Aberrant Fibrin Clot Structure Visualized Ex Vivo in Critically Ill Patients With Severe Acute Respiratory Syndrome Coronavirus 2 Infection

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OBJECTIVES: Disseminated fibrin-rich microthrombi have been reported in patients who died from COVID-19. Our objective is to determine whether the fibrin clot structure and function differ between critically ill patients with or without COVID-19 and to correlate the structure with clinical coagulation biomarkers.

DESIGN: A cross-sectional observational study. Platelet poor plasma was used to analyze fibrin clot structure; the functional implications were determined by quantifying clot turbidity and porosity.

SETTING: ICU at an academic medical center and an academic laboratory.

PATIENTS: Patients admitted from July 1 to August 1, 2020, to the ICU with severe acute respiratory syndrome coronavirus 2 infection confirmed by reverse transcription-polymerase chain reaction or patients admitted to the ICU with sepsis.

INTERVENTIONS: None.

MEASUREMENTS AND MAIN RESULTS: Blood was collected from 36 patients including 26 ICU patients with COVID-19 and 10 ICU patients with sepsis but without COVID-19 at a median of 11 days after ICU admission (interquartile range, 3–16). The cohorts were similar in age, gender, body mass index, comorbidities, Sequential Organ Failure Assessment (SOFA) score, and mortality. More patients with COVID-19 (100% vs 70%; $p = 0.003$) required anticoagulation. Ex vivo fibrin clots formed from patients with COVID-19 appeared to be denser and to have smaller pores than those from patients with sepsis but without COVID-19 (percent area of fluorescent fibrin 48.1% [SD, 16%] vs 24.9% [SD, 18.8%]; $p = 0.049$). The turbidity and flow-through assays corroborated these data; fibrin clots had a higher maximum turbidity in patients with COVID-19 compared with patients without COVID-19 (0.168 vs 0.089 OD units; $p = 0.003$), and it took longer for buffer to flow through these clots (216 vs 103 min; $p = 0.003$). In patients with COVID-19, D-dimer levels were positively correlated with percent area of fluorescent fibrin ($\rho = 0.714$, $p = 0.047$). Denser clots (assessed by turbidity and thromboelastography) and higher SOFA scores were independently associated with delayed clot lysis.

CONCLUSIONS: We found aberrant fibrin clot structure and function in critically ill patients with COVID-19. These findings may contribute to the poor outcomes observed in COVID-19 patients with widespread fibrin deposition.

KEYWORDS: COVID-19; fibrin clot structure; fibrin polymerization; fibrinogen; hypercoagulability; severe acute respiratory syndrome coronavirus 2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the COVID-19 pandemic and causes severe-to-critical illness in 19% of infected patients (1). Hypercoagulability is a common phenotype in these critically ill patients despite the use of prophylactic- or treatment-dose anticoagulation (2–5). Further, markers of fibrin polymerization (e.g., elevated D-dimer

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levels and procoagulant profiles on viscoelastic assays) have been associated with adverse outcomes in severe cases of COVID-19 (6–11). Numerous autopsy studies have reported disseminated fibrin-rich microthrombi in patients who died from COVID-19, which is again suggestive of an association between widespread fibrin deposition and poor outcomes in COVID-19 (12–15).

The pathophysiology of the augmented fibrin deposition in COVID-19 is multifactorial (16). Although many of the etiologic factors unique to the viral infection are outside of the scope of this report (17–19), there is clear evidence that patients with COVID-19 have enhanced plasma fibrin formation potential compared with critically ill patients without COVID-19 (20). Despite this evidence, changes in the fibrin clot structure in COVID-19 have not been described. Considering the proclivity for fibrin formation, we hypothesize that fibrin clots will be denser and less porous in critically ill patients with COVID-19 compared with critically ill patients without COVID-19.

Our specific objectives were: 1) to compare the fibrin clot properties in critically ill patients with or without COVID-19 and 2) to determine correlation between fibrin clot properties in experimental (plasma-based) assays and the whole-blood coagulation assays clinically used to assess hypercoagulability. The outcomes of this study have the potential to impact clinical care for patients with COVID-19 as several interventions with the potential to favorably alter fibrin clot properties have been reported (21, 22), and it is well described that the fibrin clot structure affects fibrinolysis (23–25).

MATERIALS AND METHODS

This study was approved by the Baylor College of Medicine (BCM) Institutional Review Board (IRB) protocols H-48730 and H-47607 (approved by board 4 of the BCM IRB and the Michael E DeBakey Department of Veterans Affairs Medical Center Subcommittee on Research Safety). This study is reported following Strengthening the Reporting of Observational Studies in Epidemiology reporting guidelines for observational studies (26).

Study Participants and Clinical Data

Inclusion criteria for this study were critically ill patients in the ICU at Baylor St. Luke's Medical Center between July 1 and August 1, 2020; exclusion criteria were patients in the ICU who did not have a positive reverse transcriptase-polymerase chain reaction test for SARS-CoV-2 and who did not have sepsis as defined by Sepsis-3.0 criteria (27). During this period, the first available residual venous blood sample that was drawn into sodium citrate tubes as part of routine clinical care was collected for patients meeting criteria. Platelet poor plasma (PPP) was isolated by centrifugation at 3,000 rpm for 15 minutes at room temperature and frozen at −80°C until it was used for assays. To obtain healthy human blood, informed consent was obtained from healthy donors without acute illness, and PPP was processed as above. Researchers were blinded to patient status (i.e., COVID-19 diagnosis) during the collection, processing, and experimentation stages. Additional methods are in the data supplement.

Imaging the Fibrin Clot Structure

PPP was thawed at 37°C. The fibrin clot structures were obtained as described previously (26) with the exception that PPP was supplemented with 2% human fibrinogen conjugated to Alexa Fluor 488 (Thermo Scientific, Waltham, MA), and clots were formed on a 0.15-mm chambered cover glass. The clots were imaged using laser scanning confocal microscopy at a magnification of 60×. Image analysis, specifically percent area occupied by fluorescent fibrin, was performed using Fiji (Bethesda, MD).

Flow-Through Assays

Plasma clots were formed by mixing PPP 1:5 with buffer containing 1× tris-buffered saline, 1% bovine serum albumin, 1 U of human thrombin (Sigma, St. Louis, MO), and 2.4 mM of calcium in the base of a 3-mL syringe with a stopcock to control flow-through. The syringes were incubated at 37°C for 2 hours to allow clot formation; 1 mL of buffer (1× tris-buffered saline) was gently pipetted over the formed clots. The stopcock was opened, and the duration of time required for all of the buffer to flow through the clot was recorded.

Buffer mixed with fluorescent submicron beads (Bangs Laboratories, Fishers, IN) was also used for flow-through experiments. An equal volume of each sized bead (0.2, 0.5, and 0.8 um) was added to a microcentrifuge tube, and this combined volume was diluted 1:10 with buffer containing 1× tris-buffered saline. The concentration and proportion of each sized bead within the mixture was determined by flow cytometry using the Amnis

ImageStream^{x} MkII (Luminex, Austin, TX); 1,000 events were counted, and the 0.2-, 0.5-, and 0.8-um populations were discriminated by gating via side scatter/green fluorescence. Clots were formed in the syringes as described above, and 200 uL of the bead/buffer mixture was gently added over the clots. The flow-through was collected in microcentrifuge tubes. After all 200 uL of the bead/buffer mixture was collected, flow cytometry was again performed to determine how the concentration and proportion of 0.2-, 0.5-, and 0.8-um beads within the mixture changed. The sieving coefficient was calculated by determining the concentration of 0.2-um beads in the mixture postflow-through/the concentration of 0.2 um beads in the mixture preflow-through; this calculation was repeated for the 0.5- and 0.8-um beads.

Fibrin Polymerization Assays

Fibrin polymerization and fibrinolysis were evaluated by turbidity as described (26). Briefly, plasma was mixed 1:2.5 with buffer containing 1× tris-buffered saline, 1% bovine serum albumin, and 2.4 mM of calcium in a 96-well plate. Immediately prior to inserting the plate into the spectrophotometer, an equal volume of buffer containing 1U of thrombin was added to each well. For assays that concurrently measured fibrinolysis, the buffer was supplemented with 150 ng/mL of human tissue plasminogen activator (Sigma Aldrich). The progression of fibrin clot formation and fibrinolysis was evaluated by tracking turbidity using a spectrophotometer set to λ 405 nm. Variables calculated from turbidimetric curves include maximum absorbance (optical density units), slope of fibrin polymerization, 50% clot lysis time, and slope of fibrinolysis as described (28).

Statistical Analysis

To test whether the presence of COVID-19 results in a different fibrin structure (i.e., percent area occupied by fluorescent fibrin) in critically ill subjects, we calculated a sample size of four subjects in the control group and eight subjects in the COVID-19 group. This was determined by using an estimated difference of 10% between the group means with an SD of 6%, alpha of 0.05, power of 80%, and enrollment ratio of 2:1 (COVID-19 vs non-COVID-19 with sepsis). Additional samples were collected as available due to the anticipated heterogeneity between clinical variables between the groups.

Comparison of categorical variables between groups was performed using the Pearson chi-square or Fisher exact test as appropriate. Continuous variables were expressed as mean with SD for normally distributed variables or as median with interquartile range (IQR) for variables that were not normally distributed. Comparisons between groups were conducted through the independent samples *t* test/Mann-Whitney *U* test. Associations between clinical, laboratory, and plasma-based assay parameters were analyzed using Spearman rank correlations. Agreement between correlation coefficients for the plasma-based assays between cohorts was assessed using Cohen kappa. *p* values were two-sided, and statistical significance was determined by a *p* value less than 0.05. All analyses were performed using IBM SPSS Statistics Version 26.0 (IBM Corp, Armonk, NY).

RESULTS

We obtained blood from all 42 patients meeting criteria during the study period including six healthy donors, 10 critically ill septic patients without COVID-19, and 26 critically ill patients with COVID-19. The non-COVID-19 sepsis and COVID-19 cohorts were similar in age, gender, body mass index, and preadmission antiplatelet and anticoagulant use as summarized in **Supplementary Table 1** (http://links.lww.com/CCM/H29). The COVID-19 cohort had a higher proportion of Black and Hispanic/Latino patients compared with the non-COVID-19 with sepsis cohort (80.8% vs 40.0%; $p = 0.039$). More patients with COVID-19 were diabetic, whereas more septic patients without COVID-19 had chronic obstructive pulmonary disease or chronic lung disease.

Clinical data and key outcomes are also presented in Supplementary Table 1 (http://links.lww.com/CCM/H29). There was no difference in the overall occurrence of thrombotic events between the non-COVID-19 with sepsis and COVID-19 cohorts (20% vs 30.8%; $p = 0.765$). Notably, more patients with COVID-19 received intermediate/therapeutic dose anticoagulation during hospitalization. There were no differences in need for invasive organ support (i.e., renal replacement therapy, extracorporeal membrane oxygenation, and mechanical ventilation), length of stay, or mortality between cohorts.

Plasma was collected as available from patients at a single time point during their ICU stay, and clinical characteristics at this time point were compared between the cohorts (**Table 1**). Most patients (60% of

TABLE 1.
Clinical Characteristics of Critically Ill Patients Without COVID-19 and With Sepsis or With COVID-19 on the Date Plasma Was Collected for Experimentation

Time Point: Date of Plasma Collection	Non-COVID-19 With Sepsis ($n = 10$)	COVID-19 ($n = 26$)	p
ICU day that plasma was collected (day 0 = day of ICU admission), median (IQR)	9 (2–19)	13 (3–16)	0.715
Sepsis diagnosis (Sepsis-3 criteria) (%)			
Confirmed	100.00	100.00	1
Chest radiograph findings (%)			
Normal	40.00	0.00	**< 0.001**
Unilateral opacity	0.00	0.00	
Bilateral opacities	40.00	96.20	
Interstitial infiltrates	20.00	3.80	
Respiratory support (%)			
Invasive support	60.00	73.10	0.454
Sequential Organ Failure Assessment Score, median (IQR)	8.5 (3.3–15.5)	6.5 (3–11.3)	0.689
WBC count (×10^3/uL), median (IQR)	10.5 (7.7–15.1)	10.0 (7.1–15.8)	1
Hemoglobin (g/dL), median (IQR)	8.3 (7.3–9.6)	8.8 (8.3–10.9)	0.155
Platelet count (×10^3/uL), median (IQR)	121 (57–226)	250 (184–281)	**0.023**
Prothrombin time (s), median (IQR)	18.5 (16.0–28.8)	15.1 (14.1–16.7)	**0.005**
Partial thromboplastin time (s), median (IQR)	55.4 (41.8–75.6)	45.8 (39–59.3)	0.224
D-dimer (µg/mL), median (IQR)[a]	7.09	6.0 (1.8–12.1)	na
Fibrinogen (mg/dL), median (IQR)[b]	176 (135–342)	597 (465–797)	**0.003**
Heparinase-corrected thromboelastography			
R value (min), median (IQR)[c]	6.25 (SD = 1.48)	6.5 (5.6–9.6)	NA
Fibrinogen activity angle (°), median (IQR)[c]	66.7 (SD = 8.7)	75.9 (69.8–78.2)	NA
Maximum amplitude (mm), median (IQR)[c]	57.1 (SD = 6.9)	75.6 (72.4–82.2)	NA
Clot lysis 30 min after maximum clot strength (%), median (IQR)[d]	0.05 (SD = 0.07)	0.2 (0–0.5)	NA
Thrombotic event on or before date of plasma collection (%)			
None	80.00	76.90	0.791
Ischemic stroke	0.00	0.00	
Myocardial infarction	10.00	7.70	
Acute limb ischemia	0.00	7.70	
Deep vein thrombosis	0.00	3.80	
Pulmonary embolism	10.00	3.80	
Need for renal replacement therapy on or before date of plasma collection (%)	30.00	34.60	1
Need for extracorporeal membrane oxygenation on or before date of plasma collection (%)	10.00	15.40	1
Anticoagulation (%)			
None	50.00	3.80	**0.001**
Prophylactic	0.00	0.00	
Intermediate	0.00	42.30	
Therapeutic	50.00	53.80	

(*Continued*)

TABLE 1. (*Continued*).

Clinical Characteristics of Critically Ill Patients Without COVID-19 and With Sepsis or With COVID-19 on the Date Plasma Was Collected for Experimentation

Time Point: Date of Plasma Collection	Non-COVID-19 With Sepsis ($n = 10$)	COVID-19 ($n = 26$)	p
Anticoagulation agent (%)			
None	50.00	3.80	**0.009**
Low-molecular-weight heparin	0.00	3.80	
Heparin	40.00	84.60	
Other	10.00	7.70	

IQR = interquartile range, NA = too few subjects in non-COVID-19 with sepsis cohort to do statistical comparison.
[a]$n = 1$ non-COVID-19 with sepsis, 26 COVID-19.
[b]$n = 5$ non-COVID-19 with sepsis, 16 COVID-19.
[c]$n = 2$ non-COVID-19 with sepsis (mean and SD reported), 20 COVID-19.
[d]$n = 2$ non-COVID-19 with sepsis (mean and SD reported), 7 COVID-19.
Reference ranges: WBC count: 4.3–11.3 × 10^3/μL, hemoglobin: 12–17.5 g/dL, platelet count: 150–450 × 10^3/μL, prothrombin time: 11.9–14.2 s, partial thromboplastin time: 22.5–36 s, D-dimer: < 0.5 μg/mL, fibrinogen: 225–434 mg/dL, heparinase-corrected thromboelastography results: R value: 4–7 min, fibrinogen activity angle: 61°–73°, maximum amplitude: 55–65 mm, clot lysis 30 min after maximum clot strength: 0–5%.
Sepsis diagnosis based on Sepsis-3 criteria.
Boldface font indicates $p < 0.05$.

patients without COVID-19 but with sepsis and 73.1% of patients with COVID-19; $p = 0.454$) had samples collected late in their ICU course (greater than 72 hr after ICU admission). The median day of ICU admission that the plasma was collected was 9 (IQR, 2–19) in the non-COVID-19 with sepsis cohort compared with 13 (IQR, 3–16) in the COVID-19 cohort ($p = 0.715$). On the date of plasma collection, more septic patients without COVID-19 had normal chest radiographs, but the percent of patients requiring invasive respiratory support did not differ between the cohorts. The median Sequential Organ Failure Assessment (SOFA [29]) score was similar between the cohorts. Regarding outcomes occurring at or before the date of plasma collection (Table 1), we also found no difference in the occurrence of thrombotic events or need for invasive organ support between the cohorts. At the time of plasma collection, more patients with COVID-19 were receiving intermediate or therapeutically dosed anticoagulation compared with septic patients without COVID-19 (96.2% vs 50%; $p = 0.003$).

Hematologic parameters and markers of coagulation/fibrinolysis on the date of plasma collection were consistent with prior reports for patients with COVID-19 (Table 1) (6–8). The platelet count was significantly greater in patients with COVID-19 compared with septic patients without COVID-19 (250 [IQR, 184–281] vs 121 [IQR, 57–226] ×10^3/μL; $p = 0.023$). D-dimer levels were markedly elevated in the COVID-19 cohort (6.0 μg/mL [IQR, 1.8–12.1 μg/mL], reference value less than 0.5 μg/mL), and fibrinogen was also elevated in the COVID-19 cohort relative to the reference range and in comparison with the non-COVID-19 with sepsis cohort (597 mg/dL [IQR, 465–797 mg/dL] vs 176 mg/dL [IQR, 135–342 mg/dL]; $p = 0.003$). Of all patients with thromboelastography (TEG) data, all patients with COVID-19 (20/20) demonstrated hypercoagulable TEG parameters (i.e., heparinase-corrected maximum amplitude [MA] greater than 67 mm) despite receipt of intermediate or therapeutically dosed anticoagulation, whereas no septic patients without COVID-19 (0/2) demonstrated hypercoagulable TEG parameters.

Ex Vivo Plasma Clots From Patients With COVID-19 Have Increased Fibrin Network Density

Figure 1*A* shows representative images of the fibrin structure formed in plasma from healthy donors, septic patients without COVID-19, and patients with COVID-19. The 2D images give the impression that ex vivo clots from patients with COVID-19 have smaller pores and are denser than clots from septic patients without COVID-19. Corresponding to the representative

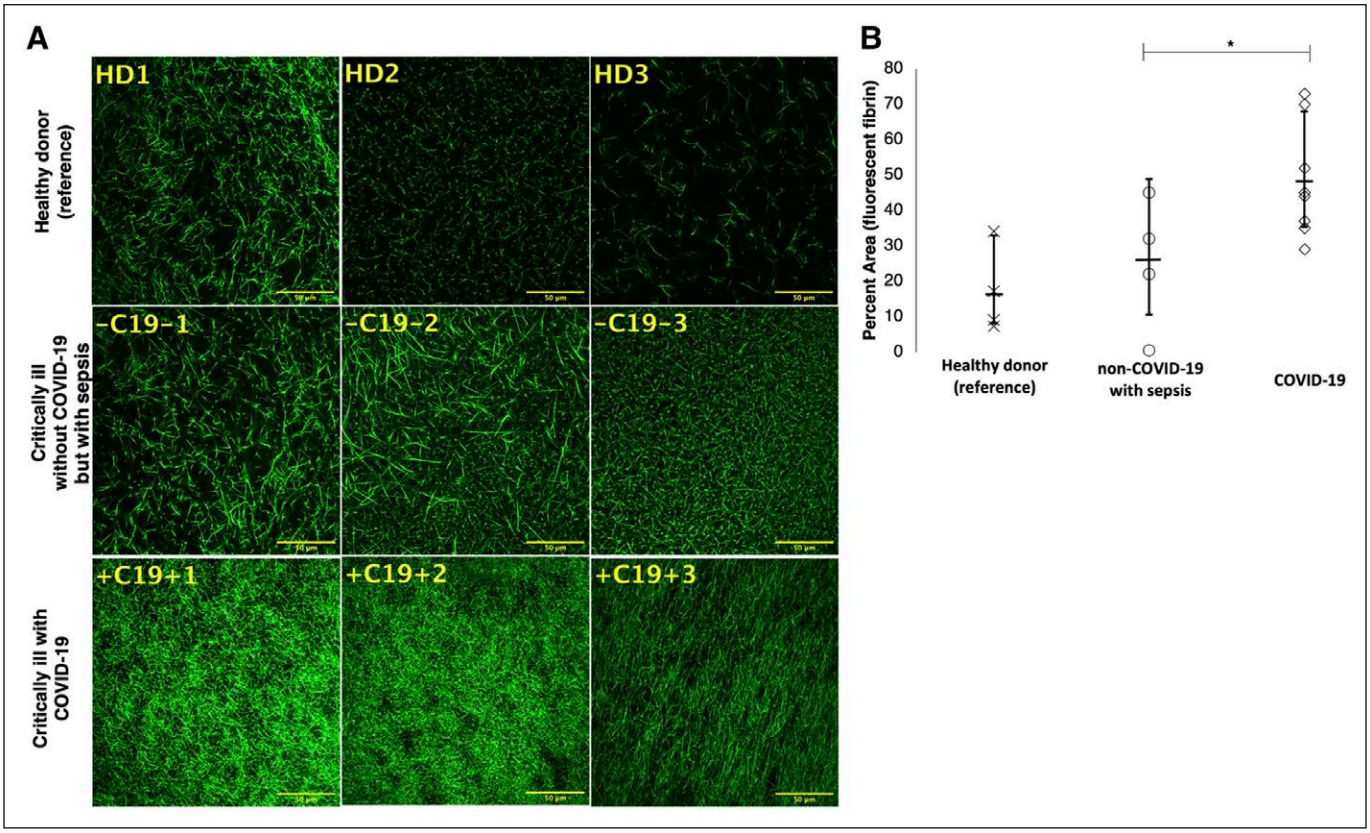


Figure 1. Aberrant fibrin clot structure in critically ill patients with COVID-19. **A**, Confocal microscopy images of fibrin clots at a magnification of ×60 formed in plasma from healthy donors (HDs), critically ill patients without COVID-19 (—C19—), or critically ill patients with COVID-19 (+C19+). Representative images from three different subjects in each cohort are displayed. Scale bars, 50 um. **B**, Quantification of confocal images of fibrin clots was performed using Fiji by ImageJ. Percent area of fluorescent fibrin was determined for healthy donors (×) to determine a reference value. Percent area was determined and compared between —C19— (*open circle*) and +C19+ (*open diamond*) patients. Lines show the mean ± 1 SD; symbols indicate values for individual subjects performed in triplicate. *$p = 0.049$.

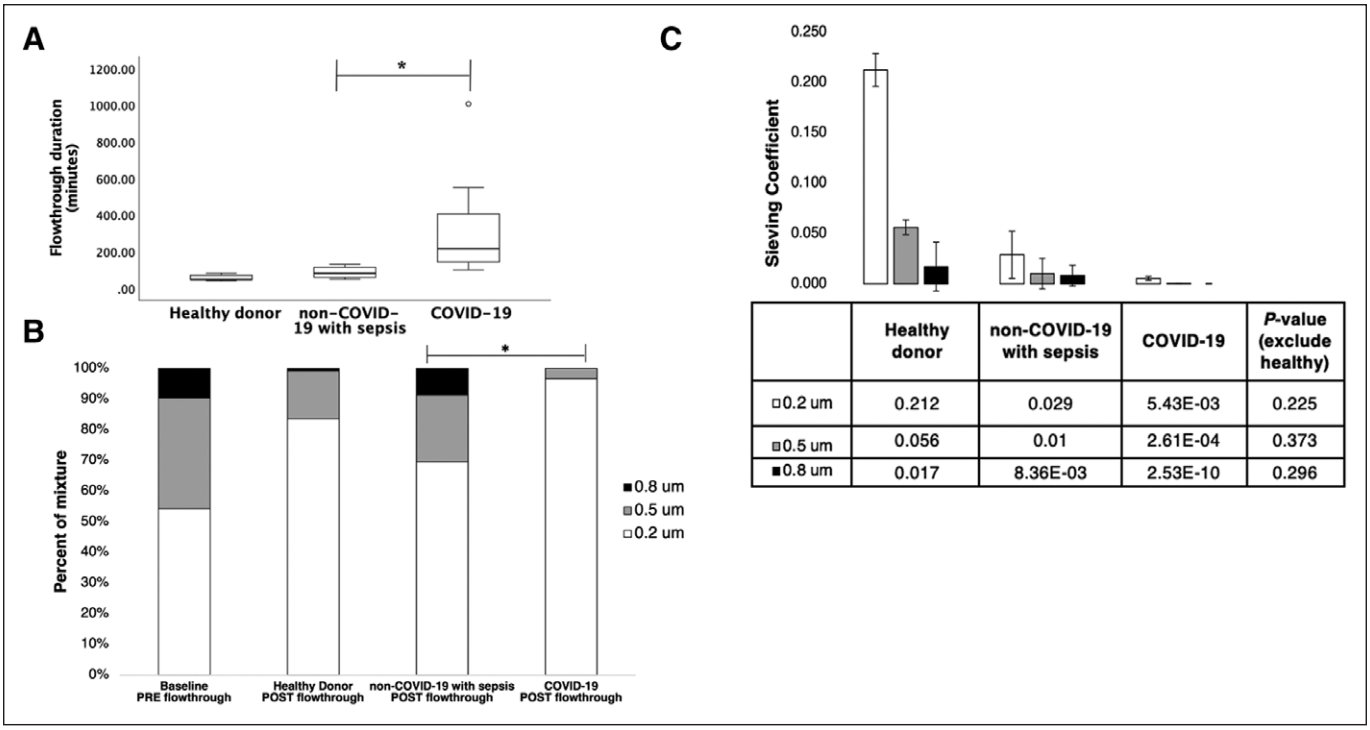
images, the average percent area occupied by fluorescent fibrin was significantly greater in patients with COVID-19 compared with septic patients without COVID-19 (48.1% [SD, 16%] vs 24.9% [SD, 18.8%]; $p = 0.049$) (**Fig. 1*B***).

Ex Vivo Plasma Clots From Patients With COVID-19 Have a Higher Resistance to Flow and Reduced Sieving Coefficient

To assess the functionality of clot porosity, we measured the resistance to flow and the sieving quality of the clots. **Figure 2*A*** shows that the median time required for buffer to flow through clots from patients with COVID-19 was significantly greater compared with septic patients without COVID-19 (216 min [IQR, 141–441 min] vs 82 min [IQR, 57–126 min]; $p < 0.001$). In a similar experiment, fluorescent submicron beads (0.2, 0.5, and 0.8 um) were added to the buffer prior to flowing the buffer through the clots. The proportion and concentration of each sized bead within the mixture was determined by flow cytometry at baseline and after clot flow-through. **Figure 2*B*** shows that the mixture became enriched for 0.2-um beads, whereas the 0.5- and 0.8-um beads were depleted after flow-through. This pattern was more pronounced in the COVID-19 cohort compared with the non-COVID-19 with sepsis cohort ($p < 0.001$). The bead concentration was used to determine the sieving coefficient that ranges from 0 (no transport) to 1 (unrestricted transport). The sieving coefficient was lower for each sized bead in the patients with COVID-19 compared with septic patients without COVID-19 (**Fig. 2*C***), but these results did not reach statistical significance.

Patients With COVID-19 Have Enhanced Ex Vivo Plasma Fibrin Formation Potential

Next, we used a turbidimetric assay to measure the kinetics of fibrin polymerization and fibrinolysis (30). The maximum absorbance change associated with fibrin polymerization was significantly greater in



	Healthy donor	non-COVID-19 with sepsis	COVID-19	*P*-value (exclude healthy)
□0.2 um	0.212	0.029	5.43E-03	0.225
▨0.5 um	0.056	0.01	2.61E-04	0.373
■0.8 um	0.017	8.36E-03	2.53E-10	0.296

Figure 2. Reduced functional porosity in fibrin clots from critically ill patients with COVID-19. **A**, Box and whisker plot representing how long it took buffer to flow through fibrin clots. Flow-through was performed in clots from five healthy donors, 10 critically ill patients without COVID-19 (–C19–), and 10 critically ill patients with COVID-19 (+C19+) in duplicate. *$p < 0.001$. **B**, Similar experiment to (**A**) but buffer contained a mixture of equal volumes of 0.2-, 0.5-, and 0.8-um fluorescent beads. The *first column* shows the submicron bead composition in the buffer at baseline (preflow-through) and the *subsequent columns* show the bead composition of the buffer after it flowed through the clots formed from healthy human donors, critically ill patients without COVID-19 (–C19–), or critically ill patients with COVID-19 (+C19+). Bead composition within the buffer was determined by FACS analysis, values represent the mean percent composition based on three clots/cohort performed in triplicate, and the composition was compared between –C19– and +C19+ cohorts using Pearson χ^2, *$p = <0.001$. **C**, The same experiment as **B** but bead concentration pre-/postflow-through was used to determine the sieving coefficient. Sieving coefficient = bead concentration postflow-through/bead concentration preflow-through. *Bars* represent the mean sieving coefficient based on three clots per cohort performed in triplicate and error bars represent 1 SD. FACS = fluorescence activated cell sorting.

patients with COVID-19 compared with septic patients without COVID-19 (**Fig. 3**, ***A*** and ***B***). This represents increased fibrin formation potential as there were no changes in the kinetics of fibrin polymerization associated with COVID-19 status.

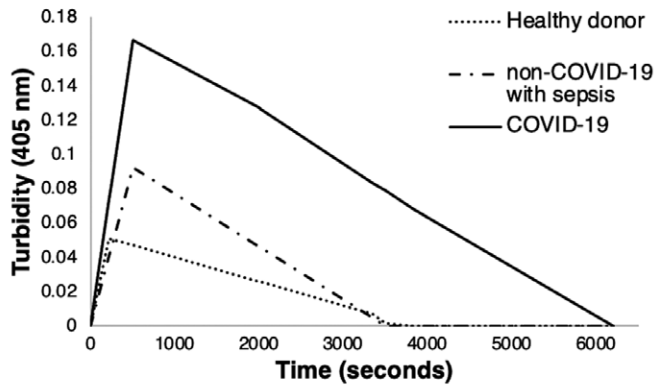
Increased Fibrin Formation Potential Is Associated With Clinical Markers of Hypercoagulability

We performed Spearman correlation analyses to explore the relationship between the ex vivo plasma-based fibrin clot assays. The heat maps for each cohort are presented in **Figure 4**, ***A*** and ***B***, and the numerical correlation coefficients and *p* values are presented in **Supplementary Tables 2** and **3** (http://links.lww.com/CCM/H29). Whereas there was a strong positive correlation between percent area and maximum absorbance in the non-COVID-19 with sepsis cohort ($\rho = 1$, $p = 0.01$), these variables were not correlated in the COVID-19 cohort ($\rho = -0.657$, $p = 0.156$). There was no linear relationship between either percent area or maximum absorbance with flow-through duration in either cohort.

Finally, we correlated the plasma-based assay results with clinical parameters and biomarkers of coagulation. D-dimer correlated positively with the SOFA score ($\rho = 0.435$, $p = 0.026$) and negatively with the Pao_2:FIO_2 ratio ($\rho = -0.486$, $p = 0.012$) in subjects with COVID-19. D-dimer also correlated strongly and positively with the percent area (fluorescent fibrin) ($\rho = 0.714$, $p = 0.047$) in this cohort.

The TEG alpha angle represents the speed of fibrin polymerization and is clinically augmented with fibrinogen. Interestingly, there was no association between

A



B

	Healthy donor	non-COVID-19 with sepsis	COVID-19	P-value (exclude healthy)
Maximum turbidity (ODU) Median (IQR)	0.051 (0.036-0.060)	0.093 (0.06-0.12)	0.172 (0.12-0.23)	**0.004**
Time to increase 0.02 ODU from baseline (seconds) Median (IQR)	90 (62.5-151.3)	80 (25-98)	50 (30-70)	0.158
Slope: fibrin polymerization (x10^-4) Median (IQR)	2.58 (1.54-3.3)	1.8 (1.1-5.0)	3.48 (1.7-5.2)	0.437
Time to 50% clot lysis (seconds) Median (IQR)	3330 (2675-3985)	2285 (2020-3898)	4000 (2465-4680)	0.2
Slope: fibrinolysis (x10^{-5}) Median (IQR)	-1.4 (-1.5- -1.31)	-3.2 (-0.6- -3.9)	-3.3 (-2.1- -3.9)	0.72

Figure 3. Increased fibrin formation potential in critically ill patients with COVID-19. **A**, Values for the combined plasma clot turbidity and lysis assay. Fibrin polymerization was performed in triplicate using plasma from six healthy donors 9 critically ill patients without COVID-19 (—C19—), and 24 critically ill patients with COVID-19 (+C19+). Fibrinolysis was performed in triplicate concurrently for two of the healthy donors, 8 of the —C19— patients, and 21 of the +C19+ patients. **B**, Representative curves constructed from the values in **A** using Microsoft Excel. IQR = interquartile range, ODU = optical density units.

the alpha angle and fibrinogen levels ($\rho = -0.049$, $p = 0.873$), nor between the alpha angle and the rate of fibrin polymerization ($\rho = 0.204$, $p = 0.403$). The TEG MA represents the highest vertical amplitude of the TEG tracing (or clot strength) and can be augmented with supplemental platelets (and to a lesser extent, fibrinogen). Consistently, there was a positive and significant correlation between platelets and TEG MA ($\rho = 0.507$, $p = 0.023$). There was no relationship between fibrinogen and TEG MA ($\rho = 0.325$, $p = 0.279$). Fibrinogen and TEG MA each correlated positively with the rate of fibrin polymerization in the turbidity assay ($\rho = 0.613$, $p = 0.02$ and $\rho = 0.527$, $p = 0.02$). In the COVID-19 cohort, there was a positive correlation between both the TEG MA and maximum absorbance with the time required for 50% clot lysis ($\rho = 0.482$, $p = 0.05$ and $\rho = 0.808$, $p < 0.001$). There was also an association between illness severity (evidence of higher SOFA) and a reduced slope for clot lysis in COVID-19.

DISCUSSION

Significant fibrin deposition has been observed in patients with COVID-19 and is thought to contribute to the morbidity and mortality of this disease (12–15). This cross-sectional study focuses on elucidating the ex vivo fibrin clot structure in critically ill patients with or without COVID-19 and correlating these findings with clinical parameters. Plasma clots from critically ill subjects with COVID-19 were denser, had greater resistance to flow, and reduced functional porosity compared with a similar cohort of patients with sepsis but without COVID-19. There was a strong correlation between the density of the fibrin clots with D-dimer levels. Thus, the aberrant clot structure and associated functional changes reported herein may explain why elevated D-dimer levels are pathologic in critically ill patients with COVID-19 (8, 11, 28, 31, 32).

Changes in the architecture of the fibrin clot, including density and pore size, are associated with a high risk for thrombosis in certain diseases (33–35). Further, a clot's proclivity to fibrinolysis is affected by the fibrin network density (21, 25, 36, 37). In this study, the fibrin network density was greater in the COVID-19 cohort (as assessed by both our plasma-based turbidity assay and whole-blood TEG), and

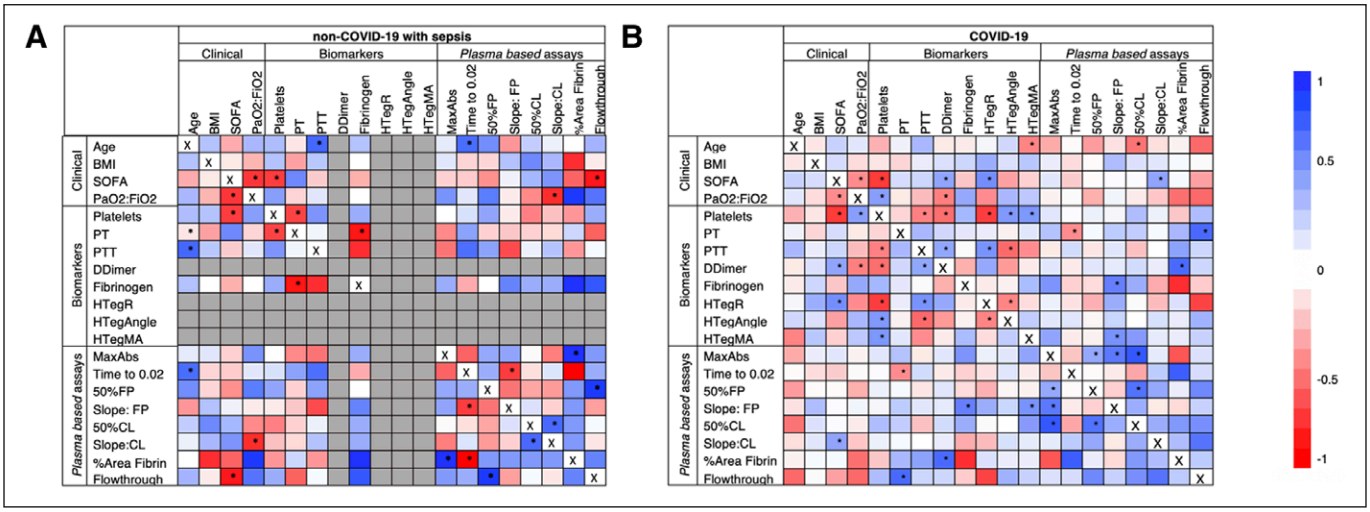


Figure 4. Increased fibrin formation potential is associated with clinical markers of hypercoagulability in correlation analysis. Spearman rank correlations were performed for clinical metrics, conventional coagulation biomarkers, and the plasma-based assays incritically ill patients without COVID-19 (**A**) and critically ill patients with COVID-19 (**B**). Positive correlations are shaded in *blue*, whereas negative correlations are shaded in *red*, as defined in the legend. *demonstrates the correlation coefficient had a p value < 0.05. BMI = body mass index, CL = clot lysis, FP = fibrin polymerization, HTEG = heparinase-corrected thromboelastography, MA = maximum amplitude, Pao_2:Fio_2 = ratio of Pao_2 to Fio_2, PT = prothrombin time, PTT = partial thromboplastin time, SOFA = Sequential Organ Failure Assessment score.

there was prolongation of the clot lysis time in patients with COVID-19 with higher SOFA scores. D-dimer levels were also associated with the percent area of fluorescent fibrin formed in the ex vivo clots—again suggesting a relationship between clot structure and fibrinolysis in COVID-19. One can argue that the altered density and porosity in the fibrin clots negatively influences fibrinolysis and possibly disease outcomes in patients with COVID-19. In fact, impaired fibrinolysis has been demonstrated in COVID-19 (20, 28). Previous reports demonstrated that the level of fibrinogen only has minor effects on fibrin structure in vitro and that other plasma proteins (e.g., vitronectin and von Willebrand factor) influence fibrin clot structure (38–40). These reports may support our finding that the level of fibrinogen was not directly associated with clot density in our ex vivo assays or TEG. More studies are necessary to investigate which other blood components contribute to the alteration of fibrin polymerization and fibrinolysis in COVID-19.

One limitation of our study is the small sample size. Our study was appropriately powered to detect a statistically significant difference in the ex vivo fibrin clot structure between critically ill patients with COVID-19 versus those with sepsis but without COVID-19. Our observation that the structure and function of ex vivo fibrin clots are altered in COVID-19 is novel. We acknowledge that the limited sample size required for this observation makes it difficult to ascertain the clinical implications with certainty. This is compounded by the significant difference in anticoagulation use between the cohorts—despite relative similarity in other key clinical variables. Nevertheless, we report some noteworthy clinical associations. Specifically, we observed that D-dimer levels positively correlated with the SOFA score and negatively correlated with the Pao_2:Fio_2 ratio in patients with COVID-19. This pattern suggests that fibrin polymerization and subsequent degradation are pathologic in COVID-19 and agrees with several previous reports (9, 11, 41).

The occurrence of thrombotic events in the patients with COVID-19 was lower than we anticipated and precluded a logistic regression analysis for this variable. Regarding the low frequency of thrombotic events in this study, we previously reported a high frequency of clinically significant thromboses in patients with hypercoagulable TEG parameters despite use of the recommended standard-dose thromboprophylaxis (6). Based on those findings, we developed and implemented a TEG-based intermediate-intensity anticoagulation protocol for patients with COVID-19 admitted to our ICU (42) (**Supplementary Fig. 1**, http://links.lww.com/CCM/H29). Thus, because all the patients with COVID-19 in this study had hypercoagulable TEG parameters, they were accordingly given intermediate or therapeutically dosed

anticoagulation; most of these subjects (65.4%) received this therapy upon admission. Whether the low frequency of thrombotic events observed in our COVID-19 cohort was due to this protocol remains to be defined, and several randomized controlled trials are currently addressing the benefits of therapeutic-intensity anticoagulation (43–46). This protocol may limit the generalizability of our results.

A final limitation is that our study used PPP under static conditions to assess ex vivo fibrin clot structure and clot formation dynamics. Factors within whole blood have an impact on clot formation (47). Hemodynamic flow also impacts fibrin deposition and fiber diameter during clot formation (48, 49). These variables may influence why there was not a stronger correlation between the turbidity assay of this study and the patients' TEG results as TEG uses whole blood and low shear stress (50).

CONCLUSIONS

This is the first report of aberrant fibrin clot structure and function in critically ill patients with COVID-19. The augmented clot density may contribute to the poor outcomes of COVID-19 patients with observed widespread fibrin deposition.

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