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Authors
Barrett, Tessa J
Cornwell, MacIntosh
Myndzar, Khrystyna
et al.

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CORONAVIRUS

Platelets amplify endotheliopathy in COVID-19

Tessa J. Barrett[1]*, MacIntosh Cornwell[1,2], Khrystyna Myndzar[1], Christina C. Rolling[1], Yuhe Xia[1], Kamelia Drenkova[1], Antoine Biebuyck[1], Alexander T. Fields[3], Michael Tawil[1], Elliot Luttrell-Williams[1], Eugene Yuriditsky[1], Grace Smith[4], Paolo Cotzia[5,6], Matthew D. Neal[7], Lucy Z. Kornblith[3], Stefania Pittaluga[3], Amy V. Rapkiewicz[8], Hannah M. Burgess[9], Ian Mohr[9], Kenneth A. Stapleford[9], Deepak Voora[10], Kelly Ruggles[2], Judith Hochman[1], Jeffrey S. Berger[1,11]*

Given the evidence for a hyperactive platelet phenotype in COVID-19, we investigated effector cell properties of COVID-19 platelets on endothelial cells (ECs). Integration of EC and platelet RNA sequencing revealed that platelet-released factors in COVID-19 promote an inflammatory hypercoagulable endotheliopathy. We identified *S100A8* and *S100A9* as transcripts enriched in COVID-19 platelets and were induced by megakaryocyte infection with SARS-CoV-2. Consistent with increased gene expression, the heterodimer protein product of *S100A8/A9*, myeloid-related protein (MRP) 8/14, was released to a greater extent by platelets from COVID-19 patients relative to controls. We demonstrate that platelet-derived MRP8/14 activates ECs, promotes an inflammatory hypercoagulable phenotype, and is a significant contributor to poor clinical outcomes in COVID-19 patients. Last, we present evidence that targeting platelet $P2Y_{12}$ represents a promising candidate to reduce proinflammatory platelet-endothelial interactions. Together, these findings demonstrate a previously unappreciated role for platelets and their activation-induced endotheliopathy in COVID-19.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an acute viral illness caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Intense systemic inflammation and hypercoagulability are hallmarks of severe COVID-19 (*1*, *2*). High rates of arterial and venous thrombosis are frequently seen in SARS-CoV-2 infection (*3*–*5*). The etiology of COVID-19–associated coagulopathy remains controversial and is likely to be complex, with contributions from many cellular and noncellular mediators.

The vascular endothelium provides a crucial interface between the blood compartment and tissues. The endothelial cell (EC) monolayer lining blood vessels prevents thrombosis and maintains hemostasis; however, following activation, ECs support the initiation of coagulation and thrombosis, hallmarks of severe COVID-19. Hospitalized COVID-19 patients have increased circulating markers of EC activation, including von Willebrand factor (vWF), plasminogen activator inhibitor 1 (PAI1), intercellular adhesion molecule–1 (ICAM-1), vascular cell adhesion molecule–1 (VCAM-1), and P-selectin (*6*–*10*). Autopsies of COVID-19 patients reveal significant EC apoptosis and loss of EC tight junction integrity in the pulmonary microvasculature (*11*). Despite the suggestion that COVID-19–associated endotheliopathy is mediated by SARS-CoV-2 EC infection (*12*, *13*), recent evidence suggests that indirect (nonviral) mechanisms drive EC activation in COVID-19 (*14*).

Platelets are anucleate blood cells classically known by their roles in hemostasis and thrombosis. Platelets also maintain vascular function and integrity by bidirectional interactions with ECs and leukocytes (*15*). Under inflammatory conditions, the cross-talk between platelets, coagulation, and the endothelium exacerbates local and systemic inflammation (*16*–*20*). Previously, we found that SARS-CoV-2–platelet interactions drive a hyperactive platelet phenotype, and in the context of viral infections, platelets initiate EC proinflammatory activation (*21*). Given that activated platelets release inflammatory factors in the local microenvironment, we hypothesized that platelet-EC interactions in COVID-19 promote endotheliopathy.

Herein we demonstrate that platelets from COVID-19 patients are hyperactive and that platelet-released factors activate microvascular ECs, promoting an inflammatory hypercoagulable phenotype. Investigation of the platelet transcriptome revealed significant differential expression of thousands of genes in COVID-19 and enrichment of pathways related to organelle/granule release, metabolism, and immune effector function. An investigation into platelet candidate genes that code for abundantly released proteins found up-regulation of *S100A8* and *S100A9* in platelets from COVID-19 patients. In vitro, we confirmed that SARS-CoV-2 could alter megakaryocyte gene expression and up-regulate *S100A8* and *S100A9*. Consistent with an increase in transcription, the heterodimer protein product of *S100A8/A9*, myeloid-related protein 8 (MRP8)/MRP14, is released to a greater extent by platelets from COVID-19 patients relative to controls. MRP8/14 activates microvascular ECs and weakens EC cell-cell contacts resulting in increased permeability of the endothelium (*22*), thus representing an important pathogenic mediator of inflammatory platelet-EC interactions in COVID-19.

Exposure of ECs to platelet releasate from COVID-19 patients induced significant transcriptomic and phenotypic changes relative to exposure with platelet releasate from controls. Consistently, we

[1]Department of Medicine, New York University Grossman School of Medicine, New York, NY, USA. [2]Institute for Systems Genetics, New York University Grossman School of Medicine, New York, NY, USA. [3]Department of Surgery, University of California, San Francisco, San Francisco, CA, USA. [4]Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA. [5]Department of Pathology, New York University Grossman School of Medicine, New York, NY, USA. [6]Center for Biospecimen Research, New York University Grossman School of Medicine, New York, NY, USA. [7]Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA. [8]Department of Pathology, NYU Langone Long Island Hospital, New York University Langone Health, Mineola, NY, USA. [9]Department of Microbiology, New York University Langone Health, New York, NY, USA. [10]Department of Medicine, Duke Center for Applied Genomics & Precision Medicine, Duke University School of Medicine, Durham, NC, USA. [11]Department of Surgery, New York University Langone Health, New York, NY, USA.
*Corresponding author. Email: jeffrey.berger@nyulangone.org (J.S.B.); tessa.barrett@nyumc.org (T.J.B.)

found a direct association between platelet-released MRP8/14 and EC activation. EC enrichment in pathways characteristic of cell-cell tight junctions, coagulation, and proinflammatory processes is correlated with the dysregulated COVID-19 platelet transcriptome and the expression of the RNA and protein product of *S100A8/9*. These transcriptomic changes were reflected in the increased production of classical inflammatory cytokines interleukin 6 (IL6) and IL8 and the correlation of the platelet and endothelial transcriptome with platelet activation. Furthermore, in a cohort of 291 hospitalized COVID-19 patients, we found that circulating MRP8/14 levels were associated with subsequent thrombosis and death following multivariable adjustment. Therapeutic targeting of platelet $P2Y_{12}$ decreases *S100A8/A9* platelet mRNA and reduced proinflammatory platelet-endothelial interactions.

Our data collectively indicate that platelets amplify EC dysfunction and mediate thromboinflammation in patients with COVID-19. Strategies to mitigate platelet activation may reduce COVID-19–associated endotheliopathy and improve clinical outcomes linked to thromboinflammation.

RESULTS

COVID-19 platelets induce dysfunction of the microvascular endothelium

Platelet hyperactivity is increasingly recognized as a hallmark of COVID-19 (*10*, *23*–*25*). Consistently, we found increased platelet reactivity in COVID-19 patients as characterized by increased platelet surface expression of P-selectin and CD40 (Fig. 1, A and B, and table S1; $n = 9$ COVID-19 patients and 7 controls) and increased circulating platelet-heteroaggregates (platelet-leukocyte, platelet-neutrophil, and platelet-monocyte aggregates; Fig. 1C). In addition, COVID-19 patients had increased spontaneous platelet aggregation and aggregation in response to submaximal ADP and epinephrine (Fig. 1D).

Patients infected with SARS-CoV-2 are noted to have an endotheliopathy (*11*, *12*). Under inflammatory conditions, platelet-endothelium cross-talk exacerbates inflammation and aberrant immunity and impairs the ability of ECs to maintain vascular homeostasis. The interconnection between platelets and the vasculature in COVID-19 is supported by autopsy data showing platelet-rich thrombi in macro- and microvasculature (*26*), characterized by platelet activation and adhesion to the endothelium (Fig. 1, E and F), increased vascular dysfunction, and thrombosis (*6*, *7*, *10*, *23*, *26*, *27*). Recent data demonstrate that direct endothelial infection of SARS-CoV-2 is unlikely to occur (*14*), suggesting an indirect mechanism. Thus, we investigated platelet-EC interactions in the setting of COVID-19.

Human microvascular ECs were incubated with platelet releasate from COVID-19 patients or controls, and EC RNA sequencing (RNA-seq) was performed (Fig. 1G and table S1; $n = 7$ COVID-19 patients and 7 controls). Treatment of microvascular ECs with COVID-19 releasate significantly altered the EC transcriptome, with 1288 transcripts differentially expressed (adj$P < 0.01$, 485 transcripts down-regulated, 803 transcripts up-regulated; Fig. 1H). Top differentially expressed transcripts separate ECs treated with COVID-19 platelet releasate from controls (Fig. 1I). Gene set enrichment analysis (GSEA) of differentially expressed genes revealed altered pathways associated with cell-cell tight junctions, coagulation, and proinflammatory processes (Fig. 1J), hallmarks of thromboinflammation. To further evaluate gene modules dysregulated in COVID-19, we used weighted correlation network analysis to obtain eigengene representing the relative coexpression of gene sets on a per sample basis (*28*). When comparing the eigengene values of these gene sets between COVID-19 and controls, the apical junction, coagulation, and tumor necrosis factor α (TNFα) signaling pathways show significantly higher expression in ECs treated with COVID-19 platelets relative to controls (Fig. 1K). EC genes involved in inflammation, immunity, and hypercoagulability (e.g., *VWF*, *MMP11*, *MMP2*, *TIMP1*, *ICAM1*, and *ICAM2*) were differentially expressed in platelet COVID-19 releasate–treated ECs (Fig. 1L).

Distinct platelet gene clusters drive microvascular EC coagulation dysfunction

Consistent with others, our group identified a prothrombotic and proinflammatory platelet transcriptome in patients with COVID-19 [GSE176480 (*23*)]. We, therefore, integrated our platelet and EC RNA-seq analyses to gain mechanistic insight underlying platelet-induced EC activation. Focusing on the coagulation pathway, we clustered differentially expressed EC-induced coagulation genes (adj$P < 0.05$) between COVID-19 patients and controls. Unsupervised clustering of these genes resulted in a clear separation of groups (Fig. 2A). To identify COVID-19–regulated platelet transcripts associated with EC coagulation, we investigated platelet transcripts identified as being differentially expressed in COVID-19 patients relative to controls (fig. S1 and table S2; $n = 8$ COVID-19 patients and 10 controls). Correlating our differentially expressed EC coagulation genes (Fig. 2A) with differentially expressed platelet genes in COVID-19 patients (Fig. 2B), we identified three distinct platelet gene clusters (C1 to C3; table S3). Hypergeometric GSEA tests were then performed on each of our three gene clusters to explore their functional makeup. C1, composed of 920 differentially expressed platelet genes, was enriched in pathways associated with organelle/granule release and oxidative phosphorylation, pathways characteristic of an activated platelet phenotype (Fig. 2C) (*29*). The C2 gene cluster (413 differentially expressed genes) was enriched in platelet pathways linked to granule secretion and immune effector functions, while the C3 gene cluster (715 differentially expressed genes) was primarily composed of pathways associated with cytokine and interferon-signaling responses and immune effector processes (Fig. 2, D and E). Collectively, these data highlight how the COVID-19–activated platelet phenotype is directly associated with EC coagulation. Consistent platelet clusters were observed for enriched platelet-mediated EC pathways of cell-cell tight junctions (fig. S2, A and B) and TNFα signaling (fig. S2, C and D).

To further explore the relationship between the transcriptomic signature of COVID-19 platelets and ECs, we repeated the above analysis across up-regulated EC pathways. Consistent in each analysis, three distinct blocks of platelet genes were identified on the basis of unsupervised clustering. Cluster one (C1) shared a 523-platelet gene signature across all EC–up-regulated enriched pathways (Fig. 2F and table S4). GSEA revealed the shared platelet EC-activating signature to be enriched in gene sets linked to membrane protein targeting, oxidative phosphorylation, and viral gene expression (Fig. 2G). These data demonstrate that platelet-activating pathways linked to granule/vesicle release, metabolism, immune effector functions, and response to viral infections induce an EC activation phenotype consistent with the described COVID-19 endotheliopathy.

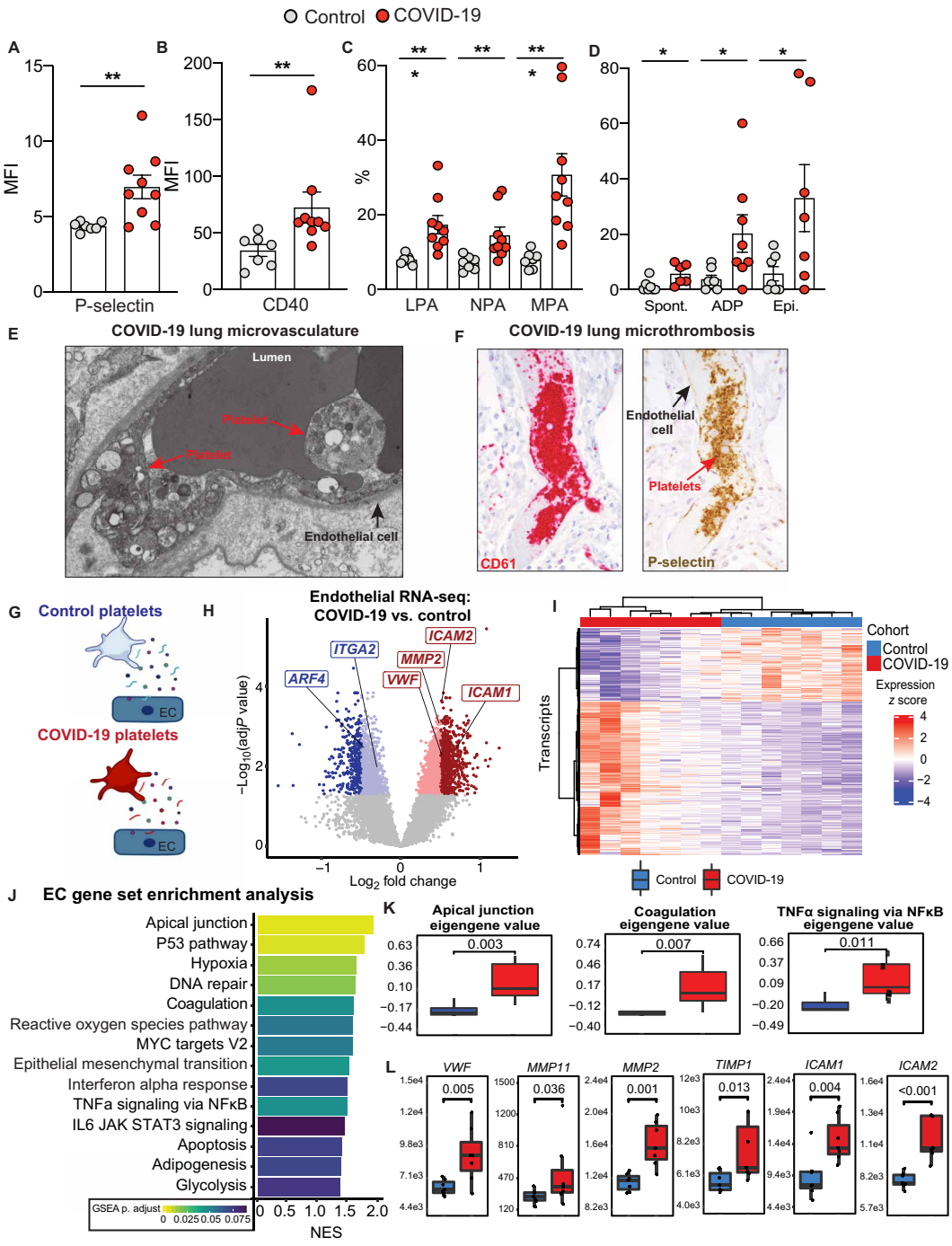

Fig. 1. Platelets from COVID-19 patients are hyperreactive and induce EC activation. (**A**) Platelet P-selectin and (**B**) platelet CD40 expression measured in whole blood by flow cytometry. (**C**) Platelet-leukocyte (LPA), platelet-neutrophil (NPA), and platelet-monocyte (MPA) aggregates measured in whole blood by flow cytometry as assessed by $CD45^{+}CD61^{+}$ events. (**D**) Quantification of platelet aggregation in response to phosphate-buffered saline (PBS) [spontaneous aggregation (Spont.)], 0.1 μM adenosine diphosphate (ADP), and 0.1 μM epinephrine (Epi.). Measurements performed in seven controls donors and nine hospitalized COVID-19 patients. (**E**) Transmission electron microscopy of an autopsy sample from a COVID-19 patient's lung, highlighting the interconnection between platelets and ECs. (**F**) CD61 (red) and P-selectin (brown) immunohistochemistry of lung tissue of a COVID-19 patient, ×40 magnification. (**G**) Schematic of endothelial RNA-seq experiment; microvascular ECs were treated with platelet releasate generated from COVID-19 platelets or controls. (**H**) Volcano plot of differentially expressed transcripts. Colored dots are adj$P < 0.05$; red dots are up-regulated genes, and blue dots are down-regulated genes. (**I**) Unsupervised hierarchical clustering heatmap of differentially expressed transcripts between EC exposed to COVID-19 releasate and control releasate (adj$P < 0.05$, $|\log_2\text{FoldChange}| > 0.5$) and (**J**) enriched pathways between COVID-19 patients and controls, with the bars depicting the normalized enrichment score. (**K**) Boxplots of relative eigengene values for each sample for the apical junction pathway, coagulation pathway, and TNFα signaling pathway. (**L**) Highlighted EC transcripts and their expression levels following exposure to COVID-19 and control patient platelet releasate. EC RNA-seq was performed with platelet releasate from seven controls and seven COVID-19 donors. Reported false discovery rate (FDR)–adjusted *P* values output from DESeq2. $^{*}P < 0.05$, $^{**}P < 0.01$, as determined by a Student's *t*-test.

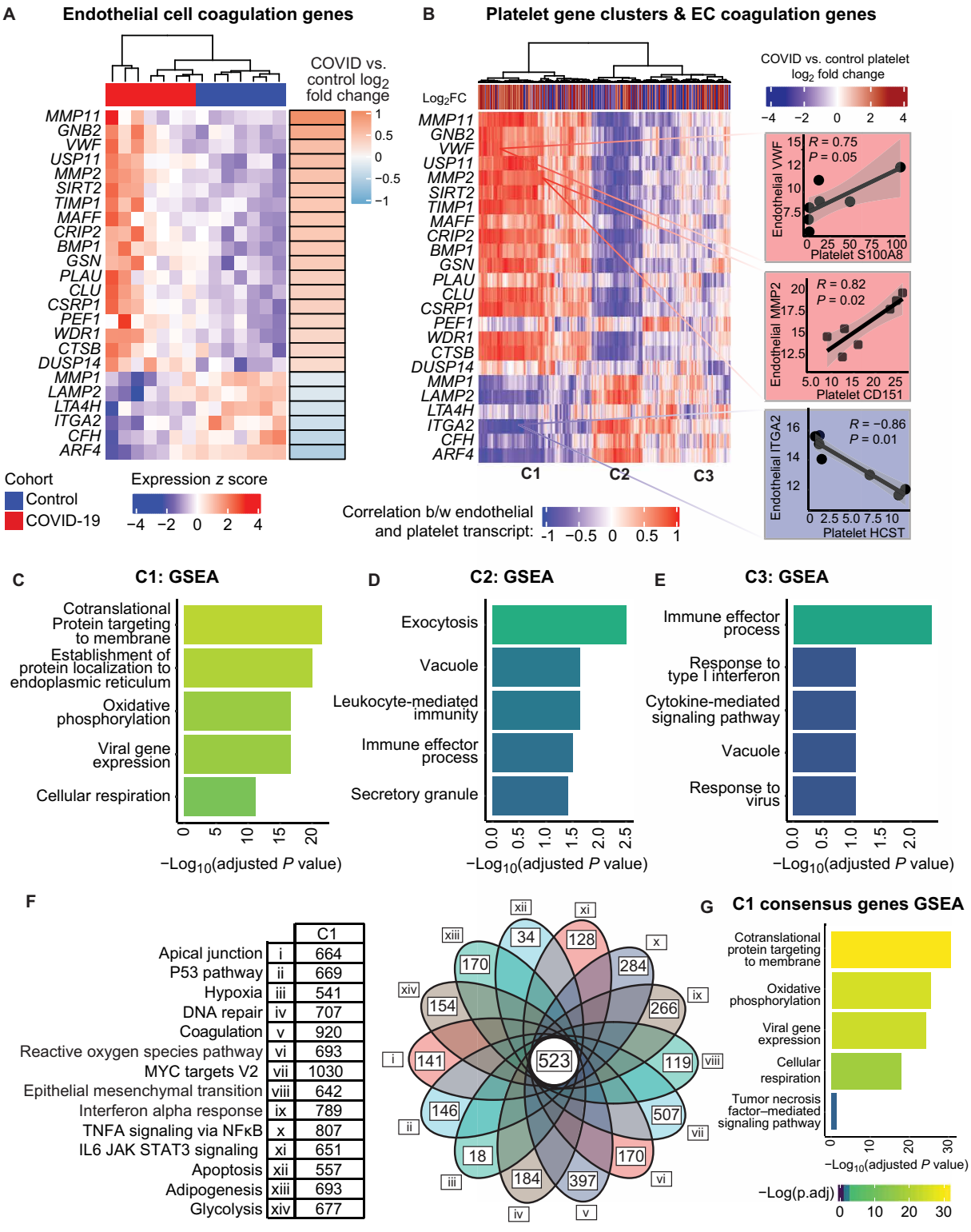

Fig. 2. COVID-19 platelet gene clusters induce a procoagulant EC phenotype. (**A**) Heatmap of differentially expressed endothelial transcripts (adj$P < 0.05$) that are a part of the coagulation pathway. (**B**) Heatmap of the correlation values for the expression of each differentially expressed platelet gene (COVID-19 versus controls) with the expression of each of the differentially expressed endothelial genes in the coagulation pathway. Clusters of interest are demarcated by C1, C2, and C3. Insets of three pairwise correlation show endothelial *VWF* versus platelet *S100A8*, endothelial *MMP2* versus platelet *CD151*, and endothelial *ITGA2* versus platelet *HCST*. (**C** to **E**) GSEA results for C1, C2, and C3. (**F**) Number of platelet genes from the C1 module across differentially expressed EC pathways, and Venn diagram showing overlap of all C1 sets. Labeled sections show the number of genes unique to each signature and the overlap of all C1 platelet gene modules. (**G**) GSEA results for the overlapping C1 gene module.

Platelet S100A8/A9 is induced by SARS-CoV-2 and increased in COVID-19 patients

We next sought to identify platelet-released factors that induce EC dysfunction. Gene ontology pathway analysis of differentially expressed transcripts reveals up-regulation of the platelet degranulation pathway in COVID-19 (fig. S3A). We therefore explored platelet candidate genes that code for abundantly secreted proteins that are differentially expressed in COVID-19 patients. Using platelet RNA-seq metrics of differential expression between COVID-19 and controls (matched for age, sex, race, and comorbidities; table S2, $\log_2$FC and adj*P* value) and overall abundance (base mean) of the gene, we evaluated the top 50 candidates (baseMean > 323, $\log_2$FC > 1.6, adj$P < 1.03 \times 10^{-4}$). Cross-referencing this list of candidate genes with a secretome database (*30*) reduced this number to six genes—*S100A8*, *CST7*, *TIMP1*, *S100A9*, *SMPD1,* and *CETP* (Fig. 3A). Of these six genes, *S100A8* and S100A9 were the only two genes that met all of the above criteria and were also members of the 523–C1 platelet gene signature (Fig. 2F). *S100A8* and *S100A9* genes code for MRP8 and MRP14 and were among the top 4 identified transcripts enriched in platelets from COVID-19 patients to have a released product (Fig. 3, A and B). MRP8 and MRP14 heterodimerize to produce the inflammatory product MRP8/14 (calprotectin), an inflammatory protein present within platelet granules and released by activated platelets (*31*, *32*).

To explore whether viral-megakaryocyte interactions could account for changes to the platelet transcriptome, human CD34-derived megakaryocytes were incubated with SARS-CoV-2 and with a coronavirus responsible for the common cold (CoV-OC43). Treatment led to the detection of SARS-CoV-2 mRNA within the megakaryocytes; however, no uptake of CoV-OC43 was found (fig. S4A). In both primary human megakaryocytes and a megakaryocyte cell line (Meg-01), treatment with SARS-CoV-2 increased *S100A8* and *S100A9* expression ($P < 0.01$; Fig. 3C and fig. S4B). Conversely, treatment with CoV-OC43 had no effect (Fig. 3C and fig. S4B), suggesting a specific SARS-CoV-2–mediated effect.

We next measured MRP8/14 release from platelets and found that COVID-19 platelets released significantly increased levels of MRP8/14 (Fig. 3D; $P < 0.001$). There were also increased circulating plasma levels of MRP8/14 in COVID-19 patients versus controls (Fig. 3E; $P < 0.001$, $n = 9$ COVID-19 patients and 7 control). A positive correlation between platelet-released MRP8/14 and plasma levels was found (Fig. 3F; $R = 0.72$, $P = 0.0037$), suggesting that platelet-released MRP8/14 contributes to the pool of circulating MRP8/14 levels. These data indicate that direct interaction with SARS-CoV-2 increases S100A8/A9 transcription, MRP8/14 production, and release from megakaryocytes and platelets in patients with COVID-19.

Platelet-released MRP8/14 is a driver of EC dysfunction

We next explored the association between platelet-released MRP8/14 and EC activation. *S100A8/A9* platelet mRNA expression significantly correlated with the expression of the dysregulated pathways seen in COVID-19–exposed ECs (Fig. 4A), highlighting the central role of MRP8/14 in driving EC dysfunction. This is further supported by the positive correlation between platelet-released MRP8/14 and COVID-19 EC-enriched pathways (Fig. 4B).

Next, we correlated various metrics of platelet activation and clinical measures to enriched EC pathways to explore further the association between platelet activation and EC dysfunction in COVID-19. In addition to platelet-derived MRP8/14, we measured factors known to be released from platelets that are increased in COVID-19 patients (IL6, P-selectin, and sCD40L) (*10*, *23*, *24*). Expression of dysregulated EC pathways following exposure to COVID-19 platelet releasate was most significantly correlated with platelet-released MRP8/14 (Fig. 4C). Assessment of well-described platelet activity measures—platelet-leukocyte heteroaggregates, surface P-selectin, CD40, PAC-1, CD42b, and platelet aggregation—revealed that although all of these measures correlated with the dysregulated transcriptomic signature, circulating monocyte platelet aggregates (MPAs) were most associated with the demonstrated transcriptomic alterations (Fig. 4C). These data are consistent with previous findings that platelet-derived MRP-14 promotes MPA formation and a proinflammatory monocyte phenotype (*31*). Circulating neutrophil platelet aggregates (NPAs) were weakly correlated with enriched EC dysfunction pathways, suggesting that neutrophils are not a significant contributor to platelet-mediated endotheliopathy in COVID-19.

MRP8/14 activates microvascular ECs and weakens EC cell-cell contacts, resulting in increased permeability of the endothelium (*22*). In further support of an activated endothelium, ECs treated with platelet releasate from COVID-19 patients secrete more significant quantities of the inflammatory cytokines IL8 and IL6 ($P < 0.05$; Fig. 4D). A positive correlation existed between platelet-released MRP8/14 and microvascular EC IL8 and IL6 production ($P = 0.001$ and $P = 0.007$, respectively; Fig. 4E), suggesting that platelet-derived MRP8/14 is a driver of EC activation. MRP8/14 is a ligand for innate receptors, including Toll-like receptor 4 (TLR4), CD36, and receptor for advanced glycation end products (RAGE) (*31*, *33*). Blocking of EC TLR4 and RAGE signaling did not suppress platelet-mediated EC IL6 or IL8 cytokine production (fig. S5). In contrast, silencing CD36 significantly suppressed COVID-19 platelet-mediated EC IL6 and IL8 cytokine production (Fig. 4F). These data suggest that platelet-released MRP8/14 mediate the proinflammatory effect of COVID-19 EC inflammation via CD36. The clinical relevance of these findings is further supported by COVID-19 autopsy data showing significant lung endothelial IL6 production (fig. S6).

MRP8/14 associates with adverse clinical events

To examine the clinical relevance of our findings, we investigated the association between MRP8/14 levels in a cohort of hospitalized COVID-19 patients. Plasma samples were obtained on the day of admission from 291 patients hospitalized with COVID-19 within the New York University (NYU) Langone Health network (Fig. 5A). The median age was 65 years, 169 (58.1%) were female, and 148 (50.9%) had hypertension (Table 1). Death or a thrombotic event occurred in 86 patients [44 deaths and 54 thrombotic events (22 deep venous thrombosis, 11 pulmonary embolism, 8 myocardial infarction, 6 stroke, and 7 others)]. COVID-19 patients were compared to a cohort of 21 patients admitted to the hospital who tested negative for SARS-CoV-2. Circulating MRP8/14 levels were significantly higher in COVID-19 patients than diseased controls (Fig. 5B and table S7). Among COVID-19 patients, MRP8/14 was significantly higher in patients who went on to develop an adverse clinical event (thrombosis, critical illness; Fig. 5, C and D, and fig. S7). Following adjustment for age, sex, race/ethnicity, body mass index (BMI), diabetes, chronic obstructive pulmonary disease (COPD)/asthma, history of coronary artery disease or cancer, antiplatelet and anticoagulant therapy, plasma MRP8/14 levels were independently associated with thrombosis [adjusted odds ratio (adjOR), 1.58; 95% confidence interval (CI), 1.27 to 1.99; $P < 0.001$] and other adverse events (Fig. 5E). When stratified into quartiles, COVID-19 patients with the highest

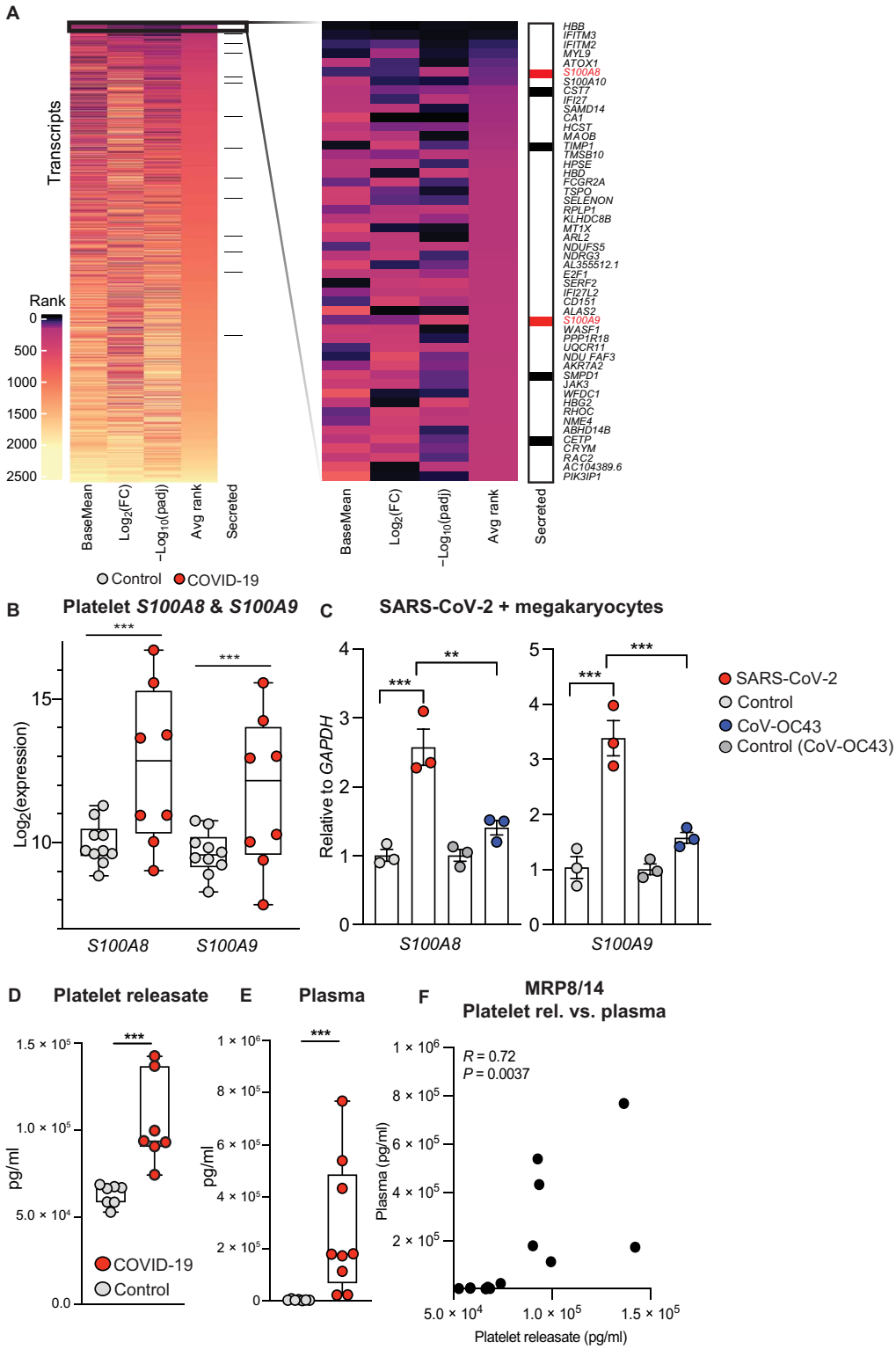

Fig. 3. SARS-CoV-2 up-regulates platelet and megakaryocyte *S100A8/A9* and MRP8/14 release. (**A**) Rank analysis of differentially expressed genes in COVID-19 platelets versus control platelets based on baseMean expression, $\log_2$ (FoldChange), and adjusted *P* value between COVID-19 and control platelets. Subset zooms in on the top 50 candidates, with *S100A8* and *S100A9* highlighted. (**B**) Platelet mRNA expression of *S100A8* and *S100A9*. *N* = 7 samples per group; ***$P < 0.001$ FDR-adjusted *P* values output from DESeq2. (**C**) CD34-derived megakaryocyte mRNA expression of *S100A8* and *S100A9* following treatment with PBS, SARS-CoV-2, or CoV-OC43 for 24 hours. *N* = 3 samples per group. **$P < 0.005$, ***$P < 0.001$ by one-way analysis of variance (ANOVA). (**D** and **E**) Platelet releasate and plasma concentration of MRP8/14. *N* = 7 to 9 samples per group; means ± SEM; **$P < 0.005$, ***$P < 0.0005$, as determined by Student's *t* test. (**F**) Correlation between plasma and platelet releasate MRP8/14.

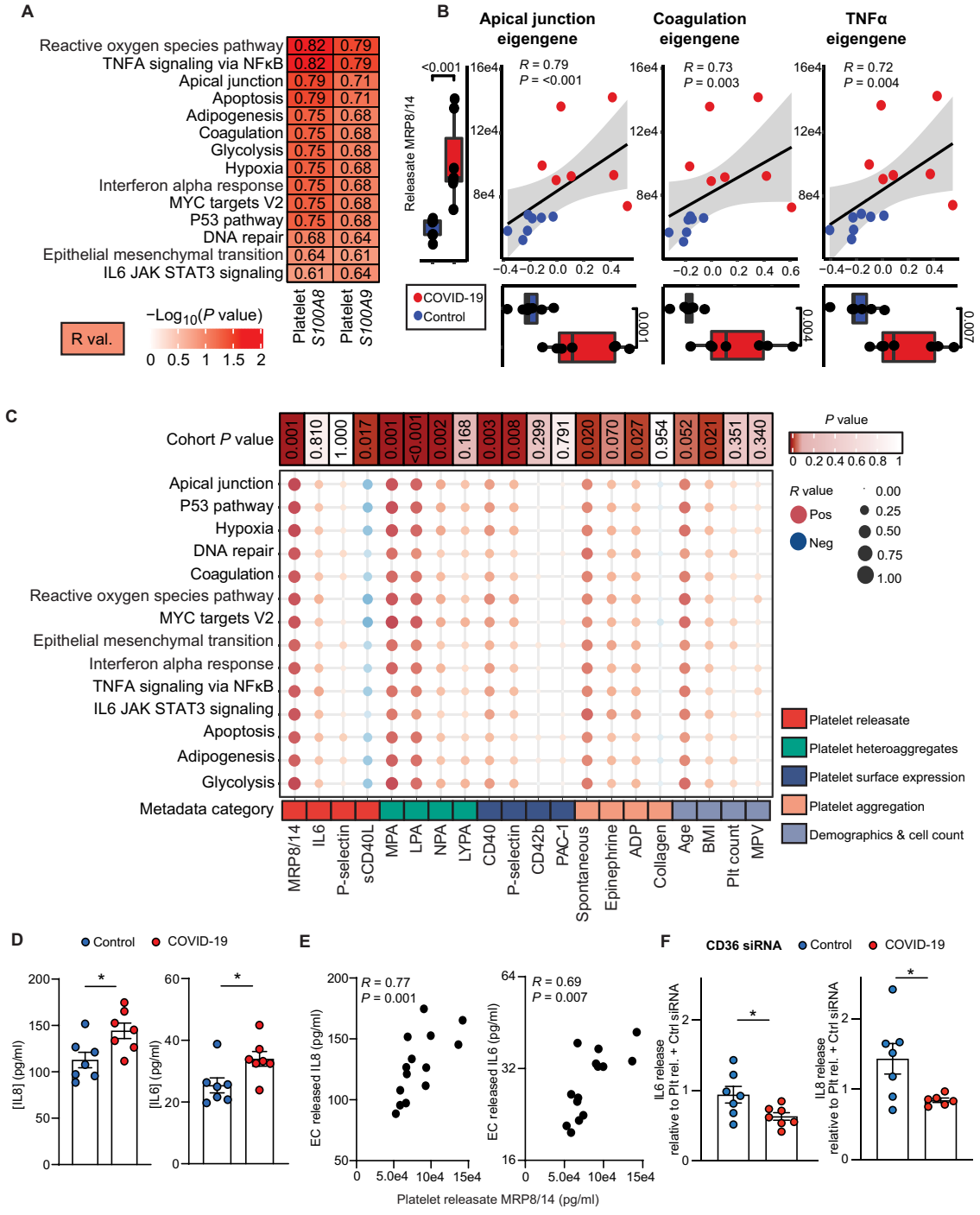

Fig. 4. Platelet-released MRP8/14 induce EC activation. (**A**) Correlation of platelet *S100A8/9* mRNA expression and the expression of identified EC pathways enriched following exposure to COVID-19–derived platelet releasate. (**B**) Boxplots of platelet MRP8/14 releasate in COVID-19 and control samples (*y* axis) and boxplots of the relative eigengene values for the apical junction, coagulation, and TNFα signaling pathways (*x* axis). Correlation of the respective values shown in scatterplots. (**C**) Dotplot depicting the correlation value of the relative eigengene expression of each pathway to its respective metadata metric. Pathways dysregulated in ECs are shown on the *y* axis, and various metadata are shown on the *x* axis. The top annotation shows the Wilcoxon *P* value for the population difference of the respective metadata metric between COVID-19 samples and controls. Platelet heteroaggregates: MPA, leukocyte platelet aggregates (LPA), NPA, lymphocyte platelet aggregates (LYPA). Platelet aggregation: Spontaneous (PBS), submaximal ADP (0.1 μM), epinephrine (0.1 μM), collagen (0.2 μM). Mean platelet volume (MPV). (**D**) Quantification of IL8 and IL6 release from microvascular ECs in response to treatment with platelet releasate isolated from COVID-19 patients or controls. Data are means ± SEM; **P* < 0.05, as determined by *t* test. (**E**) Correlation between released IL6 and IL8 by microvascular ECs and MRP8/14 concentration in platelet releasate. (**F**) Quantification of IL8 and IL6 release from microvascular ECs in response to treatment with platelet releasate isolated from COVID-19 patients or controls. CD36 silencing and EC transfection with CD36 small interfering RNA (siRNA) (or ctrl siRNA) for 72 hours before addition of platelet releasate. Data are expressed relative to each corresponding vehicle control + platelet releasate and are means ± SEM; **P* < 0.05, as determined by *t* test; *n* = 6 to 7 subjects per group.

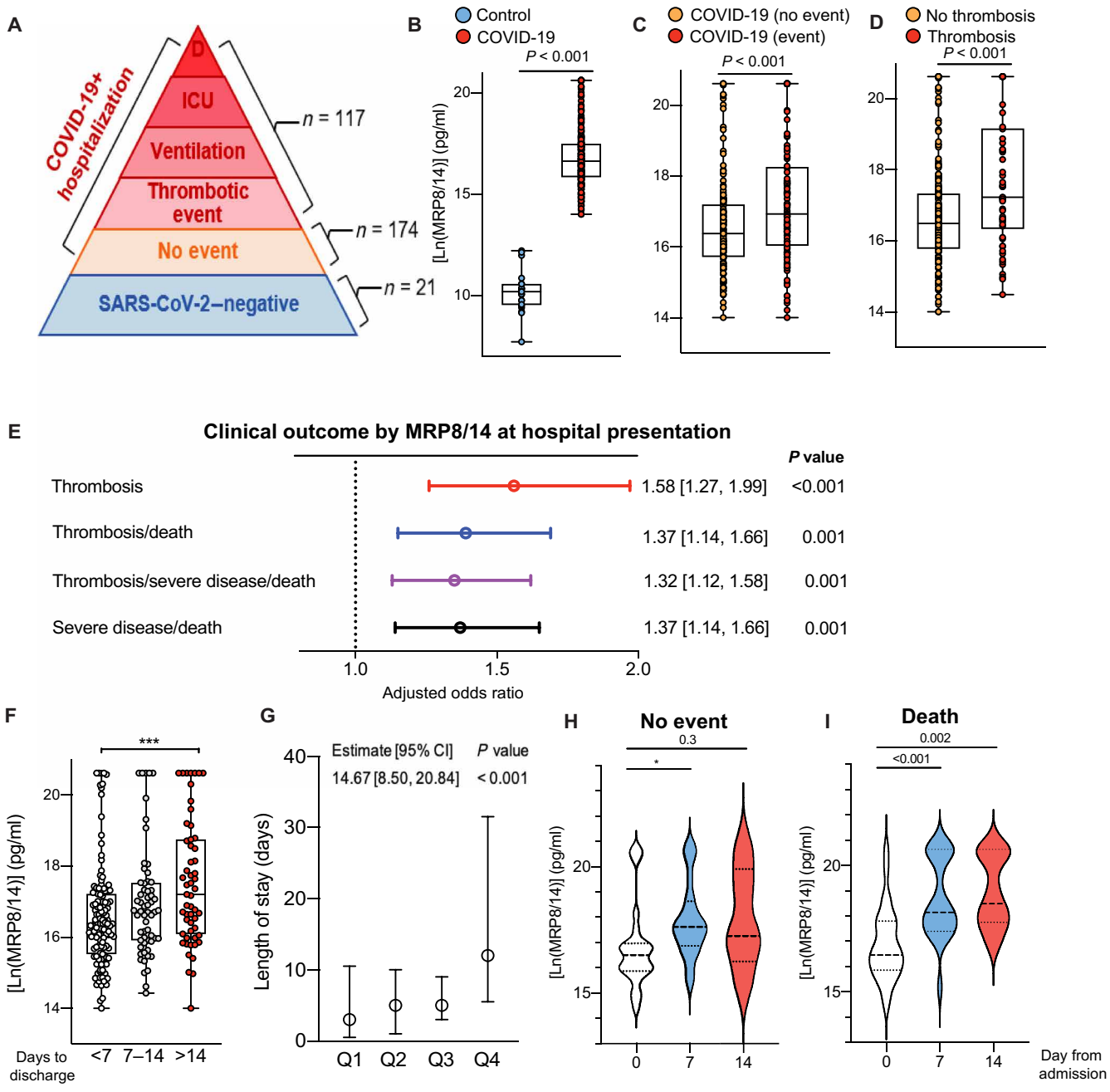

Fig. 5. Circulating MRP8/14 correlate with adverse clinical outcomes in hospitalized COVID-19 patients. (**A**) Summary of COVID-19 and control patient plasma samples collected at hospital admission. (**B** and **C**) Plasma MRP8/14 in controls ($n = 21$) versus COVID-19 patients ($n = 291$) and in COVID-19 patients stratified by adverse clinical event ($n = 174$) or no event ($n = 117$). (**D**) Plasma MRP8/14 in COVID-19 patients stratified by thrombosis (thrombosis, $n = 54$; no thrombosis, $n = 237$). (**E**) Adjusted ORs with corresponding 95% CIs for the outcomes of thrombosis, thrombosis or death, thrombosis or critically ill or death, or critically ill or death based on admission plasma MRP8/14 in COVID-19 patients. Adjusted ORs from logistic regression analysis for log-transformed biomarker levels adjusted for age, sex, race/ethnicity, BMI, diabetes, COPD/asthma, and history of coronary artery disease or cancer. (**F**) Admission MRP8/14 levels and days to discharge. (**G**) Length of stay as stratified by quartiles based on admission MRP8/14 levels, and linear regression analysis between baseline plasma MRP8/14 concentration and length of hospital stay. Longitudinal MRP8/14 measures (**H**) in those without a clinical event and (**I**) in those who subsequently died. Wilcoxon signed-rank test was performed to measure the difference of MRP8/14 levels between days 7 and 14 versus baseline. *$P < 0.05$, **$P < 0.005$.

admission MRP8/14 levels had a greater than threefold higher odds of thrombosis (adjOR, 3.36; 95% CI, 1.68 to 6.76; $P = 0.001$) and other adverse events (fig. S8). Of the 247 patients who were discharged, the mean hospital stay was 13.1 days. Circulating MRP8/14 was higher in patients with a longer length of stay (Fig. 5, F and G). After multivariable adjustment, patients in the highest quartile of MRP8/14 at baseline were in the hospital for an excess of 12.9 (6.8 to 19.0) days compared to quartiles 1 to 3.

Table 1. Demographics of COVID-19 subjects.

	Overall population (*n* = 291)
Age, median (IQR)	65 (54, 75)
Sex	
Female, *n* (%)	169 (58.1)
Race	
White, *n* (%)	148 (52.9)
African American, *n* (%)	42 (15.0)
Hispanic, *n* (%)	6 (2.1)
Asian, *n* (%)	33 (11.8)
Other, *n* (%)	49 (17.5)
Unknown or declined, *n* (%)	2 (0.7)
Comorbidities	
BMI, median (IQR)	27.4 (23.6, 31. 9)
Asthma, *n* (%)	26 (8.9)
COPD, *n* (%)	15 (5.2)
Coronary artery disease, *n* (%)	37 (12.7)
Current smoker, *n* (%)	10 (3.6)
Diabetes, *n* (%)	76 (26.1)
Hyperlipidemia, *n* (%)	75 (25.8)
Hypertension, *n* (%)	148 (50.9)
Vitals on admission	
Temperature, °C, median (IQR)	37.3 (36.8, 38.2)
SpO_2, %, median (IQR)	92.0 (86.0, 96.0)
Platelet indices on admission	
Platelet count ($10^3/\mu l$), median (IQR)	199 (157, 255)
Mean platelet volume (fl), median (IQR)	10.6 (10.0, 11.4)
Initial anticoagulant	
Prophylactic dose heparin, *n* (%)	181 (62.2)
Therapeutic dose heparin, *n* (%)	3 (1.0)
Oral anticoagulant*, *n* (%)	19 (6.5)
No record of anticoagulant, *n* (%)	88 (30.2)
Antiplatelet therapy	
Aspirin, *n* (%)	61 (20.9)
Clopidogrel, *n* (%)	7 (2.4)
Clinical outcomes	
Death, *n* (%)	44 (15.1)
ICU, *n* (%)	81 (27.8)
Ventilation, *n* (%)	70 (24.1)
Thrombosis, *n* (%)	54 (18.6)
Severe disease†, *n* (%)	107 (36.8)

*Oral anticoagulant, warfarin, apixaban, or rivaroxaban. †Severe disease (critically ill)—a composite outcome of ICU, mechanical ventilation, or death.

In a subset of 107 hospitalized COVID-19 patients, circulating MRP8/14 was measured longitudinally. In patients without a clinical event, there was no difference between admission and day 14 (Fig. 5H), while in those who subsequently died, MRP8/14 increased ≈8-fold between baseline and day 14 ($P = 0.002$; Fig. 5I). Similar trends were also found for those who went on to experience a thrombotic event and other adverse events (fig. S9, A to C). Together, these data indicate that circulating MRP8/14 levels are higher in COVID-19 patients and associate with thrombosis, critical illness, and length of stay among those hospitalized.

$P2Y_{12}$ inhibition reduces platelet *S100A8* and *S100A9* and EC activation

As COVID-19 patients remain at increased risk for thrombosis, critical illness, and death with and without anticoagulant therapy (*3*), we investigated the effect of antiplatelet therapy on platelet *S100A8* and *S100A9* mRNA and platelet-induced EC activation. A cohort of healthy controls received aspirin ($n = 63$) daily or ticagrelor ($n = 49$) twice daily for 4 weeks (table S7). Blood was collected and platelet RNA was extracted at baseline and following 4 weeks of therapy. While aspirin had no significant effect (Fig. 6A), ticagrelor led to a significant reduction in both expression of platelet *S100A8* and *S100A9* mRNA ($P < 0.0001$; Fig. 6, B and C). To assess whether antiplatelet therapy could suppress the platelet-mediated proinflammatory effects on ECs, isolated platelets were incubated with aspirin, a $P2Y_{12}$ antagonist (AZD1283), or a glycoprotein IIb/IIIa inhibitor (eptifibatide). Incubation of platelet releasate generated in the presence of different antiplatelet drugs found that the $P2Y_{12}$ antagonist had the greatest reduction in proinflammatory EC gene expression (29% reduction in *IL6*, 51% reduction in *IL8*, and 32% reduction in *CCL20*; Fig. 6D).

DISCUSSION

Severe COVID-19 is associated with a hypercoagulable state and robust inflammatory response that predisposes patients to macro- and microthrombotic events (*34*–*38*). The coordinated activation of the inflammatory and thrombotic response has been termed thromboinflammation. Levels of prothrombotic acute phase mediators, including fibrinogen, vWF, and D-dimer, are increased in COVID-19 patients, implicating the endothelium, platelets, and the coagulation system as likely mediators of thromboinflammation in COVID-19. The resulting endotheliopathy is thought to link inflammation, immune dysregulation, and thrombosis, and be a driver of poor clinical outcomes in COVID-19 patients (*39*, *40*). Despite suggestions that SARS-CoV-2 triggers endotheliopathy (*12*, *13*), recent experimental evidence supports indirect activating mechanisms driving EC injury in COVID-19 (*14*).

Platelets are first responders to vascular injury and act to bridge the immune system and thrombosis via activation and release of hemostatic and inflammatory mediators. Activated platelets secrete a host of proinflammatory mediators in the local microenvironment, altering the endothelium's inflammatory and adhesive properties (*18*, *19*). Activated platelets isolated from patients with inflammatory disease, such as systemic lupus erythematosus (SLE), HIV, and psoriasis, induce a proinflammatory EC phenotype (*16*, *20*, *21*). We and others previously reported that COVID-19 is associated with a hyperactive platelet phenotype (*23*, *24*), characterized by increased platelet activation markers and a proinflammatory, prothrombotic

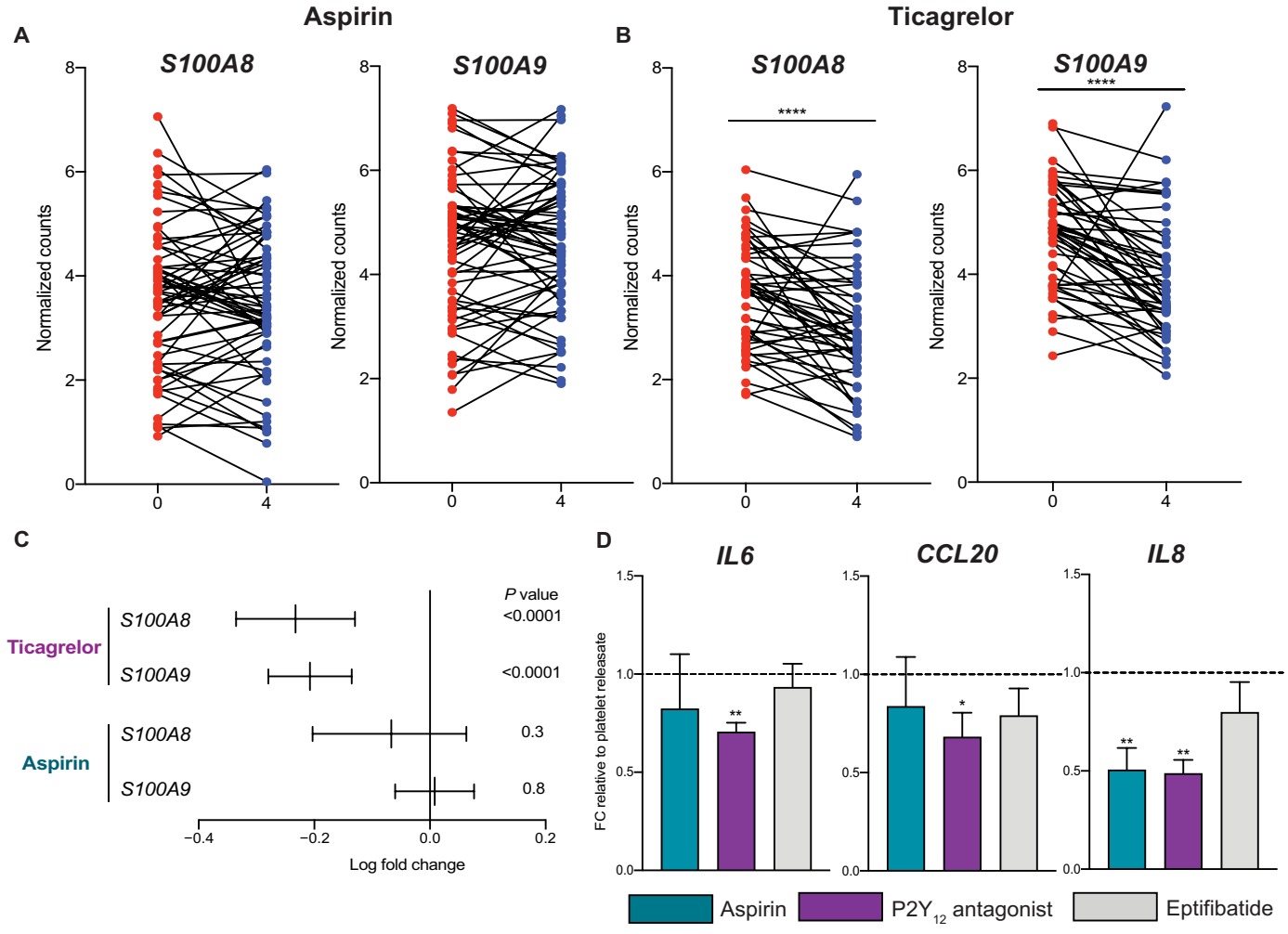

Fig. 6. $P2Y_{12}$ inhibition reduces platelet *S100A8* and *S100A9* expression and platelet-mediated EC activation. A cohort of patients received (**A**) aspirin daily ($n = 63$) or (**B**) ticagrelor (90 mg) twice daily for 4 weeks ($n = 49$). Blood was collected and platelet RNA was extracted before the commencement of the study and following 4 weeks of antiplatelet therapy. Plots depict normalized expression counts of *S100A8* and *S100A9* at baseline and following 4 weeks of antiplatelet therapy. ****$P < 0.0001$ as determined by paired *t* test. (**C**) Forest plot of log fold change (LogFC) between baseline and follow-up platelet *S100A8* and *S100A9* expression. Data are means and 95% CI. (**D**) Expression of *IL6*, *IL8*, and *CCL20* following exposure of ECs to platelet releasate generated in the presence of aspirin (1 mM), AZD1283 (1 μM, $P2Y_{12}$ inhibitor), or eptifibatide (18 μM, glycoprotein IIb/IIIa inhibitor) for 6 hours. Data expressed relative to platelet releasate–treated cells in the absence of platelet inhibitors. $n = 6$ unique releasate donors. Data are means ± SEM, expressed relative to the platelet releasate–treated ECs; *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ by paired *t* test.

transcriptome. Given the interconnection between activated platelets and the microvasculature, we explored whether platelet-released factors contribute to COVID-19 endotheliopathy.

Our study details how COVID-19 hyperactive platelets release factors that induce a proinflammatory and hypercoagulable endothelium (Fig. 7). The change in EC transcription directly correlated with alterations in the platelet transcriptome, connecting differences seen in these complementary cell types. Sequencing of the platelets used to incubate with ECs found a set of 523 platelet genes that are consistently associated with the proinflammatory EC transcriptomic pathway changes. This block of genes is potentially a unique COVID-19 platelet signature that merits future exploration and study.

Unbiased screening of candidate platelet genes differentially expressed in COVID-19 led to the identification of *S100A8* and *S100A9*, and their protein product MRP8/14, as a likely candidate of platelet-mediated endotheliopathy. Platelet *S100A8/A9* mRNA and platelet-derived MRP8/14 were increased in patients with COVID, and their expression and translation positively correlate with changes seen in EC coagulation and inflammatory pathways and the production of IL6 and IL8, proinflammatory cytokines that regulate EC adhesion molecule expression (*41*). While circulating plasma MRP8/14 was once considered only to be leukocyte-derived (*42*), platelets and megakaryocytes serve as an additional source of MRP8/14 (*31*, *32*, *43*, *44*). While circulating plasma levels of MRP8/14 alone correlated with EC coagulation and inflammatory pathways, platelet *S100A8/A9* mRNA and releasate MRP8/14 levels were more tightly associated with EC dysfunction directly, suggesting the importance of platelet-derived MRP8/14 on EC dysfunction. Our present study does not rule out other cellular and noncellular contributors of COVID-19 endotheliopathy, which may act synergistically with MRP8/14. For example, serum antiphospholipid (aPL) antibodies in COVID-19 sera were recently reported to induce adhesion

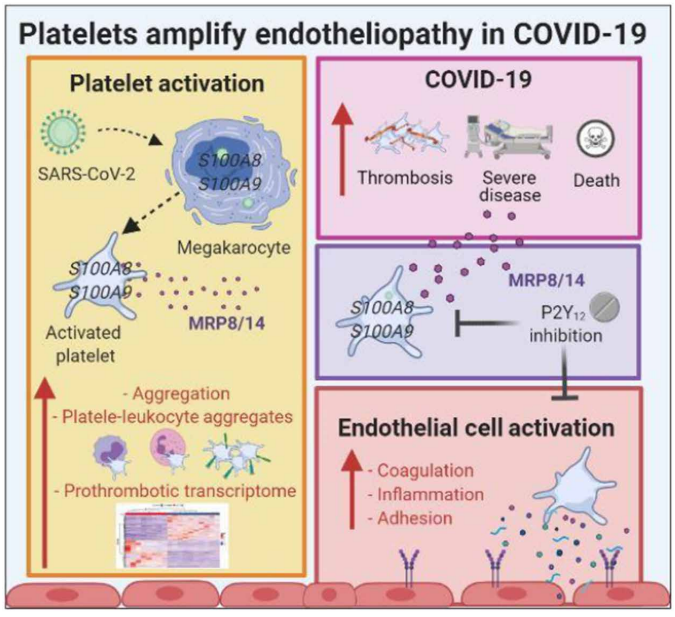

Fig. 7. Proposed mechanism. Schematic overview of the proposed mechanism of platelet-induced endotheliopathy.

molecule expression on ECs (*45*). Previous studies have found that aPL antibodies promote platelet activation and their release of MRP8/14 into the microenvironment, providing evidence of additional mechanisms by which interaction of platelets with the local inflammatory environment in COVID-19 contributes to EC dysfunction (*32*, *46*).

We find that the direct interaction of SARS-CoV-2 with primary megakaryocytes induces *S100A8*/*A9* expression, a finding that has clinical implications given our observation that megakaryocytes from COVID-19 patients contain SARS-CoV-2 virions (*26*), and single-cell sequencing data reporting up-regulation of *S100A9* in circulating megakaryocytes in COVID-19 (*27*). Intriguingly, up-regulation of *S100A8/A9* does not occur following exposure of megakaryocytes to a coronavirus responsible for the common cold (CoV-OC43), highlighting that, independent of indirect mechanisms (e.g., systemic inflammation), SARS-CoV-2 can mediate proinflammatory changes to megakaryocytes and platelets (*47*, *48*). Our data indicate that increased platelet *S100A8*/*A9* mRNA translates to increased platelet MRP8/14 release, thus indicating that direct interactions of megakaryocytes with SARS-CoV-2 enhance proinflammatory platelet-EC interactions. Consistently, MRP8/14 is increased in activated platelets and platelets isolated from patients with peripheral artery disease and ST-elevation myocardial infarction. In vitro, MRP8/14 induces platelet surface P-selectin (*31*), a major ligand in the platelet-EC interaction. In addition, MRP-14$^{-/-}$ knockout mice had reduced expression of P-selectin in response to collagen and arachidonic acid, which was independent of platelet aggregation (*44*).

The prothrombotic and proinflammatory nature of platelet MRP8/14 is supported by studies finding platelet transcript expression of *S100A8/A9*, positively correlates with plasma MRP8/14 concentrations, and is predictive of future atherothrombotic events (*31*, *43*). MRP8/14 levels are increased in patients with cardiovascular diseases, metabolic disease, and several inflammatory conditions, including rheumatoid arthritis, SLE, and psoriasis (*31*, *43*, *49*–*51*). Prior studies have found increased MRP8/14 in patients infected with SARS-CoV-2 (*52*, *53*). In the current study, we find platelet *S100A8* and *S100A9* mRNA to be increased in COVID-19 patients and a corresponding increase in platelet-released MRP8/14, all of which correlate with circulating MRP8/14. Among 291 patients hospitalized with COVID-19, circulating MRP8/14 increased during hospitalization. When measured at baseline, MRP8/14 correlates with incident thrombotic events, severe disease, and length of stay. In contrast to a small increase in MRP8/14 among patients with nonsevere illness, levels increased up to eightfold in COVID-19 patients who subsequently died.

Recently, the therapeutic potential of suppressing MRP8/14 signaling has been suggested as an approach to reduce COVID-19–associated thromboinflammation (*54*). To understand the clinical potential of our findings, we investigated the (i) effect of antiplatelet therapy on *S100A8/A9* platelet mRNA and (ii) in vitro effect of antiplatelet treatment on platelet-induced EC activation. In contrast to aspirin, targeting of $P2Y_{12}$ with ticagrelor reduced *S100A8* and *S100A9* platelet mRNA. In vitro experiments found that $P2Y_{12}$ inhibition significantly attenuated the COVID-19 platelet–mediated EC activation. The ability of aspirin to attenuate the platelet-EC interaction was less pronounced. The benefit of reducing platelet MRP8/14 release via targeting of $P2Y_{12}$ is supported by murine studies, which found that the antithrombotic effect of the S100A9 vaccination is equivalent to that with clopidogrel (*55*). Furthermore, given evidence that $P2Y_{12}$ inhibitors reduce platelet release of proinflammatory α-granule contents (*56*) and the formation of platelet-leukocyte aggregates (*57*), the beneficial effect of this approach is hypothesized to be multifaceted. These data collectively suggest that $P2Y_{12}$ inhibition will mitigate platelet activation, reduce platelet-released MRP8/14, and ultimately suppress platelet activity and EC activation and improve clinical outcomes linked to COVID-19 thromboinflammation. While observational data suggested a potential benefit of aspirin on outcomes in hospitalized COVID-19 patients (*58*, *59*), a large randomized trial of nearly 15,000 participants found that aspirin did not improve its primary end point of 28-day mortality (*60*). The clinical effect of a $P2Y_{12}$ inhibitor strategy is being investigated in ACTIV4a (NCT04505774).

Our study describes how platelets isolated from hospitalized COVID-19 patients release significant quantities of MRP8/14 that can act in a paracrine fashion on nearby ECs promoting endotheliopathy. Several limitations exist with our current study: (i) the relatively small sample size used for sequencing; (ii) the cellular source of plasma MRP8/14 cannot definitively be attributed to platelets and may originate from other cells, including neutrophils; (iii) it is unknown whether the observed MRP8/14 induced endotheliopathy occurs in other viral settings; (iv) a clinical diagnosis of thrombosis during hospitalization may be underestimated because imaging studies were limited due to concerns of transmitting infection or competing risk of death; and (v) the effect of antiplatelet therapies on the COVID-19 platelet–induced EC activation was not explored. Future studies will aim to address these limitations to further our understanding of the interaction of platelets with SARS-CoV-2 and other viral illnesses and the endothelium. An ongoing multinational trial is underway exploring the effect of $P2Y_{12}$ inhibition on clinical outcomes of hospitalized COVID-19 patients (NCT04505774).

In conclusion, we define a novel role for platelet-induced endotheliopathy in COVID-19. The activated platelet phenotype observed

in COVID-19 consistently induces a proinflammatory and dysfunctional endothelium. Platelet-derived MRP8/14 is increased in COVID-19 and induces endothelial injury. Circulating MRP8/14 has the potential to serve as a useful biomarker of thrombosis and severity of disease in patients infected with SARS-CoV-2. Platelet-directed therapy, specifically $P2Y_{12}$ inhibitors, may represent a particularly attractive therapy because of its effect on platelet *S100A8/A9* and platelet-induced endotheliopathy (Fig. 7).

MATERIALS AND METHODS

Population

Clinical cohort no. 1: A cohort of hospitalized COVID-19 patients was recruited from NYU Langone Health between 12 March and 2 May 2020. SARS-CoV-2 infection was confirmed by reverse transcription polymerase chain reaction (RT-PCR), in accordance with current standards. All COVID-19 patients and non–COVID-19 controls were recruited under study protocols approved by the NYU Langone Health Institutional Review Board. Each study participant or their legal authorized representative gave written informed consent for study enrollment in accordance with the Declaration of Helsinki. For COVID-19 patients, enrollment criteria included age greater than 18, hospital admission, positive SARS-CoV-2 testing, and informed consent. COVID-19 patients were monitored until discharge or death.

Clinical cohort no. 2: Healthy volunteers were recruited at the Duke Clinical Research Unit (DCRU; Durham, NC) (*61*). Written informed consent was obtained from each patient, and the studies were approved by the Duke University Health System institutional review board. Study participants (30 to 75 years) did not have a history of a bleeding disorder, gastrointestinal bleeding, intracranial bleeding, or known prior gastric ulcer without documented resolution; did not have known severe hepatic impairment; had not undergone surgery within the past 6 months; had not undergone prior gastric bypass surgery that could interfere with study drug absorption; and did not have an aspirin allergy or known intolerance to aspirin. Participants could not be taking any prescription medications other than oral contraceptives; could not exhibit regular use of tobacco or nicotine products or any known active use of illicit substances; and could not have a known pregnancy or be currently breastfeeding. Participants who were already taking aspirin underwent a 4-week washout period, with the approval of their primary care physician before enrollment in the study.

Platelet activity

Platelet flow cytometry

Circulating MPA, platelet P-selectin, and platelet CD40 were identified in citrate anticoagulated blood as previously described (*62*). Detailed methods are provided in the Supplementary Materials.

Platelet aggregation

Aggregation was measured using light transmission aggregometry (Helena AggRAM, Beaumont, TX) at 37°C under stirred conditions as previously described (*31*). Platelet aggregation was assessed in response to 0.1 μM adenosine diphosphate (ADP), 0.1 μM epinephrine, and phosphate-buffered saline and reported as aggregation at 180 s.

Generation of platelet releasate

Citrated anticoagulated blood samples was allowed to rest at room temperature for 15 min after phlebotomy. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 200*g* for 10 min. PRP was added to 1:10 (v/v) ACD-A [tris-sodium citrate (25 g/liter), glucose (20 g/liter), and citric acid (14 g/liter)] and spun at 1000*g* for 10 min. The platelet pellet was washed in Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 5.5 mM glucose, and 10 mM Hepes, pH 7.4) and 1 μM prostaglandin E_1 (Sigma-Aldrich), before centrifugation at 1000*g* for 10 min and resuspension at 500,000/μl in Tyrode's buffer containing 2 mM $CaCl_2$, and incubation at room temperature for 30 min. Platelet-secreted factors were then collected by isolation of the supernatant following centrifugation at 1000*g* for 3 min. Platelet supernatants were stored at −80°C before use. Protocol for generation of platelet releasate in the presence of platelet inhibitors is provided in the Supplementary Materials.

RNA sequencing

Platelet and endothelial RNA-seq details for the NYU and Duke cohorts and corresponding bioinformatic analyses are provided in the Supplementary Materials.

Plasma MRP8/14 quantification

Plasma sample collection

Blood samples were collected in PST tubes (BD Diagnostics), and plasma was collected by centrifugation, stored at 5°C for 5 days, aliquoted, and stored long term at −80°C. Plasma levels of MRP8/14 were measured by a LEGENDplex bead-based immunoassay (BioLegend, product no. 740891), according to the manufacturer's instructions.

EC cytokine production

IL6 and IL8 concentrations in the microvascular EC supernatants were measured by a LEGENDplex bead-based immunoassay (BioLegend, product no. 740907), according to the manufacturer's instructions.

Primary megakaryocyte culture

Purified peripheral blood $CD34^+$ cells were obtained from the New York Blood Center (New York, NY). Cells were cultured in StemSpan SFEM II (STEMCELL Technologies) supplemented with stem cell factor (100 ng/ml; R&D Systems) and thrombopoietin (TPO; 50 ng/ml; R&D Systems) for 5 days. Cells were cultured with TPO (50 ng/ml) only from days 5 to 11. Megakaryocytes were incubated with SARS-CoV-2 or CoV-OC43 on day 11. SARS-CoV-2 or CoV-OC43 details are provided in the Supplementary Materials.

Statistical analysis

We used Student's *t* test or the Mann-Whitney *U* test for the analyses of continuous variables, as appropriate. Differences between categorical variables were calculated with the use of chi-square or Fisher's exact test, as appropriate. Correlation analyses were performed by the Spearman rank correlation method. Logarithmic transformation of the data was performed when appropriate. Logistic regression was used to assess the association between MRP8/14 and outcomes. Results are presented as ORs along with their 95% CIs. Two-sided *P* values of <0.05 were considered statistically significant. Statistical analyses were performed with R version 3.5.2.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abh2434

View/request a protocol for this paper from *Bio-protocol*.

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