noted a marked increase in out-of-hospital cardiac arrest, early in 2020, which generally paralleled the local prevalence of COVID-19 infections (48). It was suggested that this was likely due to the SARS-CoV-2 virus, but that these deaths could not be reported as being due to COVID-19 as testing was not done on most patients before they died. The study also did not attempt to address clinical details, such as data on cardiac events.

Acute Myocardial Infarction/Acute Coronary Syndrome

The topic of COVID-19 and acute myocardial injury/ACS has been extensively reviewed (47). Among the common findings are firstly, that there is an increased risk of myocardial injury and infarction in patients with COVID-19 infection, especially in those patients with underlying cardiovascular comorbidities and/or risk factors. Secondly, there is a range of differential diagnoses with regard to the cause of myocardial injury, as noted above. Thirdly, lower rates of hospitalization have been noted worldwide for acute coronary syndrome during the COVID-19 pandemic, most likely because of patients' hesitancy to present to hospital, as well as misdiagnosis. Lastly, lack of preparation and standardized protocols for early management of ACS, in the setting of protection of healthcare professionals and the environment, have potentially led to delayed treatment of ACS.

A recent study from Sweden, indicated that when day one of the infection was excluded, the odds ratio for an AMI in the two weeks following COVID-19 infection was 3.41 [95%CI 1.58-7.36] and when day 0 was included it was 6.61 [95% CI 3.56-12.20]. The authors suggested that AMI (and ischemic stroke) represented a part of the clinical picture of COVID-19 infection, highlighting the need for prevention by vaccination (49).

In one registry-based retrospective analysis of hospitalized patients with COVID-19 who suffered an AMI, the overall mortality was 22.8% (50). The deceased were older than survivors, and patients with hypertension, worsening renal function and higher cardiac troponin T and C-reactive protein levels were more likely to die. Importantly, one observational study documented that occult infections with SARS-CoV-2 (and influenza) occurred in patients presenting with AMI, but without COVID-19 symptoms (13% of cases) and while there was no difference in mortality in those with

and without COVID-19 infection, the former group had a shorter time to a recurrent cardiovascular event (51).

Two studies investigated the clinical course and outcome of patients with AMI, with and without COVID-19 infection. The one study analyzed patients in a prospective COVID-ACS international registry, using a pre-COVID-19 cohort as the control group (52), while the other compared COVID-19-negative and -positive cases admitted to hospital with AMI early in the pandemic (53). The former study documented that patients with COVID-19 and ACS presented later and had an increased in-hospital mortality compared to the pre-COVID-19 cohort, and that there was a greater rate of cardiogenic shock, which was a major contributor to the poorer outcome. The latter study documented that patients with COVID-19 and AMI were older, had more comorbidities, and had a higher hospital mortality, compared to those without COVID-19.

Some authors have restricted their analyses to those cases with ST elevation myocardial infarction (STEMI) (54–56). The first study compared patients with confirmed COVID-19 and STEMI with an age- and sex-matched STEMI group prior to COVID-19 (54). Patients with COVID-19 infection were more likely to present with cardiogenic shock (18%), but less likely to have angiography (78%) compared to the control group. The primary outcome was a composite of in-hospital death, stroke, recurrent AMI and repeat unplanned revascularization, which occurred more commonly in the COVID-19-positive group compared to controls. The second study showed a significant increase in in-hospital mortality, stent thrombosis and cardiogenic shock after percutaneous revascularization in those patients with STEMI and COVID-19 infection compared with a contemporaneous group of non-COVID STEMI patients (55). The third study indicated that STEMI patients admitted during the first wave of COVID-19 infections in Israel, had a longer ischemic time, which translated into more severe disease on hospital admission and a higher in-hospital adverse event rate than observed in STEMI cases admitted in a corresponding period in 2018 (57).

Interestingly a literature review of COVID-19 patients who had STEMI, in the early part of the pandemic, documented that 17% of cases were due to non-obstructive coronary artery disease, which was associated with a mortality of 30% (similar to those with obstructive coronary artery disease); however, such data were based on case reports and case series only (57). The role of thrombosis, including extensive and multi-vessel thrombosis, irrespective of the presence or absence of atherosclerotic plaques has been described, and is reviewed elsewhere (58, 59).

Other studies have analyzed predominantly Type II myocardial infarction in patients with COVID-19 (60). These studies indicate that such cases may be caused by an imbalance between oxygen supply and demand, possibly due to hypoxia, increased heart rate, systemic inflammation and/or decompensated cardiac failure, and are associated with high in-hospital mortality rates.

Clearly, a number of studies, some described above, suggest that there has been a delay in health-seeking behavior, primarily due to fear of contracting SARS-CoV-2 infection. This may account for the apparent decrease in prevalence of AMI during COVID-19 and be associated with a delay in medical intervention, resulting in an alarming rate of rare complications from AMI (48, 52, 61).

THE ROLE OF PLATELETS IN ISCHEMIC CARDIAC DISEASE

Platelets play an important role in the development of thrombo-inflammatory conditions and complications and are thus recognized as critical participants in the development of AMI (62). Platelet activation is a complex and multistep process (which is beyond the scope of the current review), principally mediated by specific platelet receptors that control adhesion and activation (62). Notable amongst these are the platelet collagen receptor: Fc receptor: γ-chain (FcR γ) co-expressed with glycoprotein (GP) VI. Patients with ACS have been reported to express higher numbers of GPVI receptors (63), have altered GPVI signaling (64) and an increased aggregation response (64).

Various platelet surface activation markers, such as CD62P (P-selectin), CD40L, platelet factor 4 and GP IIb/IIIa, as well as biological markers, primarily platelet micro-particles, have been associated with inflammation, atherosclerosis, and thrombosis (65). Platelet-monocyte aggregates, which persist in peripheral blood for longer than the surface markers and are therefore viewed as more sensitive markers of *in vivo* platelet activation (65), are an early marker of acute MI and have also been linked to myocardial no-reflow in STEMI patients (66, 67). Indirect markers of platelet activation, such as increased mean platelet volume (68), as well as higher levels of von Willebrand factor (69), and serotonin (70), have also been observed during acute MI.

PLATELET INTERACTIONS WITH SARS-CoV-2

In addition to expression of the low-affinity receptor for the Fc fragment of immunoglobulin G, which may promote viral entry, platelets also express TLR7, as mentioned above, which enables these cells to interact with viral single-stranded RNA (71, 72). Both of these mechanisms, as well as others (73), may contribute to viral persistence and hyperreactivity of platelets. In the case of ACE2, the major SARS-Cov-2 cell entry receptor and its associated transmembrane serine protease 2 (TMPRSS2), expression of this viral receptor and enzyme by platelets has been reported by some (74), but challenged by others (75). Because a direct role, if any, for the spike (S) protein of SARS-Cov-2 in mediating platelet hyperreactivity and thrombocytopenia in severe disease remains to be established, this section of the review is largely focused on severe COVID-19 that is associated with endothelial infection, activation and dysfunction as significant contributors to platelet overdrive and development of vascular disease.

PLATELET INTERACTION WITH INFLAMED/DAMAGED ENDOTHELIUM

Although platelets do not normally interact with intact vascular endothelium, they nevertheless play a critical role in maintaining vascular homeostasis *via* continuous monitoring of endothelial integrity. This is accomplished by preventing and repairing vascular leaks such as those associated with leukocyte extravasation (76). However, in the setting of the excessive

endothelial activation and dysfunction that occur during serious SARS-CoV-2 infection, platelets acquire a more aggressive phenotype. Notwithstanding platelet activation mediated by thrombin and collagen, as well as autocrine activation by adenosine 5'-monophosphate and thromboxane A_2, this is augmented by interaction of platelet Toll-like receptor 7 (TLR7) with viral single stranded RNA. Platelet activation is characterized by increased expression of CD62P and tissue factor, as well as platelet/leukocyte aggregate formation, which contribute significantly to the pathogenesis of thrombotic disease (77–79).

In this setting, the interaction of platelets with stressed vascular endothelium involves most of the adhesive mechanisms that also maintain vascular homeostasis. However, the transition from homeostatic regulation to vascular disease is determined by the extent of endothelial dysfunction and the associated increase in the intensity and duration of platelet adhesion and activation that drives the recruitment of highly reactive neutrophils and monocytes.

Mechanisms that promote the adhesion of platelets to inflamed vascular endothelium are numerous and these have been extensively reviewed elsewhere (80, 81). Prominent among these are initial adhesive events that promote "rolling" of platelets along endothelium, particularly the interaction of the major platelet membrane receptor, GP1b (CD42)-1X-V with von Willebrand factor released from endothelial cells (ECs). This type of rolling action is reinforced by interactions that involve upregulated expression of CD62P, present on both cell types, with its counter receptor, P-selectin glycoprotein ligand-1 (PSGL-1), also expressed on both cell types, albeit weakly in the case of platelets (81).

Stabilization of platelet/EC interactions requires the cooperation of two key integrins and three endothelial-associated, pro-adhesive molecules. These are the integrins, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, expressed on platelets and ECs, respectively, and the proteins, fibrinogen and fibronectin, as well as the glycoprotein, von Willebrand factor, which act as bridges linking the platelet and EC integrins to achieve firm binding. This is intensified *via* the fibrinogen-dependent interactions of $\alpha_{IIb}\beta_3$ with intercellular adhesion molecule-1 (ICAM-1; CD54) expressed on ECs, as well as by the association of EC-bound GP1b-IX-V/von Willebrand factor with platelet $\alpha_{IIb}\beta_3$ (81).

In addition to these platelet-endothelium interactions, other prominent mechanisms by which prolonged platelet activation poses risks to cardiovascular health include the following:

- microvascular occlusion due to formation of large intravascular homotypic aggregates of platelets, as well as heterotypic aggregates of platelets with neutrophils and monocytes (78, 82);
- platelet-driven systemic inflammatory responses associated with activation of neutrophils and monocytes/macrophages, resulting in excessive production of proinflammatory cytokines, especially interleukin (IL)-6, as well as induction of intense intracellular oxidative stress (83, 84);
- excessive platelet-driven intravascular formation of neutrophil extracellular traps (NETs) that contribute to organ damage and dysfunction (85, 86);
- release of prothrombotic/procoagulant factors (factor V, prothrombin, fibrinogen, von Willebrand factor) from platelet α-granules (87);
- platelet-driven active and passive expression of tissue factor by monocytes and neutrophils, respectively (78, 88).

These platelet-driven inflammatory mechanisms are summarized in **Table 3**.

PLATELET-MEDIATED ENDOTHELIAL DAMAGE AND DYSFUNCTION

In addition to the direct proinflammatory/prothrombotic and cytotoxic effects of the SARS-Cov-2 S protein on vascular endothelium, as well as impairment of the renin-angiotensin-aldosterone system (83), prolonged platelet activation in severe COVID-19 infection also amplifies endotheliopathy (89) In this context, a very recent study by Barrett et al. described potential mechanisms by which activated platelets exacerbate endothelial activation and damage (90). Using a strategy based on integration of endothelial cells and platelets isolated from patients hospitalized with COVID-19, transcriptomic analysis of mRNA expression extracted from endothelial cells exposed to platelet releasate revealed alterations in processes involved in maintenance of tight junction barrier integrity, coagulation and inflammation, characteristic of an "inflammatory, hypercoaguable phenotype" (90).

Systematic genetic analysis enabled the authors to identify the genes encoding the calcium-binding proteins, SA100A8 and SA100A9, also known as myeloid-related proteins (MRP) 8 and 14, respectively, as being the most prominent platelet-derived mediators of endothelial activation. These proteins are stored in platelet granules and dimerize to form the potent, proinflammatory protein, calprotectin (MRP8/14). Interacting with CD36 on vascular endothelium, calprotectin weakens cell-cell interactions and initiates release of the proinflammatory cytokines, IL-6, and IL-8 (90–92).

Importantly, the systemic levels of MRP8/14 were found to be significantly higher in the cohort of patients hospitalized with COVID-19 relative to those of control subjects and were associated with development of thrombosis and other adverse events (90).

Very recently, Bye et al. described another mechanism operative in patients with severe COVID-19 by which platelets may augment endothelial activation and dysfunction (93). In this setting, platelets that have adhered to SARS-CoV-2-infected endothelial cells *via* von Willebrand factor make contact with surface-bound immune complexes formed between the S protein and heavily Fc region-galactosylated immunoglobulin G antibodies, which effectively bind to and activate the platelet FcγRIIA receptor (93).

ENDOTHELIITIS DUE TO SERIOUS SARS-CoV-2 INFECTION

Notwithstanding the involvement of cells of the innate and adaptive immune systems, there is increasing recognition of

TABLE 3 | Mechanisms by which activated platelets contribute to vascular and organ damage during severe COVID-19 infection.

Mechanism	Refs
• Formation of large intravascular homotypic (platelets) and heterotypic (platelets/neutrophils/monocytes) aggregates that result in microvascular occlusion	(78, 82)
• Initiation and perpetuation of systemic inflammatory responses that drive excessive production of proinflammatory cytokines and severe oxidative stress	(83, 84)
• Drive the intravascular formation of obstructive neutrophil extracellular traps	(85, 86)
• Release prothrombotic/procoagulant factors contained in α-granules	(87)
• Initiate synthesis and release of tissue factor by platelet-activated monocytes	(78, 88)

the key role played by the vascular endothelium in the development of the prothrombotic state and hypercoagulability that precede the occurrence of arterial and venous complications in patients with severe SARS-CoV-2 infection, particularly those with preexisting, procoagulant tendencies (94–97). Of the 29 SARS-CoV-2 proteins, it is the S protein in particular and, to a lesser extent, the nucleocapsid protein (NP) that drive virus-mediated endothelial damage. Of these two viral proteins, the former is cytotoxic, causing loss of endothelial structural integrity, while the latter induces a proinflammatory endothelial phenotype. Importantly, ECs do not express TLR7, excluding putative proinflammatory interactions with viral single-stranded RNA (98).

Spike Protein-Mediated Endothelial Dysfunction

This viral protein consists of two subunits, S1 and S2. These spike proteins bind initially to endothelial surface glycosaminoglycans, enabling recognition of, and interaction with, their natural cellular counter receptor, ACE2, which is expressed on several types of host cells, including vascular endothelium (99). Physical binding of ACE2 to the S protein necessitates proteolytic cleavage at the S1/S2 sites by the cellular enzyme, TMPRSS2. These events precede, and are a prerequisite, for membrane fusion and viral entry. Thereafter S protein-mediated endothelial dysfunction results from at least two distinct mechanisms, both of which are dependent on viral infection of ECs, which results from binding of the S1 region to ACE2.

Firstly, and very recently, Lei et al. engineered an RNA-free pseudovirus variant of SARS-CoV-2 that was comprised of an inert outer shell impregnated with the S protein that enabled efficient attachment to ACE2 (99). Intratracheal administration of this variant of SARS-CoV-2 to Syrian hamsters mimicked many of the features of the pulmonary damage associated with natural SARS-CoV-2 infection. These changes included downregulation of endothelial expression of ACE2 that was due to decreased stability of the virus receptor that resulted from impaired phosphorylation of the ACE2 Ser-680 amino acid residue by the endothelial enzyme, AMP-activated protein kinase (AMPK) (99). This event, in turn, targeted the dephosphorylated variant of ACE2 for proteolytic degradation by a pathway driven by the oncoprotein, MDM2 (murine double minute 2) that mediates ubiquitination of unstable ACE2 (99, 100). In addition, dysregulation of endothelial protein phosphorylative activity also resulted in interference with the activity of endothelial nitric oxide synthase (eNOS) (99).

The authors replicated these findings in a series of *in vitro* experiments in which isolated pulmonary arterial ECs were infected with the SARS-CoV-2 pseudovirus (99). In addition, they also observed that exposure of the ECs to recombinant S1 protein caused mitochondrial dysfunction and EC fragmentation, seemingly because of intense intracellular oxidative stress associated with loss of ACE2. Based on their findings, the authors concluded that SARS-CoV-2 S1 protein-mediated loss of endothelial ACE2 "may exacerbate endothelial dysfunction, leading to endotheliitis" (99).

The aforementioned findings are largely in keeping with those of another very recent study reported by Raghavan et al. (101). Using an *in vitro* cell culture-based approach, these authors investigated the effects of exposure to recombinant S1 protein on the structural integrity of primary cultures of mouse brain ECs isolated from non-diabetic and diabetic mice (101). The authors measured the pro-adhesive activities of several types of EC junctional adhesion molecules [vascular endothelial (VE) cadherin; junctional adhesion molecule-A (JAM-A) that regulates tight junctions; the gap junction protein, connexin-43; PECAM-1 (CD31) also highly expressed at EC intercellular junctions]. Following exposure to the S1 protein, the cellular levels of all four junctional adhesion molecules decreased significantly, resulting in disruption of the endothelial barrier, an effect that was most evident with ECs from diabetic mice (101). Mechanistically, S1 protein-mediated endothelial disruption appeared to result from an increased association of the junctional adhesion molecules with the membrane-associated protein, Rab5a, which is a GTPase regulator of intracellular vesicular transport that regulates the movement of membrane proteins between the plasma membrane and early endosomes (101, 102). In the clinical setting of COVID-19, loss of barrier function results not only in detachment of ECs that are detected as circulating cells floating in blood (103), but also exposure of the arterial sub-endothelium with the attendant risk of myocardial infarction and stroke.

Notwithstanding involvement in infection and death of ECs by SARS-CoV-2, the role of ACE2, if any, in S1 protein-mediated junctional adhesion molecule dysfunction does, however, remain to be established.

Spike Protein and Complement-Mediated Dysfunction of Endothelial Cells

Recently, Yu et al., albeit also using an *in vitro*-based approach, demonstrated that treatment of eukaryotic cells with either the S1 or S2 recombinant proteins, but not NP, initiated endothelial cytotoxicity *via* activation of the alternative complement pathway (104). The authors used an engineered *PIGA*null TF1 cell line that does not express ACE2 for their studies. This is an erythroleukemic cell line genetically depleted of phosphatidylinositol glycan class A (*PIGA*), which is the precursor of glycosylphosphatidylinositol

(GPI). This glycophospholipid anchors the complement regulatory proteins, CD55 (inactivates the C3 convertases of the alternative and classical pathways) and CD59 (attenuates the lytic activity of the membrane attack complex). Cytotoxicity of both the S1 and S2 proteins occurred in the presence of normal human serum measured using a colorimetric assay based on cellular metabolic activity (104). The involvement of the alternative, as opposed to the classical pathway of complement activation, was demonstrated according to the presence of increased levels of fragment Bb (derived from activation of Factor B), as well as by the protective effects of inhibition of Factor D and by the addition of Factor H (104). Although remaining to be conclusively established, the authors speculate that the S1 and S2 proteins initiate activation of the alternative complement pathway by interfering with the activity of regulatory Factor H (104).

Although of potential pathophysiological significance, studies of this type need to be repeated using endothelial cell lines with intact complement regulatory surface proteins, as well as expression of ACE2.

Activation of the NLRP3 Inflammasome by the S and Viroporin SARS-CoV-2 Proteins

The S protein of SARS-CoV-2 has also been shown to activate the proinflammatory/pro-pyroptosis NLR family pyrin domain-containing protein 3 (NLRP3) inflammasome, seemingly by a pro-oxidative mechanism in various types of eukaryotic cells, including small human hematopoietic and EC precursor cells, as well as macrophages (105, 106). In addition, the SARS-CoV-2 small membrane permeability-inducing protein, viroporin, may also potentiate activation of the NLRP3 inflammasome *via* a mechanism involving efflux of K^+ (107). Importantly, the NLRP3 inflammasome is present in vascular endothelium, activation of which triggers endothelial dysfunction and death (108). Although seemingly unexplored, putative activation of the EC NLRP3 inflammasome may also contribute to the development of SARS-CoV-2-mediated endotheliitis.

Nucleocapsid Protein

The NP of SARS-CoV-2 is a 45.6 kDa (theoretical) multivalent RNA-binding protein that initiates activation of ECs by mechanisms that are non-cytotoxic and do not involve ACE2 (109). In this context, Qian et al. recently investigated the effects of exposure of five different types of human primary EC cultures (pulmonary vascular, umbilical vein, aortic, coronary artery and dermal microvascular) to recombinant NP (0.05–1 micrograms/milliliter) for 4–24 hours *in vitro* (109). This resulted in dose- and time-related increased expression of the endothelial adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), that promoted adherence of human primary monocytes (109). Mechanistically, NP-mediated endothelial activation involved interaction of the viral protein with TLR2 expressed on ECs. This event, in turn, triggered activation of intracellular signaling mechanisms driven by nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), resulting in transcription of the genes encoding ICAM-1 and VCAM-1 (109).

Although indicative of a proinflammatory activity of the SARS-CoV-2 NP that may contribute to the endotheliitis of advanced COVID-19 infection, independent confirmation of these findings, as well as extrapolation to the pathogenesis of thrombosis in small vessels, is necessary.

These various mechanisms by which the SARS-Cov-2 spike protein, viroporin and NP promote endothelial damage and dysfunction are summarized in **Table 4**.

Given the very recent interest in the involvement of the vascular endothelium in the pathogenesis of COVID-19, only a few reports have described relationships between disease severity and outcome with elevations in systemic biomarkers of endothelial activation and dysfunction such as von Willebrand factor and thrombomodulin (110–112). The paucity of reports focused on this important topic underscores the necessity for more intensive investigation. Novel platelet-directed therapies currently under investigation include the P-selectin-targeted monoclonal antibody, crizanlizumab (113).

ADVERSE CONSEQUENCES OF A DAMAGED ENDOTHELIUM

Endothelial damage precedes the adhesion and activation of platelets, neutrophils, and monocytes. This event, in turn, results in the development of a procoagulant state due to the loss of EC-derived anti-coagulant proteins such as thrombomodulin and anti-thrombin III, as well as the prostanoid, prostacyclin. These procoagulant mechanisms are intensified, in turn, by potentiation of platelet activation *via* endothelial leakage of von Willebrand factor and by increased synthesis and expression of tissue factor by

TABLE 4 | SARS-Cov-2 proteins that mediate endothelial cell dysfunction.

Protein	Mechanisms	Refs
Spike protein	• Degradation of ACE2	(99)
	• Decreased activity of eNOS	
	• Mitochondrial dysfunction and cellular oxidative stress	
	• Displacement of endothelial junctional adhesion molecules	(101)
	• Cytotoxicity due to activation of the alternative complement pathway	(104)
	• Possible activation of the endothelial NLRP3 inflammasome potentiated by viroporin	(105–108)
Nucleocapsid protein	• Upregulation of expression of the endothelial adhesion molecules, ICAM-1 and VCAM-1, resulting in recruitment of inflammatory cells	(109)

ACE, acetylcholine esterase; eNOS, endothelial nitric oxide synthase; NLRP3, NLR family pyrin domain-containing protein 3; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

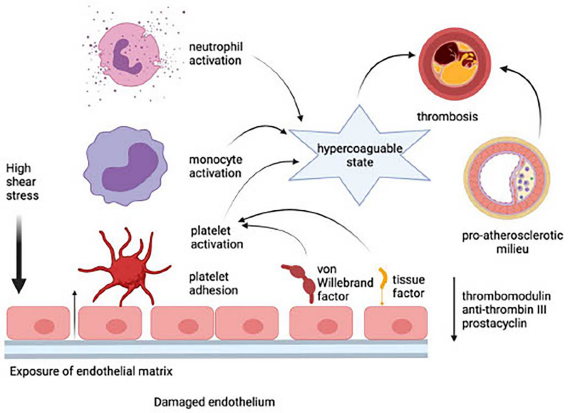

FIGURE 1 | Adverse consequences of a damaged endothelium (created in Biorender). In the setting of high shear stress, exposure of the sub-endothelial extracellular matrix leads to the loss of endothelial cell-derived anti-coagulant proteins such as thrombomodulin and anti-thrombin III, as well as the prostanoid, prostacyclin. Platelets are activated via endothelial leakage of von Willebrand factor and by increased synthesis and expression of tissue factor by endothelial cells. Platelet accumulation and interaction with monocytes and neutrophils potentiate a hypercoagulable state, which, in the presence of a pre-atherosclerotic milieu, causes thrombosis and predisposes for development of myocardial infarction and stroke.

ECs. Inflammatory events that occur on an essentially intact endothelium in the setting of a low shear stress predispose for the development of venous thrombosis (114). In the case of arterial thrombosis, disruption of the endothelial barrier in the setting of high shear stress, exposure of the sub-endothelial extracellular matrix, platelet accumulation, and, in particular, the existence of a pre-atherosclerotic milieu, are likely to predispose for development of MI and stroke. These processes are depicted in **Figure 1**.

CONCLUSION

COVID-19 is a systemic disease affecting various systems, including the cardiovascular system, where it leads to significant mortality. While our understanding of the pathogenesis of COVID-19 and associated complications is evolving, evidence is emerging about the very important role of platelets *per se* together with endotheliitis and its attendant role in platelet recruitment and activation. These events create a SARS-CoV-2-driven cycle of intravascular inflammation and coagulation, which contributes significantly to a poor clinical outcome in patients with severe disease. Clearly, the role of platelet-endothelium interactions in the pathogenesis of COVID-19 needs further intense exploration to enable optimization of the treatment of the infection and its complications with the ultimate goal of improving outcomes.

AUTHOR CONTRIBUTIONS

TR, RA, and CF contributed equally to the conceptualization and compilation of the manuscript, while all four authors (TR, RA, PM, and CF) were involved in reviewing and finalizing the manuscript and approved its submission. All authors contributed to the article and approved the submitted version.

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Signaling Through FcγRIIA and the C5a-C5aR Pathway Mediate Platelet Hyperactivation in COVID-19

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Patients with COVID-19 present with a wide variety of clinical manifestations. Thromboembolic events constitute a significant cause of morbidity and mortality in patients infected with SARS-CoV-2. Severe COVID-19 has been associated with hyperinflammation and pre-existing cardiovascular disease. Platelets are important mediators and sensors of inflammation and are directly affected by cardiovascular stressors. In this report, we found that platelets from severely ill, hospitalized COVID-19 patients exhibited higher basal levels of activation measured by P-selectin surface expression and had poor functional reserve upon *in vitro* stimulation. To investigate this question in more detail, we developed an assay to assess the capacity of plasma from COVID-19 patients to activate platelets from healthy donors. Platelet activation was a common feature of plasma from COVID-19 patients and correlated with key measures of clinical outcome including kidney and liver injury, and APACHEIII scores. Further, we

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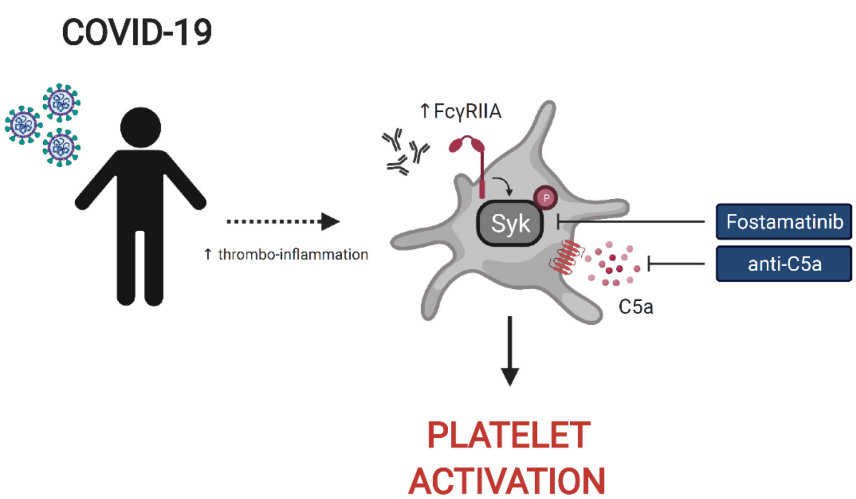
identified ferritin as a pivotal clinical marker associated with platelet hyperactivation. The COVID-19 plasma-mediated effect on control platelets was highest for patients that subsequently developed inpatient thrombotic events. Proteomic analysis of plasma from COVID-19 patients identified key mediators of inflammation and cardiovascular disease that positively correlated with *in vitro* platelet activation. Mechanistically, blocking the signaling of the FcγRIIa-Syk and C5a-C5aR pathways on platelets, using antibody-mediated neutralization, IgG depletion or the Syk inhibitor fostamatinib, reversed this hyperactivity driven by COVID-19 plasma and prevented platelet aggregation in endothelial microfluidic chamber conditions. These data identified these potentially actionable pathways as central for platelet activation and/or vascular complications and clinical outcomes in COVID-19 patients. In conclusion, we reveal a key role of platelet-mediated immunothrombosis in COVID-19 and identify distinct, clinically relevant, targetable signaling pathways that mediate this effect.

Keywords: platelet, COVID - 19, FcγRIIa, complement, fostamatinib



GRAPHICAL ABSTRACT |

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has led to a global-scale pandemic creating an unprecedented burden on human health and public health processes (1). SARS-CoV-2 infected patients present with a wide spectrum of clinical presentations, ranging from asymptomatic infections to prolonged ICU stays accompanied by significant morbidity and mortality (2–4). Although Acute Respiratory Distress Syndrome (ARDS) represents the hallmark of COVID-19 associated clinical manifestations, thrombotic events are enriched in patients with severe COVID-19 and have been linked to worse outcomes (3, 5–8). Increased levels of d-dimer and platelet dysfunction are frequently observed in COVID-19 patients (9–13), indicating a loss of homeostasis in platelet function, vascular integrity and the coagulation cascade.

Platelets are anucleated megakaryocyte-derived blood cells that play a prominent role in hemostasis and thrombus formation (14). Beyond hemostasis, platelets represent cellular mediators of inflammation and interact with the immune system in multiple ways, including priming of other immune cells and integrating extrinsic immunological stimuli (15–17). Platelets express a variety of TLR receptors, express HLA class II for antigen presentation and respond to complement activation (15, 18, 19). In COVID-19, patients with severe disease often exhibit increased platelet activation and formation of platelet-monocyte aggregates facilitated by tissue factor expression on monocytes (13). RNA sequencing of platelets in COVID-19 patients has

revealed an altered transcriptional profile with enrichment in the pathways of antigen presentation, protein ubiquitination and mitochondrial dysfunction (12). Phenotypic analysis of platelets in COVID-19 demonstrated that platelets undergo a series of functional and biochemical changes most pronounced in patients with severe disease (20). In addition, SARS-CoV-2 has been shown to associate with (21) and bind ACE2 receptors on the platelet surface (22), whereas other studies have demonstrated that platelets can internalize SARS-CoV-2 virions in a process that contributes to their hyperactivated state (23). A candidate-driven genetic association study identified putative complement and coagulation-related loci associated with severe COVID-19 (24). Finally, unbiased pathway-enrichment analysis of circulating proteins in COVID-19 patients underscored platelet degranulation and complement activation as the top pathways associated with disease severity (25). Thus, platelets have key role not only in hemostasis but also in inflammatory processes, and platelet dysregulation is central to the pathogenesis and clinical outcomes in severe COVID-19 in many patients.

Despite the importance of platelets in thrombotic events in COVID-19 patients, how heightened platelet activation is linked to clinical features of disease and the associated underlying mechanisms remain poorly understood. These gaps in our understanding of platelet function and dysfunction during SARS-CoV-2 infection limit our ability to identify patients at risk of thromboembolic events and to treat vascular complications of COVID-19 including clots. Moreover, identifying the inflammatory effector molecules and pathways that underlie the activation and dysregulation of platelets in COVID-19 could reveal novel opportunities for therapeutic intervention. To address these questions, we examined platelets and the platelet-activating potential of plasma from severely ill, hospitalized COVID-19 patients. These studies revealed an increase in basal expression of the activation marker P-selectin on platelets from severe COVID-19 patients coupled with poor response to Thrombin Receptor Activation Peptide (TRAP) stimulation, indicating loss of functional reserve, compared to platelets from convalescent and healthy donors. COVID-19 patient plasma robustly activated healthy platelets from control donors and this platelet activating potential was highest prior to the precipitation of a thrombotic event. Correlation of platelet activation induced by COVID-19 plasma with clinical features collected during patient hospitalization revealed significant associations with ferritin levels and key measures of clinical outcome including kidney and liver injury, and APACHE III scores. Moreover, proteomic analysis identified central mediators of inflammation and cardiovascular homeostasis correlating with a platelet hyperactivated state consistent with a role for platelets linking inflammatory events to thrombotic pathology. Finally, we identified a key role for Fc receptor and complement signaling in platelet activation in COVID-19 because blockade of signaling through the FcγRIIa-Syk and the C5a-C5aR axis using antibody blockade, depletion of immunoglobulin from COVID-19 plasma or the FDA approved drug fostamatinib blocked activation of healthy platelets by COVID-19 plasma. Thus, these studies identify a platelet hyperactive state associated with severe SARS-CoV-2 infection, define the underlying mechanisms, and have direct therapeutic implications for the prevention and treatment of thrombotic complications in patients with COVID-19.

RESULTS

Platelets From Hospitalized COVID-19 Patients Exhibit Increased Activation at Baseline and Poor Functional Reserve

COVID-19 is associated with heightened activation of platelets both at baseline and after pharmacologic stimulation in response to low doses of agonists (12, 13). We interrogated a cohort of hospitalized COVID-19 patients, a cohort of non-hospitalized COVID-19 recovered health care workers and a cohort of healthy control subjects recruited at the University of Pennsylvania Health System for which we had collected peripheral blood samples and clinical annotation (**Supplementary Table 1**) during the period of April to June of 2020 (26). The inpatient cohort spanned a range of severity scores with moderate and severe/critical scores being the most common. Most patients were treated in a high-acuity medicine floor or ICU setting and clinically manifested pneumonia with hypoxia.

CD62P (P-selectin) is bound to the membrane of α-granules within the cytoplasm of platelets, transported to the plasma membrane rapidly after activation and can be used as a marker of platelet activation. To evaluate the activation status of platelets from COVID-19 patients in our cohort, we assayed surface expression of CD62P directly *ex vivo* or after stimulation with TRAP that activates platelets *via* the thrombin receptor (**Figure 1A**). We compared CD62P surface expression in three separate groups: hospitalized COVID-19 patients (COVID-19 inpatient), patients previously infected with SARS-CoV-2 that recovered and reached convalescence (COVID-19 convalescent) and healthy donors. The COVID-19 inpatient group had higher expression of CD62P at baseline [median geometric mean fluorescence intensity (gMFI) 56.0] compared to the COVID-19 convalescent (median gMFI 45.2) or healthy donor (median gMFI 20.2) group (**Figures 1A, B**). Upon TRAP stimulation, CD62P expression increased for all three groups (**Figures 1A, B**). However, the ratio of TRAP/basal surface CD62P expression was highest for the healthy donor group (ratio 28.4), intermediate for the convalescent group (ratio 6.98) and lowest for the inpatient COVID19 group (ratio 5.77, **Figure 1B**). These results identify a heightened basal platelet activation state in hospitalized COVID-19 patients, but also demonstrate a reduced functional reserve in platelets from these patients revealed by *ex vivo* TRAP stimulation.

A causal link between cardiovascular disease and COVID-19 outcomes, including thrombotic complications, has been proposed previously (27–29) but how exactly such clinical events relate to platelet activation phenotypes is not clear. Thus, we first assessed CD62P expression, both basal and

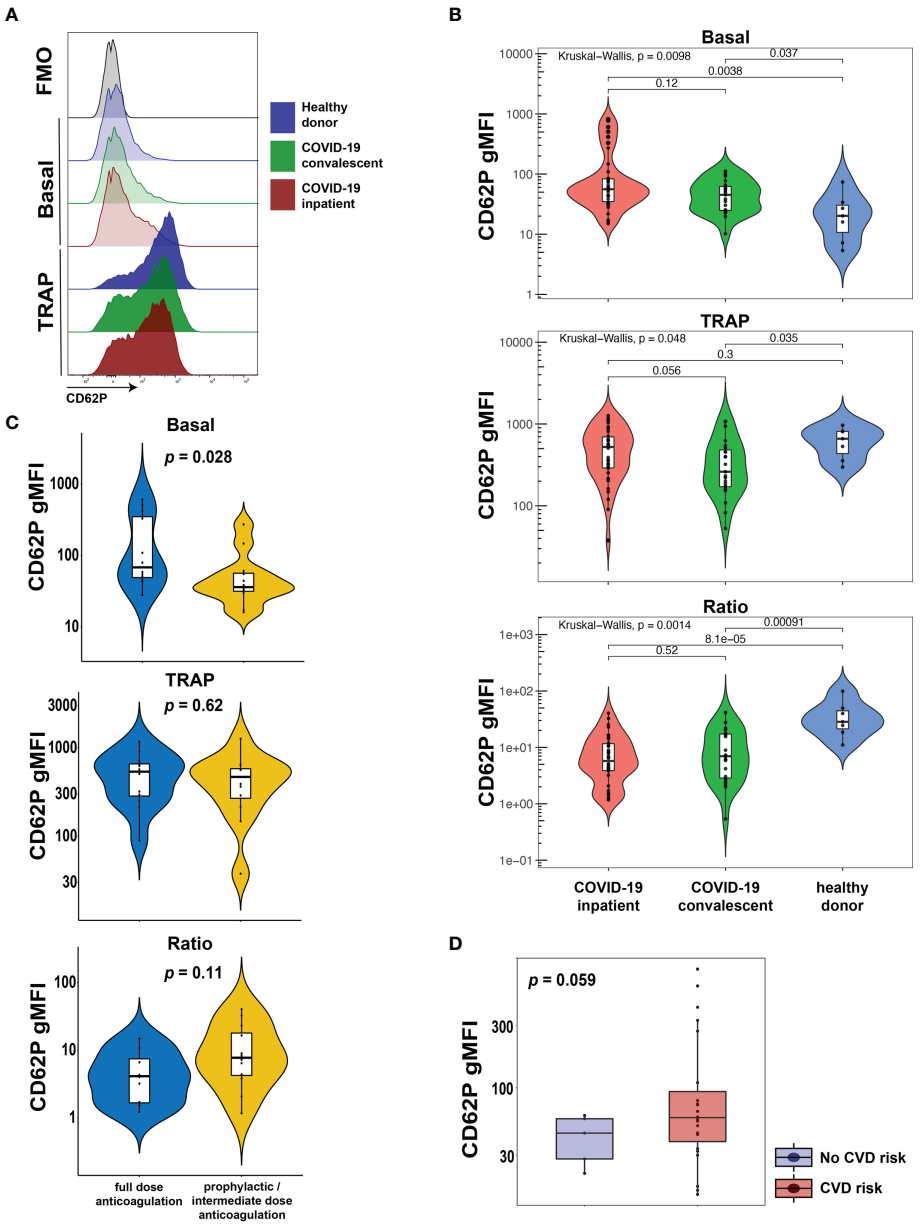


FIGURE 1 | Platelets from hospitalized COVID-19 patients exhibit increased activation at baseline and diminished functional reserve. **(A, B)** Representative histograms **(A)** and cumulative data **(B)** for CD62P surface expression of *ex vivo* isolated platelets assayed with or without TRAP stimulation for 20mins. The CD62P gMFI ratio of TRAP/basal-treated is also shown. The three patient cohorts shown are COVID-19 inpatient (n=38), COVID-19 convalescent (n=23) and healthy donors (n=7). Kruskal-Wallis non-parametric testing was used to compare all three groups and pair-wise comparisons were also performed; *p*-values are depicted. **(C)** Cumulative data for CD62P surface expression of *ex vivo* isolated platelets at baseline (basal), after TRAP activation (TRAP) and their ratio for hospitalized COVID-19 patients on full-dose (n=10) or prophylactic/intermediate dose (n=13) of anticoagulation. Wilcoxon non-parametric testing was used and the *p*-values are depicted. **(D)** Cumulative data for CD62P surface expression of *ex vivo* isolated platelets at baseline for hospitalized COVID-19 patients with (n=31) or without (n=7) cardiovascular disease risk factors. Wilcoxon non-parametric testing was used, and the *p*-value is depicted. FMO control, Fluorescence minus one control; gMFI, geometric mean fluorescence intensity; TRAP, Thrombin Receptor Activation Peptide.

following TRAP stimulation, in hospitalized COVID-19 patients that exhibited clinically evident thrombosis. However, CD62P surface levels were similar between COVID-19 inpatients that had a clinical thrombus and those who did not (**Supplementary Figure 1**). COVID-19 hospitalized patients are placed on different protocols of anticoagulation. In our cohort, several patients were on full-dose anticoagulation and others were on standard-of-care, prophylactic dose (or intermediate according to the inpatient protocol at the time of COVID-19 hospital care) anticoagulation. This distinction likely captures both the effect of

inpatient clotting incidents and pre-existing, underlying comorbidities, including pro-thrombotic states and cardiovascular etiologies, requiring anti-coagulation. We therefore used this distinction in anti-coagulant treatment to evaluate the CD62P results. This analysis revealed a significant increase in basal CD62P surface levels in patients on full-dose compared to those on prophylactic or intermediate dose anticoagulation (**Figure 1C**). Both groups, however, responded after TRAP stimulation by increasing CD62P MFI and although there was a trend for lower CD62P TRAP/basal ratio in the full-dose anticoagulation group, this difference did not reach statistical significance (**Figure 1C**). To address this question from a different perspective, we compared the platelet activation indicated by CD62P expression between subjects with and without cardiovascular disease (CVD) risk factors. COVID-19 patients with CVD risk displayed elevated basal CD62P expression on the plasma membrane of platelets compared to patients without CVD risks (**Figure 1D**). These data indicate that platelet activation in COVID-19 patients was higher in those patients on full-dose anticoagulation either due to an acquired in-hospital event or a pre-existing condition and was also associated with the presence of cardiovascular risk. Thus, defining the mechanisms and pathways contributing to the platelet activation may reveal insights into COVID-19 pathogenesis and thrombotic complications.

Plasma From Hospitalized COVID-19 Patients With High Inflammatory Index Causes Platelet Hyperactivation

COVID-19 can often manifest as a hyperinflammatory state. We, therefore, next investigated whether plasma from COVID-19 patients contained soluble mediators capable of activating platelets. To test this idea, we incubated plasma from COVID-19 patients with platelets from healthy donors and assessed platelet activation state. In addition to CD62P, we measured surface CD63 levels (also known as LAMP-3). Following response to an extracellular stimulus, platelets degranulate and during this process translocate the granule membrane bound protein CD63 to their plasma membrane, thereby allowing CD63 surface expression to be used as a marker of platelet activation. We also quantified Fcγ receptor IIa (FcγRIIa, CD32), the only Fcγ receptor on platelets, and C3aR to evaluate changes in these receptors that could underlie potential effects of immune complexes and/or anaphylatoxins on platelet activation. The control platelets were pre-gated as CD42b+ (GPIba+) single cells (for gating strategy see **Supplementary Figure 2**). Unlike our *ex vivo* analysis of platelets from COVID-19 patients, treatment of healthy platelets using plasma from COVID-19 patients did not induce higher CD62P expression than plasma from recovered or healthy donors (**Figure 2A**). In contrast, however, CD63 and CD32 were increased on control platelets following incubation with plasma from the COVID-19 inpatient group compared to platelets treated with plasma from the COVID-19 convalescent group (**Figure 2A**). C3aR levels also increased upon treatment with COVID-19 inpatient plasma when compared to the healthy donor plasma (**Figure 2A**). The reasons for the differences in CD62P between direct *ex vivo* analysis of platelets from COVID-19 patients and induction of healthy platelet activation from plasma from COVID-19 patients may reflect a different kinetics after *in vivo* versus *in vitro* activation, or non-plasma-based activation signals for CD62P, such as those that occur when platelets interact with collagen in a damaged vessel wall. Nevertheless, these results demonstrate that plasma from COVID-19 patients preferentially activates platelets indicating that this assay can be used to further gain insights into pathways underlying platelet activation and/or dysfunction associated with COVID-19 disease.

The data in **Figure 2A** implicate factors circulating in the plasma from COVID-19 patients that mediate platelet activation. However, there was considerable within-group heterogeneity. We hypothesized that this variation in platelet activation could be explained at least partially by the clinical heterogeneity documented in COVID-19 patients (30–32). To test this idea, we correlated surface expression of CD62P, CD63, CD32 and C3aR with established clinical markers used for monitoring and decision-making during the hospitalization of high-acuity patients (**Figure 2B**). These clinically measured features of disease included markers of kidney injury (incident acute kidney injury, development of end-stage renal disease, creatinine and BUN blood levels), markers of liver injury (liver transaminases AST and ALT), cell counts (neutrophils, lymphocytes, monocytes, platelets), levels of d-dimer, ferritin, lactate and lactate dehydrogenase (LDH). We also incorporated Acute Physiology and Chronic Health Evaluation (APACHE) III scoring in our analysis that affords prognostication and mortality prediction in the acute clinical setting. Analyzing these data together with platelet activation changes induced by plasma from COVID-19 patients revealed strong correlations among CD63, CD32 and C3aR, but not with CD62P (**Figure 2B**, top 4x4 square of correlations). Examining clinical features, CD63 correlated with higher levels of creatinine and BUN, both reflecting the development of kidney dysfunction. The presence of AKI, while correlating positively with CD63, did not reach nominal *p*-value significance, possibly due to the fact that AKI is exceedingly common in the ICU setting and creatinine/BUN provide a more dynamic range of kidney function. Higher levels of CD63 and C3aR correlated with AST [reaching nominal *p* value, but not our false discovery (FDR) cutoff of 0.05] that is used to monitor liver injury. Of the markers of inflammation and cellular stress, ferritin had the strongest positive correlation with the ability of plasma to activate platelets (**Figures 2B, C**). There was a statistically significant positive correlation between ferritin and CD63 or CD32 that also met our FDR cutoff (**Figure 2B**). CD32 correlated positively with LDH. Examination of cell counts revealed a strong negative correlation between platelet counts and CD62P (reaching both *p* value and FDR significance) and other correlations reached nominal *p* value, but not FDR significance (**Figure 2B**), consistent with previous studies (26). Finally, APACHE III scores positively correlated with CD63, indicating that plasma from patients with high disease severity had increased ability to activate platelets. Our findings indicate that clinical measures of disease severity and organ damage,

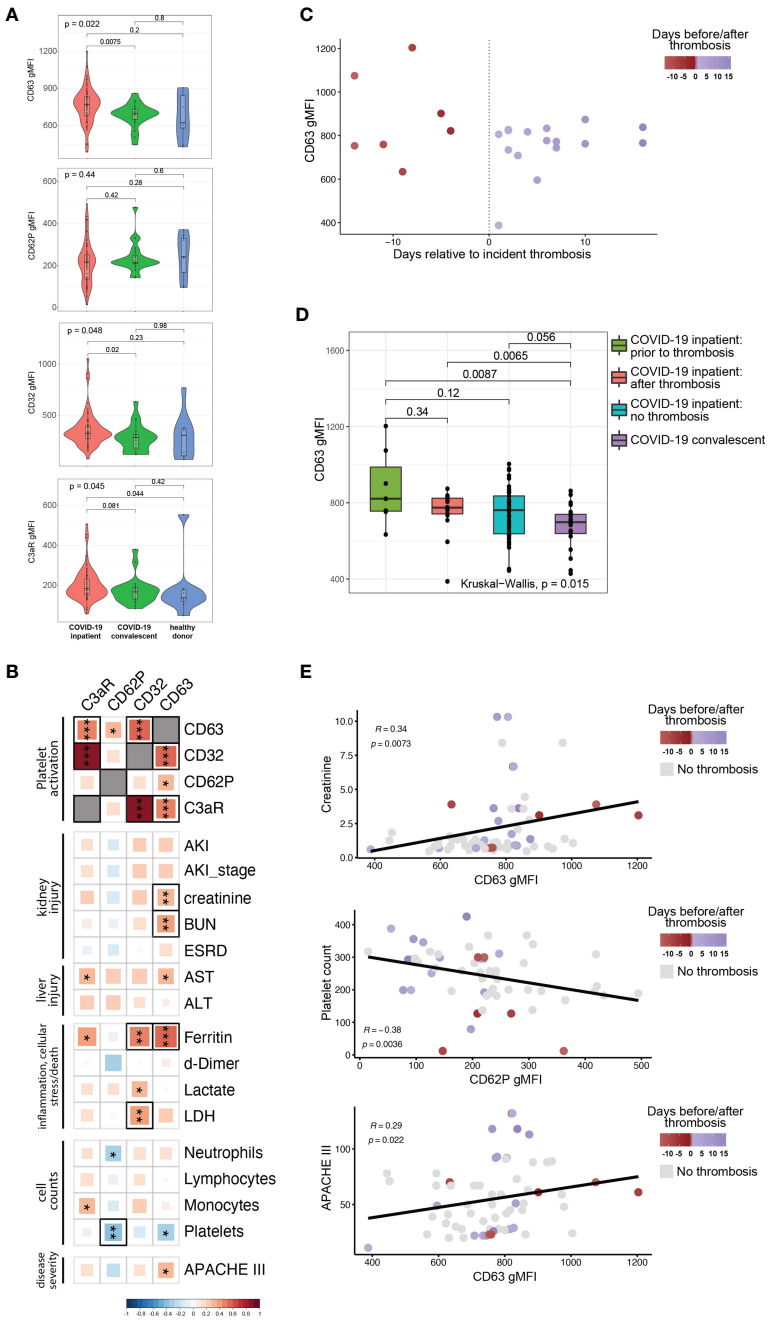


FIGURE 2 | The ability of COVID19 plasma to activate platelets is increased for patients with evidence of organ damage, high circulating levels of ferritin, and at timepoints preceding a thrombotic event. **(A)** Plasma from COVID-19 inpatient (n=63), COVID-19 convalescent (n=20) and healthy donors (n=9) was incubated with platelets isolated from healthy volunteers. The gMFI levels of CD63, CD62P, CD32 and C3aR are shown in these three groups. Kruskal-Wallis non-parametric testing was used to compare all three groups and pair-wise comparisons were also performed; *p*-values are depicted. **(B)** Spearman correlation of the gMFI surface levels of the markers used in **(A)** and selected clinical parameters of the COVID-19 inpatient group (n=63). Δ correlation coefficient shown on the key (bottom). Asterisks *, ** and *** denote p values less than 0.05, 0.01 and 0.001 respectively. Highlighted squares denote FDR values less than 0.05. **(C)** CD63 gMFI surface levels of control platelets induced by plasma derived from COVID-19 patients with thrombosis drawn at different timepoints relative to the thrombotic event. **(D)** CD63 gMFI surface levels of control platelets induced by plasma derived from COVID-19 patients prior to thrombosis and after thrombosis, COVID-19 patients without thrombosis and COVID-19 patients in convalescence. Kruskal-Wallis non-parametric testing was used to compare all four groups and pair-wise comparisons were also performed using Wilcoxon testing; *p*-values are depicted. **(E)** Representative scatter plots for creatinine vs CD63, platelet count vs CD62P and APACHE III score vs CD63. Samples are colored based on the absence (grey color) or presence of thrombosis relative to the time of draw (red before thrombosis and blue after thrombosis). The Spearman correlation co-efficient and the corresponding *p*-value are depicted.

such as kidney and liver injury, markers of inflammation and cellular stress, and cumulative disease score measures had high concordance with platelet hyperactivation.

The relationship between CD63, CD32, C3aR and clinical disease indicated by ferritin levels and organ damage biomarkers in the blood suggested that the platelet activation potential of plasma from COVID-19 patients might provide additional insights into disease pathogenesis. Thromboembolic events are associated with poor outcomes, cardiopulmonary collapse and mortality in high-severity SARS-CoV-2 infected patients (30, 33, 34). In a large metanalysis of 42 studies, thromboembolic risk was associated with higher mortality in COVID-19 patients (7). Platelets not only contribute to normal thrombus formation but in case of hyperactivation can precipitate spontaneous clotting *in situ* (35–38). Thus, we examined the COVID-19 patients who experienced a clinically evident thrombosis during their hospital stay. We subdivided the samples to those coming from patients who did not yet have a clotting incident at the time of the blood draw (but went on to develop one later in their hospital stay) and the ones that already had a clotting event at or before the time of the draw. Plotting CD63 levels of healthy platelets activated by plasma from COVID-19 patients revealed a potential relationship between platelet activation capacity and future thrombosis (**Figure 2C**). To further examine this potential relationship between platelet activation and thrombotic events, we compared CD63 induction by plasma from COVID-19 patients with future thrombotic events, patients with past thrombotic events, patients who never experience thrombotic complications and COVID-19 recovered patients (**Figure 2D**). Indeed, plasma obtained from patients prior to incident thrombotic events had the greatest capacity to induce CD63 on platelets from healthy donors compared to the other groups. Thus, platelet activation potential appears to be highest in COVID-19 patients prior to a clotting event. Samples that were drawn from patients that experienced incident thrombosis were among the ones with the highest levels of both platelet activation markers and biomarkers of clinical deterioration (**Figure 2E** and **Supplementary Figure 2B**), especially in the days leading to the thrombotic event. Additionally, we could identify clusters of patients that had their sample drawn after a thrombotic event with high creatinine levels and APACHEIII scores but intermediate CD63 levels, possibly indicating the clinical sequalae of the recent thrombosis (**Figure 2E**). Thus, the ability of a COVID-19 patient's plasma to activate platelets exogenously appears to be connected to clinical events, including future thrombotic events. This suggests that circulating factors within the plasma of COVID-19 patients might induce platelet activation, thrombosis, and other clinical outcomes.

COVID-19 Plasma-Induced Platelet Activation Is Associated With Markers of Inflammation and Cardiovascular Disease

To begin to interrogate the soluble mediators that may underlie the platelet activation ability of COVID-19 patient plasma, we performed Proximity Extension Assays (PEA) using the O-link platform. This analysis interrogated 274 analytes in the blood with a focus on cardiovascular, inflammatory and organ damage related processes. We examined which of these circulating inflammatory mediators correlated with the ability of COVID-19 plasma to induce CD63 expression on platelets from healthy donors. Indeed, the concentration of numerous proteins in circulation correlated with the induction of CD63 on platelets (**Figure 3**). The top proteins identified were mediators of inflammation, including IL-18, IL18BP, ADA, CCL15, and proteins involved in vascular and heart pathology, including VEGFA, NPPC and PCSK9 (**Figure 3**). There were also proteins suggesting neutrophil involvement such as NCF2, and potential indicators of nuclear content such as NBN, TOP2B, and EIF4EBP1 possibly consistent with release of neutrophil extracellular traps. It was notable that only positive correlations were revealed suggesting that, at least at this level of resolution, there were few if any counterregulatory pathways induced to maintain platelet quiescence. Individual positive correlations were examined more directly for CCL15, ADA, VEGFA and PCSK9 (**Supplementary Figure 3**). These findings further support the clinical metadata analysis described above (**Figure 2**) and establish the connection between increased platelet activation and circulating mediators of inflammation, neutrophil activity and/or indicators of cardiovascular disease and tissue damage in COVID-19.

FcγRIIa Activation and Complement Anaphylatoxins Mediate Platelet Activation in COVID-19

The protein analysis described above suggested a connection between the platelet activation potential of COVID-19 plasma with tissue damage, cardiovascular pathways and COVID-19 associated inflammation. Infection with SARS-CoV-2 elicits a complex immune response, characterized by patterns of cellular and soluble inflammatory mediators that may differ considerably from patient to patient (26, 39, 40). How features of this inflammatory response relate to activation of platelets is unclear, though several possibilities exist. Specifically, immune complex formation and pro-inflammatory Fcγ structures have been implicated in the pathogenesis of severe COVID-19 (41–43) and platelets express FcγRIIa (15, 18, 44). In addition, platelets express complement receptors C3aR (18) and C5aR (45) and complement components, including anaphylatoxins, have roles in the inflammatory cascade during SARS-CoV-2 infection (24, 39, 40, 46). Finally, IL-6 is often elevated and has been evaluated as a therapeutic target in COVID-19 (47–51). IL-6 may have a role in activating platelets in other settings, including through membrane-bound gp130 (52). As shown above, surface abundance of CD32 (FcγRIIa) and of C3aR increases following platelet incubation with plasma derived from COVID-19 patients with a high inflammatory signature (**Figures 2B, C**). Thus, we hypothesized that Fc receptor signaling, IL-6 signaling and/or the signaling by the anaphylatoxins C3a and C5a, might be causally involved in platelet activation by COVID-19 plasma. To test this hypothesis, we examined the effect of blocking each of these pathways on the ability of plasma from COVID-19 patients to activate platelets (**Figure 4A** and **Supplementary Figure 4A**). Blocking each

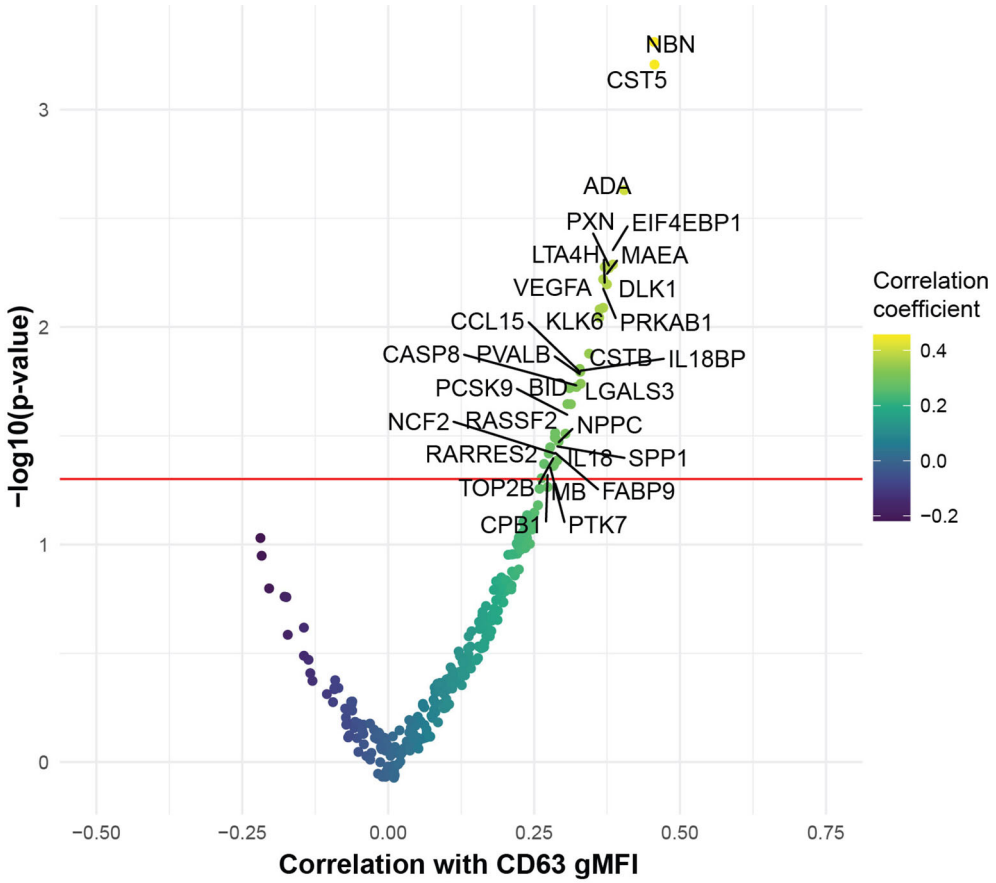


FIGURE 3 | COVID-19 plasma induced platelet activation is associated with markers of inflammation and cardiovascular disease. Volcano plot with the x-axis representing the correlation coefficient of the different analytes with CD63 gMFI induction on platelets by COVID-19 plasma (n=53) and the y-axis depicting the -log10 transformation of the corresponding *p*-value.

pathway individually inhibited platelet activation indicated by reduced induction of CD63, CD32, C3aR or CD62P. Blocking FcγRIIa had the strongest effect, followed by C5a neutralization whereas IL-6 and C3a blockade had the weakest effect and preferentially impacted CD62P and CD63, but not CD32 or C3aR (**Figure 4A**). However, blocking all 4 pathways simultaneously robustly decreased platelet activation indicated by all markers. This effect was more pronounced for samples from patients with higher ferritin (**Supplementary Figure 4B**). To evaluate how these distinct pathways might cooperate for platelet activation, we assessed different combinations of pathway blockade (**Figure 4B**). Although all pathways contributed to platelet activation indicated by CD63 induction, blockade of FcγRIIa appeared to have the most robust effect when combined with other blocking antibodies, especially in combination with anti-C5a and anti-C3a antibodies (**Figure 4B**). To further evaluate the role of FcγRIIa in platelet activation by COVID-19 plasma, we depleted IgG from the plasma samples prior to the platelet activation. IgG depletion reduced platelet activation by COVID-19 patient derived serum and this effect was accentuated further by neutralizing C5a (**Figure 4C**). Thus, these data indicate that the ability of plasma from COVID-19 patients to activate platelets occurs, at least partially, through IgG-mediated activation of FcγRIIa and this effect can be further augmented by signals from complement, including C5a.

Fostamatinib Ameliorates the Heightened Activation of Platelets Induced by COVID-19 Plasma

The FcγRIIa signals through recruitment and phosphorylation-mediated activation of Syk (53, 54). To confirm and extend the observations described above, we next investigated whether Syk inhibition impacted platelet activation by plasma from COVID-19 patients. Fostamatinib is a tyrosine kinase inhibitor that blocks the enzymatic activity of Syk and is clinically used for treatment of chronic immune thrombocytopenia (55). Syk phosphorylation was induced in as early as 1 minute following incubation of platelets from healthy donors with plasma from COVID-19 patients and increased further at 5 minutes compared to the effect of plasma from healthy control subjects (**Figure 5A**). We next assessed the impact of inhibiting Syk signaling on COVID-19 plasma mediated platelet activation. Addition of the

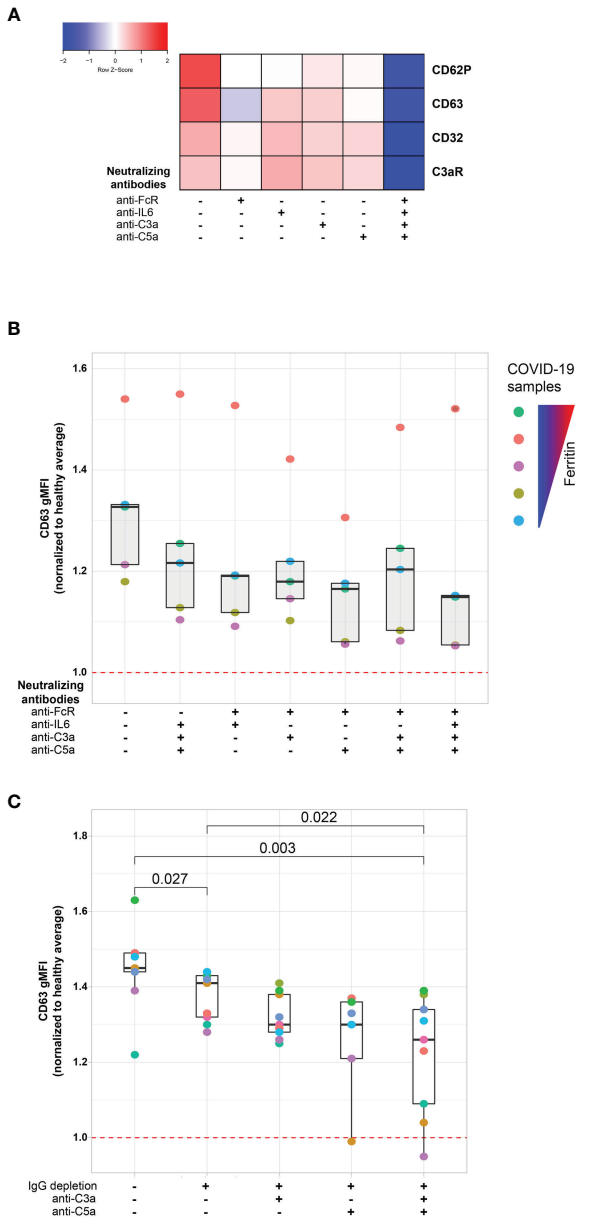


FIGURE 4 | FcγRIIa activation and complement anaphylatoxins mediate platelet activation in COVID-19. **(A)** Heatmap of gMFI expression of CD62P, CD63, CD32 and C3aR on the surface of control platelets incubated with COVID-19 plasma (n=10 patients) in the presence or absence of neutralizing antibodies to FcγRIIa, IL6, C3a and C5a, as indicated. **(B)** Boxplots of gMFI expression of CD63 on the surface of control platelets incubated with COVID-19 plasma (n=5 patients, depicted with distinct colors) with different combinations of neutralizing antibodies to FcγRIIa, IL6, C3a and C5a, as indicated. **(C)** Boxplots of gMFI expression of CD63 on the surface of control platelets incubated with COVID-19 plasma (n = 9 patients, depicted with distinct colors) with different combinations of IgG depletion and neutralizing antibodies to C3a and C5a.

fostamatinib active metabolite R406 to the platelet assay described above decreased activation of healthy platelets by plasma from COVID-19 patients (**Supplementary Figure 5**). Addition of neutralizing antibodies to C3a and C5a did not further reduce platelet activation under these conditions suggesting a dominant role of Syk signaling in this setting. These data indicate a key role for antibody-mediated activation of platelets through FcγRIIa and possibly also complement activation. Whether this effect is the result of immune complexes, only IgG or also other isotypes that result in complement activation will require future studies. Nevertheless, these data suggest potential clinical utility of Syk inhibition or drugs that block complement signaling at least in a subset of patients.

To further interrogate the prothrombotic potential of plasma from patients with COVID-19, we used a photochemical injury model in an endothelial-lined microfluidic channel. Induction of photochemical injury in this model results in activation of the endothelium with released von Willebrand factor (VWF) strands localized to the region of light exposure perfused with hematoporphyrin. We have previously described this approach in studies of platelet activation in the prothrombotic disorder of heparin-induced thrombocytopenia (56). Plasma from either normal control subjects or from patients with severe COVID-19 (1/10 final dilution) was added to calcein-labeled platelets isolated from healthy donors and perfused over injured endothelial cells. Although some platelet adhesion to the extruded VWF was observed over the subsequent 15 minutes for the channels where healthy plasma was added, this effect was markedly enhanced in those channels with added COVID-19 plasma (**Figures 5B–D**). Pre-incubation of platelets with either Syk inhibitor or antibodies that neutralize FcγRIIa, or C5a largely abrogated this enhanced platelet aggregation (**Figures 5B–D** and **Supplementary Table 2**). These data are consistent with a role for both FcγRIIa and complement activation of the platelets by the COVID-19 plasma and highlight the connection between antibody mediated platelet activation and the potential to initiate vascular thrombotic events. Moreover, the identification of these pathways dependent on Syk signaling suggests potential therapeutic opportunities.

DISCUSSION

Platelets act as a cellular connection between the immune system and hemostasis, integrating signals from different immune cell subsets, soluble inflammatory mediators and the complement cascade. As a result, platelets have the capacity to link signals from exuberant immune responses to thrombotic complications. Severe COVID-19 results in loss of immune homeostasis either due to a suboptimal, albeit persistent, immune response to SARS-CoV-2 or due to immune system hyperactivation that is disproportionate to what is necessary for efficient virus elimination (57). The data presented here indicate that plasma samples derived from COVID-19 patients with a high inflammatory index and accumulated organ damage possess a robust ability to activate healthy platelets suggesting a link between infection-induced circulating mediators and potential thrombotic events. This connection was more apparent when evaluating platelet activation through the surface expression of the lysosomal marker CD63. Of note, lysosomal markers have

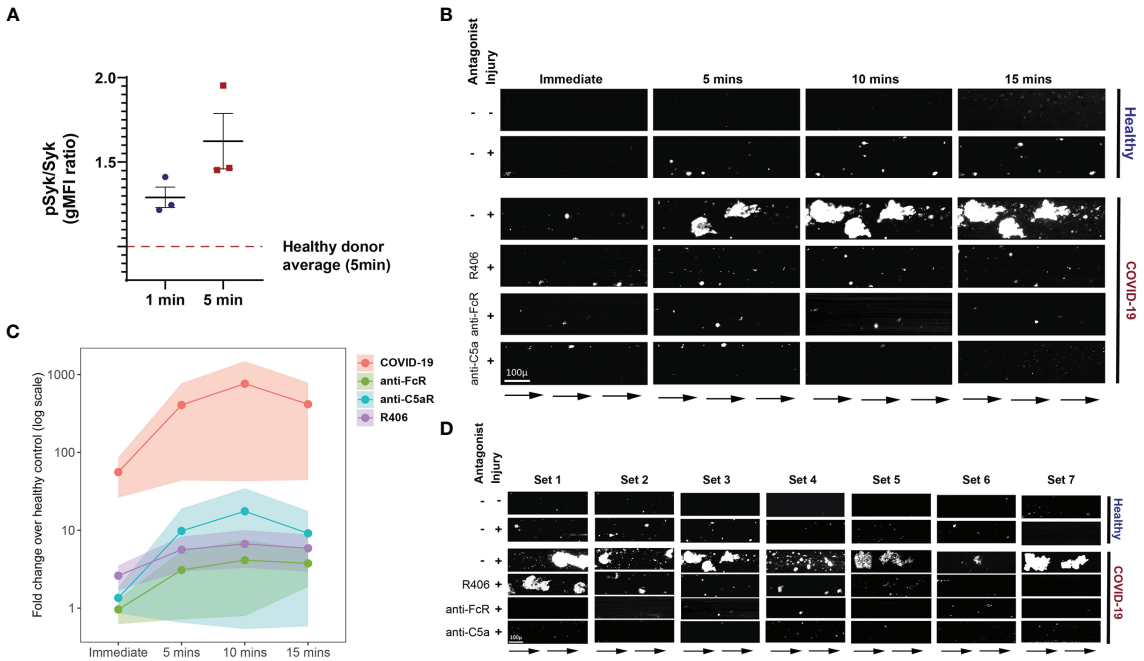


FIGURE 5 | Fostamatinib ameliorates the heightened activation of platelets induced by COVID-19 plasma. **(A)** Phospho-Syk and total Syk gMFI were measured with flow cytometry of control platelets incubated with COVID-19 plasma (n=3 patients) for 1 and 5 minutes. Incubation with healthy control plasma for 5 mins was used to normalize the data. **(B)** Representative studies of a hematoporphyrin-induced photochemical injury model in an endothelial-lined microfluidic channel. Images show platelet adhesion (in white) immediately after infusion of isolated washed healthy donor platelets, and 5 min, 10 min and 15 min after infusion. Shown is a representative study where plasma from a severe COVID-19 patient was added to the platelets. In the samples indicated, the Syk inhibitor R406, FcR blocking antibody, or an anti-C5a antibody were added prior to infusion. Direction of blood flow is indicated by arrows. Size bar indicates 100µ. **(C)** Overall data analysis from studies with plasma derived from 7 patients with severe COVID-19. The Y-axis shows accumulated platelets in each microfluidic lane done immediately after the isolated platelet suspension was added and after 5, 10 and 15 minutes (log10 scale, fold change over healthy control plasma flown in uninjured channels). **(D)** All 7 sets of experiments from D are shown at the 10-minute timepoint.

previously been associated with both inflammation and thrombosis (58). Indeed, induction of platelet activation was the highest in samples drawn prior to a clotting incident, an observation that could have future clinical utility. Further, we were able to begin to identify pathways involved in platelet activation revealing a key role for IgG mediated FcγRIIa signaling and complement, though other signals and pathways are also likely involved. Thus, our data define a key role for platelet activation potential of plasma from COVID-19 patients and link these observations to severity of the inflammatory state, clinical outcome measures, specific immunological pathways, potential thrombotic risk, and a pathway with clinical targeting opportunities.

Cardiovascular disease risk factors, including hyperlipidemia, hypertension and diabetes, have been recognized early in the pandemic as important determinants of COVID-19 outcomes (27–29). Our proteomics analysis revealed a correlation between mediators of cardiovascular health and platelet activation. Specifically, one of the most correlated proteins was VEGFA. Increased circulating VEGFA may point to an underlying strain to the cardiovascular system (59) in individuals with co-incident high levels of platelet activation. Correlation with other proteins of cardiovascular health, including NPPC (60, 61) and FABP9 (62), further support this hypothesis. Finally, among the highly correlated proteins was PCSK9 that has a central role not only in LDL metabolism (63–65) but has been shown to promote platelet activation (66) and can be found elevated in sepsis (67). As a result, our findings demonstrate a connection between platelet hyperactivation and elevated markers of cardiovascular dysregulation.

Complement activation and immune complexes that act through pro-inflammatory Fc structures have been linked to severe COVID-19 outcomes (41, 43, 68, 69). Inhibition of FcγRIIa signaling, either through receptor blockade, IgG depletion or Syk inhibition, substantially reduced the platelet hyperactivation induced by COVID-19 plasma. Fostamatinib has an FDA-approved indication as first-line treatment for chronic ITP (55). This drug has also been trialed in rheumatoid arthritis, albeit with modest effects (70). In this report, Syk inhibition using fostamatinib robustly blunted *in vitro* platelet activation induced by COVID-19 plasma. Not only do these data identify a potential mechanism of platelet activation in COVID-19 *via* antibody and/or immune complex mediated platelet Fc receptor signaling, but they also point to a therapeutic intervention opportunity in COVID-19 patients, especially those with a high risk for thrombotic complications. Indeed, fostamatinib is

currently being tested in a clinical trial in hospitalized COVID-19 patients (ClinicalTrials.gov Identifier: NCT04579393). A preliminary report posted by the manufacturer of fostamatinib suggested that Syk inhibition might protect against COVID-19 induced hypoxia. Although speculative at this stage, this information might indicate that Syk is required for the formation of pulmonary microthrombi reported in patients with COVID-19 induced hypoxia (37). In addition to Syk inhibition, inhibition of the Fc-mediated and/or complement-mediated platelet activation could be achieved through other therapeutic modalities. For example, Fc-mediated platelet stimulation could be tempered by plasmapheresis of immune complexes from COVID-19 patients (ClinicalTrials.gov Identifier: NCT04374539). In addition, it may also be relevant that of the two complement anaphylatoxin receptors tested, C5a neutralization had the largest additive effect to Fc blockade reducing platelet hyperactivation. Blocking the C5a-C5aR axis with monoclonal antibodies against C5aR reduced the activation of human myeloid cells and lung injury in a human C5aR knock-in mouse model (40). Eculizumab, a monoclonal antibody that inhibits the cleavage of C5 to C5a and C5b and previously approved for treatment of paroxysmal nocturnal hematuria (71) and atypical hemolytic uremic syndrome (72), is also currently in a clinical trial to investigate its efficacy in severe COVID-19 (ClinicalTrials.gov Identifier: NCT04355494). Other potential complement pathway inhibitors also exist and are under investigation in COVID-19 patients. The current work may provide a deeper understanding of how these interventions function to prevent thromboembolic events, suggest biomarkers of drug efficacy and even potential drug combinations that may improve outcomes in particular groups of patients. Thus, our data identify the FcγRIIa-Syk and the C5a-C5aR axes as key mediators of platelet hyperactivity in COVID-19 and highlight the therapeutic potential of targeting these mechanisms in COVID-19 patients, especially those with high-thrombotic risk.

Finally, our findings provide a framework for additional platelet-focused studies. They establish the role of thromboinflammation in COVID-19, support the role of cardiovascular disequilibrium in platelet dysfunction and indicate that there are plasma soluble factors that drive platelet hyperactivation. Moreover, given the recent concerns about very rare venous thrombosis and thrombocytopenia following adenovirus-based SARS-CoV-2 vaccination (73–76), the studies described here might suggest approaches to evaluate underlying mechanisms for these events. Indeed, although further studies are necessary to examine the beneficial potential of restoring platelet function in COVID-19 patient outcomes, our studies highlight putative therapeutic candidates to address platelet-driven clotting complications of COVID-19.

MATERIALS AND METHODS

Patients, Subjects, and Clinical Data Collection

Patients admitted to the Hospital of the University of Pennsylvania with a SARS-CoV-2 positive result were screened and approached for informed consent within 3 days of hospitalization (COVID-19 inpatient group). Health care workers were recruited at the Hospital of the University of Pennsylvania and received both a PCR test to assess for active infection and serologic testing for antibodies against SARS-CoV-2; all individuals included in this study were serologically convalescent and PCR negative (COVID-19 convalescent group). Healthy donors were recruited through word of mouth at the University of Pennsylvania and were adults with no prior diagnosis of or recent symptoms consistent with COVID-19. The sample size for all groups and experimental conditions is shown below:

- Experiments conducted on fresh platelet samples: COVID-19 inpatient (n=38), COVID-19 convalescent (n=23) and healthy donors (n=7)
- Experiments conducted using plasma samples: COVID-19 inpatient (n=63), COVID-19 convalescent (n=20) and healthy donors (n=9).

Peripheral blood was collected from all subjects. For inpatient cases, clinical data were abstracted from the electronic medical record into standardized case report forms. APACHE III scoring was based on data collected in the first 24 hours of ICU admission or the first 24 hours of hospital admission for subjects who remained in an inpatient unit. Acute kidney injury (AKI) represented incident acute kidney injury that occurred on days zero – five of ICU admission, defined according to the AKIN creatinine criteria. Eligible patients for this outcome were free of end stage renal disease at baseline (dialysis dependence, baseline creatinine > 4 mg/dL). Comorbidities including prior history of clotting (deep vein thrombosis (DVT), pulmonary embolism (PE), cerebrovascular accident (CVA), myocardial infarction (MI), or other thrombus) were collected prospectively based on the EMR. Cardiovascular disease (CVD) risk factors were considered present if patients had any of the following: diabetes, hypertension, hyperlipidemia, peripheral arterial disease, cerebrovascular disease, or known coronary artery disease. In-hospital clotting events were determined by EMR chart review and requiring a documented and date-stamped DVT, PE, CVA, MI, or other thrombus by duplex, echocardiogram, or contrast-enhanced imaging. Anticoagulation (AC) treatment at the time of research blood collection was recorded; "intermediate" dose AC was equivalent to enoxaparin 0.5 mg/kg subcutaneously twice daily in contrast to "prophylactic" dose enoxaparin of 0.5 mg/kg once daily. Clinical laboratory data were collected from the date closest to the date of research blood collection.

Sample Processing, Platelet Isolation and Activation

Peripheral blood was collected into sodium heparin tubes (BD, Cat#367874). For directly *ex vivo* assays, whole blood was diluted 10-fold with Tyrode's buffer and then used for stimulation assays with TRAP or vehicle in the presence of 50mM CaCl2. TRAP was added at a final concentration of 35uM for 15mins at 37°C. The activation was terminated by adding PBS/4% PFA for 20

mins. For *in vitro* activation assays of control platelets with COVID-19 plasma, healthy platelets were first isolated. Whole blood collected in citrate tubes was spun at 125g for 15 minutes and the supernatant was collected to isolate PRP (platelet rich plasma). PRP was spun at 330g for 10 minutes to collect pelleted platelets that were resuspended in 1x Tyrode's Buffer. Platelets were subsequently activated on a 96-well plate with 50uL of sodium heparin isolated plasma derived from COVID-19 patients or healthy volunteers.

Neutralization and Inhibition Assays

Neutralizing monoclonal antibodies against C3a (Biolegend, cat # 518105), C5a (R&D, cat # MAB2037-SP), IL-6 (Biolegend, cat # 501101) were used for the neutralization assays. Inhibition of activation of FcγRIIa by human IgG/immune complexes was achieved with the use of Human TruStain FcX (Fc Receptor Blocking Solution, cat # 422302). IgG depletion from plasma was performed using the Albumin IgG Depletion Spintrap from Millipore-Sigma (cat # GE28-9480-20) based on the manufacturer's instructions. The active metabolite of fostamatinib R406 was purchased from Selleckchem (cat # S1533), dissolved in DMSO and used at a final concentration of 5μM.

Flow Cytometry and Antibody Clones

Antibodies used for staining of whole blood and platelets:

BB700 Mouse Anti-Human CD32	BD	Cat # 745929
APC Mouse Anti-Human CD42b	BD	Cat # 551061
PE Mouse Anti-Human CD62P	BD	Cat # 555524
PE/Cyanine7 anti-human C3AR Antibody	Biolegend	Cat # 345808
BV421 anti-human CD63 Antibody	Biolegend	Cat # 353030

Samples were acquired on a 4 laser BD FACS LSR. Standardized SPHERO rainbow beads (Spherotech, Cat#RFP-30-5A) were used to track and adjust PMTs over time. UltraComp eBeads (ThermoFisher, Cat#01-2222-42) were used for compensation. Up to $1x10^5$ platelets were acquired per each sample.

Proximity Extension Assays and SARS-CoV-2 Serologic Testing

Proximity Extension Assays using plasma derived from COVID-19 patients were performed using the commercially available Olink protein biomarker platform. Specifically, three Olink Target 96 panels were used: Olink Target 96 Cardiovascular III, Olink Target 96 Inflammation and Olink Target 96 Organ Damage panel. SARS-CoV2- RBG IgG and IgM measurements were performed by enzyme-linked immunosorbent assays (ELISA) as previously described (77).

Platelet Aggregation on Photochemically-Injured Endothelium in a Microfluidic System

$6 X 10^6$ cells/channel of human umbilical vein endothelial cells (HUVECs, ATCC- PCS-100-013) were seeded into the fibronectin (50 μg/mL, Sigma-Aldrich cat. # F0895) coated channels of a 48-well microfluidic plate (Bioflux, Fluxion Biosciences) and then injured by flowing a 50ug/mL solution of hematoporphyrin (Sigma-Aldrich) with exposure to blue light using the HXP-120 C light source with 475-nm excitation and 530-nm emission filters as previously described (56). 200μL of platelet suspension ($2x10^6$ platelets in $HBSS^{Ca-Mg-}$) from healthy donors was labelled with calcein-AM (2 μg/mL final concentration, ThermoFisher Scientific Cat # C3100MP) for 15 mins and then R406, Human TrueStain FcX or anti-C5a were added into the respective tube for a 15-minute incubation. 20μl of either healthy control or COVID-19 patient plasma (1:10 final dilution) was added just prior to being flowed through the channel at 10 dynes/cm^2. Platelet accumulation in the injured endothelium field was captured by Zeiss Axio Observer Z1 inverted microscope using Montage Fluxion software and analyzed using ImageJ as described (56).

Statistics

Pairwise correlations between variables were calculated and visualized as a correlogram using the R function *corrplot* (**Figure 2B**) displaying the positive correlations in red and negative correlations in blue. Spearman *p*-value significance levels were shown.

Due to the heterogeneity of clinical and flow cytometric data, non-parametric tests of association were preferentially used throughout this study unless otherwise specified. Correlation coefficients between ordered features (including discrete ordinal, continuous scale, or a mixture of the two) were quantified by the Spearman rank correlation coefficient and significance was assessed by the corresponding non-parametric methods (null hypothesis: $\rho = 0$). Tests of association between mixed continuous versus non-ordered categorical variables were performed by Wilcoxon test (for $n = 2$ categories) or by Kruskal-Wallis test (for $n > 2$ categories). All tests were performed two-sided, using a nominal significance threshold of $p < 0.05$ unless otherwise specified. When appropriate to adjust for multiple hypothesis testing, false discovery rate (FDR) correction was performed by the Benjamini-Hochberg procedure at the FDR < 0.05 significance threshold unless otherwise specified.

Other statistical analysis was performed using Prism software (GraphPad). Other details, if any, for each experiment are provided within the relevant figure legends.

Study Approval

All participants or their surrogates provided informed consent prior to participation in accordance with protocols approved by the regional ethical research boards and the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the regional ethical research boards (University of Pennsylvania) and in accordance with protocols approved by the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SA, CSA, and EW conceived the study. SA, AmS, HG, RG, DM, MA-H, and SG carried out experiments. SA, HG, AB, AG, CA, JW, ZC, YH, AP, OK, JD, AW, CI, DD, IF, AH, LV, JR, and NM were involved in clinical recruitment, sample allocation, processing and acquisition. All authors participated in data analysis and interpretation. SA, AmS, AaS, LR, LZ, MP, CSA, and EW wrote the manuscript. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.834988/full#supplementary-material

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SARS-CoV-2 Spike Protein 1 Activates Microvascular Endothelial Cells and Complement System Leading to Platelet Aggregation

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Microvascular thrombosis is associated with multiorgan failure and mortality in coronavirus disease 2019 (COVID-19). Although thrombotic complications may be ascribed to the ability of SARS-CoV-2 to infect and replicate in endothelial cells, it has been poorly investigated whether, in the complexity of viral infection in the human host, specific viral elements alone can induce endothelial damage. Detection of circulating spike protein in the sera of severe COVID-19 patients was evaluated by ELISA. *In vitro* experiments were performed on human microvascular endothelial cells from the derma and lung exposed to SARS-CoV-2-derived spike protein 1 (S1). The expression of adhesive molecules was studied by immunofluorescence and leukocyte adhesion and platelet aggregation were assessed under flow conditions. Angiotensin converting enzyme 2 (ACE2) and AMPK expression were investigated by Western Blot analysis. In addition, S1-treated endothelial cells were incubated with anti-ACE2 blocking antibody, AMPK agonist, or complement inhibitors. Our results show that significant levels of spike protein were found in the 30.4% of severe COVID-19 patients. *In vitro*, the activation of endothelial cells with S1 protein, *via* ACE2, impaired AMPK signalling, leading to robust leukocyte recruitment due to increased adhesive molecule expression and thrombomodulin loss. This S1-induced pro-inflammatory phenotype led to exuberant C3 and C5b-9 deposition on endothelial cells, along with C3a and C5a generation that further amplified S1-induced complement activation. Functional blockade of ACE2 or complement inhibition halted S1-induced platelet aggregates by limiting von Willebrand factor and P-selectin exocytosis and expression on endothelial cells. Overall, we demonstrate that SARS-CoV-2-derived S1 is sufficient in itself to propagate inflammatory and thrombogenic processes in the microvasculature, amplified by the complement system, recapitulating the thromboembolic complications of COVID-19.

Keywords: COVID-19, SARS-CoV-2 spike protein 1, complement system, endothelial dysfunction, inflammation, thrombosis

INTRODUCTION

In December 2019, a novel coronavirus was isolated from the respiratory epithelium of patients with unexplained pneumonia in Wuhan, China. This pathogen, named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), was identified as the causative agent of Coronavirus Disease 2019 (COVID-19). As of January 2022, over 375 million confirmed SARS-CoV-2 cases had been reported, claiming almost 5.7 million lives worldwide (1).

SARS-CoV-2 is a highly cytopathic virus that, like the other members of the *coronaviridae* family, induces epithelial cells to undergo apoptotic cell death as part of its replication cycle (2). Following SARS-CoV-2 infection in target cells (3, 4), the disease can manifest as a series of different clinical conditions, ranging from asymptomatic to life-threatening cases (5). For about 80% of patients, the infection is restricted to the proximal airways of the lungs, causing mild disease with modest symptoms (5). In about 20% of patients, SARS-CoV-2 infection can expand to the distal lung and rapidly deteriorate to a severe illness, characterised by bilateral interstitial pneumonia, acute respiratory distress syndrome (ARDS), and multi-organ damage with a high fatality rate (5).

Among the distinctive features of severe COVID-19, vascular abnormalities have been among the most frequently reported complications (6–8). There is growing evidence that SARS-CoV-2 induces endotheliitis and inflammatory cell infiltration in the lungs (7, 9). Following endothelial infection, loss of vessel barrier integrity and the development of a pro-coagulative endothelium have been identified as pivotal contributors to the initiation and propagation of ARDS (7, 9). Evidence of altered coagulation parameters during COVID-19 appeared in early reports from China, as revealed by elevated levels of partial thromboplastin time, prothrombin, D-dimer, and C-reactive protein in hospitalized patients (10). Similarly, autoptic studies in lung tissues found the presence of platelet-fibrin thrombi in small vessels associated with foci of alveolar hemorrhage (11, 12), suggesting that coagulopathy is critical for the outcome of COVID-19 (13–15). In this context, microvascular injury and thrombosis have also been shown to be associated with the activation of the complement system, as revealed by the presence of C3, C3a, C5a, and C5b-9 in the lungs and in the circulation of patients who succumbed to SARS-CoV-2 infection (16–19). In line with these findings, overt disseminated intravascular coagulation of small and large vessels (20), complement activation, and venous thromboembolic complications, in particular acute pulmonary embolisms (21–24), were identified as the pathogenic features of non-survivor COVID-19 patients.

Mechanistically, several data pointed to an immune system over-reaction as the main driver of the disruption of the thromboresistant phenotype of the microvascular endothelium during SARS-CoV-2 infection (25). Additional findings also revealed that SARS-CoV-2 can induce vascular damage in the lungs by directly infecting endothelial cells. This hypothesis has been corroborated by electron microscopy and immunofluorescence analyses of post-mortem tissue that showed that SARS-CoV-2 and viral particles can be detected in endothelial cells within the lungs (7, 9), although the clinical (26–28) and experimental data (29, 30) here are controversial. Spatially resolved SARS-CoV-2 RNA was detected in the pulmonary endothelium through *in situ* hybridization (31), further suggesting potential viral replication in lung microvascular endothelial cells. Although with mixed results (32, 33), a recent study suggested that SARS-CoV-2 can be found in the circulation more abundantly than previously thought and that plasmatic viremia correlates with disease severity and mortality (34). Based on this finding, it is conceivable that endothelial cells are exposed to SARS-CoV-2 in severe COVID-19 patients.

Of all the SARS-CoV-2 components, the subunit 1 of the spike protein (S1) – which is generated following proteolysis by host proteases such as TMPRSS2 (35, 36) – has the ability to interact with different receptors on the human target cells, including angiotensin converting enzyme 2 (ACE2), *via* the receptor-binding domain (RBD) and can induce specific cellular responses (35, 36). Numerous studies have demonstrated that endothelial cells express both ACE2 and TMPRSS2 (37–39), suggesting that the S1 protein potentially has an effect on endothelial cell activation and dysfunction, possibly leading to the engagement of pro-apoptotic pathways (40). In relation to this, the deposition of S1 protein has been documented in the cutaneous microvascular endothelium (41). In line with the above findings, it has also been shown that SARS-CoV-2-derived S1 protein, but not other structural proteins of the virus, can bind endothelial cells, inducing alterations in endothelial cell phenotype by enhancing the expression of cytokines, adhesive molecules and reactive oxygen species, as well as impairing cell permeability and metabolic functions (42–46). In addition, it has been shown that S1 can directly activate the alternative pathway of complement on the cell surface by interfering with Factor H function (47). Lastly, our group recently documented that exposing sera from severe COVID-19 patients to endothelial cells induced platelet aggregation *via* the engagement of C5a/C5aR1 axis (48).

The aim of this study is to investigate whether SARS-CoV-2-derived S1 is in itself sufficient to alter the endothelial phenotype, leading to microvascular inflammatory response and thrombosis *via* activation of the complement system.

RESULTS

SARS-CoV-2-Derived Spike Protein Is Detectable in the Circulation of Patients With Severe COVID-19

We explored the presence of the spike protein in sera from uninfected subjects (n=9), mildly ill convalescent COVID-19 patients (n=9) and severely ill COVID-19 patients (n=23) with an enzyme-linked immunosorbent assay (ELISA). Patients' characteristics are summarized in **Table 1**.

None of the selected sera from uninfected subjects or mildly ill COVID-19 patients (0 out of 18; 0%) exhibited detectable levels of circulating spike protein. Conversely, we found that 30.4% (7 out of 23) of hospitalized COVID-19 patients with active disease had detectable levels of spike protein above the

TABLE 1 | Baseline characteristics of patients included in the study.

	Overall (n=41)	Negative control (n=9)	Mild COVID-19 (n=9)	Severe COVID-19 (n=23)	*p*-value
Age (years)*	62.6 ± 14.5	58.9 ± 12.1[a,b]	59.0 ± 19.0[c]	65.5 ± 13.4	[a]0.210 *vs* Severe COVID-19 [b]0.284 *vs* Mild COVID-19 [c]0.988 *vs* Severe COVID-19
Male sex (%)	26 (63.4)	6 (66.7)[d,e]	5 (55.6)[f]	15 (65.2)	[d]0.938 *vs* Severe COVID-19 [e]0.629 *vs* Mild COVID-19 [f]0.612 *vs* Severe COVID-19

**mean ± S.D.*

The superscript refers to individual p-value of each parameter.

detection range of the assay. Individual levels of the spike protein in hospitalized patients' sera are reported in **Table 2**.

When hospitalized COVID-19 patients were divided according to positivity for spike protein in the serum, we found that the mean age of spike protein-positive patients was similar to that of spike-negative patients (**Table 3**). There were no differences between the sexes regarding the rate of spike protein positivity (**Table 3**). In contrast, there was a borderline significant difference (*p-value*=0.057) in the rate of SARS-CoV-2 RNA positivity when we used RT-real-time PCR on nasopharyngeal samples at the time of blood withdrawal (**Table 3**), suggesting that spike protein-positive patients still had detectable viral load at the time of hospitalization. This was further supported by the finding – though it was not statistically significant – that spike-positive patients tended to be admitted early after symptom onset compared to spike-negative subjects (**Table 3**). Our data are in line with previous findings showing that viral peak occurs early, 2-4 days after infection, while viral shedding is almost absent after 10 days (32, 49–51).

TABLE 2 | Levels of the spike protein in the sera of hospitalized COVID-19 patients.

Patient	Age(years)	Gender	Spike protein levels*(ng/ml)
1	79	Female	45.51
2	60	Male	16.72
3	77	Male	9.43
4	78	Male	3.07
5	61	Female	2.94
6	49	Male	2.76
7	27	Male	2.73
8	76	Male	Negative
9	55	Male	Negative
10	63	Male	Negative
11	71	Male	Negative
12	66	Male	Negative
13	44	Female	Negative
14	69	Female	Negative
14	76	Male	Negative
16	67	Female	Negative
17	78	Male	Negative
18	77	Male	Negative
19	80	Female	Negative
20	76	Male	Negative
21	71	Male	Negative
22	56	Female	Negative
23	60	Female	Negative

**Assay sensitivity: > 2.7 ng/ml.*

No comorbidities were found to be associated with a positive result in the detection of the spike protein (**Table 3**). While we found no differences in most of the clinical and biochemical parameters analysed, we observed a borderline difference (*p-value*=0.054) in the levels of lactate dehydrogenase (LDH) found in spike-positive and -negative patients (**Table 3**), possibly reflecting increased, widespread tissue damage in spike-positive patients. When we measured circulating levels of C5a and sC5b-9, we found that COVID-19 patients had significantly increased levels of C5a and sC5b-9 compared to healthy subjects (C5a: 41.4 ± 20.6 vs 7.6 ± 2.2 ng/ml, *p-value*<0.0001; sC5b9: 1137 ± 432 vs 205 ± 59 ng/ml, *p-value*<0.0001). In spike-positive patients, we found significantly higher levels of circulating C5a compared to spike-negative patients (C5a: 60.4 ± 11.8 vs 27.9 ± 13.0 ng/ml; *p-value*=0.0013, **Table 3**).

We were not able to observe a statistical difference in the % of subjects experiencing thrombotic events between spike-positive and -negative patients when we analysed their medical records, although all thrombotic complications occurred in spike-negative subjects (**Table 3**). The explanation for this unexpected finding is that most spike-positive COVID-19 patients, unlike those who were spike-negative, were receiving anticoagulant treatments before hospital admission (**Table 3**), possibly counteracting the thrombogenic effect of SARS-CoV-2.

In line with a previous finding in a similar geographical area during the same time period (52), here we also found that thromboembolic complications, which occurred in 26.1% of severe COVID-19 cases, manifested at hospital admission, possibly suggesting that thrombosis is a hallmark feature of advanced disease. As for the outcome, we found no differences between the two groups regarding hospitalization length and death (**Table 3**).

SARS-CoV-2-Derived S1 Protein Binds Endothelial Cells and Alters Their Phenotype in a Dose-Dependent Manner

Having identified detectable levels of SARS-CoV-2 spike protein in the circulation of severe COVID-19 patients, we evaluated whether spike protein-derived S1 was in itself sufficient to alter endothelial cell phenotype.

First, we evaluated the effects of different S1 concentrations (53, 54) on human microvascular endothelial cell (HMEC-1) viability, which went from a concentration similar to that found in the sera of severe COVID-19 patients to higher concentrations used in previous studies (42, 47, 53, 54). As shown in **Figure 1A**,

TABLE 3 | Baseline characteristics of severe COVID-19 patients divided according to serum positivity to spike protein.

	Overall (n=23)	Spike-negative (n=16)	Spike-positive (n=7)	*p*-value
Age (years)*	65.5 ± 13.4	67.2 ± 10.5	61.6 ± 19.0	0.368
Male sex (%)	15 (65.2)	10 (62.5)	5 (71.4)	0.146
Patients with positive RT-PCR at hospital admission (%)	16 (69.6)	9 (56.2)	7 (100)	0.057
Length from symptom onset to hospital admission (days)*	8.1 ± 4.1	9.1 ± 4.3	5.9 ± 2.6	0.084
Comorbidities				
Hypertension (%)	8 (47.8)	3 (50)	3 (42.9)	0.752
Cardiovascular (%)	6 (26.1)	4 (25)	2 (28.6)	0.857
Diabetes (%)	7 (30.4)	5 (31.3)	2 (28.6)	0.898
Obesity (%)	4 (17.4)	2 (12.5)	2 (28.5)	0.349
Clinical and biochemical parameters				
BMI (Kg/m^2)*	26.5 ± 4.0	26.2 ± 4.3	27.1 ± 4.1	0.747
WBC (cells/mL)*	9315 ± 3264	9106 ± 3494	9870 ± 2756	0.637
PLTs (cells/mL)*	255826 ± 140194	264938 ± 130483	235000 ± 670248	0.575
CRP (mg/dL)*	12.2 ± 8.6	11.3 ± 8.0	14.6 ± 10.4	0.426
LDH (U/L)*	451 ± 308	348 ± 120	627 ± 449	0.054
D-dimer (ng/mL)*	3065 ± 8089	4013 ± 9442	600 ± 345	0.439
PF (mmHg)*	148 ± 47	147 ± 53	151 ± 26	0.271
Complement components				
C5a (ng/ml)*	41.4 ± 20.6	27.9 ± 13.0	60.4 ± 11.8	0.001
sC5b-9 (ng/ml)*	1137 ± 432	987 ± 292	1347 ± 539	0.164
Patients receiving antithrombotic treatments before admission (%)	7 (30.4)	3 (18.7)	4 (57.1)	0.066
Hospitalization length (days)*	41.8 ± 33.7	46.8 ± 32.4	30.4 ± 36.3	0.293
Deaths (%)	7 (30.4)	5 (31.3)	2 (28.6)	0.898

**mean ± S.D.*

BMI, body mass index; CRP, c-reactive protein; LDH, lactate dehydrogenases; PF, PaO_2/FiO_2 ratio; PLTs, platelets; WBC, white blood cell.

at the concentrations of 0.5 and 10 nM, S1 did not affect endothelial cell count after 24 h exposure, unlike when a concentration of 50 nM S1 was used, which markedly reduced cell vitality at 24 h. These findings were corroborated by data obtained with a crystal violet viability assay, showing a significant decrease in endothelial cell viability only after exposure to 50 nM S1 (**Supplementary Figure 1A**).

Having established the sub-toxic concentrations of S1, we elected to use 0.5 and 10 nM S1 in the subsequent experiments. First, we evaluated whether S1 can bind and activate endothelial cells. Through immunofluorescence analysis, we found a significant dose-dependent binding of the S1 protein on the apical surface of HMEC-1 treated with 0.5 and 10 nM S1 (**Figure 1B**). Then, we evaluated whether the binding of S1 could alter the phenotype of endothelial cells by analysing the expression of the pro-inflammatory adhesive molecule for leukocyte, intercellular adhesion molecule 1 (ICAM-1) (55), and the pro-thrombogenic protein von Willebrand factor (vWF) (56). We found that both S1 concentrations were able to significantly upregulate ICAM-1 protein expression (**Figure 1C**) in a dose-dependent manner and enhance vWF deposition (**Figure 1D**). Given the critical thrombogenic role of vWF when shuttled from Weibel-Palade to the endothelial luminal surface (56), we elected to analyse its localization through a 3D reconstruction of z-stack slices acquired with confocal microscopy. In resting HMEC-1 co-stained with green cell tracker, we found that vWF was detectable in the cell cytoplasm, in contrast with 10 nM S1-activated endothelial cells, which exhibited remarkable vWF staining on the luminal surface (**Supplementary Figure 1B**), reflecting the ability of S1 to promote the release of vWF a key player in the formation of platelet adhesion and aggregation.

Collectively, these findings highlight a novel mechanism triggered by SARS-CoV-2-derived S1 alone that can propagate the pro-inflammatory and pro-thrombotic effects on the endothelium.

Given that we found detectable levels of the spike protein in the sera of hospitalized patients, it is conceivable that the total burden of circulating S1 on endothelial cells could be significantly higher in the early course of the disease. SARS-CoV-2 viral load peacks early after symptom onset, while it decreases at 10 days, when patients are usually hospitalized (32, 49–51). For this reason, we used the sub-toxic concentration of 10 nM S1 in subsequent experiments to mimic the prolonged exposure of endothelial cells to S1 that occurs during the early phase of COVID-19.

SARS-CoV-2-Derived S1 Protein Induces Pro-Inflammatory and Pro-Thrombotic Responses in Endothelial Cells Through the Engagement of ACE2

Several reports have suggested that, among the different host cell receptors, ACE2 could be the key surface protein for SARS-CoV-2 binding and entry into endothelial cells (57). Thus, we first investigated whether HMEC-1 express ACE2 and whether S1 could affect its expression. As shown in **Figure 2A**, HMEC-1 constitutively express ACE2 protein which, however, was not modulated by exposure to 10 nM S1. This finding was also confirmed in Vero cells exposed to 10 nM S1, used here as a positive control (**Supplementary Figures 2A, B**).

Then, we explored whether S1 engagement with ACE2 was instrumental to the observed changes in endothelial cell phenotype. To this end, we studied whether the upregulation

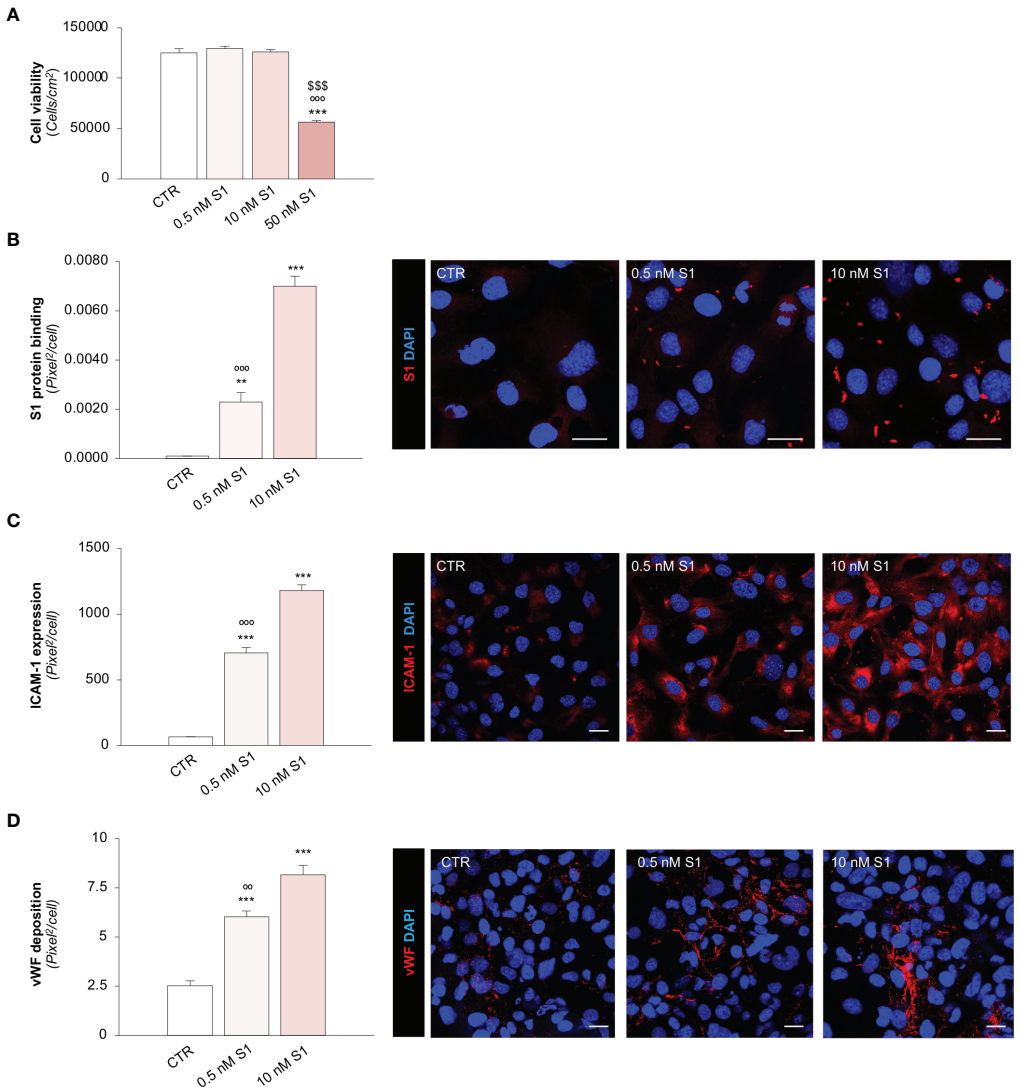

FIGURE 1 | SARS-CoV-2-derived Spike 1 (S1) protein affects microvascular endothelial viability and phenotype *in vitro*. **(A)** Quantification of cell viability in HMEC-1 exposed for 24 h to medium alone (CTR) or S1 at the concentration of 0.5 nM, 10 nM, or 50 nM. **(B)** Quantification and representative images of the binding of the S1 protein (red) to HMEC-1 treated with medium alone (CTR) or S1 at the concentration of 0.5 nM and 10 nM for 24 h. **(C, D)** Quantification and representative images of ICAM-1 expression [**(C)**, red] and vWF deposition [**(D)**, red] on HMEC-1 treated with medium alone (CTR) or S1 at the concentration of 0.5 nM and 10 nM for 24 h. All experiments were repeated 3 times. Data represent mean ± SEM and were analysed with Tukey's multiple comparison test. ***p-value*<0.01, and ****p-value*<0.001 *vs* CTR; $^{\$\$\$}$*p-value*<0.001 *vs* 0.5 nM S1; °°*p-value*<0.01, and °°°*p-value*<0.001 *vs* 10 nM S1. All the slides were counterstained with DAPI (blue). Scale bar 20 μm.

of adhesive and pro-thrombotic molecules induced by S1 was prevented by the inhibition of ACE2. In our setting, we observed that the upregulation of ICAM-1 induced by S1 was reduced significantly by the functional blocking antibody anti-ACE2 (ACE2) but not by an irrelevant (Irr) antibody (**Figure 2B**). Then, we studied P-selectin, an adhesive molecule that participates with ICAM-1 in endothelial cell/leukocyte interaction and in promoting pro-thrombotic processes (58, 59). We observed increased expression of P-selectin on the surface of S1-treated endothelial cells, which was inhibited significantly by ACE2 antibody (**Figure 2C**). Similarly, functional blockade of ACE2, but not an Irr Ab, significantly reduced vWF staining on the endothelial surface (**Figure 2D**). When we studied the expression of the endothelial adhesive molecule vitronectin receptor (αVβ3), we found that S1 failed to modulate its expression on endothelial cells (**Supplementary Figure 3A**). Lastly, we investigated the expression of thrombomodulin, a glycoprotein that confers cytoprotective, anti-inflammatory and thromboresistant properties to endothelial cells (60, 61). We observed that S1 challenge significantly reduced thrombomodulin (CTR: 703 ± 35 *vs* 10 nM S1: 258 ± 22, pixel^2/cell; **Supplementary Figure 3B**), which

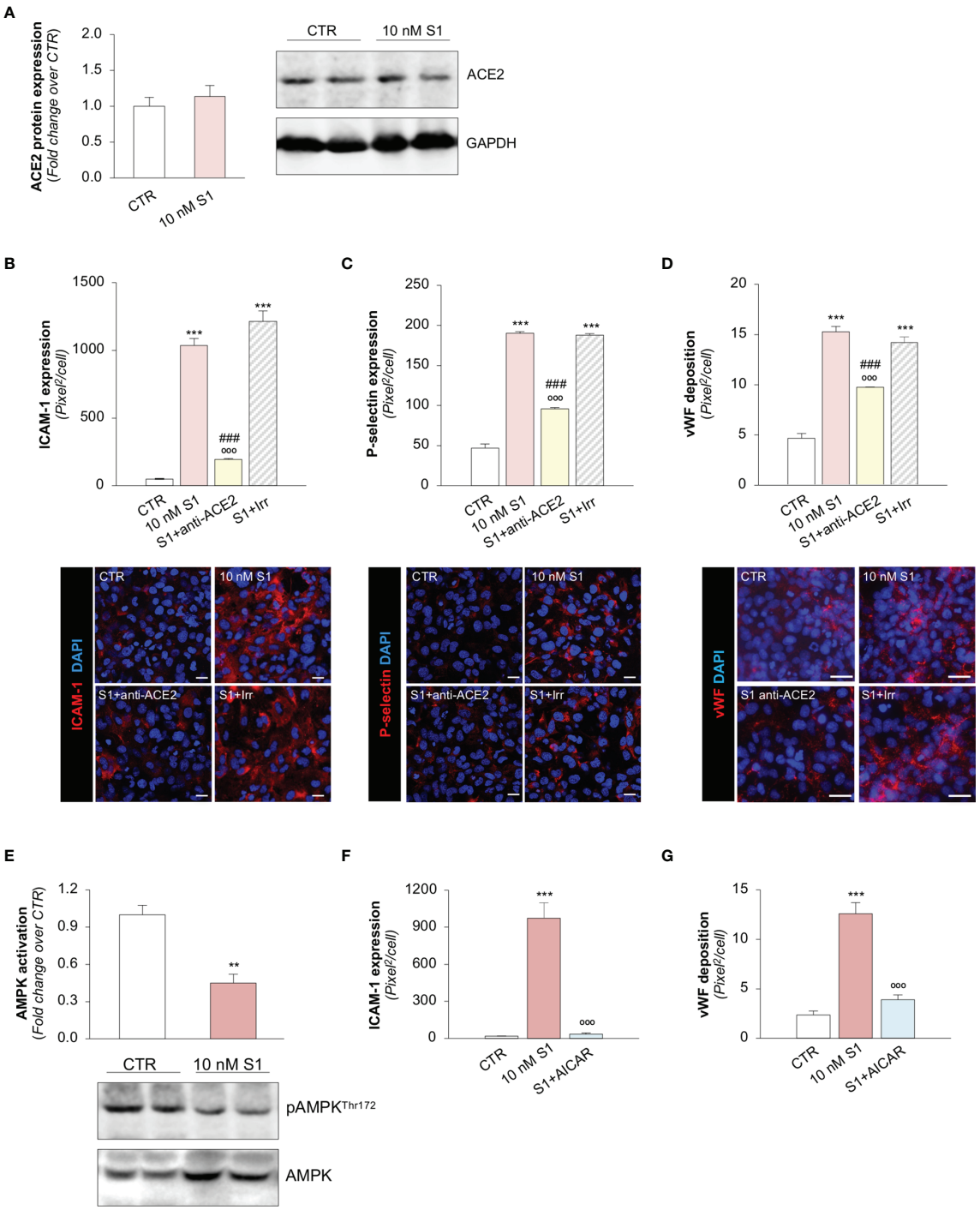

FIGURE 2 | S1, through ACE2, upregulates adhesive molecules on HMEC-1 by impairing AMPK signalling. **(A)** Quantification and representative Western Blots of ACE2 protein expression in HMEC-1 exposed for 24h to medium alone (CTR) or S1 (10 nM). GAPDH was used as a sample loading control. **(B–D)** Quantification and representative images of ICAM-1 expression [**(B)**, red], P-selectin expression [**(C)**, red], and vWF deposition [**(D)**, red] on HMEC-1 incubated with medium alone (CTR) or with S1 (10 nM) in the presence of anti-ACE2 Ab (2 μg/ml) or Irr Ab (2 μg/ml). **(E)** Quantification and representative Western Blots of AMPK activation, evaluated as the ratio between the expression of pAMPK^{Thr172} and total AMPK in HMEC-1 exposed for 24h to medium alone (CTR) or S1 (10 nM). **(F, G)** Quantification of ICAM-1 expression **(F)** and vWF deposition **(G)** on HMEC-1 incubated with medium alone (CTR) or with S1 (10 nM) in the presence or absence of AMPK agonist AICAR (2 mM). All experiments were repeated at least 3 times. Data represent mean ± SEM and were analysed with unpaired t-test or Tukey's multiple comparison test, as appropriate. ***p-value*<0.01, and ****p-value*<0.001 *vs* CTR; $^{°°°}$*p-value*<0.001 *vs* 10 nM S1; $^{###}$*p-value*<0.001 *vs* 10 nM S1+Irr. All slides were counterstained with DAPI (blue). Scale bar 20 μm for **(B, C)** and 50 μm for **(D)**.

was inhibited significantly by ACE2 functional blockade (10 nM S1+ACE2: 536 ± 102 vs S1+Irr: 228 ± 47, pixel^2/cell; representative images are shown in **Supplementary Figure 3B**).

Taken together, these data suggest that S1, by targeting ACE2, plays a critical role in inducing the activation of the microvascular endothelium, driving the shift toward a pro-inflammatory and pro-thrombotic endothelial phenotype.

SARS-CoV-2-Derived S1 Protein Impairs AMPK Signaling

To find the molecular mechanisms behind the phenotypic changes in microvascular endothelial cells that S1 induces, we investigated whether AMPK signaling is involved (42). As shown in **Figure 2E**, we observed a significant reduction in AMPK activity in HMEC-1 upon 10 nM S1 exposure, as revealed by the decreased ratio of phospho AMPK (pAMPK) and total AMPK by Western Blot analysis. Given that AMPK plays a major role in regulating the expression of adhesive molecules (62–64), we studied the effect of the AMPK agonist AICAR on S1-treated HMEC-1. In this setting, AICAR exerted a beneficial effect on endothelial cells by reducing the expression of ICAM-1 and vWF induced by S1 (**Figures 2F, G**, and **Supplementary Figure 4A, B**), providing direct evidence that AMPK signaling plays a protective role in mediating the endothelial cell response to the S1 protein.

SARS-CoV-2-Derived S1 Protein Induces Endothelial Dysfunction, Leading to Aberrant Complement Activation *via* ACE2

Recent evidence suggests that the complement system may play a substantial role in promoting microvascular inflammation, which appears to contribute to the severity of COVID-19 (65–67). The finding that the upregulation of adhesive molecules, such as P-Selectin (58, 59), or the loss of thrombomodulin (59, 68), may drive the complement attack on the endothelium, prompted us to investigate whether S1 protein-induced endothelial dysfunction could modulate complement activation and deposition on the microvascular endothelium. To this end, we exposed HMEC-1 to S1 and then to human serum (HS) from a pool of healthy volunteers as a source of complement. As shown in **Figure 3A**, we observed massive C3 deposition on HMEC-1 exposed to 10 nM S1, compared to unstimulated cells. When endothelial cells were exposed to S1 in the presence of anti-ACE2 Ab, there was a significant decrease in C3 staining, compared to the deposits observed on cells treated with an Irr Ab (**Figure 3A**), demonstrating that the S1/ACE2 axis plays a key role in mediating complement activation at the endothelial level. Pre-treating human serum with complement C3 inhibitor compstatin led to a significant decrease in C3 staining that was comparable to that of unstimulated HMEC-1 (**Figure 3A**). A significant inhibitory effect was found in the presence of complement 1 inhibitor, which blocks the classical pathway (**Figure 3A**). Notably, blocking the endothelial C3a receptor with a specific antagonist led to a significant decrease in C3 deposits on endothelial cells, whereas C5a receptor blockade had no effect (**Figure 3A**).

In our assay, we also demonstrated that complement activation on HMEC-1 exposed to 10 nM S1 protein proceeded to the formation of the terminal membrane attack C5b-9 complex, as shown in **Figure 3B**. The massive C5b-9 staining that had been observed on endothelial cells exposed to S1 protein was largely prevented by anti-ACE2 Ab but not by Irr Ab (**Figure 3B**). Blocking C3 activation with compstatin led to a profound inhibition of C5b-9 formation on the surface of HMEC-1 (**Figure 3B**). The partial effect of C1 inhibitor on C5b-9 staining was like that obtained on C3 deposits (**Figure 3B**). Blockade of the C3a and C5a receptors led to an inhibition of C5b-9 formation on HMEC-1 (**Figure 3B**).

Overall, these data indicate that endothelial dysfunction induced by SARS-CoV-2-derived S1 protein triggers exuberant complement deposition on activated microvascular endothelial cells and that the anaphylatoxins C3a and, to a lesser extent, C5a, further amplify the complex process of complement activation that fuels inflammation in response to S1.

SARS-CoV-2-Derived S1 Protein Induces Endothelial Dysfunction, Promoting Leukocyte Adhesion *via* ACE2

To investigate the functional impact of the observed endothelial phenotypic changes, we first determined whether the activation of HMEC-1 promoted by S1 was instrumental to the recruitment of leukocytes under flow conditions. Exposure of HMEC-1 to 10 nM S1 markedly induced leukocyte adhesion on HMEC-1 in a parallel plate flow chamber (**Figure 4A**). This effect was like that obtained by a positive control such as Shiga Toxin 2 (Stx2, **Supplementary Figure 5A**). Data that the functional blocking antibody against ICAM-1 robustly reduced the number of adherent leukocytes (**Supplementary Figure 5B**) confirmed that the upregulation of ICAM-1 on S1-activated HMEC-1 was accountable for leukocyte stable adhesion. We also found that ACE2 affected the adhesive properties of S1-activated endothelial cells, as demonstrated by the significant decrease in leukocyte adhesion by ACE2 functional blocking antibody, unlike with the Irr antibody (**Figure 4A**). Pre-exposure of leukocytes to 10 nM S1 resulted in more adherent leukocytes on S1-treated HMEC-1 (**Figure 4B**). Co-staining of histone H3 citrullinated (citHH3, green) and neutrophil elastase (NE, red) revealed that neutrophils adhered to S1-treated HMEC-1 and were activated to release NE in extracellular traps (NETs, inset) when they were challenged with 10 nM S1 (**Figure 4C**, insets). No detectable NETs were found when unstimulated leukocytes were perfused on S1-activated HMEC-1 (**Figure 4C**, insets).

A recent study has suggested that aberrant complement activation during COVID-19 plays an important role in the activation of circulating neutrophils (65). Therefore, we proceeded to evaluate whether C3a generated during the S1-induced C3 activation amplifies the process of leukocyte-endothelial interaction induced by S1. Notably, we found that the number of adherent S1-treated leukocytes on S1-activated HMEC-1 rose significantly after endothelial exposure to C3a, compared to endothelial cells exposed to S1 alone (adhesion of S1-treated leukocytes on: 10 nM S1-treated HMEC-1, 35 ± 2 *vs* 10 nM S1- and C3a-treated HMEC-1, 46 ± 2, adherent leukocytes/mm^2).

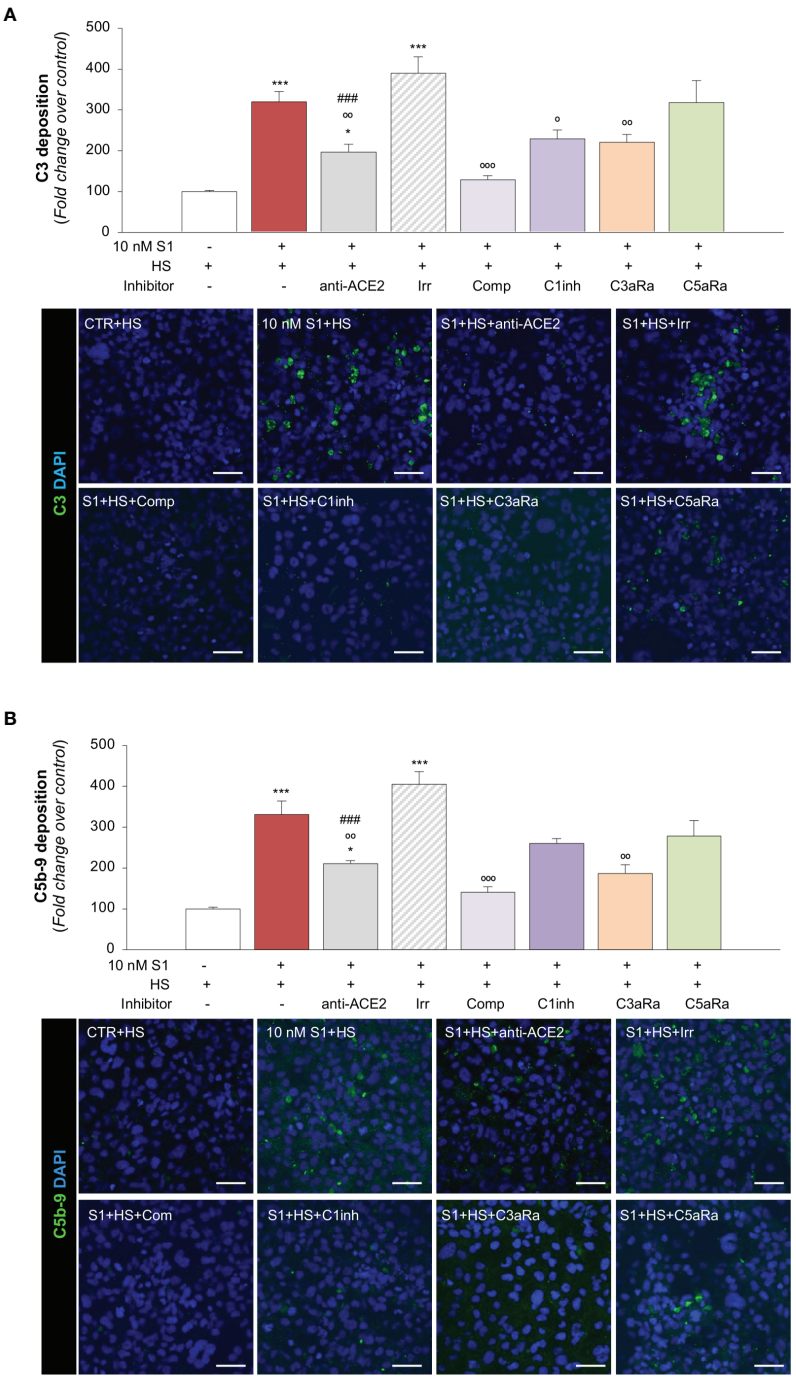

FIGURE 3 | S1 induces C3 and C5b-9 deposition on HMEC-1 by interacting with ACE2. **(A)** Quantification and representative images of C3 deposition (green) on HMEC-1 pre-exposed for 24h to medium alone (CTR) or to S1 (10 nM) in the presence of anti-ACE2 Ab (2 μg/ml) or the corresponding Irr Ab (2 μg/ml) and then incubated with human serum (HS, 50%) for 2h in the presence or in the absence of complement inhibitors (Compstatin, Comp; C1 inhibitor, C1inh; C3a receptor antagonist, C3aRa; C5a receptor antagonist, C5aRa). **(B)** Quantification and representative images of C5b-9 formation (green) on HMEC-1 pre-exposed for 24h to medium alone (CTR) or S1 (10 nM) in the presence of anti-ACE2 Ab (2 μg/ml) or Irr Ab (2 μg/ml) and then incubated with HS 50% in the presence or in the absence of complement inhibitors (Comp, C1inh, C3aRa, and C5aRa). All experiments were repeated at least 3 times. Data represent mean ± SEM and were analysed with Tukey's multiple comparison test. *p-value<0.05, and ***p-value<0.001*vs* CTR; °p-value<0.05, °°p-value<0.01, and °°°p-value<0.001 *vs* 10 nM S1; ###p-value<0.001 *vs* 10 nM S1+Irr. All slides were counterstained with DAPI (blue). Scale bars 50 μm.

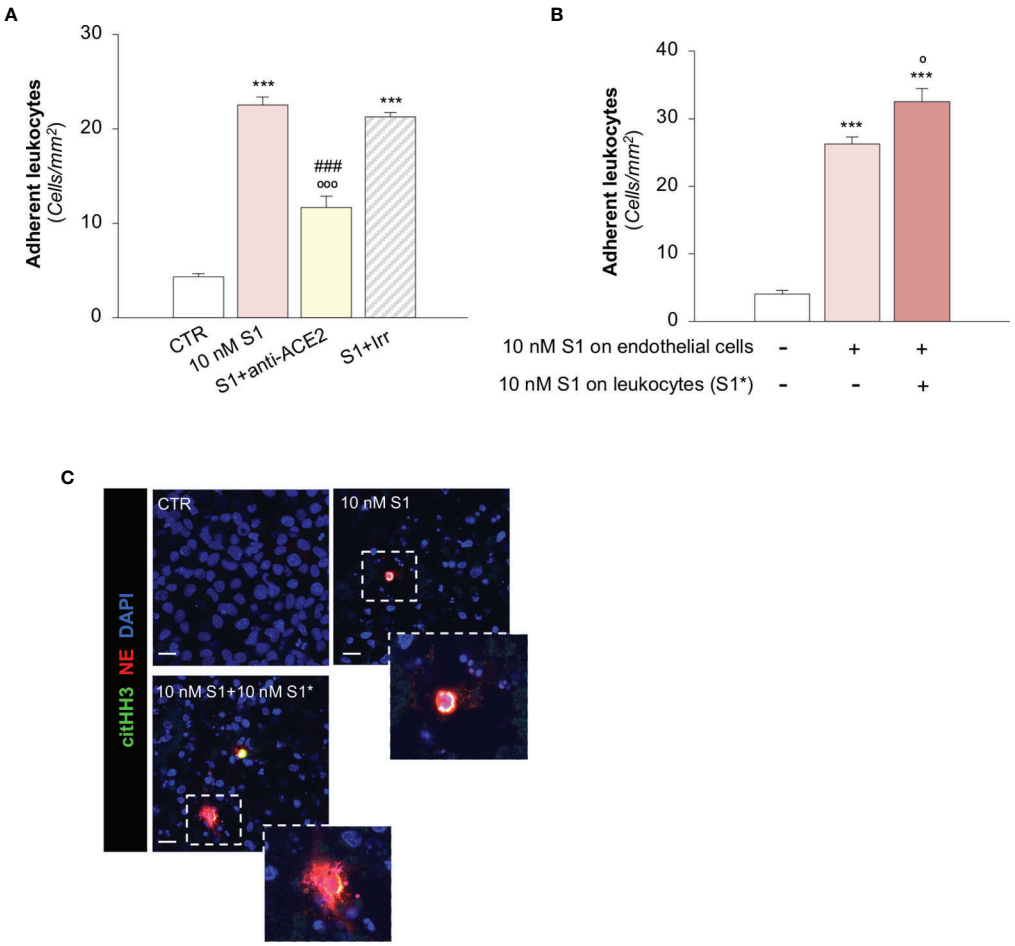

FIGURE 4 | S1 promotes leukocyte adhesion and NET formation on HMEC-1 under flow. **(A)** Quantification of leukocyte adhesion under flow conditions on HMEC-1 exposed for 24h to medium alone (CTR) or to subtoxic concentration of S1 (10 nM) in the presence of anti-ACE2 functional blocking Ab (ACE2, 2 μg/ml) or the corresponding Irr Ab (2 μg/ml). **(B)** Adhesion of leukocytes, incubated for one hour with control medium or with S1 (S1*, 10 nM) and perfused under flow conditions (1.5 dynes/cm^2) on HMEC-1 exposed to medium alone (CTR) or with S1 (10 nM). **(C)** Representative images of leukocytes treated with medium alone (CTR) or S1 (S1*, 10 nM), which adhered to HMEC exposed for 24h to medium alone or to S1. In this setting, neutrophils were co-stained with histone H3 citrullinated (citHH3, green) and neutrophil elastase (NE, red). The release of neutrophil extracellular traps (NETs) was observed only when leukocytes were activated with S1 (10 nM S1+ 10 nM S1*, inset). All experiments were repeated at least 3 times. Data represent mean ± SEM and were analysed with Tukey's multiple comparison test. ****p-value*<0.001 *vs* CTR; $^{\circ}$*p-value*<0.05, and $^{\circ\circ\circ}$*p-value*<0.001 *vs* 10 nM S1; $^{\#\#\#}$*p-value*<0.001 *vs* 10 nM S1+Irr. Slides were counterstained with DAPI (blue). Scale bar 20 μm.

SARS-CoV-2-Derived S1 Protein Induces Platelet Aggregates, Exacerbated by Complement Activation, on Microvascular Endothelial Cells

We then moved on to evaluating whether the endothelial dysfunction induced by the S1 protein also has functional relevance in disrupting the thromboresistant phenotype of the microvascular endothelium. To this end, we perfused heparinized whole blood on S1-activated HMEC-1 in a parallel plate flow chamber. Our data showed that 10 nM S1 exposure on HMEC-1 promoted significant platelet deposition and aggregation on the cell surface compared to unstimulated cells under flow (**Figure 5A**). The S1-induced platelet aggregate formation was comparable to that observed following Stx2 exposure (Stx2: 2277 ± 129 pixel^2/field), used as a positive control (69).

To demonstrate how complement activation contributes to amplifying S1-dependent platelet aggregation, S1-treated HMEC were exposed to HS as a source of complement. Here, we found that when perfused with whole blood under flow conditions (**Figures 5B, C**), HMEC-1 exposed to 10 nM S1 protein exhibited a more than 10-fold increase in the cell area covered by platelet aggregates compared to unstimulated cells. The addition of anti-ACE2 Ab during incubation with 10 nM S1 protein almost normalized platelet aggregate formation on the endothelial cell surface, which remained unaffected after the addition of Irr Ab (**Figures 5B, C**). The pivotal role of the complement system was demonstrated by the remarkable

inhibition of platelet aggregation detected on 10 nM S1-treated HMEC-1 in the presence of HS incubated with C3 inhibitor compstatin, C3aR or C5aR antagonists, respectively (**Figures 5B, C**).

We then evaluated whether complement activation affects vWF deposition on HMEC-1. In this experimental setting, exposure of endothelial cells to 10 nM S1 and HS also induced a significant increase in vWF on the endothelial cell surface (**Figures 5D, E**). Conversely, the addition to HS of the C3 inhibitor compstatin, C3aR, or C5aR antagonists, significantly decreased vWF staining on HMEC-1 exposed to 10 nM S1 (**Figures 5D, E**).

Overall, these data indicate that S1 promotes a robust microvascular endothelial cell response, which is a determinant in the propagation of pro-inflammatory and thrombogenic processes both amplified by the activation of the complement system.

SARS-CoV-2-Derived S1 Activates Pulmonary Endothelial Cells to Express Adhesive and Pro-Thrombotic Molecules by Binding to ACE2

To provide proof-of-concept that the above mechanisms are also shared by microvascular endothelial cells in the lungs, we further studied the effect of S1 on the expression of ACE2, ICAM-1, vWF and AMPK signalling on primary human pulmonary microvascular endothelial cells (HPMECs). First, we found that S1 bound to the surface of HPMEC (**Figure 6A**). We confirmed that HPMEC constitutively express ACE2, whose expression was not affected by S1 challenge (**Figure 6B**). Notably, we found that HPMEC expressed ACE2 to a similar extent as Vero cells, used as a positive control, while they expressed higher ACE2 levels than HMEC-1 (**Supplementary Figure 6**). Exposure of HPMEC to S1 elicited a marked increase in ICAM-1 and vWF stainings, which were limited by the addition of ACE2 blocking antibody (**Figures 6C, D**). Lastly, we found that S1 impaired AMPK signalling (**Figure 6E**). The finding that AICAR prevented ICAM-1 and that vWF increased expression induced by S1 (**Figures 6C, D**) demonstrated that AMPK signalling plays a critical, protective role in HPMEC activation.

All these data suggest that S1 induced a robust response in HPMEC, even more than HMEC-1, and caused a loss in the thromboresistant phenotype *via* ACE2.

DISCUSSION

In this study, we describe a mechanism that has never been reported through which SARS-CoV-2-derived S1 protein alone induces a pro-inflammatory and pro-thrombotic phenotype of human microvascular endothelial cells of dermal and pulmonary origin, possibly recapitulating the systemic microvascular complications observed in severe COVID-19 cases.

The first finding of this study is that the spike protein of SARS-CoV-2 can be found in the sera of patients with COVID-19. Indeed, using ELISA we found that spike protein is detected in at least 30% of hospitalized patients with severe disease. Although this assay cannot discriminate between intact SARS-CoV-2 virus and free spike protein, a recent study suggests that SARS-CoV-2 can be found in the circulation in a similar proportion (30-40%) of severe COVID-19 patients and that plasmatic viremia correlates with disease severity and mortality (34). Our finding that patients positive for plasmatic spike protein had a borderline significant increase in the rate of SARS-CoV-2 RNA positivity suggests that spike protein-positive patients may have had a higher viral load at the time of hospitalization than spike protein-negative subjects. However, we cannot exclude the possibility that the presence of spike protein could be the result of systemic viral particle release during COVID-19. At this stage of the disease, elevated viremia could contribute to the diffusion of viral particles that may damage microvascular endothelial cells in the lungs and in more distal organs, leading to systemic thrombotic complications. In line with this possibility, a study in mice showed that circulating S1 could localise in the microvascular endothelium (54). Furthermore, a recent report documented that circulating nucleocapsid protein can be detected in the sera of COVID-19 positive subjects, particularly in those who remain negative for anti-SARS-CoV-2 antibodies, suggesting that systemic shedding of viral components likely occurs soon after infection (70). However, our serological analysis was performed on the sera of hospitalized patients, who are generally admitted to the hospital 10 days after symptom onset (32, 49–51). All available data regarding the kinetics in SARS-CoV-2 viral load reveal that the viraemic peak occurs early, 2-4 days after infection, and viral shedding is almost absent 10 days after infection (32, 49–51). In line with this finding, we found that patients positive for plasmatic spike protein tended to be admitted earlier after symptom onset. The presence of spike protein in the sera of hospitalized patients suggests that the total burden of circulating S1 on endothelial cells could be significantly higher early during COVID-19.

Based on our data and the available published studies, we can infer that, during the early phase of the infection, the lung microvasculature is the first target of SARS-CoV-2. Indeed, the high replication rate of the virus in lung epithelial cells could particularly affect the local microvascular endothelial cells lining the capillaries in the alveoli. In this phase, pulmonary embolism has been identified as the hallmark feature of microvascular thrombosis in severe COVID-19 cases (71, 72). As the disease progresses, sustained shedding of viral protein may target microvascular endothelial cells in organs distal to the lungs, possibly leading to systemic thrombotic complications (73).

Following these observations, here we investigated whether S1 protein could induce endothelial cell activation and dysfunction to understand the underlying mechanisms that may recapitulate the microvascular thrombotic complications observed in COVID-19. We provide data that S1 alone was able to activate human microvascular endothelial cells, thus promoting leukocyte adhesion. The ability of S1 to induce inflammatory cell recruitment on endothelial cells was the result of the upregulation of endothelial P-selectin and ICAM-1, which are

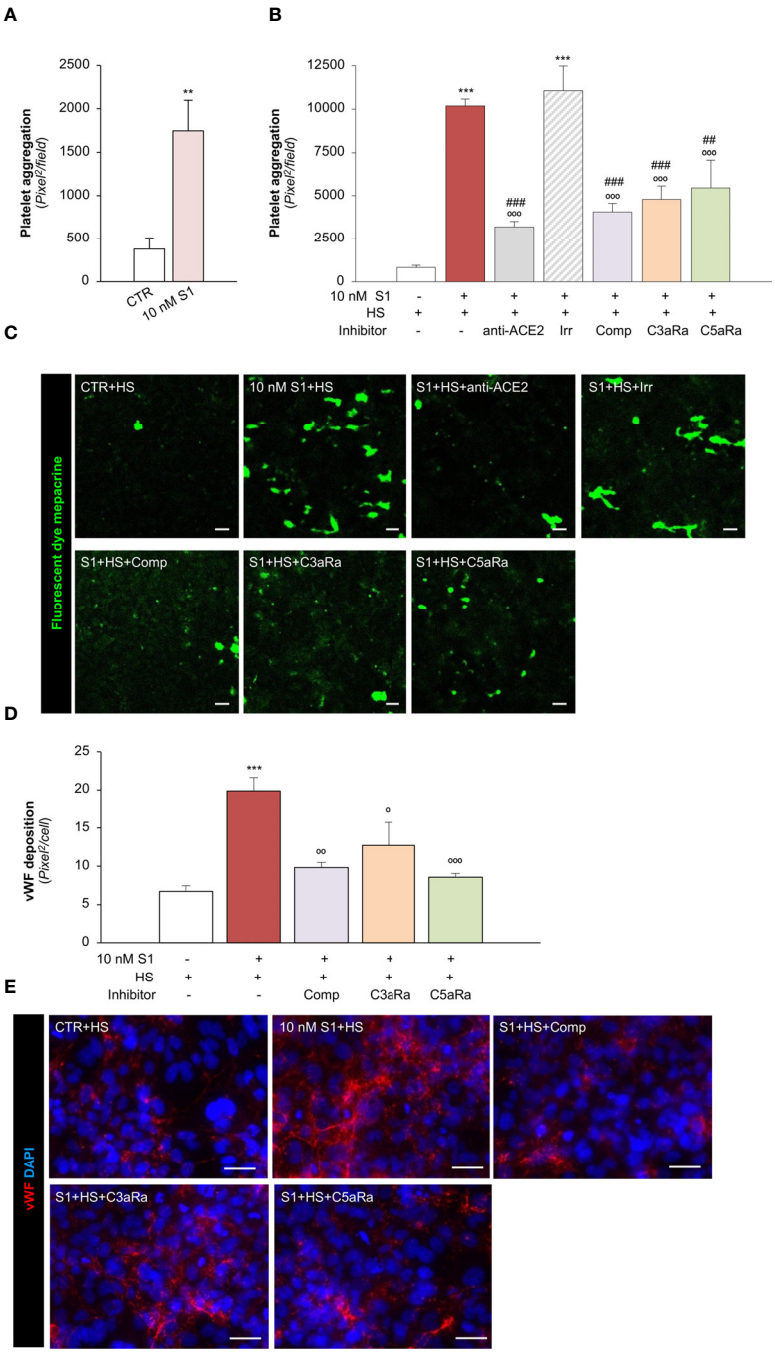

FIGURE 5 | S1 activates the complement system amplifying platelet aggregate formation on HMEC-1 through ACE2. **(A)** Quantification of platelet aggregate formation on HMEC-1 pre-exposed for 24h to medium alone (CTR) or S1 (10 nM). Platelets aggregates on HMEC-1 perfused with heparinised blood under flow conditions (60 dynes/cm^2) were evaluated and expressed as pixels^2/field analysed. **(B, C)** Quantification **(B)** and representative images **(C)** of platelet aggregate formation on HMEC-1 pre-exposed for 24h to medium alone (CTR), S1 (10 nM), or 10 nM S1 in the presence of anti-ACE2 Ab (2 μg/ml) or the corresponding Irr Ab (2 μg/ml) and then incubated with 50% human serum (HS) for 2h. In selected samples, S1-treated HMEC-1 were incubated with 50% HS in the presence of complement inhibitors (Compstatin, Comp; C3a receptor antagonist, C3aRa; C5a receptor antagonist, C5aRa). Platelet aggregate formation on HMEC-1 under flow conditions (60 dynes/cm^2) was quantified and expressed as pixel^2/field analysed. **(D, E)** Quantification **(D)** and representative images **(E)** of vWF deposition on HMEC-1 pre-exposed for 24h to medium alone (CTR) or S1 (10 nM) and then incubated with 50% HS in the presence or absence of complement inhibitors (Comp, C3aRa, and C5aRa). All experiments were repeated at least 3 times. Data represent mean ± SEM and were analysed with unpaired t-test or Tukey's multiple comparison test, as appropriate. $^{**}p$-*value*<0.01, and $^{***}p$-*value*<0.001 *vs* CTR; $^{\circ}p$-*value*<0.05, $^{\circ\circ}p$-*value*<0.01, and $^{\circ\circ\circ}p$-*value*<0.001 *vs* 10 nM S1; $^{\#\#}p$-*value*<0.01 and $^{\#\#\#}p$-*value*<0.001 *vs* 10 nM S1+Irr. Scale bars 50 μm.

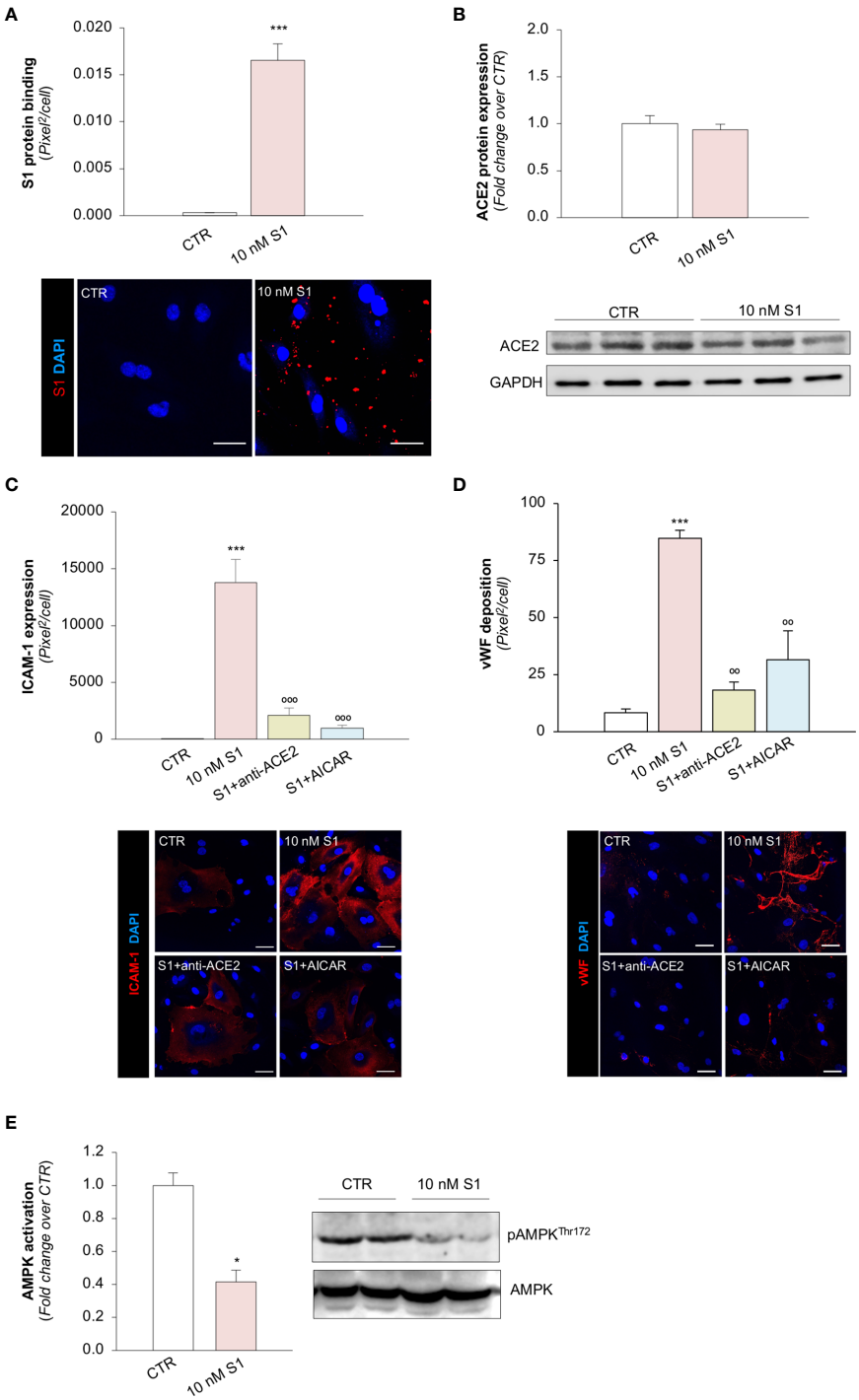

FIGURE 6 | S1, through ACE2, upregulates adhesive molecules on HPMEC by impairing AMPK signalling. **(A)** Quantification and representative images of the binding of the S1 protein (red) to HPMEC treated with medium alone (CTR) or S1 (10 nM) for 24 h. **(B)** Quantification and representative Western Blots of ACE2 protein expression in HPMEC exposed for 24h to medium alone (CTR) or S1 (10 nM). GAPDH was used as a sample loading control. **(C, D)** Quantification and representative images of ICAM-1 expression [**(C)**, red], and vWF deposition [**(D)**, red] on HPMEC incubated with medium alone (CTR) or with S1 (10 nM) in the presence or absence of anti-ACE2 Ab (2 μg/ml) or AICAR (2 mM). **(E)** Quantification and representative Western Blots of AMPK activation, evaluated as the ratio between the expression of $pAMPK^{Thr172}$ and total AMPK in HPMEC exposed for 24h to medium alone (CTR) or 10 nM S1. All experiments were repeated at least 3 times. Data represent mean ± SEM and were analysed with unpaired t-test or Tukey's multiple comparison test, as appropriate. **p-value*<0.05, and ****p-value*<0.001 *vs* CTR; °°*p-value*<0.01, and °°°*p-value*<0.001 *vs* 10 nM S1. All slides were counterstained with DAPI (blue). Scale bar 50 μm.

known to be key players in the paradigm of rolling and stable adhesion of inflammatory cells on the endothelium (55).

To demonstrate that the S1 directly elicits cell signalling *via* interaction with its cognate receptor, we showed that the functional blockade of ACE2 was sufficient to inhibit S1-dependent increased endothelial adhesiveness to leukocytes. The hypothesis that ACE2 plays a fundamental role in S1-endothelial cell interaction is supported by the available data, which confirms that soluble human recombinant ACE2 halted SARS-CoV-2 infection in engineered human blood vessel organoids (74). However, we cannot rule out the possibility that other endothelial receptors, such as neuropilin-1 (75), dipeptidyl peptidase 4 (76), and CD147 (77) contribute to S1-induced endothelial cell injury.

Although it is important to consider that S1 can induce exocytosis from Weibel-Palade bodies, contributing to increased expression of P-selectin on endothelial cells, it is possible that the engagement of S1 with ACE2 activates endothelial gene transcription of P-selectin and ICAM-1. Our *in vitro* studies support this hypothesis because they clearly demonstrated that S1-ACE2 interaction impaired AMPK signalling, inducing an increase in the adhesive properties of endothelial cells. Indeed, AMPK inhibits NF-κB (78), the main transcription factors involved in the expression of proinflammatory and adhesive proteins (79). Notably, the importance of AMPK signalling is a common feature of the microvascular endothelial cells of different origin.

Our data also revealed an additional mechanism through which S1 potentiates microvascular endothelial injury *via* the direct activation of neutrophils. Indeed, leukocytes challenged with S1 further increased their recruitment on S1-activated endothelial cells and promoted neutrophil NET release. The clinical relevance of our data rests on the findings that both the accumulation of neutrophils activated by SARS-CoV-2 (80), and the formation of NETs in damaged tissue are associated with a poor COVID-19 prognosis (81, 82). Collectively, our findings highlight that S1 plays a direct role in fuelling the process of inflammation in microvascular endothelial cells.

Exposing HMEC-1 to S1 significantly promoted the deposition of platelet aggregates under flow at high shear stress, which demonstrates that S1 directly affects the endothelial thromboresistant phenotype. This phenomenon was likely the result of the S1-induced alterations in the complex interplay between the surface expression of P-selectin and vWF, due to increased exocytosis. However, the contribution of blood-derived vWF should also be considered, as revealed by the lower level of vWF expression in the serum-free experimental setting. It is well known that endothelial P-selectin participates in the process of thrombosis by binding directly to platelets or by interacting with vWF, which further supports our data. At high shear stress, this latter fundamental adhesive substrate enables the deposition of platelets, tethering their GPIb and then αIIβ3 receptors (56, 69).

Furthermore, the ability of S1 to induce the loss of thrombomodulin – a cofactor that prevents local fibrin formation and that is also an inhibitor of complement activation (59) – may amplify the thrombotic effects triggered by the viral protein. Considering that S1 was able to promote exocytosis in endothelial cells, the loss of thrombomodulin could be the result of its shedding by newly exposed proteases on the cell surface.

Our *in vitro* study highlights the direct role that S1-ACE2 interaction plays in engaging intracellular signalling, contributing to the impairment of vascular integrity and development of a pro-thrombotic state that may have pathophysiological implications in COVID-19.

Earlier studies have shown that alterations in the endothelial thromboresistant phenotype, including the overexpression of endothelial P-Selectin (58, 69) and the loss of thrombomodulin (59, 68), contribute to complement activation, which increases the risk of thrombosis. In line with these reports, we have provided evidence that S1 interaction with ACE2 led to marked C3 and C5b-9 deposition on endothelial cells, which was associated with increased formation of platelet aggregates. The complement system plays a major role in exacerbating this phenomenon, as confirmed by data that showed that the specific C3 inhibitor compstatin, as well as the inhibitors of C3a and C5a receptors, robustly inhibited platelet deposition on S1-activated endothelial cells. Furthermore, several pieces of evidence support the hypothesis that the terminal complement pathway plays a role in exacerbating the inflammatory reaction on the endothelium by promoting neutrophil and macrophage recruitment and their activation to generate an oxidative burst (83). Additionally, C3a and, to a lesser extent, C5a – generated following S1 exposure – were the key mediators in the amplification of the complement cascade and, together with C3, contributed to S1-dependent microvascular thrombosis. There is evidence that C3a and C5a, generated following complement activation, are driving factors in altering endothelial thromboresistance (59, 84, 85). Further proof-of-concept that C5a has thrombogenic effects on endothelial cells comes from data showing that C5a inhibition halts the platelet aggregation induced by sera from severe COVID-19 patients, possibly through the exocytosis of vWF and P-selectin (48). Finally, data from the UK show that genetic predisposition to complement dysregulation is a risk factors for morbidity and death from SARS-CoV-2 infection, which indicates that hyperactivation of complement is a hallmark feature of the pathophysiology of severe COVID-19 (86, 87).

Based on the above, inhibiting the complement system could be a potential treatment for COVID-19 patients. A recent case report on a patient with ARDS due to COVID-19 pneumonia showed that treatment with a C3 inhibitor was safe and associated with a favourable outcome (88). Much larger case series have shown that the C5 inhibitor, eculizumab, and a MASP-2 inhibitor, narsoplimab, may have also some therapeutic efficacy (89–91).

Limitation of the study: one of the major limitations of our study is the small sample size of patients, which may affect the analysis outcomes. Additionally, the analysis of circulating spike protein was performed exclusively in serum from COVID-19 patients obtained at the time of hospital admission, making it

impossible to study the early phase kinetics of circulating spike proteins, as well as the temporality of the thrombotic phenomena in COVID-19. Furthermore, these patients were in an advanced phase of the disease and thrombosis was mainly diagnosed based on the analysis of medical records. Additionally, thrombosis in COVID-19 is often difficult to detect, particularly in mechanically ventilated patients with severe pneumonia. Finally, the ELISA assay used to detect the spike protein in human sera cannot differentiate between intact SARS-CoV-2 virus and free spike protein.

In summary, our study documented that: 1) circulating spike protein can be found in severe COVID-19 patients; 2) S1 directly induced the activation of multifaceted deleterious processes that lead to endothelial cell dysfunction; 3) engagement of ACE2 by S1 is sufficient to alter the adhesive properties of microvascular endothelial cells by altering AMPK signalling, resulting in the recruitment of inflammatory cells; 4) the S1-induced inflammatory phenotype favours exuberant C3 and C5b-9 deposits on microvascular endothelial cells, and the generation of C3a and C5a further amplified the complement activation induced by S1; 5) all these events promote a loop of reciprocal activation, ultimately leading to increased platelet aggregates on microvascular endothelial cells.

Overall, these data provide novel insights that can help to identify more effective therapies to inhibit the complement system in patients with severe COVID-19.

MATERIALS AND METHODS

Ethics Statement

Sera from convalescent COVID-19 patients with mild disease or negative subjects were selected from a previous study by our group in the same geographical area and during the same period of time (92). These subjects were selected in order to obtain age- and sex-matched controls for the severe COVID-19 cases. Patients with severe COVID-19 who were admitted to the COVID Unit of the Azienda Socio Sanitaria Territoriale (ASST) Papa Giovanni XXIII hospital in Bergamo (Italy) between March and June 2020 because of severe respiratory distress due to COVID-19 diagnosed on the basis of the 19 March 2020 WHO Interim guidance criteria (93). Sera from severely ill COVID-19 patients were collected at hospital admission. The study was approved by the Ethical Committee of the Azienda Sanitaria Locale Bergamo, Italy. Written informed consent was obtained from all enrolled patients. All patients' characteristics are summarized in **Table 1**.

Detection of Spike Protein and Complement Components in Human Samples

To detect SARS-CoV-2 spike protein in human sera, the specific COVID-19 Spike Protein ELISA Kit (Abcam, ab274342) was used, following the manufacturer's instructions. Briefly, microwell plates were coated with SARS-CoV-2-derived spike antibody and incubated with human sera. Antigen detection was performed by incubation with an anti-SARS-CoV-2 spike antibody, followed by streptavidin-HRP conjugate. Measurement of OD at 450 nm was performed on the multimode microplate reader TECAN Infinite M200® PRO.

Plasmatic levels of sC5b-9 and C5a were evaluated using MicroVue sC5b-9 Plus EIA (Quidel) and MicroVue C5a EIA (Quidel). Blood was collected in ice-cold EDTA tubes and immediately centrifuged at 4°C to avoid *ex vivo* complement activation. Plasma was quickly separated and frozen at -80°C until assay.

Endothelial Cell Cultures and Experimental Design

A large body of the literature reported the use of different types of immortalized endothelial cell lines to study SARS-CoV-2 infection (47, 94–96). We chose to study the human microvascular endothelial cell line of dermal origin (HMEC-1; RRID: CVCL_YJ39), obtained from Dr Edwin Ades and Francisco J. Candal (Centers for Disease Control and Prevention) and Dr Thomas Lawley from Emory University (97). Cells were cultured and validated as described previously (69). In our setting, HMEC-1, obtained from different batches, were used at low passages (max 20^{th}) in order to retain all the endothelial functions during culture (69).

For cell viability studies, HMEC-1 were exposed to MCDB 131 medium (Invitrogen) supplemented with 2% Fetal Calf Serum (FCS, Invitrogen) in the presence or absence of SARS-CoV-2-derived spike protein 1 (S1) at a concentration of 0.5 nM (37.5 ng/ml), 10 nM (750 ng/ml), and 50 nM (3750 ng/ml) for 24 h and then a cell count was performed. The S1 used for all the experiments is a commercially available recombinant SARS-CoV-2 S1 purified by metal ion affinity chromatography (230-01101, RayBiotech). The above range of concentrations was chosen on the basis of previous studies (47, 53, 54).

All the experimental designs are summarized in **Supplementary Figures 7A, B**. For the leukocyte adhesion assay, leukocyte suspensions were incubated for 1h with control medium or S1 (10 nM) before perfusion on unstimulated or S1-treated HMEC-1 (24 h, 10 nM). In selected experiments, HMEC-1 were incubated with S1 for 24 h in the presence of anti-ACE2 functional blocking antibody (Ab, 2 μg/ml, Adipogen, AG-20A-0037PF), anti-ICAM-1 functional blocking Ab (10 μg/ml, Merck, MAB2146), or the corresponding irrelevant (Irr) Ab (IgG mouse, Santa Cruz, sc2025) at the proper concentration (2 and 10 μg/ml) 1 h before S1 incubation (**Supplementary Figure 7A**). In additional experiments, HMEC-1 were incubated with 1 μM C3a (A118, Complement Technology; for 4 h) or 50 pM Shiga Toxin 2 (Stx2, for 24 h) – used here as a positive control (98) – before leukocyte perfusion.

For immunofluorescence studies, HMEC-1 were incubated for 24h with control medium or S1 (10 nM) in the presence or absence of anti-ACE2 Ab (2 μg/ml, Adipogen) or the corresponding Irr Ab (IgG mouse, Santa Cruz). In additional experiments, after 24 h incubation with control medium or S1, cells were exposed for 2 hours to a pool of human serum from healthy donors (HS) diluted 1:2 with test medium (HBSS with 0.5% BSA) in the presence or in the absence of compstatin (100

μM, Tocris bioscience, 2585/1) to block the complement component C3, or complement inhibitor 1 (36 μg/ml, Merck, SRP3318) to block the classical complement pathway. In this setting, the addition of a C3a receptor antagonist (1 μM, Merck, 559410) or a C5a receptor antagonist (10 μM, Merck, 234415) were tested (**Supplementary Figure 7B**). In additional samples, AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, Toronto Research Chemicals Inc, A611700) was used at a concentration of 2 mM.

For the analysis of vWF localization, HMEC-1 were stained with 1 μM cell tracker green CMFDA (Life Technology, C7025) for 30 minutes at 37°C at the end of incubation with control medium or 10 nM S1.

For the platelet adhesion assay under flow conditions, HMEC-1 were incubated for 24h with control medium or S1 (10 nM) before blood perfusion. In additional experiments, after 24h of incubation with medium or S1, HMEC-1 were exposed for 2 hours to HS diluted 1:2 in the presence or in the absence of anti-ACE2 Ab (2 μg/ml, Adipogen) or the corresponding Irr Ab (IgG mouse, Santa Cruz), or C3 inhibitor compstatin (100 μM, Tocris bioscience). In this setting, a C3a receptor antagonist (1 μM, Merck) or a C5a receptor antagonist (10 μM, Merck) were also added in HS diluted 1:2.

Human primary pulmonary microvascular endothelial cells (HPMEC; Lonza, CC-2527) were grown in EGM™-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit™ (Lonza, CC-3202) following the manufacturers' instructions. According to the manufacturer's validation, HPMEC express CD31/105, von Willebrand Factor VIII, are positive for acetylated low density lipoprotein uptake, and are PECAM positive. HPMEC were incubated with S1 at a concentration of 10 nM for 24h in the presence or absence of 2 μg/ml anti-ACE2 Ab or 2 mM AICAR.

As a positive control for the determination of S1 binding and ACE2 expression, Vero CCL-81 cells (ATCC, CCL-81; RRID: CVCL_0059) were cultured in Eagle's minimal essential medium (EMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S, Invitrogen).

Crystal Violet Viability Assay

HMEC-1 were seeded 10000 cells/well in 96-well plates in MCDB 131 in the presence of 2% FCS and, when confluent, were exposed for 24 h to different concentrations of S1 (0.5 nM, 10 nM and 50 nM). At the end of incubation, cells were fixed and stained with 0.5% crystal violet in 20% methanol. The stain was eluted with a 1:1 solution of ethanol and 0.1M sodium citrate, the absorbance was measured at 595 nm on the multimode microplate reader (Victor3, 1420 Multilabel counter, PerkinElmer). Cell viability was evaluated as live cells stained with crystal violet after subtraction of baseline absorbance. Data are expressed as percentage of viable cells.

Leukocyte Adhesion Assay Under Physiologic Flow Conditions

Leukocytes were isolated from blood collected on EDTA (final concentration 5 mmol/L) as we previously described (98). For adhesion experiments, we used a parallel-plate flow chamber connected to a perfusion system (98). HMEC-1 slides were flowed with leukocyte suspension (10^6 cells/ml) with a shear stress of 1.5 dynes/cm^2 to reproduce the circulation of post-capillary venules for 10 minutes (98). Images of adhering leukocytes on the HMEC-1 surface were acquired during the perfusion experiments, digitized, and processed using Image J software. The number of adherent leukocytes was determined on a series of 16 consecutive images. Adherent leukocytes were identified and counted at the end of the 10 min perfusion (98).

Platelet Adhesion Assay Under Flow Conditions

Perfusion of heparinized whole blood (10 UI/ml) obtained from healthy subjects (prelabelled with the fluorescent dye mepacrine, 10 μM) was performed in a flow chamber at 60 dynes/cm^2, as encountered in the microvasculature (59, 69). After 3 min of perfusion, the endothelial cell monolayer was fixed in acetone. Fifteen fields – systematically digitalized *per* sample of platelets, deposited along the endothelial surface – were acquired using an inverted confocal laser microscope (Leica TCS SP8, Leica Microsystems), and areas occupied by platelet aggregates were evaluated using Image J software and expressed as pixel^2 *per* field analysed. For each sample, after excluding the lowest and the highest value, the mean was calculated based on the remaining 13 fields.

Immunofluorescence Analysis

The slides were fixed with 3% paraformaldehyde (Società Italiana Chimici). After blocking, cells were incubated with the specific antibodies: mouse anti-SARS-CoV-2 RBD (1:1000, Abcam, ab277624), mouse anti-P-selectin (1:10, R&D Systems, BBA30), mouse-anti ICAM-1 (1:100, Merck, MAB2146), FITC-conjugated rabbit anti-C3c-complement (1:300, Dako, F0201), rabbit anti-complement C5b9 complex (1:200, Calbiochem, 204903), rabbit anti-vWF (10 μg/ml, Dako, A0082), mouse anti-thrombomodulin (1:50, R&D Systems, MAB3947) followed by the corresponding secondary antibodies (Jackson ImmunoResearch Laboratories). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Digital images were acquired using an inverted confocal laser microscope (Leica TCS SP8) or ApoTome Axio Imager Z2 (Zeiss).

The quantification of S1 deposition on endothelial cells was performed by analysing 10 fields/sample using Image J software and expressed as the area covered by the fluorescence *per* cell number (pixel2/cell).

The quantification of endothelial P-selectin and ICAM-1 stainings was performed by analysing 10 fields/sample using Image J software and expressed as the area covered by the fluorescence *per* cell number (pixel^2/cell).

For C3, and C5b-9, fifteen fields, systematically digitized along the surface, were acquired using a computer-based image analysis system. The area occupied by the fluorescent staining was evaluated by automatic edge detection, using built-in specific functions of Image J software and expressed as pixel^2 *per* field analysed. vWF was quantified as above and expressed as pixel^2 *per* cell. For each sample, after excluding the lowest and the highest values, the mean was calculated on the remaining 13 fields.

NET formation, at the end of the leukocyte adhesion assay, was studied on HMEC-1 fixed with 3% paraformaldehyde, and permeabilised with 0.1% Triton X-100 (Sigma-Aldrich, T8787). After blocking, cells were incubated with rabbit anti-Histone H3 (citrulline R2+R8 R17, 1:100, Abcam, ab5103) and mouse anti-Neutrophil Elastase (1:100, Abcam, ab254178), followed by the corresponding secondary antibodies. Nuclei were counterstained with DAPI.

Protein Extraction and Western Blot Analysis

HMEC-1, Vero CCL-81, and HPMEC were sonicated in CelLytic M (Sigma-Aldrich, C2978) supplemented with protease inhibitor cocktail (Sigma-Aldrich, P8340). Following centrifugation at 16000xg for 10 minutes at 4°C, lysates were collected and total protein concentration was determined using DC™ assay (Bio-Rad Laboratories, 5000112).

Equal amounts of proteins (30 μg) were separated on 12% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad Laboratories). After blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) supplemented with 0.1% Tween-20, membranes were incubated overnight at 4°C with the following antibodies: rabbit anti-ACE2 (1:1000; abcam, ab272500), rabbit anti-phospho AMPKα Thr172 (1:1000; Cell signaling, 2531), and rabbit anti-AMPKα (1:1000; Cell signaling, 2532). Mouse anti-GAPDH (1:5000; Origene Technologies, TA802519) was used as sample-loading control. The signals were visualised on an Odyssey®FC Imaging System (LiCor) by infrared (IR) fluorescence using a secondary goat anti-rabbit IRDye 680LT antibody (1:1000; LiCor, FE3680210) and a goat anti-mouse IRDye 800CW (1:1000; LiCor, FE30926210). Bands were quantified through densitometry using the Image Studio Lite 5.0 (LiCor) software.

Statistical Analysis

For studies in human subjects, data were expressed as mean ± standard deviation (SD) or as number of patients (%). Comparisons of binary characteristics in positive *vs* negative participants were performed using the chi-squared test, while age and continuous levels were compared with unpaired t-test. All analyses were carried out using SAS (Version 9.4). All *p-value*s were 2-sided.

For *in vitro* studies, all experiments were performed in at least three distinct biological samples (15 replicates for each sample). Data are presented as the mean ± standard error of the mean (SEM). Data analysis was performed using Prism Software (GraphPad Software Inc). Comparisons were made using unpaired t-test or ANOVA with Tukey *post hoc* test, as appropriate. Normality assumption was verified with the Shapiro-Wilk test. Statistical significance was defined as $p\text{-value}<0.05$.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Azienda Sanitaria Locale Bergamo, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: LP and MM. Investigation, data curation, and formal analysis: LP, MM, MG, APez, SG, and BI. Clinical data analysis: APer and PR. Evaluation of plasmatic complement components: RD. Writing of the original draft: LP, MM, MG, and BI. Supervision and final approval: AB and GR. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.827146/full#supplementary-material

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The COVID Complex: A Review of Platelet Activation and Immune Complexes in COVID-19

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Coronavirus disease 2019 (COVID-19) is a highly prothrombotic viral infection that primarily manifests as an acute respiratory syndrome. However, critically ill COVID-19 patients will often develop venous thromboembolism with associated increases in morbidity and mortality. The cause for this prothrombotic state is unclear but is likely related to platelet hyperactivation. In this review, we summarize the current evidence surrounding COVID-19 thrombosis and platelet hyperactivation. We highlight the fact that several studies have identified a soluble factor in COVID-19 patient plasma that is capable of altering platelet phenotype *in vitro*. Furthermore, this soluble factor appears to be an immune complex, which may be composed of COVID-19 Spike protein and related antibodies. We suggest that these Spike-specific immune complexes contribute to COVID-19 platelet activation and thrombosis in a manner similar to heparin-induced thrombocytopenia. Understanding this underlying pathobiology will be critical for advancement of future research and therapeutic options.

Keywords: COVID-19, platelet, antigen-antibody complex, immune complex, thrombosis, thrombocytopenia, heparin, VITT

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a respiratory infection caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1, 2). It has resulted in a global pandemic and is characterized by a highly inflammatory and prothrombotic state. Pulmonary involvement is the primary clinical manifestation but subsequent multi-organ failure and death can occur in severe cases (3–5). The prevalence of COVID-19 thromboembolism is quite variable across studies but appears to be highest in the critical care population, where estimates range from 20-65% (5–9). These can be both arterial and venous thromboses, making COVID-19 a unique prothrombotic state. Although the exact mechanisms underlying thrombosis are likely multifactorial, mounting evidence suggests that platelets play a crucial role.

Platelets have long been known to function as mediators of thrombosis and hemostasis but have only recently gained recognition in their role as immune mediators (10, 11). These anucleate cells mediate various immune related roles throughout the body, from antigen presentation to immune complex signaling. Of particular interest is their role in viral infection, where platelets are able to internalize and degrade pathogen as well as release soluble immune mediators (12). This likely contributes to their

important role in COVID-19 and explains, at least in part, how platelet hyperactivation increases thrombotic risk.

In this review, we summarize the general thrombotic nature of COVID-19 and the importance of understanding this process in regards to therapeutic options. Subsequently, we review platelet physiology and their current recognized functions as immune cells. We also review platelet activation in heparin-induced thrombocytopenia (HIT) and how this parallels platelet activity in COVID-19. Finally, we discuss the novel entity of vaccine-induced thrombotic thrombocytopenia (VITT) and the role of platelets in this unique COVID-19 associated disorder.

COVID-19 AND THROMBOSIS

COVID-19 is a severe viral infection that was identified in Wuhan, China in late 2019. It initially presents as a respiratory tract infection, including fever, dyspnea, and myalgias, but can rapidly progress to a more severe form (1). These critically ill COVID-19 patients are characterized by excess inflammation and a prothrombotic state. COVID-19 thrombosis features both arterial and venous thromboembolic events, often with concomitant thrombocytopenia (7, 13). In one retrospective study of 1476 hospitalized COVID-19 patients, 20.7% were found to have thrombocytopenia (where 125 x 10^9/L was the lower limit of normal) (14). The degree of thrombocytopenia has also been shown to independently associate with mortality outcomes, suggesting that platelet activation plays an important role in disease severity (14).

Thrombosis is particularly prevalent amongst critically ill COVID-19 patients, with some studies identifying deep vein thrombosis in up to 79% of patients through ultrasound screening (9). Unusual thrombi are also more prevalent amongst COVID-19 patients, including ischemic stroke, limb ischemia, and aortic thrombi (15). Up to 27.6% of thromboembolic events in critically ill patients occur even in the setting of prophylactic anticoagulation, emphasizing the extreme nature of this thrombotic state (16). Therapeutic dosing of anticoagulation may thus be required in COVID-19 hospitalized patients.

This observation has resulted in several randomized clinical trials demonstrating a benefit of therapeutic-dose low molecular weight heparin in hospitalized COVID-19 patients. In the ATTACC, ACTIV-4a, and REMAP-CAP multi-platform trial, therapeutic doses of heparin increased the probability of organ support-free days in non-critically ill patients (odds ratio 1.27, 95% credible interval 1.03-1.58) (17). However, there was no significant benefit for survival until hospital discharge, although there was a trend towards benefit (adjusted odds ratio 1.21, 95% credible interval 0.87-1.68). Interestingly, the RAPID trial did demonstrate a significant mortality benefit from therapeutic heparin in hospitalized, non-critically ill patients (odds ratio 0.22, 95% confidence interval 0.07-0.65) (18). Therefore, therapeutic anticoagulation is likely to benefit COVID-19 patients who are hospitalized without critical illness. It should be noted, however, that therapeutic anticoagulation showed no benefit to mortality or reduced organ support in critically ill COVID-19 patients (19). It may be that anticoagulation in these patients was introduced at an overly advanced stage of disease. Nonetheless, it implies that critically ill patients differ significantly in their underlying physiology and require unique therapies.

PLATELETS AND THEIR ROLE AS IMMUNE CELLS

Prior to delving into platelet activation in COVID-19, it is important to gain a basic understanding of how platelet synthesis and function are intimately related to immunity. Platelets are produced in the bone marrow from progenitor cells, termed megakaryocytes, through a complex process of hematopoietic stem cell differentiation (20). Their production is primarily driven by the cytokine mediator, thrombopoietin (TPO), which is synthesized by both the liver and kidneys. TPO is known to be upregulated by inflammatory cytokines, such as IL-6, and contributes to the rapid platelet production seen with inflammation (21). This is secondary to a subgroup of "pre-differentiated" stem cells that are biased towards the megakaryocyte lineage and rapidly differentiate on TPO exposure (22–24). The hematopoietic system is thus efficiently designed to produce platelets in the context of infection, suggesting an important role in immunity.

Once released into circulation, platelets are equipped with various intracellular materials (over 300) to mediate their effector functions (25). These include inflammatory cytokines (e.g. IL-1β), procoagulant factors (tissue factor, serotonin), and angiostatic molecules (platelet-factor 4/PF4) (26–28). These molecules are released upon platelet activation, which is mediated through various cell surface receptors. Many of these cell surface receptors also contribute to immune cell interaction and function. For example, the GPIb receptor is normally involved in platelet adhesion at sites of vascular injury through von Willebrand factor binding. However, GPIb is also capable of binding to von Willebrand factor exposed on immune cells infected with bacterial pathogen, such as hepatic Kupffer cells (29). It has been shown in a mouse model that this interaction is crucial for platelet aggregation around infected cells and host survival. P-selectin is another platelet surface receptor that is known to be upregulated with platelet activation. It is capable of binding to leukocytes through the P-selectin glycoprotein ligand-1 to mediate intracellular leukocyte signaling and neutrophil rolling (30–32). This process is crucial for leukocyte mobilization and concentration at sites of infection. Indeed, the P-selectin dependent interaction between neutrophils and platelets has been shown to contribute to acute lung injury in mouse models (33, 34). Platelet depletion or P-selectin inhibition both reduced subsequent neutrophil recruitment and lung injury. Platelets are thus equipped, through both intracellular and cell surface proteins, to mediate various immune functions. These interactions may contribute to the lung pathology seen in COVID-19 through immune cell recruitment.

IMMUNE COMPLEXES ARE CAPABLE OF PLATELET ACTIVATION

Immune complexes are important initial defenses against pathogen infection and are formed from antibody binding to soluble antigen. They often consist of immunoglobulins (Ig) of the IgG or IgM type but can also be IgA (35, 36). Immune complexes primarily mediate function through binding to cell surface receptors found on various cell types, including platelets. Most binding occurs through the Fcγ receptors II (FcγRII) and III, which are either activating (a) or inhibiting (b), respectively (35). Platelets contain only one Fc receptor (FcγRIIa) on their surface and thus are able to bind IgG-specific immune complexes (37). Immune complex binding to the platelet receptor leads to subsequent activation and release of intracellular molecules such as serotonin. This promotes a prothrombotic state and has been implicated in various autoimmune conditions. The most well-characterized platelet-mediated immune complex disorder is heparin-induced thrombocytopenia (HIT) (38, 39).

HIT is a prothrombotic autoimmune disorder characterized by the presence of thrombocytopenia (low platelets) and thrombosis that shares many features with COVID-19. It most commonly presents in hospitalized patients who are receiving unfractionated heparin anticoagulation and is characterized by antibodies targeting platelet factor 4 (PF4)-heparin complexes (39). PF4 is a positively charged protein released from platelets that is capable of binding negatively charged molecules, such as heparin (40). Certain individuals develop anti-PF4/heparin IgG antibodies that form immune complexes. These immune complexes activate the FcγRIIa on platelets resulting in thrombocytopenia that is often accompanied by thrombosis, which is secondary to the release of serotonin and other procoagulant platelet microparticles. Circulating anti-PF4/heparin antibodies can be found in up to 50% of patients exposed to heparin (41, 42). However, only a minority of these will be functional and lead to disease presentation. This is secondary to the unique epitope specificity required for immune complex formation and platelet activation (43). Therefore, it is important to use functional platelet activation assays to diagnose HIT.

One of the international reference assays for diagnosing HIT, developed at our institution, is the serotonin release assay (SRA). Briefly, platelets from healthy donors are incubated with radioactive ^{14}C-serotonin allowing uptake into platelets. These modified platelets are subsequently exposed to patient plasma, in the presence and absence of heparin, which allows formation of anti-PF4/heparin immune complexes (44). These immune complexes subsequently activate platelets through the FcγRIIa leading to release of ^{14}C-serotonin, which is then measured by beta radioactivity. The addition of exogenous heparin is vital for this activation in HIT to facilitate formation of appropriate antigen complexes. However, certain samples tested in the SRA for HIT will demonstrate heparin-independent platelet activation (45). This is inconsistent with a diagnosis of classical HIT, meaning that the assay may detect additional mechanisms of platelet activation. Therefore, the SRA can be modified to study platelet hyperactivation in novel diseases, such as COVID-19 (see **Figure 1**).

PLATELETS ARE HYPERACTIVATED IN CRITICALLY ILL COVID-19 PATIENTS

To this point, many studies have confirmed that platelets in COVID-19 patients display a hyperactivated phenotype with altered gene expression. In a cohort of 115 COVID-19 patients, featuring both non-severe and severe disease, platelets secreted increased IL-1beta and soluble CD40 ligand compared to healthy controls (46). Furthermore, circulating levels of serotonin and PF4 were increased in patient serum, suggesting platelet degranulation. Common cell markers of platelet activation, including P-selectin and CD63, are also increased in critically ill COVID-19 patients, but not those with mild disease (47). Platelets from critically ill COVID-19 patients also demonstrated increased markers of apoptosis, such as phosphatidylserine externalization and cleaved-caspase 9, which correlate with thromboembolic events (48). In addition to platelet activation, there is evidence of unique transcriptome changes that occur in platelets from COVID-19 patients. Using RNA-seq analysis on platelets from 10 COVID-19 patients, Manne et al. demonstrated significant upregulation of genes involved in antigen presentation (49). Platelets are thus significantly altered to a more active phenotype in COVID-19, particularly in critically ill patients, and may contribute to clinical presentation.

One mechanism by which platelets may contribute to COVID-19 presentation is through neutrophil recruitment and aggregation. As previously noted, platelet P-selectin is able to bind neutrophil ligands to induce rolling and aggregation at sites of activation (50). This interaction can lead to prothrombotic platelet-neutrophil aggregates as well as the formation of neutrophil extracellular traps. For example, plasma from hospitalized COVID-19 patients demonstrates increased circulating platelet-neutrophil aggregates on flow cytometry compared to healthy controls (51). Furthermore, autopsies in COVID-19 patients confirm the presence of microvascular thrombi consisting of neutrophil extracellular traps and platelets (52, 53). These platelet-neutrophil interactions are more prominent in critically ill COVID-19 patients, where there is evidence of a hyperactivated platelet phenotype (52, 54). Therefore, hyperactivated platelets in COVID-19 also contribute to neutrophil activation, which fuels the thrombo-inflammatory milieu.

It is still unclear as to what triggers such drastic platelet changes in critically ill patients. Some have hypothesized that SARS-CoV-2 directly interacts with platelets to mediate these observed effects. Evidence for this is supported by the presence of viral RNA in platelets of infected individuals, although this is only seen in up to 24% of patients (46, 49, 55). However, aside from a single study (55), multiple studies have failed to demonstrate ACE2 expression on the platelet surface or evidence of ACE2 RNA in platelets (46, 49). The cause of this discrepancy is unclear and may be related to different techniques for platelet isolation (56). Regardless, SARS-CoV-2 RNA has been consistently found within platelets and thus suggests that ACE2-independent mechanisms of entry exist. Interestingly, when critically ill COVID-19 patient plasma is incubated with platelets from healthy volunteers, there is a similar increase in platelet activation markers (P-selectin, CD63) (47). While circulating virus may account for this change as well, other soluble mediators should be considered.

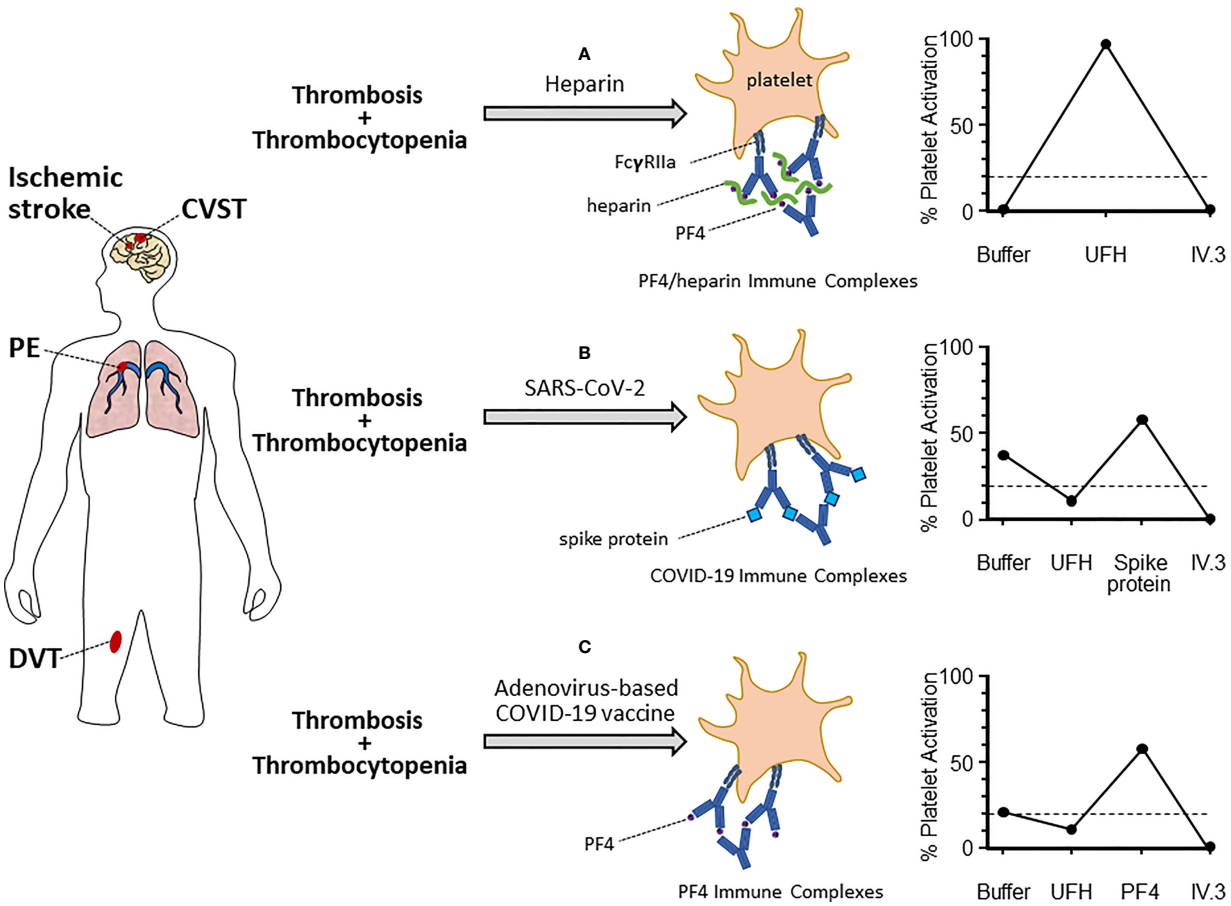

FIGURE 1 | Platelet activation mechanisms and assay patterns in various thrombotic-thrombocytopenic syndromes. Patients who present with evidence of thrombosis (arterial or venous) and thrombocytopenia should be considered for hematology referral and specialized testing for platelet activation disorders. These can include heparin-induced thrombocytopenia (HIT, **A**), COVID-19-related platelet activation **(B)**, and vaccine-induced thrombotic thrombocytopenia (VITT, **C**). The suspected platelet activation disorder will depend on the clinical context and known exposure to antigen (e.g. heparin, SARS-CoV-2, or adenovirus-based COVID-19 vaccine). Each of these disorders is characterized by platelet activation through the FcγRIIa *via* unique immune complexes. These include: anti-PF4/heparin (HIT), unidentified immune complexes (COVID-19), or anti-PF4 (VITT). Serum testing from patients will also feature unique platelet activation schemas in functional activation assays, such as the serotonin release assay (examples shown on the right). In the classic HIT-SRA (top right), addition of exogenous UFH significantly increases immune complex formation and platelet activation, which is inhibited by IV.3. Contrastingly, UFH inhibits immune complex formation in COVID-19 and VITT thrombotic patients. Instead, alternate antigens (Spike protein and PF4, respectively) are required for significant platelet activation. Dashed lined represents 20% platelet activation, which is the positive cut-off for the SRA.

THE "COVID COMPLEX" – IMMUNE COMPLEX MEDIATED PLATELET ACTIVATION

Immune complexes are one potential circulating factor that could contribute to platelet activation in COVID-19. As previously mentioned, immune complexes activate platelets through the FcγRIIa and may be formed from antibodies against self or exogenous antigens. Viral illnesses are well documented to produce antibodies against self-antigens, such as antiphospholipid antibodies, through a process called molecular mimicry. Early reports in COVID-19 patients highlighted the presence of these antibodies in association with thrombosis, including anti-beta-2 glycoprotein and non-specific inhibitor (57–59). Injection of the serum IgG fraction from these patients into mice resulted in significantly increased thrombus formation compared to controls (59). However, this thrombus formation was also seen with COVID-19 patient serum that had low levels of antiphospholipid antibodies. This suggests that antiphospholipid antibodies are not the sole antibodies associated with this prothrombotic state.

Another potential hypothesis is that HIT antibodies are contributing to the IgG-mediated platelet activation seen in COVID-19 patients. This is supported by the observations that COVID-19 and HIT share many clinical similarities; COVID-19 patients are often exposed to heparin in the context of hospitalization; and a high proportion of COVID-19 patients test positive for anti-PF4/heparin antibodies on further testing (60, 61). However, in a cohort of ten critically ill COVID-19 patients with high suspicion of HIT, we found no evidence of

platelet-activating HIT antibodies, which has been replicated by others (61, 62). Interestingly, six of these samples were able to activate platelets in the serotonin release assay in the absence of heparin. This activation was inhibited by IV.3, an FcγRIIa inhibitor, thus confirming immune complex mediated platelet activation. Furthermore, all patients with platelet activation also contained anti-Spike IgG antibodies targeting SARS-CoV-2. It is plausible that Spike-specific IgG antibodies bind circulating Spike protein in viremic, critically ill patients to form platelet-activating immune complexes. This mechanism has previously been shown to occur with H1N1 influenza virus whereby influenza antibodies bind to virus to form immune complexes (63). These immune complexes activate platelets through the FcγRIIa and likely contribute to the pulmonary thrombosis seen with H1N1 infection (64). Most recently, one *in vitro* study confirmed that recombinant anti-Spike IgG is able to activate platelets through the FcγRIIa (65). This was determined through *in vitro* thrombus measurement using microfluidic flow chips and confocal microscopy. Thrombus formation only occurred in the presence of Spike protein and an "inflammatory signal" (von Willebrand factor in this study). Interestingly, anti-Spike IgG and Spike protein alone did not lead to significant thrombus formation. How exactly von Willebrand factor interacts to promote platelet activation is unclear but may be through facilitating platelet aggregation. Glycosylation status of anti-Spike IgG was also found to be a significant factor in the ability of these complexes to activate platelets. Therefore, certain anti-Spike IgG activate platelets in the context of COVID-19 infection, but this remains to be validated in the clinical context.

VACCINE INDUCED THROMBOTIC THROMBOCYTOPENIA (VITT)

It would be remiss to avoid a discussion of vaccine-induced thrombotic thrombocytopenia (VITT) in the context of platelet activation and COVID-19. Although this platelet activation is not directly related to SARS-CoV-2 viral infection, it has important clinical and public health implications. VITT is a novel, "drug" related disorder attributed to vaccination by adenoviral vector-based SARS-CoV-2 vaccines. This primarily includes the ChAd-Ox1 (produced by AstraZeneca) and Ad26.COV2.S (Johnson and Johnson) vaccines. VITT was first described in eleven patients, predominantly female, who presented with unusual thromboses (cerebral venous sinus thrombosis, splanchnic-vein thrombosis) and thrombocytopenia (66). Patients often present a median of 14 days from vaccination and can be critically ill – estimated mortality is 22% in one cohort of 220 VITT cases (67). This mortality is significantly reduced from initial reports (55%), likely due to a combination of increased recognition and better treatment implementation (66). Given the parallels to HIT, it was hypothesized that similar platelet activating antibodies may be the underlying cause. This proved to be the case, with all patients featuring high titers of anti-PF4/heparin antibodies that were able to activate platelets in functional assays (66). Interestingly, these antibodies did not require the presence of heparin to form immune complexes and thus are able to target PF4 independently (i.e. anti-PF4 antibodies). The binding site for these antibodies is located in the heparin-binding site on PF4, as shown by alanine-scanning mutagenesis, thus allowing them to form tetrameric immune complexes and activate platelets through FcγRIIa (68). This competitive binding to the heparin site likely explains why heparin inhibits VITT platelet activation *in vitro* (unlike in HIT, where heparin facilitates activation; see **Figure 1**). As previously mentioned, anti-PF4/heparin antibodies do not appear to be responsible for the thrombosis and platelet activation seen in COVID-19. In a cohort of 222 COVID-19 patients with thrombosis, only nineteen (8.6%) tested positive for anti-PF4/heparin antibodies (69). None of these were able to activate platelets in the functional platelet assay. Therefore, anti-PF4 antibodies are likely not responsible for the thrombosis seen in COVID-19 patients and do not demonstrate cross reactivity with the Spike protein. This understanding is important for future vaccine development and management of these rare cases.

SUMMARY

Although the field of COVID-19 thrombosis is in its infancy, there is sufficient evidence to support a major role for platelets in disease pathogenesis. Platelets have been shown to be hyperactivated in critically ill patients and secrete excessive procoagulant molecules. Furthermore, they are able to interact with other immune cells to mediate the immune response. However, this excess inflammation may also contribute to tissue damage and ultimately, mortality. Targeting these pathways in order to dampen the excess immune response may thus present attractive therapeutic targets.

Immune complexes also appear to contribute significantly to these platelet changes in a manner similar to HIT and other immune-complex mediated disorders. The antigen specificity and additional characteristics of these immune complexes remain to be determined but will be crucial to the development of therapeutic targets. Identifying the specific antibodies involved may also allow clinicians to risk stratify patients who are at high risk of severe disease or thrombosis, thus offering the potential for prophylactic anticoagulation.

AUTHOR CONTRIBUTIONS

Both SJ and IN contributed equally to this work, including initial manuscript preparation, editing, and final drafting. All authors contributed to the article and approved the submitted version.

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Antiphospholipid antibodies in patients with stroke during COVID-19: A role in the signaling pathway leading to platelet activation

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Background: Several viral and bacterial infections, including COVID-19, may lead to both thrombotic and hemorrhagic complications. Previously, it has been demonstrated an "*in vitro*" pathogenic effect of "antiphospholipid" antibodies (aPLs), which are able to activate a proinflammatory and procoagulant phenotype in monocytes, endothelial cells and platelets. This study analyzed the occurrence of aPL IgG in patients with acute ischemic stroke (AIS) during COVID-19, evaluating the effect of Ig fractions from these patients on signaling and functional activation of platelets.

Materials and methods: Sera from 10 patients with AIS during COVID-19, 10 non-COVID-19 stroke patients, 20 COVID-19 and 30 healthy donors (HD) were analyzed for anti-cardiolipin, anti-β2-GPI, anti-phosphatidylserine/prothrombin and anti-vimentin/CL antibodies by ELISA. Platelets from healthy donors were incubated with Ig fractions from these patients or with polyclonal anti-β2-GPI IgG and analyzed for phospho-ERK and phospho-p38 by western blot. Platelet secretion by ATP release dosage was also evaluated.

Results: We demonstrated the presence of aPLs IgG in sera of patients with AIS during COVID-19. Treatment with the Ig fractions from these patients or with polyclonal anti-β2-GPI IgG induced a significant increase of phospho-ERK and phospho-p38 expression. In the same vein, platelet activation was supported by the increase of adenyl nucleotides release induced by Ig fractions.

Conclusions: This study demonstrates the presence of aPLs in a subgroup of COVID-19 patients who presented AIS, suggesting a role in the mechanisms contributing to hypercoagulable state in these patients. Detecting these antibodies as a serological marker to check and monitor COVID-19 may contribute to improve the risk stratification of thromboembolic manifestations in these patients.

KEYWORDS

antiphospholipid antibodies, β2-GPI, COVID-19, thrombosis, stroke.

Introduction

Several infections have been shown to play a role in the triggering of anti-phospholipid antibodies (aPLs) (1, 2); indeed, it was demonstrated an overall increased risk of developing anti-cardiolipin (aCL) antibodies and an association between viral infections and presence of anti-β2-Glycoprotein I (anti-β2-GPI) antibodies (3). aPLs are often transient during infections, but in some cases, they may be associated with thromboembolic events (4). The most common mechanism involved in the infectious origin of the aPLs is molecular mimicry between β2-GPI and/or other Antiphospholipid Syndrome (APS) antigens and infectious agents (5). Another well-studied mechanism is the two-hit hypothesis, which attempts to explain the persistent presence of aPLs without the occurrence of thrombotic events. Detection of aPLs might represent the first hit, whereas several findings underline the role of infections as a potential second hit. This hypothesis is supported by data that demonstrate, at the same time, the involvement of innate immunity receptors in APS pathogenesis mechanism(s) (6). The association between aPLs and infectious agents was first described in syphilis, but many other viral, bacterial and parasitic infections have been found to induce aPLs. The most common "triggering" factors are skin infections, human immunodeficiency virus (HIV), pneumonia, hepatitis C virus (HCV), hepatitis B virus (HBV) (7–9).

In patients affected from coronavirus disease 2019 (COVID-19) thromboembolic events involving arterial, venous and microcirculation have been frequently reported. Therefore, this syndrome is now described as a viral pulmonary infection with respiratory complications, and more precisely as a multiple-organ disorder accompanied by hypercoagulability (10–12).

Furthermore, it has been reported that, even in young patients, neurological manifestations, in particular ischemic stroke, may arise in the context of COVID-19 (13–15). Interestingly, some papers have described the presence of aPLs together with increasing proinflammatory cytokine levels in COVID-19 patients with ischemic stroke (16). aPLs, including IgG and/or IgM aCL antibodies, IgG and/or IgM anti-β2-GPI antibodies, and Lupus Anticoagulant (LA) are established as laboratory criteria for diagnosis of the APS, characterized by arterial and venous thrombosis and recurrent abortions (17). In addition, some "non-criteria" aPLs have shown promising clinical utility, especially in "seronegative" APS (SN-APS) patients, with a clinical picture typical of APS, but persistently negative for routine aPL antibody tests. They include mainly anti-phosphatidylserine/prothrombin (aPS/PT) and anti-vimentin/CL (aVim/CL) antibodies (18).

Reports in COVID-19 patients described the association of aPLs with thrombotic events (19), demonstrating that injection of the serum IgG fraction from these patients into mice resulted in significant increase of thrombus formation in addition to neutrophil hyperactivity, higher platelet counts and lower clinical estimated glomerular filtration rate. The mechanisms underlying the prothrombotic state are more complex and still unclear, but likely related to platelets, acting as mediators of thrombosis and hemostasis, but also as immune mediator. In fact, in critically ill COVID-19 patients, platelets are hyperactivated with an excessive secretion of procoagulant molecules (20–22). Activated platelets also secrete platelet factor 4 (PF4), a member of the C-X-C chemokine family with high affinity for heparin and other anionic glycosaminoglycans (e.g., endothelial cell surface or platelet surface GAGs). PF4 has a proven procoagulant role but appears to have also anticoagulant effects (23, 24).

A role of platelets has been also demonstrated in aPL-related thrombosis by experimental models, which revealed that platelets are activated following the infusion of anti-β2-GPI antibodies (25). Platelet activation is increased when anti-β2-GPI–β2-GPI complexes bind to the platelet thrombus and the activation-signaling pathways are mediated by interaction with phospholipids on the cell surface (phosphatidylserine, phosphatidylethanolamine) or with receptors of platelet membrane (26, 27). Their combination in complexes contributes to the activation of two platelet receptors for β2-GPI, apolipoprotein E receptor 2' (apoER2') - and glycoprotein Ib α (GPIb α), that in turn cross-link anti-β2-GPI antibodies (28, 29). In our previous paper we showed that aPLs, in particular anti-β2-GPI antibodies, were able to induce intracellular signals in platelets, which involve IRAK phosphorylation and NF-κB activation, leading to the up-regulation of Tissue Factor (TF), the major initiator of the clotting cascade (30).

Moreover, anti-β2-GPI antibody binding induces the activation of a platelet prothrombotic phenotype expressed by p38 mitogen-activated protein kinases (MAPK) phosphorylation, GP IIb/IIIa conformational change, P-selectin expression, and thromboxane B2 production (31). Therefore, MAPK pathways, including ERK kinases, activated by various stimuli, are important intracellular signaling in the activation of platelets as pivotal component of arterial and venous thrombosis (31, 32).

Since COVID-19 infection triggers the production of aPLs and the thrombotic events observed in severe COVID-19 resemble hypercoagulation seen in APS, especially as regards the catastrophic variant, it is reasonable to assume that aPLs may be potential mediators of cerebrovascular events in patients with COVID-19. From this assumption, our study analyzed the occurrence of aPLs in patients with COVID-19 and concomitant acute ischemic stroke (AIS), evaluating the effect of Ig fractions from these patients on signaling and functional activation of platelets.

Materials and methods

Patients

We enrolled 10 patients, referred to Emergency Department of Umberto I Polyclinic of Rome, positive for PCR Sars-CoV-2 and affected by large vessel occlusion AIS [mean age 72.8 (S.D. 17.6); 7 males, 3 females]. Clinical and demographic characteristics of patients are reported in Table 1. As control groups, matched for age and sex, we evaluated 10 non-COVID-19 stroke patients [mean age 71.5 (S.D. 17.5); 6 males, 4 females], 20 COVID-19 patients without vascular thrombosis [mean age 68.5 (S.D. 15.8); 13 males, 7 females] and 30 healthy donors (HD) [mean age 65.0 (S.D. 16.2); 21

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TABLE 1 Demographics and clinical characteristics of the COVID-19/AIS patients.

	AIS patients *n* =10
Demographics, vascular risk factors and pre-stroke medications	
Age, mean (SD)	72.8 (17.6)
Female/Male	3/7
Stroke clinical and radiological characteristics	
Stroke on awakening/unknown time of onset	2 (20)
Infarct location - Right Left - Bilateral - Subtentorial	4 (40) 6 (60) 0 0
Occlusion site - Top of ICA - Tandem occlusion - MCA-M1 - MCA-M2 - MCA-M3-M4 - Posterior circulation - No occlusion	1 (10) 0 4 (40) 3 (30) 0 0 2 (20)
NIHSS at baseline, median (IQR)	17 (4.75-23)
NIHSS at 24 h, median (IQR)	8 (4-19)
IV thrombolysis	1 (10)
Mechanical thrombectomy	4 (40)
Hemorrhagic transformation	3 (30)
TICI - 2a - 2b - 3	1/4 (25) 0 3/4 (75)
Infarct volume, cm^3 - mean (SD) - median (IQR)	73.47 (108.25) 25.95 (7.80-105.35)
Characteristics of COVID-19	
Time from COVID-19 diagnosis and stroke (days) - mean (SD) - median (IQR)	6.5 (8.7) 4 (0-12.25)
Chest CT	9 (90)
Pneumonia - <20% (mild) - 20-50% (moderate) - >50% (severe) - Bilateral pleural effusion	9 (90) 1/9 (11.1) 3/9 (33.3) 3/9 (33.3) 2/9 (22.2)
SOFA	
- 0	1/9 (11.1)
- 1	2/9 (22.2)
- 2	2/9 (22.2)
- 3	2/9 (22.2)
- 4	2/9 (22.2)
Laboratory data (reference values)	
Lymphocytes, $x10^3/\mu L$ (1-3.2)	1.31 (0.81)
PLT, $x10^3/\mu L$ (150-450)	284.7 (112.41)
LDH, UI/L (135-225)	447.89 (340.54)
Myoglobin, ng/ml (28-72)	294 (283.95)
CRP, mg/dL (0-0.5)	6.59 (15.74)
D-dimer, µg/L (0-550)	3115.40 (1719.73)
Fibrinogen, mg/dL (200-400)	481.0 (91.35)
INR (0.8-1.2)	1.07 (0.09)
aPTT (0.8-1.2)	0.89 (0.18)

(Continued)

TABLE 1 Continued

Values are expressed as means (SD) and percentages.

males, 9 females]. Sera were collected and stored at −20°C until use. This study was conducted in compliance with the Helsinki declaration and approved by the local ethic committees (number NCT04844632); participants gave written informed consent.

Ig fractions were isolated from sera of patients or healthy donors using $(NH4)_2SO_4$ (ammonium sulfate, Sigma-Aldrich, St Louis, MO, USA) precipitation (33), slightly modified. In particular, following a preliminary purity reduction step by precipitation with caprylic acid (octanoic acid, Sigma-Aldrich) (34), saturated $(NH4)_2SO_4$ to a final concentration of 33% was slowly added to sera and incubated for 1 h at 4°C. Then, the samples were centrifuged at 3000 x g for 30 min at 4°C and carefully the supernatant was decanted into a fresh tube. Again, saturated $(NH4)_2SO_4$ (one-third of the supernatant volume) was added to bring its final concentration to ∼50% and incubated for 1 h at 4°C. The resulting precipitated Ig were isolated by centrifugation at 3000 x g for 30 min at 4°C after removing the supernatant. The pellets were resuspended with PBS equal to the original volume of sera. Finally, samples were dialyzed overnight against $(NH4)_2CO_3$ (ammonium carbonate) through a dialyzing membrane to remove remaining ammonium sulfate, lyophilized and resuspended in sterile PBS.

Detection of aPL antibodies

All the patients and HD sera were analysed for the presence of aCL and anti-β2-GPI antibodies (IgG and IgM) by ELISA using QUANTA Lite™ detection kit (INOVA Diagnostic Inc., San Diego, CA, USA) and confirmed by chemiluminescence assay using Zenit RA Immunoanalyzer (A. Menarini Diagnostics, Florence, Italy), according to manufacturer's instructions.

Detection of "non-criteria" aPL antibodies

All the sera were also tested for the presence of "unconventional" ("non-criteria") aPLs: IgG and IgM antibodies specific for PS/PT were

assessed by ELISA using a QUANTA Lite™ detection kit (INOVA Diagnostic Inc.) according to manufacturer's instructions; IgG and IgM aVim/CL antibodies were tested by ELISA assay, as previously reported (18).

Detection of anti-PF4 antibodies

We additionally tested patients and HD sera for antibodies against PF4/polyanion, using a commercial enzyme immunoassay (Immucor, Lifecodes, Waukesha, WI). Sera with an optical density > 500 arbitrary units (AU) were considered as positive.

Platelet preparation

Platelets were obtained from blood samples [in presence of acid citrate dextrose (ACD) as anticoagulant] of healthy donors, that signed informed consent from Transfusional Center of Policlinico Umberto I, Sapienza University of Rome.

Platelet-rich plasma (PRP) was preliminary separated from the whole blood by centrifugation at 150 x g for 15 min at 20°C. Two thirds of the PRP, with the addition of ACD, to prevent platelet activation, were transferred into a new another sterile tube, without disturbing the buffy coat layer, in order to avoid contamination. PRP was centrifuged at 900 x g for 10 min at 20°C (with no brake applied). Platelet-poor plasma (PPP) was discarded and platelet pellets were resuspended in Tyrode's buffer, containing 10% (v:v) ACD. Then, after washing, as above, platelets pellets were resuspended in Tyrode's buffer, containing Bovine Serum Albumin (BSA, 3 mg/ml).

Platelet were counted by a hemocytometer (Coulter, Beckman Coulter, Brea, California, USA), which gives that leukocyte contamination was < 1 leukocyte/10^7 platelets. The purity of the isolated platelets was analyzed and confirmed by staining with a fluorescein isothiocyanate (FITC)–conjugated anti-CD41 antibody (Beckman Coulter) and using flow cytometry (Coulter Epics, Beckman Coulter; data not shown).

In vitro incubation of human platelets and western blot analysis

After isolation, human platelets (3×10^8/ml), were seeded in into 6-well cell culture plates and incubated with Ig fractions (200 µg/ml) from sera of patients described above, with Ig fractions from sera of healthy donors, with polyclonal anti-β2-GPI IgG (200 µg/ml, Affinity Biologicals, Ancaster, Ontario, Canada) or with control IgG (Sigma Aldrich, cod. NI02, 200 µg/ml), for 10 min at 37°C, according to the methods previously described (35, 36). After the treatment, platelets were lysed in a buffer prepared with 20 mM HEPES, pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4 and protease inhibitors cocktail (Sigma-Aldrich). Protein extracts, in equal amount, were analyzed by western blot and, for this purpose, they were first subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Richmond, CA, USA). Membranes, after blocking with Tris-buffered saline Tween 20 (TBS-T) 3% BSA, were incubated with polyclonal rabbit anti-phospho-ERK1/2 (Cell Signaling, Inc., Danvers, MA, USA) and polyclonal rabbit anti-phospho-p38 antibodies (Cell Signaling, Inc.). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma-Aldrich) and then enhanced chemiluminescence western blot system (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used to visualize antibody reactions. As a control of loaded protein content, phospho-ERK1/2 and phospho-p38 membranes were stripped and reprobed with rabbit anti-ERK1/2 and rabbit anti-p38 (Cell Signaling, Inc.) respectively. Densitometric scanning analysis was performed using a NIH Image 1.62 software (National Institutes of Health). The density of each band (absolute value) in the same gel was analyzed.

Platelet activation

Platelets from healthy donors were prepared as reported above. Platelets were incubated for 1 h, with a mix of agonists composed by U-446619 (1 µM; synthetic thromboxane A2 receptor agonist, Helena Biosciences Europe) plus epinephrine (10 µM; Helena Biosciences Europe) used as positive control, or, alternatively, with Ig fractions (200 µg/ml) from sera of patients or healthy donors and then analyzed by a luciferin/luciferase method (ATP lite, PerkinElmer, Waltham, MA) to analyze platelet secretion by ATP release dosage. ATP release was calculated as a percentage of max response from the U-446619 plus epinephrine positive control. This method was performed as previously reported (36–38).

In parallel experiments, platelet function was also investigated evaluating platelet aggregation. Briefly, after treatment, platelets were added in appropriate wells and, immediately after, the plate was placed in a Plate Reader Victor 3 (PerkinElmer). The absorbance and platelet aggregation (PA) percentage were assessed using the following formula: PA% = (sample Absorbance Units – PRP Absorbance Units)/(PPP Absorbance Units– PRP Absorbance Units) × 100, as previously described (36).

Statistical analysis

For western blot analysis the statistical procedures were performed by GraphPad Prism software Inc. (San Diego, CA, USA). D'Agostino-Pearson omnibus normality test was used to assess the normal distribution of the data. Normally distributed variables were summarized using the mean ± standard deviation (SD). Differences between numerical variables were tested using Paired t-test.

For ATP dosage, the level of significance was determined by unpaired, 2-tailed Student's t test by KaleidaGraph Software 3.6. Results (showed as mean + SD) are considered statistically significant: *$p \leq 0.05$, **$p \leq 0.01$ ***$p \leq 0.001$, ****$p \leq 0.0001$.

Results

Serum detection of antiphospholipid antibodies

The study included 10 patients defined as COVID-19/AIS. Clinical and demographic characteristics of patients are reported in Table 1.

This study analyzed the occurrence of aPLs in these patients. In particular, aCL IgG were detected in 5/10 (50%) patients and aCL IgM in 2/10 (20%). The patients who tested positive for IgG and IgM anti-β2-GPI antibodies were 3/10 (30%) and 2/10 (20%) respectively. All positive samples were confirmed after 12 weeks. Two out of 10 (20%) resulted positive for IgG aPS/PT antibodies; the prevalence of aVim/CL antibodies was of 5/10 (50%) for IgG (Tables 2, 3).

In the control group of non-COVID-19 stroke no patients were positive for the aPL tests used, whereas in the COVID-19 patients without vascular thrombosis 2/20 (10%) resulted positive for IgM aCL and anti-β2-GPI antibodies (Table 3). None of these 2 samples was confirmed as positive after 12 weeks.

No one of the HD sera studied resulted positive for all the aPLs tested.

Our results indicated that no patient or HD was positive for anti-PF4/polyanion antibodies (Table 3).

Ig fractions isolated from patients with AIS during COVID-19 induce ERK1/2 and p38 phosphorylation

Since anti-β2-GPI antibodies are the main candidate to trigger platelet activation, platelets from healthy donors were treated with Ig fractions isolated from patients with AIS during COVID-19, with Ig fractions from healthy donors or with polyclonal anti-β2-GPI IgG. As shown in Figure 1, treatment of platelets with the Ig fractions from patients with AIS during COVID-19, as well as with polyclonal anti-β2-GPI IgG, induced a significant increase of both phospho-ERK (Figure 1A) and phospho-p38 (Figure 1B) expression, as compared to control untreated platelets or platelets treated with Ig fractions from healthy donors, non-COVID-19 stroke patients and COVID-19 patients without vascular thrombosis ($p < 0.0001$). In Figure S1 the results of all ten patients with AIS during COVID-19 are reported.

Ig fractions isolated from patients with AIS during COVID-19 trigger ATP release and platelet aggregation

In order to investigate the effect of Ig fractions isolated from patients with AIS during COVID-19 on functional platelet activation, platelets from healthy donors were treated with the Ig fractions. As shown in Figure 2A, results highlighted a significant increase of ATP release in samples stimulated with the Ig fractions from patients with AIS during COVID-19 as compared to those from healthy donors ($p < 0.001$). The analysis revealed that 7 out of the patients with AIS showed a significant increase of ATP release compared to healthy donors, with different values (Table 2), summarized in Figure 2A. Interestingly, the three patients unable to induce ATP release were resulted negative for all aPL tests. However, the strongest ATP release was with the Ig fraction from the AIS/COVID-19 patient with no aPL Ab, suggesting that antibodies other than aPLs present in sera of COVID-19 patients may activate platelets.

This finding was supported by the analysis of platelet aggregation, which revealed that Ig fractions isolated from AIS/COVID-19 patients induced in platelets from healthy donors an

TABLE 2 Positivity of aPL antibodies in the 10 patients with acute ischemic stroke during COVID-19.

Patient *n*	aCL IgG (GPL)	aCL IgM (MPL)	aβ2-GPI IgG (UA/ml)	aβ2-GPI IgM (UA/ml)	aPS/PT IgG (UA/ml)	aPS/PT IgM (UA/ml)	aVim/CL IgG	aVim/CL IgM	ATP release (%)	Platelet aggregation (%)
1	47.0	neg	neg	neg	neg	neg	pos	neg	8	22
2	neg	neg	neg	neg	neg	neg	pos	neg	6	21
3	69.7	neg	32.1	neg	35.9	neg	pos	neg	10	20
4	59.6	55.6	28.6	25.0	neg	neg	pos	neg	11	32
5	141.6	neg	27.1	neg	38.0	neg	pos	neg	7	24
6	neg	neg	neg	neg	neg	neg	neg	neg	3	15
7	neg	neg	neg	neg	neg	neg	neg	neg	17	25
8	neg	neg	neg	neg	neg	neg	neg	neg	3	15
9	neg	neg	neg	neg	neg	neg	neg	neg	2	17
10	70.0	62.3	neg	32.1	neg	neg	neg	neg	16	29

Ig, immunoglobulin; aCL, anti-cardiolipin; aβ2-GPI, anti-β2-Glycoprotein I; aPS/PT, anti-phosphatidylserine/prothrombin; aVim/CL, anti-vimentin/cardiolipin.
The cut-off level used is 20 GPL or MPL units for aCL IgG or IgM, 15 UA/ml for aβ2-GPI IgG or IgM and 30 UA/ml for aPS/PT according to manufacturer's instructions.

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TABLE 3 Prevalence of autoantibodies in patients and healthy donors.

Autoantibodies	COVID-19/AIS (10) *n* (%)	Non-COVID-19/stroke (10) *n* (%)	COVID-19 (20) *n* (%)	Healthy donors (30) *n* (%)
aCL IgG	5 (50)	0 (0)	0 (0)	0 (0)
aCL IgM	2 (20)	0 (0)	2 (10)	0 (0)
aβ2-GPI IgG	3 (30)	0 (0)	0 (0)	0 (0)
aβ2-GPI IgM	2 (20)	0 (0)	2 (10)	0 (0)
aPS/PT	2 (20)	0 (0)	0 (0)	0 (0)
aVim/CL	5 (50)	0 (0)	0 (0)	0 (0)
aPF4	0 (0)	0 (0)	0 (0)	0 (0)

AIS, acute ischemic stroke; Ig, immunoglobulin; aCL, anti-cardiolipin; aβ2-GPI, anti-β2-Glycoprotein I; aPS/PT, anti-phosphatidylserine/prothrombin; aVim/CL, anti-vimentin/cardiolipin; aPF4, anti-platelet factor 4.

average platelet aggregation of 21.5 + 5.67%, significantly higher of that induced by Ig fractions from healthy donors ($p < 0.001$), that virtually did not induce platelet aggregation (Figure 2B).

Discussion

This study demonstrates the presence of aPLs in a subgroup of COVID-19 patients who presented acute ischemic stroke during the SARS-CoV-2 infection. Our results converge with the data describing an increased prevalence of aPLs in COVID-19 patients, suggesting a role in the mechanisms contributing to hypercoagulable state observed in patients with critical ill, such as those presenting ischemic stroke (12, 15–17).

Several viral and bacterial infections may lead to both thrombotic and hemorrhagic complications, emphasizing a clear correlation between inflammation and coagulation. Pathogens, as well as inflammatory cells and mediators, are responsible of TF induction, the main initiator of the coagulation cascade (39, 40). In previous works it has been demonstrated an "*in vitro*" pathogenic effect of aPLs, which are able to activate a proinflammatory and procoagulant phenotype in monocytes, endothelial cells and platelets, triggering a signal transduction pathway, leading to proinflammatory cytokines and TF release (41). The severe thrombo-inflammatory manifestations of COVID-19 patients are likely related to pathogenic mechanisms of viral infection and circulating aPLs. Several studies demonstrated platelet activation in COVID-19 patients (42); in particular it was shown that immune-mediated activation of platelets may contribute to the prothrombotic state in these patients (43, 44). With the aim to explore and verify a mechanism directly involved in the amplification of severe COVID-19 disease, we analyzed the effect

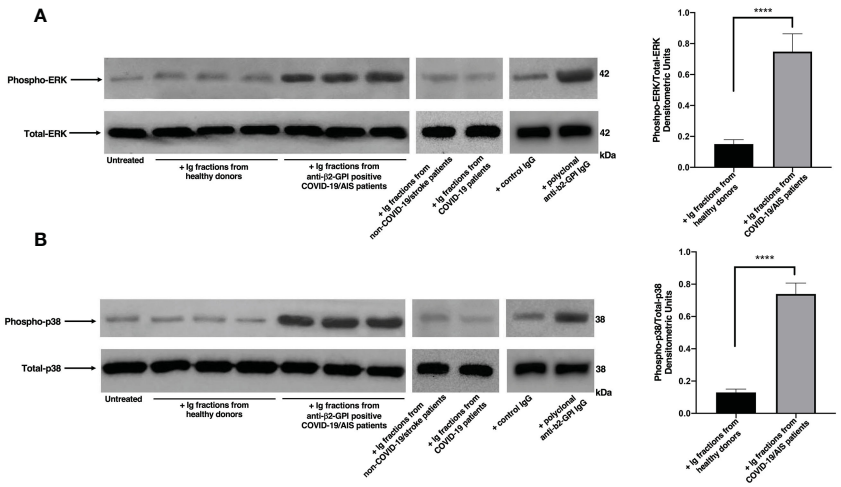

FIGURE 1
Ig fractions from patients with AIS during COVID-19 induce ERK and p38 phosphorylation. Human platelets from healthy donors were treated for 10 min with Ig fractions (200 μg/ml) from AIS/COVID-19 patients, non-COVID-19 stroke patients, COVID-19 patients without vascular thrombosis and from healthy donors or with polyclonal anti-β2-GPI IgG (200 μg/ml). Protein extracts were separated by SDS-PAGE and analyzed by western blot to investigate phosphorylated ERK **(A)** and p38 MAPK **(B)** using anti-phospho-ERK1/2 and anti-phospho-p38 antibodies, respectively. Samples from three representative healthy donors, three anti-β2-GPI IgG positive patients with AIS during COVID-19 (n. 3, 4, 5 of Table 2), one representative non-COVID-19 stroke patient and one representative COVID-19 patient are shown. Densitometric values of phospho-ERK and phospho-p38 levels, calculated in all healthy donors and patients, are represented and summarized by histograms. A statistically significant difference between expression of phospho-ERK and phospho-p38 in patients with AIS during COVID-19 *vs* healthy donors was found (****$p < 0.0001$).

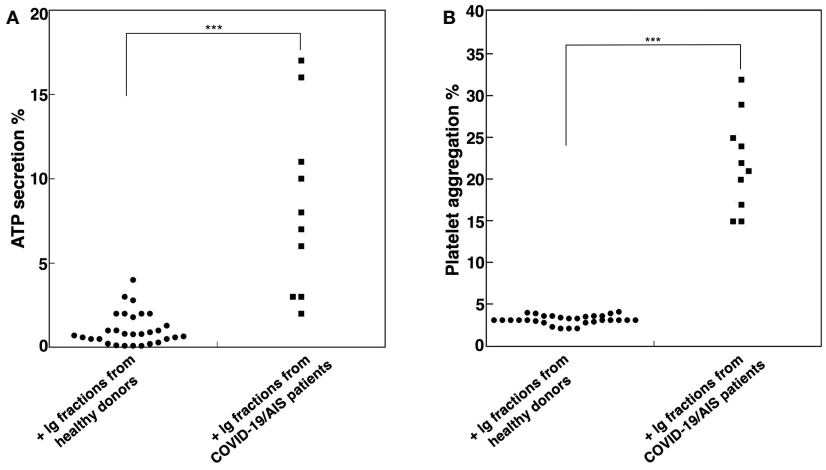

FIGURE 2
Ig fractions from patients with AIS during COVID-19 induce ATP release and platelet aggregation. Human platelets from healthy donors were treated with Ig fractions (200 μg/ml) from 10 patients with AIS during COVID-19 and from 30 healthy donors. After treatments, platelets were analyzed: **(A)** for ATP release dosage by a luciferin/luciferase method to evaluate platelet secretion; **(B)** for platelet aggregation. Statistical analysis indicates: ***p < 0.001 *vs* healthy donors.

on a pivotal signaling pattern in human platelets and investigated the functional activation of these cells. Our results showed an activation of MAPK pathways, referred to an increased phosphorylation of ERK1/2 and p38 proteins, following incubation with Ig fractions from sera of patients with AIS during COVID-19, compared to untreated platelets or treated with healthy donor Ig fractions. Moreover, our results highlighted an increase of adenyl nucleotides release, as well as platelet aggregation after treatment with Ig fractions from sera of patients with AIS during COVID-19.

For several years, the homeostatic role of PF4 has been ascertained, since it is released from the alpha granules of activated platelets and it transfers from plasma to the high affinity heparan sulphate on endothelial cells, inhibiting local antithrombin activity (AT), thus promoting clotting (45). In this concern, we additionally tested patient and healthy donor sera for antibodies against PF4/polyanion and no one resulted positive.

It is known that heparin-induced thrombocytopenia (HIT) is caused by antibodies that recognize the PF4 and they have also been found associated with vaccine-induced thrombosis with thrombocytopenia (VITT) syndrome (46). In a recent paper, we analyzed autoantibody specificities in sera from patients affected by VITT, showing the presence of aPF4/polyanion antibodies, as well as aPLs, suggesting a possible pathogenic role (47). Indeed, the aPF4/polyanion antibodies seem to play a role mainly in the thrombotic syndrome occurring after vaccines administration, probably resembling an autoimmune-HIT, where aPF4/polyanion antibodies activate platelets in the absence of heparin. Interestingly, the data of the present study indicate the absence of aPF4/polyanion antibodies in patients with AIS during COVID-19. Thus, in this case, the effect on platelet activation may be triggered by anti-β2-GPI antibodies. However, patients other than anti-β2-GPI positive may activate the signaling pathway. It may be due to the positivity for other antibody specificities (48), including aCL and non-criteria antibodies. In addition, we cannot exclude that antibodies other than aPLs present in sera of COVID-19 patients may activate platelets (49).

In conclusion, our findings indicate that aPLs, including "non-criteria" aPLs, are present in a cohort of patients with AIS during COVID-19, suggesting that these antibodies may represent a thromboembolic risk factor. Moreover, the data on functional and signaling activation in platelets would seek to explain the link between autoimmune response and SARS-CoV-2 viral infection. Further studies will certainly be needed to elucidate the role of aPLs and other immunological mediators in triggering the immunopathological pathways that create the pro-inflammatory and pro-coagulant conditions found in COVID-19. However, detecting these antibodies, as a serological marker to check and monitor COVID-19, may contribute to improve the risk stratification, and drive a personalized treatment with the aim of preventing thromboembolic complications.

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 Local ethical committee (Policlinico Umberto I) number NCT04844632. The patients/participants provided their

written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

FP and MS designed and performed the research. MD and AF selected the patients. GR, SR and VM performed experiments. AL and VM provided and analyzed the data. AC, RM and TG wrote the paper. MS supervised the research and edited the paper. All authors read, edited, participated in the revision, and approved the manuscript. All authors contributed to the article and 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1129201/full#supplementary-material

SUPPLEMENTARY FIGURE 1.
Ig fractions from patients with AIS during COVID-19 induce ERK and p38 phosphorylation. Human platelets from healthy donors were treated for 10 min with Ig fractions (200 mg/ml) from 10 AIS/COVID-19 patients. Protein extracts were separated by SDS-PAGE and analyzed by western blot to investigate:
(A) phosphorylated and total ERK, using anti-phospho-ERK1/2 and anti-total-ERK1/2 antibodies.
(B) phosphorylated and total p38 MAPK using anti-phospho-p38 and anti-total-p38 antibodies.

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Platelet-neutrophil interaction in COVID-19 and vaccine-induced thrombotic thrombocytopenia

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Coronavirus disease 2019 (COVID-19) is known to commonly induce a thrombotic diathesis, particularly in severely affected individuals. So far, this COVID-19-associated coagulopathy (CAC) has been partially explained by hyperactivated platelets as well as by the prothrombotic effects of neutrophil extracellular traps (NETs) released from neutrophils. However, precise insight into the bidirectional relationship between platelets and neutrophils in the pathophysiology of CAC still lags behind. Vaccine-induced thrombotic thrombocytopenia (VITT) is a rare autoimmune disorder caused by auto-antibody formation in response to immunization with adenoviral vector vaccines. VITT is associated with life-threatening thromboembolic events and thus, high fatality rates. Our concept of the thrombophilia observed in VITT is relatively new, hence a better understanding could help in the management of such patients with the potential to also prevent VITT. In this review we aim to summarize the current knowledge on platelet-neutrophil interplay in COVID-19 and VITT.

KEYWORDS

COVID-19, platelet activation, coagulopathy, VITT, neutrophil extracellular traps (NET), immunothrombosis

1 Introduction

Coronavirus disease 2019 (COVID-19) is caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The disease was initially recognized as a predominantly respiratory illness after its first appearance in the city of Wuhan, China in late 2019, but the presence of the virus at extrapulmonary sites and its fatal effects were subsequently demonstrated (1–3).

COVID-19 patients often suffer from coagulopathy in addition to mortality due to respiratory failure. These are mainly consequences of the prothrombotic state, especially in moderate and severe cases. Venous thromboembolism (VTE), thrombocytopenia and disseminated intravascular coagulation (DIC) were early described as common complications in SARS-CoV-2 infected patients (4–7). Agarwal et al. calculated the

overall prevalence of VTE to be as high as 20.7%, with the risk being doubled in COVID-19 cases admitted to the intensive care unit (8). A large retrospective analysis of more than 370,000 cases from England found that 86% of hospitalized COVID-19 patients with VTE also suffered a concomitant pulmonary embolism, highlighting the coagulation-related risks in COVID-19 (9).

The pathophysiology of COVID-19-associated coagulopathy (CAC) is still under investigation and both cellular and plasmatic constituents of the coagulation system appear to be affected by infection with SARS-CoV-2. It has been established that hyperactivated platelets play a major role in CAC (10–14). Recently, the contributions of cells of the immune system during thrombus formation have been discussed in the setting of immunothrombosis. Histopathological examinations of thrombi from COVID-19 patients have demonstrated an increased deposition of neutrophils within the thrombus matrix in the lung vasculature suggesting that platelet-neutrophil interplay may be crucial in initiation and perpetuation of thrombosis in (hyper-) inflammatory diseases such as COVID-19 (13, 15–18). Several studies have already shown an increase in platelet-neutrophil aggregates (PNAs) circulating in blood of COVID-19 patients. However, the exact mechanisms of interaction between platelets and neutrophils remain unclear as little research was conducted yet on how these cells interact in promoting CAC. Next to their ability of phagocytosis and secretion of antimicrobial enzymes, neutrophils are capable of releasing neutrophil extracellular traps (NETs) mainly consisting of DNA (19). NETs serve as attachment structures for enzymes such as myeloperoxidase (MPO) or neutrophil elastase but also trap pathogens and allow their degradation by the antimicrobial substances. NETs have been studied extensively in the last decade for their impact on thrombus formation (20, 21). Various prothrombotic conditions including DIC in septic patients (22), neoplasms (23) and heparin-induced thrombocytopenia (HIT) (24, 25) have been found to be associated with elevated levels of NETs. SARS-CoV-2 was also demonstrated to directly induce NETosis (26–28). Thus, the detrimental consequences of severe COVID-19 have been partially attributed to both direct and indirect effects of NETs.

Up until now several vaccine candidates have been approved worldwide to mitigate the burden on society and healthcare caused by the COVID-19 pandemic. Among the first vaccine platforms authorized in Europe were mRNA-based vaccines and vaccines using adenoviral vectors. Shortly after the rollout of immunization programs, cases of thrombocytopenia accompanied by thrombotic events have been reported in individuals recently vaccinated with Vaxzevria (ChAdOx1 nCoV-19 vaccine, AstraZeneca) or Janssen Covid-19 vaccine (Ad26.COV2.S, Johnson & Johnson) – both vaccines relying on the adenoviral vector technique. For the first (or unknown) immunization with Vaxzevria, the UK´s Medicines and Healthcare products Regulatory Agency (MHRA) calculated the overall reported incidence of thromboembolic events associated with thrombocytopenia to be 15.9 cases/million doses (29). This specific syndrome, termed vaccine-induced thrombotic thrombocytopenia (VITT), is caused by the formation of antibodies against platelet factor 4 (PF4). Thrombosis and particularly cerebral venous sinus thrombosis (CVST) is the key finding of VITT with the case fatality rate estimated to be approximately 18% (30, 31). The diagnosis of VITT usually requires a history of immunization with an adenoviral vector anti-SARS-CoV-2 vaccine (mainly Vaxzevria or Janssen) minimum 4 days prior, detection of anti-PF4 antibodies in serum and additional more specific platelet aggregation tests (32–34). Treatment options in VITT include non-heparin anticoagulants, administration of intravenous immunoglobulins (IVIG) as well as supportive care (35, 36).

Here, we give an overview of the current state of research on the interaction between platelets and neutrophils in CAC and VITT.

2 Platelets and neutrophils in CAC

2.1 Altered platelet functionality in COVID-19

Besides a reduction in platelet count, the functional properties of platelets are reportedly deranged during COVID-19. Platelets of patients infected with SARS-CoV-2 were found to have higher expression of activation markers than non-COVID-19 controls (11, 12, 37–40). Table 1 lists markers for platelet and neutrophil activation described in COVID-19. Moreover, platelets in COVID-19 had an increased tendency towards aggregation and showed greater responses to stimuli as ADP, thrombin receptor activator peptide 6 (TRAP-6) or thrombin itself (10, 14, 37, 38). This indicates both a hyper-active and hyper-reactive platelet phenotype during infection with SARS-CoV-2.

Direct and indirect aspects of platelet activation have been proposed. Zhu et al. confirmed the presence of SARS-CoV-2 RNA within platelets. Six out of the seven patients with this finding deceased shortly after. On the contrary, only one out of 24 COVID-19 patients from the survivor group was found to have RNA positive platelets (47). The principal mechanism of cellular uptake of SARS-CoV-2 is assumed to occur *via* the angiotensin-converting enzyme (ACE2) receptor in combination with the transmembrane serine protease/serine subfamily member 2 (TMPRSS2) (48). However, whether ACE2 and TMPRSS2 are expressed on platelets is still under debate and other mechanisms of viral entry have also been proposed (37, 49, 50). Furthermore, CD147 may serve as a site of direct interaction between platelets and SARS-CoV-2 and was also described to be a mediator of viral entry into cells *via* endocytosis (51, 52). Additionally, glycoprotein Ib (or CD42b) was identified as a receptor used by the spike protein of SARS-CoV-2 (53). Furthermore, the direct effects of SARS-CoV-2 on platelets appear to be mediated through the upregulation of both caspase-dependent (apoptosis) and caspase-independent pathways (necroptosis) (54, 55).

Examples for indirect mechanisms of platelet activation during SARS-CoV-2 infection include specific immunoglobulins found in sera of COVID-19 patients that induce procoagulant platelets *via* FcγRIIa signaling (11, 12), endothelial dysfunction with increased expression of von Willebrand factor (vWF) (56, 57) and stimulation of platelets by proinflammatory markers during the cytokine storm complicating severe cases of COVID-19 (58, 59). Tissue factor (TF)

TABLE 1 Markers of platelet and neutrophil activation described in COVID-19.

	Found in/on	Clinical significance	Citations
Platelets			
P-Selectin/CD62P	α granules	parameter of platelet activation	(12, 13, 37–39, 41)
Phosphatidylserine (PS)	cell membrane	procoagulant platelets defined as $PS^+/CD62P^+$	(11, 12)
CD63	δ granules	parameter of platelet activation	(39)
PAC-1	activated GP IIb/IIIa	parameter of platelet activation	(13, 37)
Mitochondrial membrane potential, ΔΨm (e.g., TMRE)	mitochondria	loss of ΔΨm is seen in procoagulant and apoptotic platelets	(11)
Neutrophils			
CD66b	granulocyte membrane	general PMN marker, but also increases with activation/ degranulation	(42, 43)
CD11b and CD18	Mac-1	markers of activation	(13, 38, 42–45)
Citrullinated histone (CitH3)	formed in process of NETosis, part of NETs	markers for NETosis	(40, 41, 46)
Myeloperoxidase (MPO) or MPO-DNA complexes	Primary (azurophilic) granules, part of NETs		

secreted from SARS-CoV-2 infected cells such as epithelium also indirectly activates platelets *via* thrombin-mediated signaling (59, 60). Moreover, thrombopoietin (TPO), which promotes *in vitro* platelet hyperresponsiveness and platelet-leukocyte interaction, is found to be increased in COVID-19 patients (61, 62) Additionally, platelets also secrete cytokines during SARS-CoV-2 infection themselves and consequently contribute to the hyperinflammatory state increasing the risk of CAC (63, 64).

2.2 The role of neutrophils and NETs in CAC

Leukocytosis and thus, neutrophilia are common laboratory findings in COVID-19 as the mobilization of immune cells from the bone marrow is one of the earliest responses to combat pathogens (65). The phenotype of neutrophils changes during the infection with SARS-CoV-2. As expected, serum levels of typical markers of neutrophil activation (degranulation and NETosis) such as MPO-DNA complexes or citrullinated histone H3 (Cit-H3) in COVID-19 patients were found to be correlating with disease severity (41, 46). Furthermore, TF increases on neutrophils isolated from patients with severe COVID-19 (66). This suggests how among other pathways primed neutrophils potentially promote or even elicit thrombus formation. As mentioned, NETs are composed of DNA, DNA-associated structures (e.g., histones) and contents of neutrophil granules. Noubouossie et al. reported on the ability of neutrophil DNA to induce thrombin generation (TG) in both platelet-rich and platelet-free plasma although histone-mediated TG appeared to require the presence of platelets (67). For the latter, the toll-like receptors (TLR) 2 and 4 on platelets seem to be of importance in mediating the increase in TG (68).

In a mouse model for SARS-CoV-2 infection, Sung et al. demonstrated the importance of TLR2 and C-type lectin domain family 5 member (CLEC5A) in neutrophils for NETosis and the release of proinflammatory cytokines such as interleukin 6 (IL-6). Interestingly, further *in vitro* experiments with SARS-CoV-2 and mice neutrophils showed accumulation of MPO, Cit-H3 and DNA within the neutrophilic cytoplasm after 5 hours. However, marked NETosis became evident only when incubated with autologous platelets. This suggests that platelet presence could be necessary for further neutrophil activation and NETosis in the case of SARS-CoV-2 infection. Interestingly, in contrast to former evidence with the Dengue virus where NET formation was found to be thread-like, the authors concluded that NETosis elicited by SARS-CoV-2 had a different, more aggregated appearance (69).

Additionally, low-density neutrophils (LDNs) appear to be increasing in number in COVID-19 more than other neutrophil subpopulations (44). These cells - termed $CD16^{int}$ due to their behavior to only intermediately stain with anti-FcγRIII (CD16) – also display an upregulation of genes that are related to NETosis when compared to the $CD16^{high}$ neutrophils. As expected, the authors reported spontaneous *in vitro* formation of NETs in these LDNs (42). Schulte-Schrepping et al. have further elaborated on the myeloid response in severe COVID-19 giving rise to distinct neutrophil precursor subclasses which are characterized by different gene activation signatures including genes involved in NETosis (70). Previously, LDNs have commonly been described in rheumatological diseases as systemic lupus erythematosus or anti-phospholipid syndrome for their proinflammatory effects although consensus on their precise characterization in terms of origin, function and fate has not been reached (71, 72).

As a side note, neutrophils contribute to CAC by releasing a variety of immune mediators causing a cytokine storm and DIC

(73). For instance, Kaiser et al. proposed a vicious cycle of IL-8 released from neutrophils in severe COVID-19 which further attracts and activates additional neutrophils (74).

2.3 Direct and indirect interplay between platelets and neutrophils in COVID-19

2.3.1 Platelet-neutrophil aggregates as the result of direct interaction

The first quantifiable endpoint of platelet and neutrophil interaction in CAC is represented by complex formation. As mentioned, such platelet-neutrophil aggregates (PNAs) are abundant in SARS-CoV-2 positive patients (37, 41, 45, 75–77). COVID-19 disease severity correlates with blood levels of PNAs (78). Both normal-density neutrophils (NDNs) and LDNs form PNAs although complexes of platelets and $CD16^{int}$ had significantly higher expression of P-selectin (CD62P) and CD40 than PNAs with $CD16^{high}$ neutrophils (42). This could be an additional hint for the hyperactive properties of LDNs and their dominant role in mediating a potential synergy between activated platelets and neutrophils in thrombosis.

In the following, we provide an outline of the most relevant receptors in the process of aggregate formation. Figure 1 illustrates the direct and indirect aspects of platelet-neutrophil interaction in COVID-19.

2.3.1.1 P-selectin and PSGL-1

Platelet CD62P (P-selectin) and neutrophil P-selectin glycoprotein ligand-1 (PSGL-1, CD162) are a long-known interaction site for platelets and granulocytes (79, 80). Wang et al. demonstrated the major role of PSGL-1 in the coagulopathy associated with systemic inflammation suggesting that CD62P-PSGL-1 coupling also is involved in CAC (81). P-selectin has been identified as the major platelet receptor for monocyte-platelet aggregation in COVID-19 patients (82). Interestingly, platelets *in vitro* activated by SARS-CoV-2 spike protein were shown to cause activation of monocytes *via* CD62P-PSGL-1 coupling (53). Non-specific gene signature analysis of whole blood from severe to critical COVID-19 patients additionally has shown an upregulation of SELPG, the gene encoding PSGL-1 (83).

2.3.1.2 Mac-1

The macrophage-1 antigen (Mac-1) is made up of the two integrins αM (CD11b) and β2 (CD18) and serves several purposes including binding complement and regulation of leukocyte extravasation (84). Despite the name, neutrophils also express Mac-1 and determination of CD11b is considered a typical marker of neutrophil activation beside CD66b (Table 1). Previously, it was shown that Mac-1 interacts with platelet glycoprotein Ibα (vWF receptor) in mediating thrombosis (21, 85). Increased expression of both CD11b and CD18 on neutrophils isolated from COVID-19 patients was noted when compared to healthy volunteers suggesting one potential mechanism of platelet activation *via* Mac-1 binding of platelet GP Ib (43, 45). Additionally, the behavior of three different neutrophil subpopulations in COVID-19 patients was investigated by Reyes et al. First, neutrophils isolated by density gradient centrifugation from both the PMN and PBMC layer were separated into NDNs

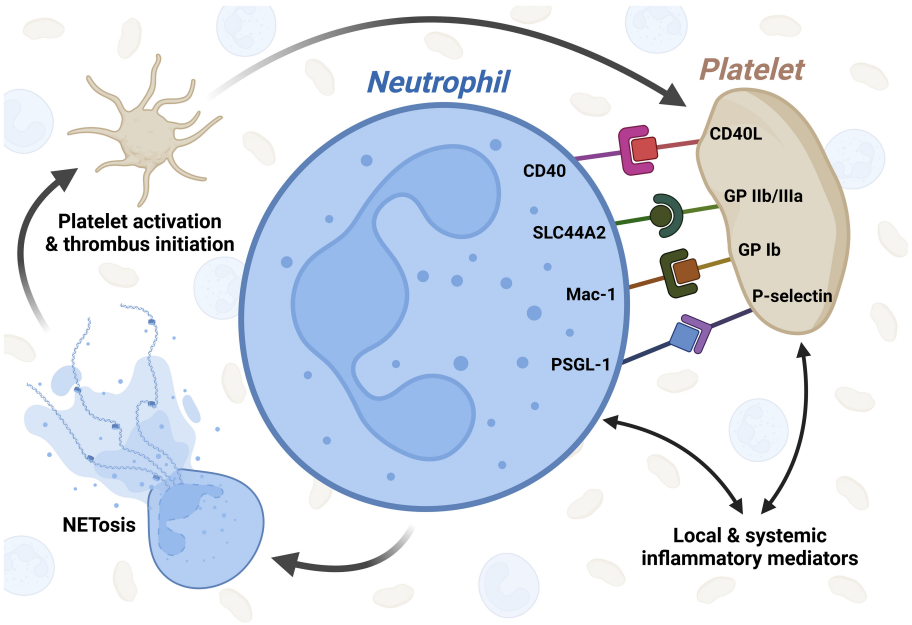

FIGURE 1
Graphical illustration of platelet-neutrophil interaction in COVID-19. Inflammatory mediators released from immune cells as well as endothelial cells during COVID-19 activate both platelets and neutrophils independently. After activation, several receptors are known to be involved in platelet-neutrophil interaction in COVID-19 which in turn leads to NETosis, subsequent platelet activation and thrombus initiation. CD40L - CD40 ligand; SLC44A2 - solute carrier family 44 member 2; GP IIb/IIIa - glycoprotein IIb/IIIa; Mac-1 - macrophage-1 antigen complex; GP Ib - glycoprotein Ib; PSGL-1 - P-selectin glycoprotein ligand-1. Created with BioRender.com.

and LDNs. LDNs were further characterized for maturity based on expression of CD10 and CD16. Interestingly, mature LDNs ($CD16^+/CD10^+$) showed high levels of Mac-1 similar to NDNs but formed more complexes with platelets than NDNs. On the other hand, immature LDNs ($CD16^-/CD10^-$) showed lower levels of Mac-1 and appeared to form fewer PNAs (43).

As discussed later, Mac-1 also recognizes several chemokines secreted from platelets including PF4.

2.3.1.3 GP IIb/IIIa and SLC44A2

The platelet glycoprotein IIb/IIIa (integrin α2bβ3, CD41/CD61) is known to interact with the widely distributed choline transporter-like protein 2 (CTL2, SLC44A2) presenting on neutrophils. The importance of SLC44A2 in hemostasis and particularly VTE has already been established in both genetic and animal studies (86–88). Constantinescu-Bercu et al. highlighted the neutrophil SLC44A2 - platelet integrin α2bβ3 axis as an important communication channel of NETosis. Neutrophils were shown to form NETs when infused through GP IIb/IIIa-coated microchannels although simple incubation without flow resulted in a significant decrease in NETosis (89). This implies that formation of NETs also depends on mechanistic effects. From studies on platelet-monocyte interactions, Hottz et al. reported that *in vitro* inhibition of GP IIb/IIIa with abciximab limited the ability of platelets from COVID-19 patients to activate TF expression by monocytes (82). Up to this point there is no data available on SLC44A2 in COVID-19 and how it could potentially impact CAC except for fundamental proteomic data which suggests a significant downregulation of SLC44A2 in neutrophils from severe COVID-19 patients (90).

2.3.1.4 CD40 and CD40L

Apart from many immune responses which are regulated by CD40 and its ligand CD40L (CD154), platelets and neutrophils were also demonstrated to use this pathway (91, 92). Both CD40L expressed on the platelet membrane and soluble CD40L released from platelets (sCD40L) were found to be critical for neutrophil activation in animal models (93, 94). It was also established that CD40L is not uniquely limited to bind CD40 as it also interacted with Mac-1 (95). In general, neutrophil adhesion to platelets was shown to be enhanced by CD40L but this effect was dependent on Mac-1 as its inhibition with anti-CD11b reversed the bonding affinity of neutrophils for platelets (96). Blood from COVID-19 patients had significantly higher concentrations of sCD40L than healthy volunteers (97, 98). However, this was not consistent with the report of Blasi et al. where no significant difference in plasma sCD40L was evident between COVID-19 patients and healthy controls (99). Interestingly, Al-Tamimi et al. showed soluble CD40L levels peaking with moderate disease followed by a decline when disease severity increases (100). This may explain, at least in part, the inconsistencies observed at different time points in the course of COVID-19. *In vitro* stimulation of platelet-rich plasma with the receptor-binding domain of SARS-CoV-2 caused the levels of soluble CD40L to increase (101). This suggests a direct viral effect on platelets causing sCD40L secretion which in turn could induce neutrophil activation. Li et al. showed that spike protein-activated platelets interacted with monocytes using CD40L (53). This clearly highlights the substantiality of platelet presence in fully unfolding the effects of SARS-CoV-2.

On the other hand, the expression of CD40 on LDNs ($CD16^{int}$) correlated with both disease severity and the concentration of D-dimers (42). As described previously, such LDNs are thought to be pro-NETotic. Increased expression of CD40 on LDNs as binding site for platelet surface CD40L and sCD40L released from platelets among other cells (e.g., endothelium) could render these LDNs more susceptible to PNA formation and subsequent platelet-mediated neutrophil activation. For further information on the role of CD40/CD40L in thromboinflammation we refer to the review by Cognasse et al. (102).

2.3.2 Indirect pathways of platelet-neutrophil interaction: inflammatory mediators and microvesicles

Numerous indirect ways of communication between platelets and neutrophils have been reported. Precise dissection of these pathways is challenging and often ambiguous. Most importantly, inflammatory mediators (e.g., cytokines) secreted from both cell types and so-called microvesicles (MVs) are thought to participate in indirect platelet-neutrophil interaction.

Microvesicles are released from cells through membrane budding and usually contain intracellular contents. Platelets are well known to release such extracellular vesicles into circulation in various situations including COVID-19 (63, 64, 103). High levels of platelet-derived MVs expressing TF were found in COVID-19 patients highlighting the thrombotic diathesis of SARS-CoV-2 infection (58, 104). Neutrophils from COVID-19 patients also release MVs which are an important source of TF. Skendros et al. suggested that this platelet-neutrophil-TF axis may be the critical link between immune defense and both plasmatic and cellular hemostasis, leading to CAC (66). Previously, a circular relationship between MVs from neutrophils and platelets has been proposed where direct interaction *via* P-selectin/PSGL-1 coupling initiated platelet-induced arachidonic release from neutrophils. In turn, after uptake into the platelet interior, thromboxane A2 (TxA2) is generated and released causing endothelial activation and subsequently leukocyte rolling and diapedesis (105). Furthermore, TxA2 has been previously reported to play a role in NET formation in the pathogenesis of transfusion related acute lung injury (TRALI) (106). However, whether this applies to NETosis in COVID-19 as well is yet to determine.

The cytokine response to SARS-CoV-2 viremia is complex and sometimes progresses to an hyperinflammatory state with excessive cytokine release ("cytokine storm"). Multiple cell types participate in this process including neutrophils and platelets. As mentioned, platelets in COVID-19 may secrete soluble CD40L but also other non-cytokine mediators such as the positively charged PF4 in COVID-19 (64). PF4 or CXCL4 is known to interact with the neutrophil Mac-1 receptor and also directly with NETs as by their anionic nature. In general, its effects are diverse but include neutrophil chemotaxis, stimulation of NET formation and NET

compaction (107–112). The exact role of PF4-mediated platelet-neutrophil interaction in COVID-19 was not investigated further albeit a single study on COVID-19 patients that reported elevated levels of both PF4 and RANTES (Regulated and Normal T cell Expressed and Secreted), a chemokine released from platelets (76). High-mobility group box 1 (HMGB1) also plays a role among the mediators of platelet-neutrophil interplay and high HMGB1 levels were shown to be associated with COVID-19 mortality (113, 114). HMGB1 is a damage-associated molecular pattern (DAMP) protein which can be released from activated or necrotic cells. The function of HMGB1 in thrombosis has only been superficially covered but findings from acute myocardial infarction patients suggested that platelet-derived HMGB1 acts on neutrophils and stimulates the release of NETs. Here, the RAGE receptor (Receptor for Advanced Glycation End products) is of importance (115). An animal study from Vogel et al. has further elaborated on the essential role of HMGB1 in passing prothrombotic signals from platelets to neutrophils (116).

Another example of a relevant mediator in platelet-neutrophil interplay is represented by IL-6 which has already been identified as a main target in counteracting the hyperinflammatory state observed in severe cases of COVID-19 (58, 63). Interestingly, IL-6 blockade in COVID-19 plasma with tocilizumab significantly reduced the high levels of TF+-platelet MVs and PNAs when compared to control plasma (58).

Additionally, neutrophils may release calprotectin or S100A8/A9 upon activation, a protein with the potential to induce procoagulant platelets *via* GP Ibα *in vitro*. COVID-19 patients showed high levels of S100A8/A9 correlating with disease severity (117, 118). Again, differences in upregulation of both S100A8 and S100A9 gene were noted among distinct neutrophil precursor subclasses in severe COVID-19 (70). Additionally, calprotectin deposits have been identified on lung autopsies of COVID-19 deaths (119). The procoagulant effects of neutrophil cathepsin G on the other hand is more certain and direct interaction between this serine protease and platelets is thought to be mediated by protease-activated receptors, PAR-1 and/or PAR-4 (120–123). High levels of cathepsin G are found in COVID-19 but also pneumonia with acute respiratory distress syndrome of different etiologies (124, 125).

2.4 Platelets, neutrophils and NETs in VITT

Although little is known about platelet-neutrophil interplay in VITT yet, recent evidence suggests the importance of this relationship in initiating and perpetuating vaccine-induced thrombosis. Direct platelet-neutrophil interaction in the form of PNAs was already found to be upregulated in VITT resulting in higher levels of PNAs as compared to control (126). On a single cellular level, both platelets and neutrophils have been demonstrated to be directly activated *in vitro* by VITT antibodies (126–128). Several case reports have highlighted the presence of NETs within thrombi of VITT patients indicating that NETs are involved in vaccine-induced thrombosis (129, 130). Additionally, increased plasma levels of NET markers (e.g., Cit-H3, MPO-DNA complex) were observed in VITT patients (126, 129). This is in line with recent findings in other prothrombotic conditions such as COVID-19 and HIT. Furthermore, the severity of side effects of the immunization has been correlated to serum histone 3 levels as well (131).

For neutrophils, the proportion of NET-releasing granulocytes was found to be significantly higher in VITT patients compared to control groups (126). Again, LDNs appear to be particularly involved here. NETosis from LDNs in VITT was significantly higher than NET release from NDNs (126, 129). Further research should be directed towards functions and significance of this peculiar neutrophil subpopulation to eventually identify potential pharmaceutical targets in counteracting NET formation in immunothrombosis. Interestingly, Greinacher et al. reported that *in vitro* incubation of isolated neutrophils with VITT serum and PF4 did not lead to NETosis unless platelets were also added to the experiment (128). Thus, it could be concluded that platelets have a crucial role in neutrophil activation and NET formation also in the setting of VITT. Here, microvesicles released from platelets seem to be of importance for the prothrombotic milieu and could help in explaining the cerebral venous tropism of VITT thrombosis (132). However, future efforts are needed to investigate the exact direct and indirect mechanisms of intercellular communication and interplay of neutrophil and platelets in VITT.

Recent studies have focused on neutrophil-activating peptide 2 (NAP2) or CXCL7 released from platelets stimulated with VITT antibodies which in turn activated neutrophils (133–135). Hundelshausen et al. proposed the use of Bruton tyrosine kinase inhibitors (BTKi) in VITT as they were shown to limit platelet P-selectin expression, reduce neutrophil activation and inhibit platelet-neutrophil aggregation (136, 137). Apart from stored VITT sera mainly from spring 2021, future VITT models could rely on chimeric anti-PF4 antibodies mimicking vaccine-induced thrombotic thrombocytopenia such as 1E12 (138).

3 Conclusions

In general, both platelets and neutrophils on their own are considered to be major actors in the prothrombotic state seen in COVID-19 and VITT. With our understanding of thromboinflammation still evolving, further efforts should be directed towards dissecting the precise mechanisms of direct and indirect platelet-neutrophil interplay. From our point of view, this relationship should be seen as bidirectional with both types of cells closely interacting and potentiating the thrombotic cascade of initiation, formation and extension of thrombosis. Detailed insights into this interaction and its pathways could be used to design targeted therapies that reduce the occurrence of life-threatening thrombotic complications in COVID-19 and VITT. Additional research on the role of LDNs may help limit the consequences of hyperinflammation associated with COVID-19. *In vitro* thrombosis models, such as microfluidic systems, might be helpful in this regard to understand the role of different cells in the development of thrombosis in patients with COVID-19 as well

as VITT.

Author contributions

JH, GU and TB wrote the manuscript. JH, GU and AS performed the literature review and data collection. JH and JZ designed the Figures. JH, GU, JZ, AS and TB revised the manuscript. 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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Annexin-V positive extracellular vesicles level is increased in severe COVID-19 disease

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Objectives: To evaluate extracellular vesicles levels in a cohort of SARS-CoV-2's patients hospitalized in an intensive care unit with and without COVID-19 associated thromboembolic events.

Methods: In this study, we aim to assess endothelial and platelet membrane-derived extracellular vesicles levels in a cohort of SARS-CoV-2 patients with and without COVID-19-associated thromboembolic events who were hospitalized in an intensive care unit. Annexin-V positive extracellular vesicles levels were prospectively assessed by flow cytometry in one hundred twenty-three critically ill adults diagnosed with acute respiratory distress syndrome associated with a SARS-CoV-2 infection, ten adults diagnosed for moderate SARS-CoV-2 infection and 25 healthy volunteers.

Results: On our critically ill patients, thirty-four patients (27.6%) had a thromboembolic event, Fifty-three (43%) died. Endothelial and platelet membrane-derived extracellular vesicles were drastically increased in SARS-CoV-2 patients hospitalized in the ICU compared to healthy volunteers. Moreover a slighty higher small/large ratio for platelets membrane-derived extracellular vesicles in patients was linked to thrombo-embolic events.

Conclusion: A comparison between total annexin-V positive extracellular vesicles levels in severe and moderate SARS-CoV-2 infection and healthy controls showed a significant increase in patients with severe infection and their sizes could be considered as biomarkers of SARS-CoV-2 associated thrombo-embolic events.

KEYWORDS

extracellular vesicles, microparticles, COVID-19, thrombosis, intensive care unit

Introduction

Coronavirus 2019 (COVID-19) infection can lead to severe acute respiratory syndrome due to coronavirus 2 (SARS-CoV-2). This disease is also associated with cardiovascular complications such as thrombo-embolic events (1). During the course of the disease, some macro or micro thromboembolic events, and disseminated intravascular coagulation have been described. Indeed, viral diseases can damage the endothelium in many ways. Endothelial cells are key regulators of vasomotricity, oxidative stress, coagulation, inflammation and are an important mediator of atherosclerosis and vascular disease (2). The virus can bind the angiotensin-converting enzyme 2 (ACE-2) receptor expressed notably by type 2 alveolar cells, bronchial epithelial cells, and endothelial cells (1). Viral elements have been found inside endothelial cells, and associated to an accumulation of inflammatory cells, leading to endothelial and inflammatory cell death (3).

Extracellular vesicles (EVs) are lipid-bound vesicles secreted into the extracellular space. EVs are heterogeneous membranous vesicles with different sizes, functions and origins. They include exosomes, microvesicles, and apoptotic bodies, which contain lipids, nucleic acids and proteins (4). Exosomes are released continuously from cells, whereas microvesicles and apoptotic bodies are released predominantly by activated or apoptotic cells. EVs are generated by plasma membrane blebbing with externalization of phosphatidylserine. Indeed, in physiological conditions, phosphatidylserine is usually located only in the cytoplasmic leaflet (4). EVs can transfer these molecules to target cells in a communication system between neighboring and distant cells and tissues. EVs secreted by endothelial cells have some effects such as antioxidant effects by including for example glutathione transferases and peroxidases which may contribute to maintaining blood plasma redox state (5, 6), effects on endothelial cell activation through for example miRNA (7), role in oxidative stress-mediated dysfunction of endothelial cells. EVs from platelets play a role in coagulation, linked to the surface exposure of negatively charged phospholipids (8).

In this study, we aim to assess endothelial and platelet membrane-derived extracellular vesicles levels (eEVs and pEVs, respectively) in a cohort of SARS-CoV-2 patients with and without COVID-19-associated thromboembolic events who were hospitalized in an intensive care unit.

Materials and methods

Patients

Consecutive patients ($n = 131$) referred to the medical intensive care medicine department in the Amiens University Hospital at, France between February 2020 and October 2021 who were diagnosed with acute respiratory distress syndrome associated with a SARS-CoV-2 infection (severe disease) were prospectively enrolled in this study. Severe disease was defined by at least one visceral failure (most often respiratory failure) requiring transfer to ICU. Eight patients were excluded in relation to a negative or questionable COVID-19 test (RT-PCR). Only data from 123 patients were analyzable for our study. This study "Thromboembolic events in hospitalized patients with COVID-19 serious acute pneumopathy (THROMBOCOVID1)" was reviewed and approved by the Institutional Review Bord (IRB) for the local Ethics Committee of Amiens Nord-Ouest II and registered on clinicaltrials.gov with the identifier NCT04377490. The need for IRB approval and informed consent was waived on May 4, 2020. All procedures were followed in accordance with the ethical standards of the responsible committee on institutional human experimentation and with the Helsinki Declaration of 1975. As some patients were recruited during the vaccination campaign, 8 patients were vaccinated (4 with one dose, 2 with two doses, 2 with three doses).

We also selected 10 consecutive patients referred to our medicine department, for moderate SARS CoV-2 infection who did not meet the criteria used to define patients with severe disease (no organ support, no invasive ventilation, no vasoactive drugs, no visceral failure). All patients or next-of-kin signed an informed consent.

Healthy individuals

Healthy volunteers ($n = 25$) were selected from a bone marrow donation center. None of the volunteers took any drugs at that time or had chronic disease. They had no clinical signs of viral infection. COVID-19 tests were performed in the week within the selection and were negative. All participants signed an informed consent.

Investigations

All patients underwent a complete clinical examination, with laboratory and morphological measurements at Day 0, Day 7, Day 14, Day 21, and Day 28. Examinations included Doppler ultrasound in the case of a suspected venous thrombosis and a pulmonary CT angiography in the case of a suspected pulmonary embolism.

Endothelial and platelet membrane-derived extracellular vesicles (eEVs and pEVs)

Samples for EVs measurement were collected at Day 0, Day 7, Day 14, Day 21, and Day 28 according to the patients' availability. EVs were isolated from whole blood using a common differential centrifugation assay described in the literature (9). The citrated tubes were quickly centrifuged (2,500 × g for 15 min at room temperature). Supernatant was collected and platelet poor plasma was stored at −80°C. After thawing, we performed a second centrifugation of the supernatant (25,000 × g for 30 min at room temperature) and this platelet poor plasma was used to study EVs concentration and phenotype.

EV detection was then performed on thawed samples using a Cytoflex cytometer (Beckman Coulter Life Sciences, Villepinte, France). Annexin V-BV510 (Brillant Violet 510) as the EV marker (BioLegend, Paris, France), anti-CD41-FITC (IM0649U) as platelets marker and anti-CD144-PE (A07481) as endothelial marker (Beckman Coulter Life Sciences, Villepinte, France) were used for MP detection. Megamix-plus FSC beads (BioCytex, Marseille, France) calibrated from 0.1 to 0.9 µm were used to define an analysis window consistent with the size of EVs. EVs were quantified using EV Count Beads (BioCytex, Marseille, France): [EV counts × (EV-count beads)]/EV count beads counted. In order to discriminate EVs and aggregates, we set the signal detection at peak height (V-SSC-H and FSC-H) and peak width.

Statistical analysis

Categorical variables were presented as frequencies and continuous variables were presented as the median and 25th to 75th percentiles. Statistical analyses were performed using the nonparametric Mann–Whitney and Kruskal–Wallis tests for testing the hypothesis that the distributions of each of two groups was close for quantitative variables and Fisher's exact test for frequencies to assess the existence of statistical differences between groups. For EVs follow-up with this measurement at more than two time points, we used a repeated measures Anova test. A p-value <0.05 was considered statistically significant.

Results

Demographics, clinical characteristics, and biological parameters of the study population

One hundred twenty-three patients were included in this study. The clinical and biological characteristics of all included patients are listed in Table 1. Mean age was 68 years, age range 58.5–72 years, 88 (71%) were male. Ninety patients (73%) required mechanical ventilation for a median of 18 days. Thirteen patients (10.5%) required extracorporeal circulation, 53 patients (43%) died. Thirty-four patients (27.6%) had a thromboembolic event: 25 deep vein thrombosis, 2 arterial thrombosis, 15 pulmonary embolisms (including the 6 associated with deep vein thrombosis). Four thromboses were detected on foreign material (2 on central catheter, one on extracorporeal circulation cannula, one on arterial cannula).

TABLE 1 Demographic characteristics of patients with severe form of COVID-19.

Patients (n = 123)	
Age years, median (range)	67 (58.5–72)
Male	35 (71.5%)
Female	54 (28.5%)
BMI, mean (SD)	29.4 (25.3–33.6)
Comorbidities	
Arterial hypertension	60 (49%)
Auricular fibrillation	6 (5%)
Ischemic cardiopathy	18 (14.5%)
Thromboembolism	8 (6.5%)
Oral anticoagulants	14 (11%)
Diabetes	37 (30%)
Renal impairment	18 (14.5%)
Chronic dialysis	2 (1.5%)
Cancer	17 (14%)
Obstructive sleep apnea	7 (5.5%)
Chronic obstructive pulmonary disease	5 (4%)
BMI > 30	57 (46%)
Follow-up in the intensive care medicine department	
Sepsis-related organ failure assessment (SOFA), mean	5 (3–11)
Stay in the intensive unit (meantime in days)	19 (8.5–29.5)
Endotracheal intubation	90 (73%)
Invasive ventilation, meantime in days	18 (10–33.5)
Extracorporeal membrane oxygenation, number of patients	13 (10.5%)
Thromboembolic events	34 (27.6%)
Pulmonary embolism	15 (12%)
Dexamethasone use	109 (88.5%)
Tocilizumab use	8 (6.5%)
Death	53 (43%)

Endothelial and platelet membrane-derived extracellular vesicles are drastically increase in SARS-CoV-2 patients hospitalized in an intensive care unit compared to healthy volunteers

Circulating annexin-V positive EV subsets were quantified by sensitive flow cytometry using annexin-V forward scatter gating to identify annexin-V positive EVs of interest by size. This allows for the discrimination of small and large EVs (Supplementary Figure S1). They were drastically increased in patients with severe disease [median 1944/μL, 25th to 75th percentile (837.5–3595)] compared with those of patients with moderate disease [median 410.5/μL, 25th to 75th percentile (275.5–995.5)] $p = 0.004$ and healthy volunteers [median 247/μL, 25th to 75th percentile, (157.5–429.5)] $p < 0.0001$ (Figure 1; Supplementary Table S1) and this continued for the duration of the ICU stay (follow-up Day 1 to Day 28). Platelet (pEVs) and endothelial-derived (eEVs) extracellular vesicles were also increase in patients with severe disease compared with moderate disease and healthy controls (Figure 2; Supplementary Table S1). By analyzing annexin-V positive EVs with forward scatter, we identified several profiles of EVs according to their small and large sizes. Small and large total EVs were increased in patients with severe disease, especially pEVs (Supplementary Table S1).

Endothelial and platelet membrane-derived extracellular vesicles levels are not linked to thromboembolic events in SARS-CoV-2 in-patients

To better understand the degree to which EVs are associated with severe disease, we compared the data between severe disease with and without thromboembolic events, $n = 34$ and $n = 89$, respectively. Unfortunately, none of the studied EV subsets showed any significant difference between these two populations (total, endothelial and platelet-derived small and large size EVs). However, we observed a trend of a slightly higher small/large ratio for total EVs ($p = 0.04$) and pEVs ($p = 0.02$) in patients with thrombo-embolic events (Supplementary Table S2). For a limited number of patients, we established the follow-up of annexin-V positive EV levels before and after the thrombo-embolic events ($n = 13$). These events did not appear to induce a significant and reproductible change in the levels of our annexin-V positive EV subsets (Figure 3). This distinction was difficult to study because of the small number of patients who could be followed up for more than two weeks.

Discussion

Circulating annexin-V positive EVs were quantified by sensitive flow cytometry in a cohort of patients diagnosed with acute respiratory distress syndrome associated with a SARS-CoV-2 infection referred to an intensive care medicine department. Our data demonstrate a higher total annexin-V positive EV level in severe disease compared to moderate SARS-CoV-2 infection and healthy controls, with a significant increase in eEVs and particularly in pEVs.

In our study, we have included our patients over a large period of time including several pandemic waves. However the virus-variant status did not appear to be a factor of heterogeneity for our cohort. Indeed a previous study showed high similar counts especially of pEVs in two SARS-CoV-2 positive cohorts enrolled during both the first and the second pandemic waves (10).

In several studies, pEVs had already been found in association with viral infections such as the H1N1 virus (11), HIV (12), and dengue virus (13). They point to EVs and pEVs as potential biomarkers in COVID-19. Indeed, increased levels of circulating pEVs have been observed in patients with SARS-CoV-2 infection (10, 14, 15). In the same way, high plasma concentrations of eEVs were also observed (15). In particular, patients with severe disease (intubated group) exhibited increased eEVs concentrations and mean size compared to an uninfected group. These eEVs also induced endothelial expression of pro-adhesive proteins (16), in line with a procoagulant and hyperinflammation setting. Indeed, the most severe forms of COVID-19 involve endothelial damage, clot formation, microthrombi, and multiple organ failure, suggesting a central role of the vascular endothelium (17). However, in our study, although we demonstrated a clear increase in total EV levels in severe COVID-19 infection, we failed to distinguish, in this way, thromboembolic events in this

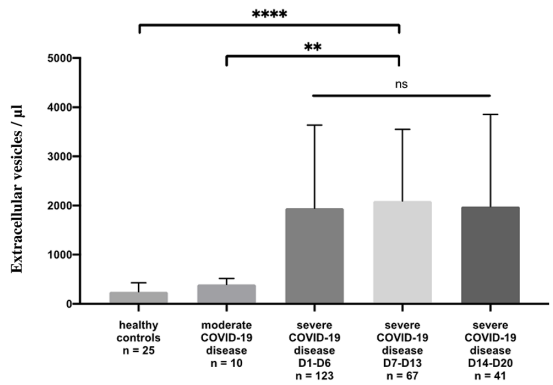

FIGURE 1
Annexin-V positive EV levels in healthy controls and COVID-19 patients. Plasma levels (/µL) of annexin-V positive EVs in healthy controls compared to moderate and severe COVID-19 disease. For severe disease, three measure points are performed: Day 1 to Day 6, Day 7 to Day 13, and Day 14 to Day 20, the duration of the ICU stay.

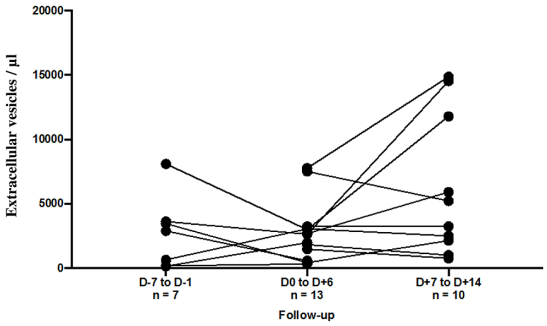

FIGURE 3
Follow-up of annexin-V positive EV levels (/µL) according to the thromboembolic event. For 6 patients, EV levels were assessed at Day 7 to Day 1 before and at Day 1 to Day 6 after the thromboembolic event. For 10 patients, EV levels were assessed at Day 1 to Day 6 and at Day 7 to Day 14 after the thromboembolic event.

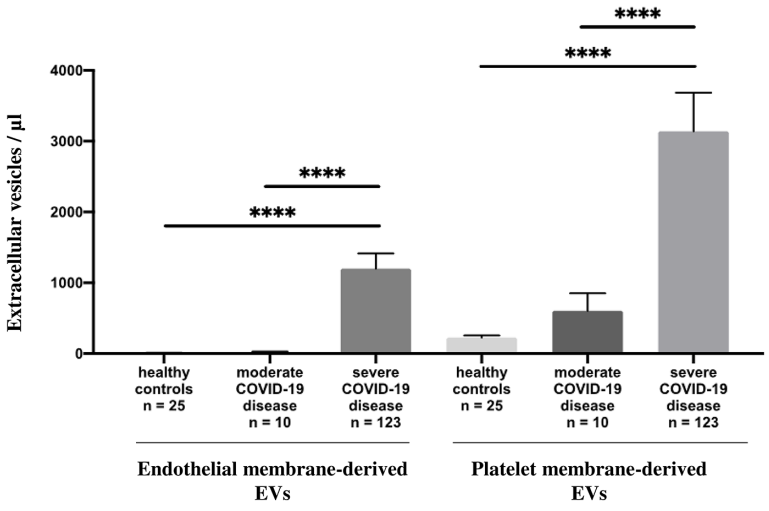

FIGURE 2
Annexin-V positive EV levels in healthy controls and COVID-19 patients. Plasma levels (/µL) of annexin-V positive endothelial and platelet membrane-derived EVs in healthy controls compared to moderate and severe COVID-19 disease.

specific population. Campello et al. (15) showed that baseline levels of pEVs were significantly associated with the development of thromboembolic events with a low OR (1.07) in a cohort of mild and moderate COVID-19 infection. In contrast, Guervilly et al. (18) did not show differences in EV subsets between the moderate and severe forms of COVID-19, but the activity of tissue factor linked to these EVs was significantly increased in patients with severe disease, but also in patients with symptomatic clinical thromboembolic events within 28 days after sampling. Another study showed that plasma procoagulant extracellular vesicles (tissue factor activity) were elevated ~nine-fold in severe COVID-19 patients (19). These data could explain the strong procoagulant imbalance in severe infection not only linked to the levels of EVs, but also by a high level of tissue factor activity. The systemic inflammation in severe COVID-19 disease leads to a wide range of hemostatic derangements in which the study of EVs is of interest. Moreover, we showed a slightly higher small/large ratio, especially for pEVs ($p = 0.02$) in patients with thrombo-embolic events. This observation could be linked with previous studies showing that stimuli can activate platelets, resulting in the genesis of pEVs. The quantity, the structure and the size of the pEVs clearly depend on the type of activation. After activation, platelets undergo a conformational change, releasing effector molecules and EVs (20). Then, collagen and ADP activation induced spherical and smooth EVs production. Moreover with collagen or thrombin activation, many more EVs are produced. But, in case of thrombin activation, MPs are smaller, both spherical and elongated form (21, 22). Surface exposure of phosphatidylserine can explain the procoagulant effects of pEVS. Moreover, pEVs were more procoagulant when they were activated by a combination of collagen and thrombin (8). In COVID-19 infection, platelets internalized SARS-CoV-2 virions leading to EVs release (23). However, platelets activation was not directly induced by SARS-CoV-2 or purified spike but rather to the release of active tissue factor by infected cells (24).

Conclusion

Total annexin-V EV levels were increased in severe disease compared to moderate SARS CoV-2 infection and healthy controls, with a significant increase in eEVs, and particularly pEVs. In patients with thromboembolic events, although pEV and eEV levels were not linked to thromboembolic events, we nevertheless showed a slightly higher small/large ratio, especially for pEVs which seems to be an interesting way to explore in the link between severe COVID disease and thrombo-embolic events.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Bord (IRB) for the local Ethics Committee of Amiens Nord-Ouest II and registered on clinicaltrials.gov with the identifier NCT04377490. The patients/participants provided their written informed consent to participate in this study.

Author contributions

VJ, AL, BS and YZ: acquisition of data, analysis and interpretation of data, revising the article critically for important intellectual content, final approval of the version to be submitted. MD: analysis and interpretation of data, final approval of the version to be submitted. SS, EB, IS and JM: acquisition of data, final approval of the version to be submitted. MS and NG: conception and design of the study, interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be submitted.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1186122/full#supplementary-material

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Comparison of platelet-and endothelial-associated biomarkers of disease activity in people hospitalized with Covid-19 with and without HIV co-infection

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Introduction: SARS-CoV-2 elicits a hyper-inflammatory response that contributes to increased morbidity and mortality in patients with COVID-19. In the case of HIV infection, despite effective anti-retroviral therapy, people living with HIV (PLWH) experience chronic systemic immune activation, which renders them particularly vulnerable to the life-threatening pulmonary, cardiovascular and other complications of SARS-CoV-2 co-infection. The focus of the study was a comparison of the concentrations of systemic indicators o\f innate immune dysfunction in SARS-CoV-2-PCR-positive patients (n=174) admitted with COVID-19, 37 of whom were co-infected with HIV.

Methods: Participants were recruited from May 2020 to November 2021. Biomarkers included platelet-associated cytokines, chemokines, and growth factors (IL-1β, IL-6, IL-8, MIP-1α, RANTES, PDGF-BB, TGF-β1 and TNF-α) and endothelial associated markers (IL-1β, IL-1Ra, ICAM-1 and VEGF).

Results: PLWH were significantly younger (p=0.002) and more likely to be female (p=0.001); median CD4+ T-cell count was 256 (IQR 115 -388) cells/μL and the median HIV viral load (VL) was 20 (IQR 20 -12,980) copies/mL. Fractional inspired oxygen (FiO2) was high in both groups, but higher in patients without HIV infection (p=0.0165), reflecting a greater need for oxygen supplementation. With the exception of PDGF-BB, the levels of all the biomarkers of innate immune activation were increased in SARS-CoV-2/HIV-co-infected and SARS-CoV-2/HIV-uninfected sub-groups relative to those of a control group of healthy participants. The magnitudes of the increases in the levels of these biomarkers were comparable between the SARS-CoV-2 -infected sub-groups,

the one exception being RANTES, which was significantly higher in the sub-group without HIV. After adjusting for age, sex, and diabetes in the multivariable model, only the association between HIV status and VEGF was statistically significant (p=0.034). VEGF was significantly higher in PLWH with a CD4+ T-cell count >200 cells/μL (p=0.040) and those with a suppressed VL (p=0.0077).

Discussion: These findings suggest that HIV co-infection is not associated with increased intensity of the systemic innate inflammatory response during SARS-CoV-2 co-infection, which may underpin the equivalent durations of hospital stay, outcome and mortality rates in the SARS-CoV-2/HIV-infected and -uninfected sub-groups investigated in the current study. The apparent association of increased levels of plasma VEGF with SARS-CoV-2/HIV co-infection does, however, merit further investigation.

KEYWORDS

SARS-CoV-2, COVID-19, HIV, platelets, cytokines, chemokines, vascular endothelial growth factor

1 Introduction

Cytokine release syndrome, also referred to as hypercytokinemia or a 'cytokine storm', is a consequence of a dysregulated immune response and has been linked to the pathology seen in individuals presenting with severe COVID-19 (1). High levels of pro-inflammatory cytokines cause symptoms such as fever, hemodynamic instability, coagulopathy, splenomegaly, hepatitis, and multi-organ failure, which can be fatal (1). One of the common causes of mortality in COVID-19 is hypercoagulability (2), with some patients presenting with strokes, myocardial infarcts, pulmonary emboli, mesenteric ischemia, and limb thrombosis (3). Microemboli have also been associated with myocarditis and cardiac failure (2).

While it is still unclear whether the virus itself has intrinsic procoagulant effects, coagulopathy likely stems from the inflammatory response observed in these individuals and the ensuing endothelial activation or damage (4). Some authors have suggested that COVID-19 should be viewed and treated as a 'true vascular disease' and the importance of endothelium-platelet interactions is increasingly recognised (5, 6).

Although the main function of platelets is to maintain vascular integrity through coagulation and angiogenesis, their role in both the innate and adaptive immune response is also well recognised (7). Platelets are involved in the first-line response to invading pathogens, activating an innate immune response through Toll-like receptors (TLRs) (7). Toll-like receptor-7 plays an important role in protecting against viral infections in the innate immune system (8). SARS-CoV-2, a single-stranded ribonucleic acid (ssRNA) virus, binds to TLR-7 within endosomal compartments of cells, including platelets (9). Following binding of ssRNA viruses to TLR-7, the MyD88-dependent pathway is initiated, resulting in upregulated expression of inflammatory cytokines, most notably interleukin (IL)-1, IL-6, IL-10, IL-12, and tumor necrosis factor (TNF)-α, by various immune cells including T-cells, B-cells, macrophages, monocytes, fibroblasts, plasmacytoid dendritic cells, and endothelial cells (10). The upregulated expression of these cytokines is consistent with a skewing of the immune response towards a T helper (Th)2 or Th17 phenotype (10).

It has been shown that platelets interact directly with SARS-CoV-2 and enhance uptake of the virus into various cell types (9). Platelets are hyperactivated during SARS-CoV-2 infection through mechanisms as diverse as epithelial damage, hypoxia, neutrophil extracellular trap formation, interactions between SARS-CoV-2 spike protein and platelets, autoimmune responses, and autocrine activation (6). Activated platelets undergo degranulation and release a number of inflammatory mediators that are stored in their granules (11). In addition, activated platelets also exhibit upregulated expression of adhesion molecules, including platelet-derived growth factor (PDGF) and regulated upon activation normal T-cell expressed and secreted (RANTES), which are both stored in and secreted from platelet α-granules (12).

South Africa has one of highest prevalences of HIV infection in the world, estimated at 13% in the general and 18.7% in the adult population (13). From the start of the pandemic, it was feared that HIV-associated immunodeficiency might predispose people living with HIV (PLWH) to infection with SARS-CoV-2, and that the persistent, systemic immune activation caused by HIV might exacerbate hyperinflammation during COVID-19. Both scenarios could lead to poor outcomes. Notably, an increase in thrombotic events (up to ten-fold higher) has been reported in PLWH receiving antiretroviral therapy (ART) compared to those of the healthy, uninfected population (14). This vulnerable population is, therefore, potentially at an even higher risk of developing thrombosis if co-infected with SARS-CoV-2.

Nevertheless, despite the existence of an extensive body of literature, predominantly encompassing clinical studies and

systematic reviews focused on comparisons of outcomes of patients hospitalized with COVID-19 without and with HIV co-infection, few of these studies have included detailed comparisons of the levels of biomarkers of systemic innate immune activation and their associations with organ dysfunction and outcome. To address this issue, the present study compared associations between the systemic levels of biomarkers of platelet and endothelial activation with clinical parameters and treatment outcomes in people living with and without HIV infection, admitted to hospital with COVID-19 in Tshwane, South Africa.

2 Materials and methods

2.1 Study population and sample collection

In this study, 174 patients who were admitted with COVID-19 (parameters of disease severity can be found in Supplementary Table 1) were recruited from May 2020 until December 2021 from Steve Biko Academic and Tshwane District Hospitals, Pretoria, South Africa. For each patient, approximately 20 milliliters (mL) of blood were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant on the first day of admission, before treatment was commenced. The inclusion criteria were as follows: SARS-CoV-2 polymerase chain reaction (PCR)-positive; 18 years or older; and willing and able to provide informed consent to participate. Samples were processed and stored on the day of venipuncture. Plasma was stored in sterile tubes at -80°C until assayed. Results of routine pathology tests were extracted from the National Health Laboratory Service (NHLS) Trakcare database. Clinical data were recorded by trained clinicians using RedCap (v9.5.36). The study was approved by the University of Pretoria Faculty of Health Sciences Research Ethics Committee (ref. 247/2020). Nine healthy volunteers with no prior history of SARS-CoV-2 or HIV were included as controls for biomarker testing.

2.2 Quantification of cytokines, chemokines, and growth factors

Circulating levels of cytokines were determined in plasma samples using a Bio-Plex Human Cytokine/Chemokine Magnetic Bead Panel Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The concentrations of the following cytokines, chemokines, and growth factors were determined: interleukin (IL)-1β, IL1-receptor antagonist (IL-1Ra), vascular endothelial growth factor A (VEGF-A, also known as just VEGF), IL-6, tumor necrosis factor (TNF)-α, macrophage inflammatory protein-1 alpha (MIP-1α), platelet-derived growth factor (PDGF)-BB, and IL-8. Plasma samples were diluted four-fold and the experimental procedure was followed as per the manufacturer's instructions. Briefly, prepared magnetic beads (50 µL) were added to each of the 96 wells, and the plate was washed twice using an automated magnetic microplate washer (Bio-Rad Laboratories, Inc.). The diluted samples, blanks, standards, and controls (50 µL) were added to appropriately designated wells. The plate was sealed and incubated (protected from light) at room temperature for 45 minutes with gentle agitation on an orbital plate shaker (Thomas Scientific, Swedesboro, NJ, USA). Following the incubation period, the plate was washed three times as described above and detection antibody (25 µL) was added to each well. The plate was sealed and incubated for 30 minutes at room temperature with gentle agitation. The plate was again washed three times followed by the addition of streptavidin-phycoerythrin (50 µL). The plate was sealed and incubated for 15 minutes at room temperature with gentle agitation. The plate was then washed a final three times. The beads were resuspended in 125 µL assay buffer and shaken vigorously for two minutes on a Cooke AM69 microplate shaker (Dynatech AG, Bleichestrasse, ZUG, CH) prior to assay on a Bio-Plex Suspension Array platform (Bio-Rad Laboratories, Inc.). Bio-Plex Manager Software 6.0 was used for bead acquisition and analysis of median fluorescence intensity. Results are presented as picograms per milliliter (pg/mL).

2.2.1 Preparation and dilution of: regulated-on activation, normal T-cell expressed and secreted, intracellular-adhesion molecule-1, and transforming growth factor-β1

The regulated-on activation, normal T-cell expressed and secreted (RANTES) concentrations were determined using the Human RANTES enzyme-linked immunoassay (ELISA) kit (E-EL-H6006, Elabscience Biotechnology, Inc., Houston, TX, USA). Intercellular adhesion molecule 1 (I-CAM-1) levels were measured using the Human I-CAM-1/CD54 ELISA kit (E-EL-H6114, Elabscience, Biotechnology, Inc.). Samples for both assays were thawed at room temperature and diluted to a ratio of 1:20 prior to analysis.

Transforming growth factor-β1 (TGF-β1) levels were determined using the Human TGF-β1 ELISA kit (E-EL-0162, Elabscience Biotechnology, Inc.). Latent TGF-β1 was activated to the immunoreactive form by adding 40 µL 1N hydrochloric acid to 240 µL plasma (diluted 1:8). After mixing thoroughly, the samples were incubated for 10 minutes at room temperature. The samples were then neutralized with the addition of 40 µL 1.2 N sodium hydroxide and mixing thoroughly. The assay was performed immediately.

2.2.2 Procedure for sandwich enzyme-linked immunosorbent assays

Levels of RANTES, I-CAM-1 and TGF-β1 were determined as per the manufacturer's instructions described briefly below.

The standards and appropriately prepared plasma samples (100 µL) were added to the designated wells. The plate was sealed and incubated for 1.5 hours at 37°C. Following the incubation period, the plate contents were discarded followed by the addition of biotinylated detection antibody (100 µL) to each well and the plate incubated for 1 hour at 37°C. The plate was then washed three times using an automated plate washer (BioTek Instruments, Inc., Winooski, VT, USA) followed by the addition of horseradish

peroxidase (HRP) conjugate (100 µL) to each well. The plate was incubated for an additional 30 minutes at 37°C. The plate was washed a further five times as described above followed by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate reagent (100 µL) and a further 15-minute incubation period at 37°C, protected from light. The reaction was stopped by the addition of 50 µL stop solution and the optical density (OD) read at a wavelength of 450 nm using a plate spectrophotometer (BioTek Instruments Inc.). The concentration of the analyte present in each sample was determined using the appropriate generated standard curve and the results are presented as nanograms (ng)/mL.

2.3 Statistical analysis

Clinical information was captured from patient files and entered into a Microsoft excel spreadsheet. Results of routine laboratory tests were obtained from the NHLS. Results from specialized immunological tests were exported from the instrument to the spreadsheet. Double data entry by two independent researchers ensured the accuracy of the records. Data were exported to Stata 17 for analysis. Data were assessed for distribution and appropriate tests applied. The student's t-test and Kruskal-Wallis test were used to compare continuous variables between groups, while Pearson's chi-square and Fisher's exact test were used for univariate comparison of categorical variables. Stepwise, backward, multivariable logistic regression analysis was used to examine associations with HIV status after appropriate transformation of predictor variables. Spearman's correlation test, with Bonferroni correction for multiple comparisons, was used to assess correlations between continuous variables.

3 Results

3.1 Demographic and clinical parameters at the time of hospital admission

A total of 174 patients was recruited. Their mean age was 52 (SD ±14) years and just over half (53.5%) were male. Comorbidities were common: hypertension (42.8%), diabetes mellitus (34%), heart disease (14.5%), kidney disease (9.9%), lung disease (8.9%), and cancer (3.1%). Of the group, 37 (21%) were PLWH. Twenty-six (70.3%) PLWH were on ART, with one having started 3 days before admission. The median CD4+ T-cell count was 256 (IQR 115 – 388) cells/µL and the median HIV viral load (VL) was 20 (IQR 20 – 12,980) copies/mL. Nineteen PLWH (51.4%) had a suppressed VL (i.e. VL <20 copies/mL) at the time of admission. PLWH were significantly younger and more likely to be female. They were also less likely to have diabetes mellitus as a comorbidity, but more likely to have had, or to currently have, active TB. There was no difference in their duration of stay in the hospital, disease severity, or in their outcome. The in-hospital mortality rate for the cohort was 18.6%. The mortality rate in the PLWH group was 12.5% compared to 20% in the non-PLHIV group and the difference was not significant. These data are summarized in Table 1.

With respect to cardiac and pulmonary function on admission, both groups had an elevated pulse and respiratory rate. Patients

TABLE 1 Clinical characteristics of the participants.

Variable		COVID-19 PLWH (n=37)	COVID-19 HIV-uninfected (n=137)	P-value
Age (years)		45.6 (± 11)	53.7 (± 14.6)	**0.002**
Gender (male)		11/37 (29.7%)	82/137 (59.9%)	**0.001**
Hypertension		12/37 (32.4%)	56/122 (45.9%)	0.147
Diabetes		6/37 (16.2%)	48/122 (45.9%)	**0.009**
Heart disease		4/37 (10.8%)	19/122 (15.6%)	0.599
Lung disease		2/37 (5.4%)	12/121 (9.9%)	0.523
Kidney disease		2/37 (5.4%)	15/125 (12%)	0.080
Cancer		2/37 (5.4%)	3/122 (2.5%)	0.330
Current TB		3/36 (8.3%)	1/125 (0.8%)	**0.015**
Past TB		5/36 (13.8%)	2/125 (1.6%)	**0.004**
Overweight		9/35 (25.7%)	36/117 (30.8%)	0.256
Duration of admission		8 (5 – 10)	8 (5 – 12)	0.722
Disease severity:	mild moderate severe	13/35 (37.1%) 18/35 (51.4%) 4/35 (11.4%)	23/114 (20.2%) 62/114 (54.4%) 29/114 (25.4%)	0.061
Outcome (died)		4/32 (12.5%)	22/108 (20%)	0.395

Age is shown as mean (± standard deviation) and duration of admission as median (interquartile range). All other variables are shown as number (percentage).
People living with HIV (PLWH), Tuberculosis (TB).
Values in bold are significant.

without HIV had lower levels of saturation on room air, although this association just missed statistical significance. Fractional inspired oxygen (FiO2) was high in both groups, but significantly higher in patients without HIV infection, reflecting a greater need for oxygen supplementation. The ratio of arterial oxygen partial pressure (PaO2 in mmHg) to FiO2, known as the PF ratio, was low in both groups, indicating moderate acute respiratory distress syndrome (ARDS) (Supplementary Table 2).

Access to specialized scans was unfortunately limited during the pandemic and only ten cases of clinically detectable thrombotic events were reported: eight in people without HIV (8/137 - 5.8%) and two in PLWH (2/37 - 5.4%) (p=0.640).

Routine blood parameters measured at the time of hospital admission are shown in Table 2. PLWH and HIV-uninfected patients had significantly elevated, but equivalent, levels of C-reactive protein (CRP). While ferritin levels were elevated in both groups, PLWH had significantly lower levels of this biomarker. In addition, D-dimers were similarly elevated in both groups, with no differences observed between the groups. Apart from significantly lower hemoglobin levels in PLWH, all other hematological markers were similar. All patients had a notable lymphopenia, but platelet counts were within normal limits. A total of 16 patients had thrombocytopenia: 4 living with and 12 without HIV (p=0.714).

3.2 Systemic concentrations of biomarkers of platelet and endothelial activation

Cytokines, chemokines, and growth factors representative of platelet (IL-8, MIP-1α, RANTES, IL-1β, IL-6, TNF-α, PDGF-BB, TGF-β1) and endothelial cell (VEGF, IL-1Ra, IL-1β, ICAM-1) activation were compared between patients with COVID-19, with and without HIV infection, and healthy control participants (n=9) by means of univariate analysis. The median age of the control group was 44 years (IQR 41 – 47) with a male:female ratio of 2:1. As shown in Table 3, all biomarkers, with the exception of PDGF-BB, were significantly different between the three groups with all markers being higher in patients with COVID-19, with or without HIV infection, than in controls. The only significant difference observed between patients with COVID-19 with and without HIV infection was RANTES, which was significantly lower in PLWH (p=0.034). RANTES levels were significantly higher in people with acute kidney disease [117.78 (36.42-166.66) p=0.0435] and narrowly missed significance in patients with hypertension [96.41 (46.17-163.92), p=0.0511]. In patients with cancer, RANTES levels were significantly lower [56.76 (23.32-64.99), p=0.040]. Male patients had significantly higher levels of RANTES [118.53 (54.17-204.44)] than female patients [68.19 (42.11-114.51), p=0.0016]. The results of the linear regression indicated that age explained 3.35% of the variation in RANTES levels [F(1,169)= 6.89, p=0.0095].

Stepwise backward multivariable logistic regression of all the markers revealed significant associations between HIV and PDGF-BB (OR=0.65; p=0.035), as well as between HIV and VEGF (OR=1.32; p=0.034). After adjusting for age, sex, disease severity, and diabetes in the multivariable model, the only significant association that remained was between HIV status and VEGF (Table 4). Levels of VEGF were higher in people living with hypertension (28.75 [IQR 4.22 - 150.01] versus 4.22 [IQR 4.22 - 72.88]; p=0.0312) but not in those with diabetes (p=0.2181), pre-existing lung disease (p=0.5558), kidney disease (p=0.6602) or cancer (p=0.3564).

TABLE 2 Routine blood parameters at admission.

Variable	COVID-19 PLWH (n=37)	COVID-19 HIV-uninfected (n=137)	Reference range	P-value
CRP (mg/L)	116 (57 – 189.5	105.5 (53 – 195)	<10	0.7819
Ferritin (ug/L)	269.5 (79 – 855)	742.5 (325 – 1576)	5 – 204	**0.0020**
D-dimer (mg/L)	0.77 (0.5 – 5)	0.66 (0.4 – 1.5)	0.00 – 0.25	0.2523
Trop I (ng/L)	10 (10 – 24)	11 (10 - 35)		0.4390
Hb (g/dL)	11.4 (± 2.96)	13.5 (± 2.6)	11.6 – 16.4	**0.0001**
Platelets (L x 10^9/L)	277.5 (208– 359)	263 (202.5 – 324.5)	186 – 454	0.4841
WCC (H x 10^9/L)	7.8 (5.7 – 9.8)	9.3 (6.8 – 11.8)	3.90 – 12.60	0.0509
Neutrophil count (H x 10^9/L)	6.8 (5.4 – 7.7)	6.8 (4.7 – 10.3)	1.60 – 8.30	0.4214
Lymphocyte count (L x 10^9/L)	1.06 (0.75 – 1.61)	1.16 (0.74 – 1.75)	1.40 – 4.50	0.9271
NLR	6.4 (2.8 – 9.7)	6.4 (3.5 – 10.3)		0.7959
PNR	43.2 (32.2 – 70.5)	38.9 (17.0 – 37.1)		0.2970
PLR	310.8 (156.7 – 419.3)	253.9 (153.6 – 347.1)		0.3921

All variables are shown as median (interquartile range) except for Hb which is shown as mean (± standard deviation)
C-reactive protein (CRP), Hemoglobin (Hb), neutrophil lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR), People living with HIV (PLWH), platelet neutrophil ratio (PNR), troponin I (Trop I), white cell count (WCC).
Values in bold are significant.

TABLE 3 Immunological parameters at admission.

Variable	COVID-19 PLWH (n=37)	COVID-19 HIV-uninfected (n=137)	Controls (n=9)	P-value*
ICAM-1 (ng/mL)	153.88 (132.09 – 170.56)	144.19 (121.61 – 183.81)	112.23 (78.97 – 112.36)	**0.0099**
TGF-β1 (ng/mL)	7.35 (5.2 – 11.57)	8.08 (5.82 – 11.85)	5.02 (3.10 – 5.73)	**0.0476**
RANTES (ng/mL)	65.10 (51.62 – 104.93)	98.93 (45.63 – 181.51)	57.29 (36.51 – 60.35)	**0.0371**
IL-1β (pg/mL)	1.95 (1.46 – 2.66)	1.95 (1.46 – 2.89)	1.22 (0.98 – 1.71)	**0.0492**
IL-1Ra (pg/mL)	894.53 (725.59 – 1133.51)	812.8 (631.27 – 1089.68)	237.73 (214.08 – 407.8)	**0.0002**
IL-6 (pg/mL)	5.79 (1.82 – 12.0)	4.32 (1.6 – 9.4)	0.49 (0.3 – 0.54)	**0.0007**
IL-8 (pg/mL)	19.69 (15.32 – 28.36)	17.5 (10.73 – 23.48)	3.58 (2.8 – 6.47)	**0.0001**
MIP-1α (pg/mL)	4.10 (2.69 – 5.17)	3.29 (2.47 – 4.72)	1.3 (0.95 – 1.3)	**0.0001**
PDGF-BB (pg/mL)	460.42 (192.38 – 955.7)	504.48 (253.69 – 1044.48)	392.41 (31.74 – 494.58)	0.0926
TNF-α (pg/mL)	93.16 (73.41 – 118.40)	83.87 (68.6 – 102.39)	55.49 (50.67 – 68.6)	**0.0009**
VEGF (pg/mL)	17.71 (4.22 – 165.71)	4.22 (4.22 – 87.07)	4.22 (4.22 – 4.22)	**0.0480**

All variables are shown as median (interquartile range).
intracellular-adhesion molecule-1 (ICAM-1), transforming growth factor-β1 (TGF-β1), regulated-on activation, normal T-cell expressed and secreted (RANTES), interleukin (IL), IL1-receptor antagonist (IL-1Ra), macrophage inflammatory protein-1 alpha (MIP-1α), platelet-derived growth factor BB (PDGF-BB), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF).
*P-values represent the overall difference between the 3 groups according to the Kruskal-Wallis test. Values in bold indicate significance. P-values for the differences between respective groups, according to the post-hoc Dunn test, are shown in Supplementary Table 1.

In order to determine whether the association with VEGF was due to HIV alone or rather secondary to a SARS-CoV-2/HIV interaction, PLWH with COVID-19 were compared to nine PLWH without COVID-19. The median age of this group was 47 years (IQR 39 - 61); 5 were female and 4 were male. Eight were on ART. The median CD4 count was 196 cells/mm3 (IQR 126 - 349), VL was 79 copies/mL (IQR 20 - 19,900); three had a suppressed VL (≤20 copies/mL) and six an unsuppressed VL. VEGF was significantly higher in PLWH with COVID-19 than in PLWH without COVID-19 (17.71 [IQR 4.22 - 165.71] versus 4.22 [IQR 4.22 - 4.22]; p=0.019). There was no difference in VEGF between the virally suppressed and unsuppressed PLWH without COVID-19 (p=0.2642), although it should be kept in mind that the numbers in these sub-groups were small.

Many variables were significantly correlated with one another, but no correlations were found between the cytokines and either the CD4+ T-cell count or the HIV VL, or between CD4 T-cell count and HIV VL. (Supplementary Figure 1). The heatmap presented in

TABLE 4 Multivariable logistic regression model.

HIV	Odds ratio	Standard Error	Z	P>z	95% confidence interval	
Age	0.9612	0.0159	-2.39	**0.017**	0.9305	0.9929
Sex	0.2580	0.1138	-3.07	**0.002**	0.1087	0.6125
Diabetes	0.2777	0.1484	-2.40	**0.016**	0.0975	0.7913
PDGF-BB	0.7800	0.1828	-1.06	0.289	0.4927	1.2347
VEGF	1.3537	0.1931	2.12	**0.034**	1.0236	1.7902
Estimated baseline odds	10.1942	14.4829	1.63	0.102	0.6296	165.0657

platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF).
Values in bold indicate significance.

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FIGURE 1
Correlation plots of cytokine interactions. **(A)** COVID-19 PLWH, statistically significant positive correlations with rho above 0.5 were identified between the following cytokines: IL-8 and ICAM-1; TGF-β1 and MIP-1α; TGF-β1 and TNF-α; MIP-1α and IL-1β; IL-1β and VEGF; IL-1Ra and IL-6; IL-1Ra and IL-8; MIP1-α and VEGF; and TNF-α and VEGF. **(B)** COVID-19 HIV-uninfected, statistically significant positive correlations with rho above 0.5 were identified between the following cytokines: IL-1Ra and PDGF-BB; MIP-1α and VEGF; PDGF-BB and VEGF; and TNF-α and VEGF. *: $p < 0.05$, **: $p < 0.01$, ***: $p< 0.001$.

Figure 1A represents the correlations in the COVID-19/PLWH group while the heatmap in Figure 1B depicts the results in people with COVID-19 but without HIV.

Just under half of PLWH (17/36 – 47.2%) with CD4+ T-cell count data (36/37 – 97.3%) had a level ≤200 cells/μL. These individuals had non-significantly lower levels of saturation on O2 (94% [IQR 90.5% - 96%] versus 96% [IQR 95% - 98%]; p=0.0564), and equivalent levels of FiO2 (p=0.1647), CRP (p=0.7185) and ferritin (p=0.750) when compared with individuals with counts above 200 cells/μL. They also had significantly higher levels of IL-6 and lower levels of VEGF. Although levels of RANTES and IL-8 were both higher in PLWH with a CD4 ≤200 cells/μL, these differences missed statistical significance (Table 5). Four out of the 17 (23.5%) COVID-19/PLWH patients with a CD4 count ≤200 cells/μL died, compared to none of the 15 (with outcome data) and a CD4 count >200 cells/μL. Levels of all the markers tested differed significantly between COVID-19/PLWH and PLWH controls, suggesting that all the changes observed were induced by COVID-19 in the context of HIV infection.

Eighteen (18/37 – 48.65%) PLWH had a detectable VL (>20 copies/mL). Compared to PLWH with an undetectable VL, they were significantly younger (39.9 ± 11 years versus 51.1 ± 7.7 years; p=0.037), less likely to have hypertension (16.7% versus 47%; p=0.046), diabetes (0% versus 31.6%, p=0.009) or be overweight (0% versus 47.4%; p=0.001). Patients with a detectable VL were also more likely to have active TB, but this difference was not statistically significant (17.6% versus 0%; p=0.095). They had significantly lower levels of TGF-β1, IL-1β, PDGF-BB, TNF-α and VEGF (Table 6). After multivariable logistic regression, only the association between VL and age (p=0.024) and VEGF (p=0.043) remained.

No significant differences in the levels of VEGF could be found between healthy PLWH controls and PLWH co-infected with SARS-CoV-2 with detectable VL. PLWH (19/37-51.35%) with an undetectable VL had significantly higher levels of VEGF than healthy PLWH controls with undetectable VL (126.12 [IQR 4.22–269.27] versus 4.22 [IQR 4.22– 4.22]; p=0.0117) and detectable VL (126.12 [IQR 4.22– 269.27] versus 4.22 [IQR 4.22– 4.22]; p=0.0200).

All four of the PLWH who died had a CD4+ T-cell count of <200 cells/μL, 3/4 had a detectable VL, and two were known not to be on ART.

4 Discussion

As expected, COVID-19 was characterized by high levels of systemic inflammation, as reflected by the acute phase reactant, CRP, which is produced by the liver in response to increased expression of IL-6. Increased levels of CRP lead to elevated concentrations of IL-1β and TNF-α that, in turn, lead to the increased expression of adhesion molecules such as ICAM-1 (15). All the platelet and endothelial markers, with the exception of PDGF-BB, were significantly higher in people with COVID-19 than in the group of healthy controls, indicating the extent of platelet and endothelial activation elicited by SARS-CoV-2 (6).

Overall, levels of inflammation were equivalent between COVID-19 patients with and without HIV infection. While the levels of pro-inflammatory markers, such as CRP, IL-6, IL-1Ra, and TNF-α, were slightly higher, and anti-inflammatory cytokines, such as TGF-β1, were lower in COVID-19/PLWH than in those without HIV, none of these differences was significant. The platelet

TABLE 5 Comparison of immunological markers in PLWH with COVID-19 with low and higher CD4 counts.

Variable	COVID-19 PLWH CD4 ≤200 cells/μL (n=17)	COVID-19 PLWH CD4 >200 cells/μL (n=19)	P-value*
ICAM-1 (ng/mL)	169.192 (144.02–178.61)	153.88 (132.09 –169.78)	0.1303
TGF-β1 (ng/mL)	7.35 (5.08 –11.57)	7.98 (5.20 –17.06)	0.2736
RANTES (ng/mL)	70.22 (59.71–107.02)	64.99 (32.98–82.01)	0.0890
IL-1β (pg/mL)	2.03 (1.71–2.43)	1.95 (1.35–3.69)	0.9494
IL-1Ra (pg/mL)	952.91 (747.98–1175.93)	894.53 (655.64–1133.51)	0.6345
IL-6 (pg/mL)	13.18 (5.39–72.3)	4.32 (1.25–7.28)	**0.0028**
IL-8 (pg/mL)	23.81 (15.32–31.61)	18.59 (14.45–20.24)	0.0769
MIP-1α (pg/mL)	4.8 (3.83–5.17)	3.38 (2.69–5.57)	0.2163
PDGF-BB (pg/mL)	578.07 (222.09–920.74)	378.77 (181.27–1040.34)	0.9873
TNF-α (pg/mL)	93.16 (76.85–116.12)	84.6 (73.41–124.78)	0.8121
VEGF (pg/mL)	4.22 (4.22–72.88)	123.35 (4.22–269.27)	**0.0141**

All variables are shown as median (interquartile range).
intracellular-adhesion molecule-1 (ICAM-1), transforming growth factor-β1 (TGF-β1), regulated-on activation, normal T-cell expressed and secreted (RANTES), interleukin (IL), IL1-receptor antagonist (IL-1Ra), macrophage inflammatory protein-1 alpha (MIP-1α), platelet-derived growth factor BB (PDGF-BB), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF).
Values in bold indicate significance.

chemokine, RANTES, was significantly lower in PLWH but, after adjusting for other factors significantly associated with RANTES, such as age and sex, this difference lost significance. The initial difference observed was therefore likely due to the dissimilar demographic profiles of the patients with and without HIV infection.

The aforementioned systemic, pro-inflammatory biomarker data reflected, and probably explain, the essentially comparable clinical data, which characterized the groups of SARS-CoV-2-HIV-infected and –uninfected patients. While patients with COVID-19 without HIV infection appeared to have a more coordinated immune response, as reflected by the larger number of significant correlations between the immunological markers, this did not translate into differences in levels of inflammation or clinical outcomes. There were, however, a few exceptions. PLWH had lower levels of the acute phase reactant, ferritin, possibly secondary to less hypoxia (supported by the fact that PLWH needed less oxygen supplementation). In contrast, PLWH had higher levels of the coagulation marker, INR, together with non-significantly elevated levels of D-dimer. D-Dimer levels increase on recurrent cycles of coagulation and fibrinolysis, are associated with thrombosis and thromboembolic conditions and are, therefore, considered an important predictor of these events in COVID-19 patients (16). A correlation between D-Dimer and various physiological processes recognized in PLWH have been reported. These include endothelial dysfunction, microbial translocation, and active viral replication, as measured by VL (17–19). Interestingly, an equivalent number of clinical thrombotic events was reported in the two groups, although an important caveat is the absence of specialized scans that could have enabled diagnosis of occult thrombosis.

After multivariable logistic regression that accounted for age, gender, and differences in the prevalence of diabetes between the groups, VEGF emerged as a marker of interest. VEGF is a potent angiogenic factor and inducer of vascular permeability (20). It is released during hypoxia or in inflammatory conditions in response to endothelial injury and has been shown to be significantly increased in patients with COVID-19, correlating with disease severity (20, 21).

The early stage of endothelial activation is characterized by increased expression of ICAM-1 on the surface of endothelial cells to allow trans-endothelial migration of leukocytes to sites of inflammation (20). This is followed by elevation of plasminogen activator inhibitor-1 (PA-1) and soluble thrombomodulin that initiate alterations in the coagulation process, as well as VEGF, which drives angiogenesis (20). The interaction of these biomarkers with leukocytes, smooth muscle cells, and other proinflammatory cytokines, leads to remodeling of the vessel wall and subsequent

TABLE 6 Comparison of immunological markers in PLWH with COVID-19 with detectable and undetectable HIV viral loads.

Variable	COVID-19 PLWH VL >20 copies/mL (n=18)	COVID-19 PLWH VL ≤20 copies/mL (n=19)	P-value*
ICAM-1 (ng/mL)	155.59 (132.09–175.55)	162.49 (134.75–170.56)	0.4158
TGF-β1 (ng/mL)	6.49 (4.41–10.51)	9.15 (6.86–15.12)	**0.0268**
RANTES (ng/mL)	66.31 (58.65–104.93)	64.78 (26.58–83.23)	0.1370
IL-1β (pg/mL)	1.90 (1.46–2.03)	2.43 (1.47–4.93)	**0.0442**
IL-1Ra (pg/mL)	886.04 (439.52–990.82)	979.5 (844.02–1462.14)	0.0833
IL-6 (pg/mL)	4.03 (1.25–31.69)	7.99 (4.32–13.18)	0.2626
IL-8 (pg/mL)	18.29 (12.89–28.23)	20.24 (17.47–29.62)	0.2016
MIP-1α (pg/mL)	3.52 (2.02–4.87)	4.57 (2.99–5.57)	0.0605
PDGF-BB (pg/mL)	307.17 (148.02–578.07)	704.67 (343.84–1052.76)	**0.0167**
TNF-α (pg/mL)	73.96 (60.28–107.82)	113.84 (81.64–148.85)	**0.0025**
VEGF (pg/mL)	4.22 (4.22–66.99)	126.12 (4.22–269.27)	**0.0021**

All variables are shown as median (interquartile range).
intracellular-adhesion molecule-1 (ICAM-1), transforming growth factor-β1 (TGF-β1), regulated-on activation, normal T-cell expressed and secreted (RANTES), interleukin (IL), IL1-receptor antagonist (IL-1Ra), macrophage inflammatory protein-1 alpha (MIP-1α), platelet-derived growth factor BB (PDGF-BB), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF).
Values in bold indicate significance.

vasculopathy (22). In this regard, it is interesting to note that VEGF was strongly and positively correlated with the pro-inflammatory cytokines, IL-1β, TNF-α and the chemokine, MIP-1α, in both groups, indicating the recruitment of inflammatory cells to the newly formed vessels.

HIV has long been known to be associated with higher levels of VEGF than uninfected controls (23). This is generally assumed to be detrimental and associated with the presence of Kaposi's sarcoma, as well as HIV-associated encephalopathy (24). It has, however, also been postulated that VEGF may play a role in maintaining vasculature and protecting against the development of age-related cognitive decline, and may hence represent an appropriate response under conditions of hypoxia (25). In this regard, it is interesting to note that, in our study, VEGF was higher in PLWH but that their oxygen saturation was higher and FiO2 lower, indicating less hypoxia. This suggests that the association between VEGF and HIV is unrelated to hypoxia, a contention also supported by others (26). It is intriguing to consider whether the higher levels of VEGF observed in PLWH could be related to the lower oxygen requirement in this population.

It would seem that the elevated levels of VEGF were caused by the interaction between HIV and SARS-CoV-2, since levels were significantly higher in the presence of co-infection than with HIV infection alone. This interaction was, however, evident only in patients with good HIV control, since levels in the co-infected group with active viral replication had median VEGF levels at the lower limit of detection of the assay. This is further supported by the fact that the difference in VEGF between COVID-19/PLWH and control PLWH was only evident in the presence of an undetectable VL. It is therefore interesting to note that some authors have proposed that VEGF might be a biomarker of a more preserved immune system (27). In this regard, it is noteworthy that PLWH with a low CD4 count and unsuppressed VL, common features of those who had demised, had significantly lower levels of VEGF.

COVID-19/PLWH with a CD4 count ≤200 cells/μL could be distinguished from those with higher CD4 counts by lower levels of VEGF and higher levels of IL-6. While their inflammatory markers were similar, it is notable that all four of the COVID-19/PLWH who died were in this category. This finding should be considered in light of the significant lymphopenia observed in all COVID-19 patients in this study.

5 Conclusion

In conclusion, this study of patients hospitalized with COVID-19 demonstrated a relatively low mortality and good outcomes overall, both in people with and without HIV infection. Although

significantly elevated, no significant differences were observed in the levels of cytokines, chemokines, and growth factors for platelet- and endothelial-associated markers between patients with and without HIV infection, which is in keeping with the clinical findings. The exception was VEGF, which was higher in PLWH. VEGF is a potent angiogenic factor, which could be advantageous under hypoxic conditions. Interestingly, VEGF concentrations were lower in PLWH with a low CD4 count and unsuppressed VL, which could be an indication that these patients are less able to mount an appropriate immune response.

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

Ethics approval was obtained from the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (247/2020). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained for participation from the participants or the participants' legal guardians/next of kin.

Author contributions

TR, FA and VU conceptualized the study. MM, ZB and HS prepared samples and performed the biomarker assays. FA, VU and ZB recruited participants and collected clinical data. MM, ZB, HS, RA and TR wrote the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1235914/full#supplementary-material

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