

BRIEF REPORT

COVID-19 patients exhibit reduced procoagulant platelet responses

Frederik Denorme¹  | Bhanu Kanth Manne¹  | Irina Portier¹ | Aaron C. Petrey^{1,2} | Elizabeth A. Middleton^{1,3} | Benjamin T. Kile⁴ | Matthew T. Rondina^{1,2,3,5}  | Robert A. Campbell^{1,3}  

¹University of Utah Molecular Medicine Program, Salt Lake City, UT, USA

²Division of Microbiology and Immunology, Department of Pathology, University of Utah, Salt Lake City, UT, USA

³Department of Internal Medicine, University of Utah, Salt Lake City, UT, USA

⁴Faculty of Health and Medical Sciences, The University of Adelaide, Adelaide, SA, Australia

⁵George E. Wahlen VAMC Department of Internal Medicine and GRECC, Salt Lake City, UT, USA

Correspondence

Robert A. Campbell, Eccles Institute of Human Genetics, University of Utah Health Sciences Center, 15 North 2030 East, Bldg 533, Suite 4225, Salt Lake City, UT 84112, USA.

Email: rcampbell@u2m2.utah.edu

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Abstract

Background: Emerging evidence implicates dysfunctional platelet responses in thrombotic complications in COVID-19 patients. Platelets are important players in inflammation-induced thrombosis. In particular, procoagulant platelets support thrombin generation and mediate thromboinflammation.

Objectives: To examine if procoagulant platelet formation is altered in COVID-19 patients and if procoagulant platelets contribute to pulmonary thrombosis.

Patients/Methods: Healthy donors and COVID-19 patients were recruited from the University of Utah Hospital System. Platelets were isolated and procoagulant platelet formation measured by annexin V binding as well as mitochondrial function were examined. We utilized mice lacking the ability to form procoagulant platelets (CypD^{plt-/-}) to examine the role of procoagulant platelets in pulmonary thrombosis.

Results and Conclusions: We observed that platelets isolated from COVID-19 patients had a reduced ability to become procoagulant compared to those from matched healthy donors, as evidenced by reduced mitochondrial depolarization and phosphatidylserine exposure following dual stimulation with thrombin and convulxin. To understand what impact reduced procoagulant platelet responses might have in vivo, we subjected mice with a platelet-specific deletion of cyclophilin D, which are deficient in procoagulant platelet formation, to a model of pulmonary microvascular thrombosis. Mice with platelets lacking cyclophilin D died significantly faster from pulmonary microvascular thrombosis compared to littermate wild-type controls. These results suggest dysregulated procoagulant platelet responses may contribute to thrombotic complications during SARS-CoV-2 infection.

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KEYWORDS

COVID-19, infections, mitochondria, platelet activation, thrombosis

1 | INTRODUCTION

Thrombotic complications are common in patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).¹⁻³ In particular, intravascular pulmonary thrombosis is believed to contribute to acute respiratory distress syndrome (ARDS) and mortality in COVID-19 patients.⁴ Previously, we demonstrated that neutrophil extracellular traps (NETs) and platelets accumulate in the lung microvasculature of COVID-19 patients.⁵ Furthermore, platelets from COVID-19 patients were hyperresponsive to agonist stimulation and interacted more with leukocytes at baseline, indicating crosstalk between platelets and leukocytes during SARS-CoV-2 infection.⁶

Upon strong activation by classical agonists, a subpopulation of platelets becomes procoagulant. These procoagulant platelets have distinct properties, including the ability to support thrombin generation, an increased tendency to interact with leukocytes, and a reduced capacity to aggregate.⁷ Recently, we and others described a critical role for procoagulant platelets in the formation of detrimental platelet-neutrophil aggregates during reperfusion injury.^{8,9} Interestingly, in septic patients,¹⁰ as well as patients infected with human immunodeficiency virus (HIV)¹¹ and dengue,¹² elevated levels of procoagulant platelets have been reported. Whether procoagulant platelet responses are altered in COVID-19 patients is currently unknown.

In this study, we report our observation that procoagulant platelet responses are reduced in COVID-19 patients, compared to healthy donors. Additionally, mice with impaired procoagulant platelet responses died faster in a model of pulmonary microvascular thrombosis. These results warrant further investigation into the role of procoagulant platelets in the pathophysiology of COVID-19.

2 | METHODS

2.1 | Study design

Eleven patients with acute SARS-CoV-2 infection were recruited from 17 to 20 March 17 2020 at the University of Utah Health Sciences Center in Salt Lake City. SARS-CoV-2 infection was confirmed molecularly by reverse transcription polymerase chain reaction (RT-PCR), in accordance with current standards (Table 1).¹³ All COVID-19 patients were identified under study protocols approved by the Institutional Review Board (IRB) of the University of Utah (IRB#: 00102638, 00093575). Eleven healthy, age- and gender-matched donors were enrolled under a separate IRB protocol (IRB#: 0051506). Each study participant or their legal authorized

Essentials

- Procoagulant platelet formation regulates thrombin generation and thromboinflammation.
- COVID-19 patients have increased thrombosis and inflammation, but the contributions of procoagulant platelets to the pathophysiology of COVID-19 is unknown.
- We observed less procoagulant platelet formation in COVID-19 patients due, in part, to mitochondrial dysfunction.
- Mice lacking the ability to form procoagulant platelets surprisingly had increased mortality in a pulmonary thrombosis model.

representative gave written informed consent for study enrollment in accordance with the Declaration of Helsinki. Enrollment criteria for COVID-19 patients included age >18 years, respiratory symptoms (cough, shortness of breath) or fever, hospital admission, positive SARS-CoV-2 testing, and written informed consent. The standard of care at the University of Utah does not include screening ultrasound, and no clinically diagnosed thrombotic events occurred in our patient population. From each COVID-19 patient or healthy donor, we collected ~60 mL of anticoagulant citrate dextrose (ACD)-anticoagulated whole blood via venipuncture for assays described below.

2.2 | Flow cytometry

To detect phosphatidylserine positive (PS+) platelets, washed platelets were resuspended in Medium 199 (12-117 F; Lonza) containing calcium and stimulated for 15 minutes with thrombin (1.0 U/mL, final, Thermo Fisher) and convulxin (250 ng/mL, final, Santa Cruz) in the presence of fluorescein isothiocyanate (FITC)-labelled annexin V and anti-human CD41 Allophycocyanin (APC). In some experiments, platelets were stimulated with A23187 (50 μ mol/L, final, Sigma Aldrich) in the presence of FITC-labelled Annexin V and anti-human CD41 APC. To measure mitochondrial membrane potential and mitochondrial reactive oxygen species, washed platelets were resuspended in M199 in the presence of mitotracker green (200 nmol/L, final) and tetramethylrhodamine methyl ester (TMRM; 60 nmol/L, final) or MitoSox Red (200 nmol/L, final) for 40 minutes at 37°C in the presence or absence of thrombin and convulxin. Samples were immediately run on a Beckman Coulter Cytoflex. TMRM and MitoSox Red median fluorescence

TABLE 1 Clinical characteristics of COVID-19 patients

	Healthy donors (n = 11)	Hospitalized COVID-19 patients (n = 11)	Reference range ^a
Age (mean, \pm SD)	55.6 (\pm 15.8)	60.7 (\pm 11.5)	–
Male (%)	45.5%	45.8%	–
Hispanic/Latino (%)	9%	54.5%	–
Diabetes (%)	0%	45.5%	–
Hypertension (%)	0%	54.5%	–
SOFA score (median [range])	–	4 [0-9]	–
ARDS (%)	–	63.6%	–
Mechanical ventilation (%)	–	18.2%	–
Survival to date (%)	–	91%	–
Platelet count (K/ μ L, mean \pm SD)	–	225.8 \pm 43.8	159-439 K/ μ L
MPV (fL, mean \pm SD)	–	10.20 \pm 0.84	8.6-12.3 fL
Aspirin (%)	0.0%	0.0%	–
Hydroxychloroquine (%)	–	9%	–
Remdesivir (%)	–	18.2%	–
Convalescent plasma (%)	–	9%	–

Abbreviations: ARDS, acute respiratory distress syndrome; MPV, mean platelet volume; SD, standard deviation; SOFA, sequential organ failure assessment.

^a Reference range from ARUP Laboratories as of 26 August 2020.

intensity (MFI) were normalized to MitoTracker Green MFI. For TMRM, subsequently the fold change relative to baseline was calculated.

2.3 | Pulmonary thrombosis model

All animal experiments complied with the regulatory standards of the University of Utah. Pulmonary thrombosis experiments were performed as described previously in mice with a platelet specific deficiency in CypD (CypD^{plt^{-/-}}) and their littermate controls (CypD^{plt^{+/+}}).¹⁴ Mice lacking CypD are unable to form procoagulant platelets. A mixture of collagen (0.4 mg/kg; Chronolog) and epinephrine (30 mg/kg; Sigma-Aldrich) in 100 μ L of phosphate buffered saline (PBS) was administered through retro-orbital injection. Time until cessation of respiration (time needed to the onset of respiratory arrest that lasted at least 2-3 minutes) was recorded.

2.4 | Statistical analyses

For all analyses, continuous variables were assessed for normality with skewness and kurtosis tests. Summary statistics were used to describe the study cohort and clinical variables are expressed as the mean \pm standard deviation or as a number and percentage (%). Parametric two-tailed t-tests or analysis of variance were used for continuous variables. Statistical analyses were performed by using GraphPad Prism (version 7, San Diego, CA). A two-tailed P-value < .05 was considered statistically significant.

3 | RESULTS AND DISCUSSION

Pulmonary thrombotic events significantly contribute to COVID-19 pathology.^{4,15,16} As platelets are key players in thrombosis under systemic inflammatory settings, we investigated procoagulant platelet responses in COVID-19 patients. Unlike dengue¹² or HIV-infected patients,¹¹ COVID-19 patients had similar levels of annexin V positive, procoagulant platelets at baseline compared to healthy donors (Figure 1A-B). To assess procoagulant platelet formation, platelets from COVID-19 patients or healthy donors were stimulated with thrombin and the glycoprotein VI (GPVI) agonist convulxin, and phosphatidylserine (PS) exposure was assessed using annexin V binding. Interestingly, dual agonist stimulation induced significantly less PS exposure in COVID-19 patients compared to healthy donors (Figure 1A-B). Procoagulant platelet responses were similarly reduced in non-intensive care unit (ICU) and ICU patients ($P = .7$, Figure 1B) and did not correlate with disease severity (data not shown). In contrast, platelets from healthy donors and COVID-19 patients stimulated with the Ca²⁺ ionophore A23187 displayed similar levels of PS exposure (Figure 1C-D). The latter result suggests that reduced procoagulant platelet responses observed in COVID-19 patients are likely not due to defective or impaired PS scramblase activity.^{17,18}

Formation of the mitochondrial permeability transition pore is a critical event in the generation of procoagulant platelets^{19,20} and results in a decrease in mitochondrial membrane potential and the subsequent release of mitochondrial Ca²⁺ initiating PS exposure. To investigate whether dysfunction of the mitochondrial permeability transition pore contributes to our observations in COVID-19

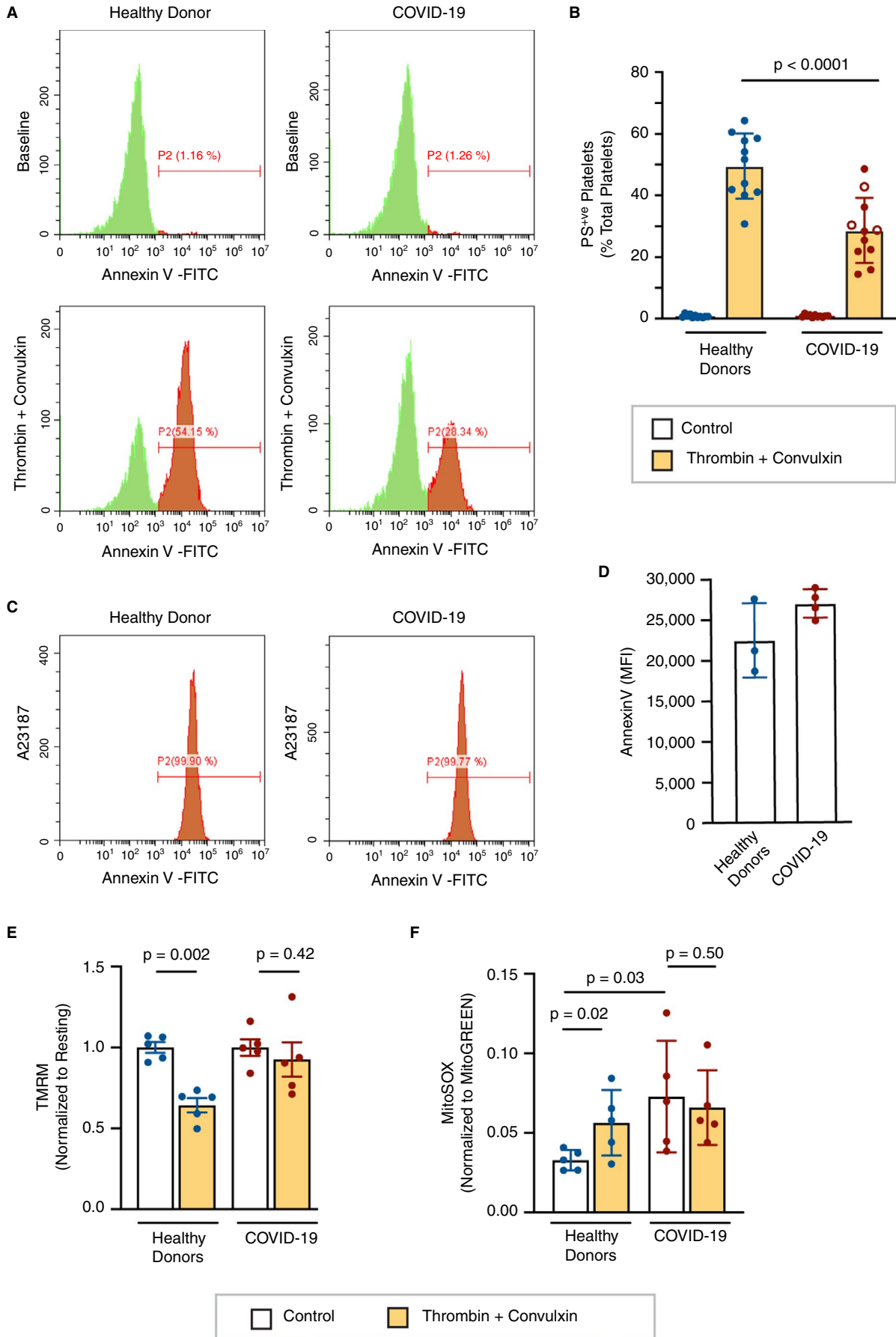


FIGURE 1 Altered mitochondrial function reduces procoagulant platelet formation. Platelets were isolated from healthy donors and COVID-19 patients. A, Phosphatidylserine (PS) exposure was assessed by flow cytometry using annexin V binding under resting conditions and after dual agonist stimulation (1 U/mL thrombin + 250 ng/mL convulxin). Representative flow plots are shown. B, The percentage of platelets with high PS exposure was quantified ($n = 11$). Open circles represent non-intensive care unit (ICU) patients, closed circles represent ICU patients. C, Platelet PS exposure in response to 50 $\mu\text{mol/L}$ A23187 was measured by annexin V binding. Representative flow plots are shown. D, Mean fluorescence intensity (MFI) of annexin V staining after A23187 treatment ($n = 3-4$). E, Platelet mitochondrial membrane potential was measured by tetramethylrhodamine methyl ester staining analyzed by flow cytometry under resting conditions and after dual agonist stimulation (1 U/mL thrombin + 250 ng/mL convulxin; $n = 5$). F, Mitochondrial reactive oxygen species was measured by MitoSOX staining analyzed by flow cytometry under resting conditions and after dual agonist stimulation (1 U/mL thrombin + 250 ng/mL convulxin; $n = 5$)

platelets, we measured changes in mitochondrial membrane potential following dual agonist stimulation by TMRM staining. As expected, dual agonist stimulation induced a significant decrease in mitochondrial membrane potential compared to resting platelets from healthy donors. Consistent with the lack of PS exposure, we did not observe a significant loss of mitochondrial membrane potential upon dual agonist stimulation of platelets from COVID-19 patients (Figure 1E). These results indicate dysregulated procoagulant platelet responses in COVID-19 patients are due to defects upstream or at the point of the mitochondrial permeability transition pore.

Because PS exposure is dependent on mitochondrial function^{18,21} and alterations in mitochondrial function are known to alter procoagulant platelet formation,²² we next assessed the level of mitochondrial reactive oxygen species (ROS), a marker of mitochondrial function. Platelets from COVID-19 patients exhibited higher mitochondrial ROS levels at baseline compared to those from healthy donors (Figure 1F), consistent with mitochondrial dysfunction in platelets during SARS-CoV-2 infection. Furthermore, dual agonist stimulation of platelets from COVID-19 patients did not elicit further

increases in mitochondrial ROS generation (Figure 1F). This aligns with a defect in mitochondrial permeability transition, as opening of the mitochondrial permeability transition pore is known to result in increased mitochondrial ROS.²²

Pulmonary microvascular thrombosis is a common finding in COVID-19 patients. To investigate the contribution of reduced procoagulant platelets to this phenomenon, we subjected mice lacking the ability to form procoagulant platelets ($\text{CypD}^{\text{plt}^{-/-}}$, Figure 2A) to a model of pulmonary microvascular thrombosis, in which platelets are the primary driver of vascular occlusion.²³ To induce pulmonary thrombosis, collagen and epinephrine are injected intravenously and the time until breathing cessation is recorded. We observed that mice lacking procoagulant platelet responses died significantly faster after induction of pulmonary thrombosis, relative to littermate wild-type controls in which procoagulant platelet formation was intact (Figure 2B).

While classically recognized for their prothrombotic properties, several studies have indicated an important role for procoagulant platelets in the regulation of thrombotic events.^{20,24,25} Indeed, while procoagulant platelets have an excellent ability to

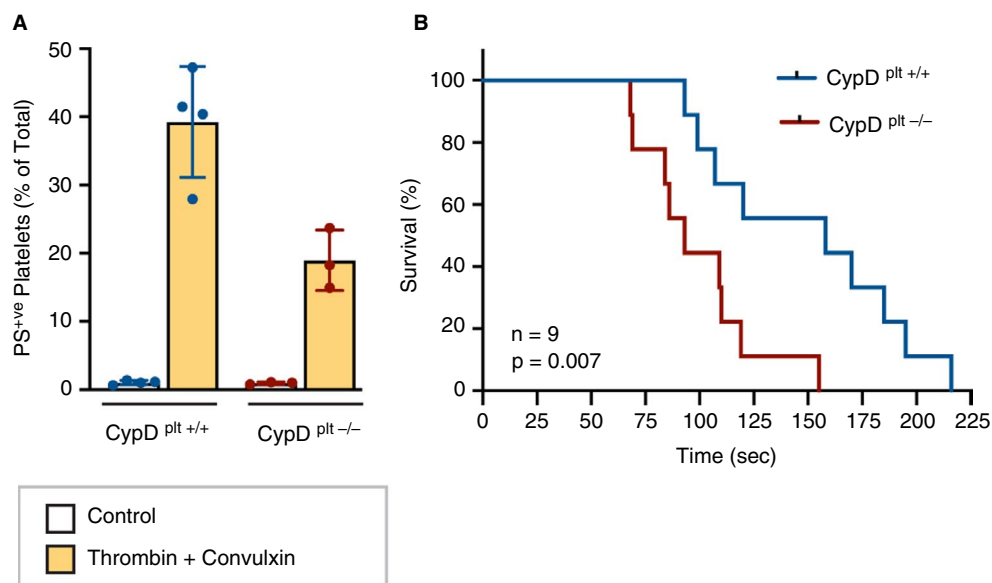


FIGURE 2 Mice deficient in procoagulant platelet formation die faster in a pulmonary thrombosis model. A, Platelets were isolated from wild-type ($\text{CypD}^{\text{plt}+/+}$, $n = 4$) and platelet specific cyclophilin D knockout mice ($\text{CypD}^{\text{plt}^{-/-}}$, $n = 3$) and stimulated with 1 U/mL thrombin + 250 ng/mL convulxin and annexin V staining was measured. B, Pulmonary thrombosis was induced in anesthetized mice by intravenous injection of a mixture of collagen (0.4 mg/kg; Chronolog) and epinephrine (30 mg/kg; Sigma-Aldrich) in 100 μL of phosphate buffered saline. Time to death was monitored. Shown is the Kaplan–Meier survival curve ($n = 9$ per genotype).

bind coagulation factors, they also have a reduced ability to aggregate.²⁶ In particular, CypD-dependent procoagulant platelet formation initiates a negative-feedback mechanism that limits platelet aggregation and subsequent thrombosis.²⁴ The reduced ability to form procoagulant platelets in COVID-19 patients could therefore potentially result in hyper-aggregating platelets, as previously described.⁶ Consistent with this hypothesis, cyclosporin, a potent inhibitor of CypD and procoagulant platelet formation, has been reported to increase the risk of thromboembolic events in renal transplant patients.^{27,28} Our current findings support these observations, as mice lacking the ability to form procoagulant platelets through CypD deletion died faster in a pulmonary thrombosis model. Interestingly, also in a model of venous thromboembolism, CypD-deficient animals developed larger thrombi and in a more rapid manner.²⁹

Mitochondrial depolarization through CypD and the mitochondrial permeability transition pore are necessary for the formation of procoagulant platelets.^{19,20} However, only minor depolarization of platelets from COVID-19 patients was observed upon dual agonist stimulation, coinciding with a lack of PS expression. Ingenuity Pathway Analysis on RNA-seq performed on platelets from COVID-19 patient demonstrated significant enrichment of genes involved in mitochondrial dysfunction.⁶ It is unclear why COVID-19 induces significant alterations in gene expression related to mitochondrial function. Viral infections are known to induce mitochondrial dysfunction¹² and our results suggest SARS-CoV-2 infection alters platelet gene expression, although the mechanism remains unknown at this time. As platelets and megakaryocytes do not express ACE2, the receptor necessary for SARS-CoV-2 infection³⁰ and we have not detected SARS-CoV-2 virus within platelets from COVID-19 patients,⁶ alterations in the platelet transcriptome may be due to systemic changes related to the infection.

Finally, our study has several limitations. First, only a limited number of patients were included in this study and therefore our results will need to be validated in larger COVID-19 cohorts. Second, the small sample size in our study precluded correlation analysis with thrombotic biomarkers or thrombotic events due to limited statistical power and the lack of screening for asymptomatic thrombotic events in COVID-19 patients at our institution. Last, our animal studies were performed in the absence of a viral challenge, and in a microvascular thrombosis model primarily driven by platelets. This limits translation of our murine findings to clinical COVID-19 pathophysiology.

While we recognize the limitations of our clinical observations and animal models of thrombosis, these results warrant further investigation into the role of dysregulated procoagulant platelet responses and thrombotic events observed in COVID-19 patients.

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CONFLICTS OF INTEREST

Frederik Denorme, Bhanu Kanth Manne, Irina Portier, Aaron Petrey, Elizabeth Middleton, Benjamin Kile, and Robert Campbell have nothing to disclose. Matthew Rondina serves on the Scientific Advisory Board for Acticor Biotech SAS.

AUTHOR CONTRIBUTIONS

FD, BKM, IP, ACP, EAM, and RAC designed and performed experiments; FD, BKM, and RAC analyzed results and made the figures; FD, BKM, IP, ACP, EAM, BTK, MTR, and RAC wrote the paper. All authors reviewed and critically edited the manuscript.

ORCID

Frederik Denorme  <https://orcid.org/0000-0003-1442-3568>

Bhanu Kanth Manne  <https://orcid.org/0000-0001-6848-4484>

Matthew T. Rondina  <https://orcid.org/0000-0003-3455-3730>

Robert A. Campbell  <https://orcid.org/0000-0003-0027-694X>

TWITTER

Robert A. Campbell  @racampbell04

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