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# Immunothrombosis Biomarkers for Distinguishing Coronavirus Disease 2019 Patients From Noncoronavirus Disease Septic Patients With Pneumonia and for Predicting ICU Mortality

**IMPORTANCE:** Coronavirus disease 2019 patients have an increased risk of thrombotic complications that may reflect immunothrombosis, a process characterized by blood clotting, endothelial dysfunction, and the release of neutrophil extracellular traps. To date, few studies have investigated longitudinal changes in immunothrombosis biomarkers in these patients. Furthermore, how these longitudinal changes differ between coronavirus disease 2019 patients and noncoronavirus disease septic patients with pneumonia are unknown.

**OBJECTIVES:** In this pilot observational study, we investigated the utility of immunothrombosis biomarkers for distinguishing between coronavirus disease 2019 patients and noncoronavirus disease septic patients with pneumonia. We also evaluated the utility of the biomarkers for predicting ICU mortality in these patients.

**DESIGN, SETTING, AND PARTICIPANTS:** The participants were ICU patients with coronavirus disease 2019 ( $n = 14$ ), noncoronavirus disease septic patients with pneumonia ( $n = 19$ ), and healthy age-matched controls ( $n = 14$ ).

**MAIN OUTCOMES AND MEASURES:** Nine biomarkers were measured from plasma samples (on days 1, 2, 4, 7, 10, and/or 14). Analysis was based on binomial logit models and receiver operating characteristic analyses.

**RESULTS:** Cell-free DNA, d-dimer, soluble endothelial protein C receptor, protein C, soluble thrombomodulin, fibrinogen, citrullinated histones, and thrombin-anti-thrombin complexes have significant powers for distinguishing coronavirus disease 2019 patients from healthy individuals. In comparison, fibrinogen, soluble endothelial protein C receptor, antithrombin, and cell-free DNA have significant powers for distinguishing coronavirus disease 2019 from pneumonia patients. The predictors of ICU mortality differ between the two patient groups: soluble thrombomodulin and citrullinated histones for coronavirus disease 2019 patients, and protein C and cell-free DNA or fibrinogen for pneumonia patients. In both patient groups, the most recent biomarker values have stronger prognostic value than their ICU day 1 values.

**CONCLUSIONS AND RELEVANCE:** Fibrinogen, soluble endothelial protein C receptor, antithrombin, and cell-free DNA have utility for distinguishing coronavirus disease 2019 patients from noncoronavirus disease septic patients with pneumonia. The most important predictors of ICU mortality are soluble thrombomodulin/citrullinated histones for coronavirus disease 2019 patients, and protein C/cell-free DNA for noncoronavirus disease pneumonia patients. This hypothesis-generating study suggests that the pathophysiology of immunothrombosis differs between the two patient groups.

**KEY WORDS:** coagulation; coronavirus disease 2019; endothelial dysfunction; immunothrombosis; mortality; NETosis

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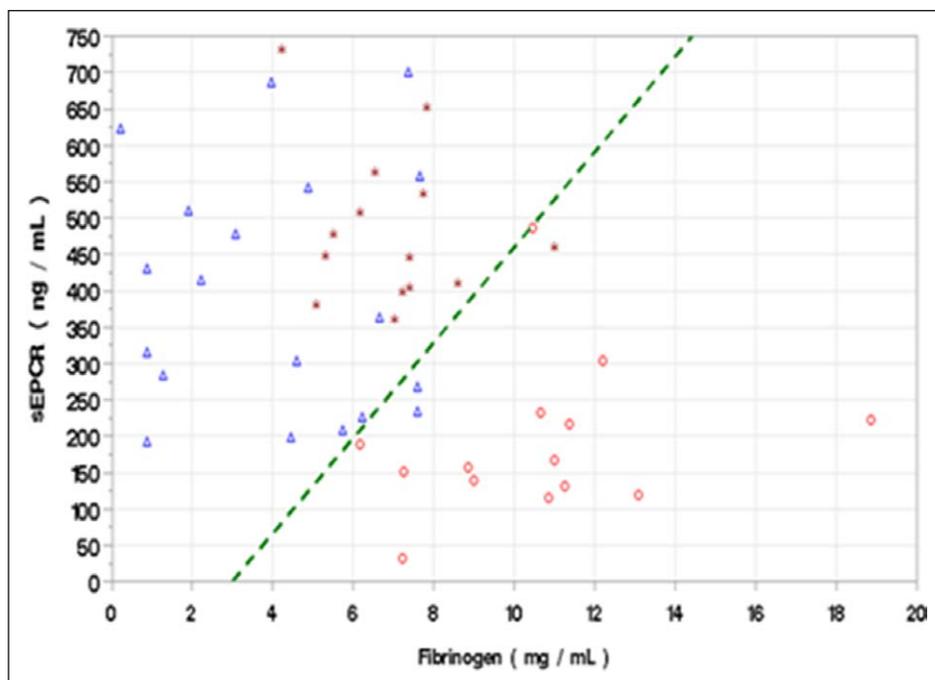
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**Figure 1.** Separation of coronavirus disease 2019 (COVID-19) patients ( $n = 14$ ), non-COVID septic pneumonia patients ( $n = 19$ ), and healthy volunteers ( $n = 14$ ) with respect to baseline (day 1) values of soluble endothelial protein C receptor (sEPCR) and fibrinogen. Based on the outputs of single markers binomial logit models, the *green border line* maximize the sum of sensitivity and specificity between each pair. Symbols: COVID-19 patients (*red circles*), septic pneumonia patients (*blue triangles*), and healthy volunteers (*brown stars*).

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus has infected over 244 million people and resulted in over 4.9 million deaths worldwide (1). Risk factors associated with death from coronavirus disease 2019 (COVID-19) infection include older age, male sex, and comorbidities such as diabetes, hypertension, and obesity (2, 3). The virus uses the angiotensin-converting enzyme 2 (ACE-2) receptor to infect alveolar epithelial cells as well as extrapulmonary tissues including heart, kidney, and blood vessels (4). A flu-like illness can progress to severe hypoxemic respiratory failure, sepsis, and multiple organ failure in some individuals (2).

Among the numerous complications associated with this virus, patients who have required hospitalization are at an increased risk of thrombotic complications such as microvascular thrombosis and venous/arterial thrombosis (5, 6). A number of earlier studies noted the presence of abnormal coagulation and inflammatory parameters in hospitalized patients with COVID-19 as well as undiagnosed thrombosis in nonsurvivors (7–11). The prothrombotic state is likely initiated by the infection of vascular endothelial cells by the SARS-CoV-2

virus via the ACE-2 receptor (12, 13). This can lead to endothelial injury, coagulation activation, and neutrophil extracellular trap (NET) formation that collectively culminates in immunothrombosis that predominantly affects the microvasculature (14). For example, COVID-19 infection leads to degradation of the endothelial glycocalyx that can lead to increased von Willebrand factor (vWF) release and platelet activation (15–17). Serum from COVID-19 patients contains elevated levels of NET components such as cell-free DNA (cfDNA), myeloperoxidase-DNA complexes, and citrullinated histones (H3-Cit) (18). Autopsies of COVID-19 patients revealed neutrophil infiltration and blood clots in pulmonary capillaries (19) as well as in the proximal lung vasculature (14).

While previous studies have provided insights into longitudinal changes in cytokine and growth factor levels ICU patients with COVID-19 (20, 21), few studies have investigated longitudinal changes in immunothrombosis biomarkers such as those of coagulation, endothelial dysfunction, and NET formation. Furthermore, the extent to which longitudinal changes in these biomarkers differ between ICU COVID-19 patients and ICU non-COVID patients with sepsis from pneumonia has not been examined. Inclusion of the latter in biomarker studies may help to identify pathologic pathways that are unique in COVID-19 patients.

The objective of this study is to compare time-dependent changes immunothrombosis biomarkers between COVID-19 patients and non-COVID septic patients with pneumonia. We focused on biomarkers that have been shown to be associated with poor outcome in ICU COVID-19 patients or in ICU non-COVID patients with sepsis from pneumonia (22–27). Specifically, we measured biomarkers of blood coagulation (thrombin-antithrombin [TAT] complexes), anticoagulation (protein C

[PC], antithrombin), endothelial injury (soluble thrombomodulin [sTM] and soluble endothelial protein C receptor [sEPCR]), fibrinolysis (fibrinogen, D-dimer), and NET formation (extracellular DNA, H3-Cit). We investigated the ability of these biomarkers to distinguish between COVID-19 patients and non-COVID patients with sepsis from pneumonia. A secondary objective is to evaluate the utility of the biomarkers for predicting ICU mortality in the two patients groups.

## MATERIALS AND METHODS

### Patients and Selection Criteria

Patients with SARS-CoV-2 infection ( $n = 14$ ) were recruited from a prospective cohort study conducted at a single ICU at an academic tertiary care hospital in London, Ontario, Canada. Patients were recruited from March 16, 2020, to April 24, 2020, corresponding to the initial COVID-19 outbreak in the London health region. The study was approved by the Western University Human Research Ethics Board (REB Number 6963) (15). Consecutive patients ( $\geq 18$  yr) admitted to the ICU with suspected COVID-19 infections were enrolled based on the Centers for Disease Control and Prevention criteria (28). COVID-19 status was confirmed by two positive polymerase chain reaction tests for the SARS-CoV-2 virus. All patients met the inclusion criteria for sepsis (29). Enrolled patients ranged in high-flow oxygen via nasal cannula, to non-invasive ventilation, to mechanical ventilation.

ICU patients with sepsis from pneumonia ( $n = 19$ ) were recruited from a pan-Canadian multicenter prospective observational study (the DNA as Prognostic Marker in ICU patients Study, ClinicalTrials.gov Identifier: NCT01355042) (25). The study was approved by the Hamilton Integrated Research Ethics Board (REB Number 10-532). Patient details are provided in **Supplemental Text 1** (<http://links.lww.com/CCX/A861>).

### Clinical Data

Baseline characteristics included age, sex, comorbidities, and presenting chest radiograph findings. Disease severity was classified using Sequential Organ Failure Assessment and Multiple Organ Dysfunction Scores. Clinical data were prospectively collected including the lowest or worst  $P_{aO_2}/F_{iO_2}$  ratio, mean arterial pressure, and standard laboratory values. Recorded

interventions included the use of antibiotics/antivirals, corticosteroids, vasopressors, respiratory support, renal replacement, antiplatelet agents, and anticoagulants. Patients that survived to hospital discharge were considered survivors for the purposes of these analyses.

### Collection of Plasma Samples From Patients and Healthy Volunteers

Whole blood was processed within 2 hours of blood collection (25). The plasma was stored at  $-80^{\circ}\text{C}$  and thawed at the time of assays. For COVID-19 patients and non-COVID patients with sepsis from pneumonia, blood was collected within 24 hours of ICU admission (day 1), then daily up to day 7, and then every 3 days until death or discharge. Blood samples from ICU non-COVID septic patients with pneumonia were collected within 24 hours of meeting the inclusion criteria for sepsis (25). Previously collected blood samples from a healthy control group were assembled from age- and sex-matched participants held at the Translational Research Center in London, Ontario.

### Assays to Measure Levels of Biomarkers in Plasma Samples

sTM was measured using the Human Thrombomodulin/BDCA-3 Quantikine Enzyme-Linked Immunosorbent Assay (ELISA) Kit from R&D Systems (Minneapolis, MN). D-dimer was measured using the Human D-dimer ELISA from RayBiotech (Peachtree Corners, GA). TAT complexes, antithrombin, fibrinogen, PC were measured using kits from Affinity Biologicals (Ancaster, ON, Canada). sEPCR was measured using an in-house ELISA using JRK 1535 (monoclonal anti-sEPCR) and JRK 1495-horse radish peroxidase conjugate kindly provided by Dr. Charles Esmon. Plasma levels of H3-Cit were measured according to Thålin et al (30) except that the anti-histone biotin was diluted 1:20 in the incubation buffer and plasma samples were diluted 1:5 in phosphate buffered saline/1% bovine serum albumin. Plasma levels of cfDNA were measured using the Quant-iT PicoGreen double-stranded DNA Assay Kit from Invitrogen (Carlsbad, CA). The DNA used for standards was obtained from genomic DNA isolated from the blood of healthy donors using the Qiagen PAXgene Blood DNA Kit (Hilden, Germany).

## Statistical Analyses

Data analysis was based on binomial logit models and receiver operating characteristic (ROC) analysis. The LOGISTIC procedure of SAS (Version 9.4; SAS AI & Analytics, Cary, NC) was used to perform most of the computations. The ROC analysis not only generates the area under the curves (AUCs) and their 95% CIs for indicating the predictive (distinguishing) powers of biomarkers but also identifies the cutoff values for maximizing the sums of sensitivity and specificity. In order to let the lengths of the CIs better reflect the effect of sample size, we modified the method of Hanley and McNeil (31) by choosing the critical value from a *t* distribution rather than the standard normal distribution.

Since the sample sizes are small, we used mostly single-marker and two-marker binomial logit models to assess the distinguishing and predictive powers of potentially useful biomarkers. For distinguishing the patients with COVID-19 from healthy volunteers and from non-COVID septic patients with pneumonia, we let the dependent variable be the probability of having COVID-19 and used the day 1 values of all biomarkers. For predicting mortality, we let the dependent variable be the probability of dying and used the values of all biomarkers on the “last” day, which is defined as the most recent day on which a nonmissing value was available. Our choice of the last-day values over the day 1 values of biomarkers for ICU mortality analysis was based on our previous finding that longitudinal changes in biomarker levels are more important than the day 1 biomarker values in determining their predictive powers on ICU mortality (27). We also report additional findings from multivariate logit models via the “forward stepwise method” (Supplemental Text 2, Supplemental Tables 5–10, and Supplemental Fig. 2, <http://links.lww.com/CCX/A861>) as well as whether SOFA has distinguishing and/or prognostic utility (Supplemental Text 3, <http://links.lww.com/CCX/A861>) as well as whether SOFA has distinguishing and/or prognostic utility (Supplemental Text 3, <http://links.lww.com/CCX/A861>).

## RESULTS

### Baseline Characteristics of COVID-19 Patients and Non-COVID Patients With Sepsis From Pneumonia

The baseline characteristics of ICU patients with COVID-19 infection ( $n = 14$ ) and non-COVID ICU

patients with sepsis from pneumonia ( $n = 19$ ) as well as age- and sex-matched healthy control subjects ( $n = 14$ ) are shown in **Table 1**. Most of the characteristics are similar between the two patient groups. The 31-day ICU mortality rate for the COVID-19 patients and for the septic patients with pneumonia was 50% and 32%, respectively.

### Identification of Biomarkers That Can Distinguish COVID-19 Patients From Healthy Volunteers and From Non-COVID Septic Patients With Pneumonia

Summary statistics of the day 1 values of the nine biomarkers are shown in **Supplemental Table 1** (<http://links.lww.com/CCX/A861>). To identify biomarkers that can distinguish COVID-19 patients from healthy volunteers and from non-COVID patients with pneumonia, a binomial logit model was applied to the day 1 values of the biomarkers. A biomarker is considered to have a significant power to distinguish the COVID-19 patients from either healthy volunteers or from non-COVID septic patients with pneumonia if the AUC has the lower limit of the 95% CI being greater than 0.50 (i.e., better than random assignments).

**Table 2** reports the findings on those biomarkers with significant distinguishing powers. With AUC values of 1.00, cfDNA and D-dimer have the strongest power to distinguish COVID-19 patients from healthy volunteers (Table 2, top panel). Using the cutoff values of 2.4  $\mu\text{g/mL}$  for cfDNA and 1.3  $\mu\text{g/mL}$  for D-dimer, the combination of sensitivity = 1 and specificity = 1 was achieved. sEPCR, PC, and sTM also have strong distinguishing powers with AUC values of 0.95, 0.95, and 0.89, respectively. The distinguishing power is moderate for fibrinogen (AUC = 0.86) and modest for both H3-Cit and TAT (AUC = 0.81).

The lower panel of Table 2 shows that four of the biomarkers have significant powers to distinguish between COVID-19 patients and non-COVID patients with sepsis from pneumonia. The most powerful biomarker is fibrinogen (AUC = 0.95) with COVID-19 patients having higher levels of fibrinogen compared with non-COVID patients with sepsis from pneumonia. sEPCR and antithrombin also have high distinguishing power with AUC values of 0.89. With AUC = 0.83, cfDNA has a modest distinguishing power.

Next, we determined if a combination of the biomarkers can be used to separate the COVID-19 patients

**TABLE 1.**  
**Baseline Characteristics of Coronavirus Disease 2019 Patients, Noncoronavirus Disease Septic Patients With Pneumonia, and Sex- and Age-Matched Healthy Volunteers**

Patient Characteristics	COVID-19 Patients (n = 14)	Non-COVID Septic Patients With Pneumonia (n = 19)	p	Healthy Controls (n = 14)
Age	61 (54–67)	65 (49–73)	0.5530	58 (54–63)
Male	6 (43%)	10 (52.6%)	0.5926	6 (42.9%)
Comorbidities				
Diabetes	5 (35.7%)	6 (31.6%)	0.8107	
Hypertension	7 (50.0%)			
Coronary artery disease	2 (14.3%)			
Congestive heart failure	0	0	1	
Chronic kidney disease	2 (14.3%)	2 (10.5%)	0.7530	
Cancer	2 (14.3%)	4 (21.1%)	0.6314	
Chronic obstructive pulmonary disease	1 (7.1%)			
Types of infection				
Severe acute respiratory syndrome coronavirus 2	14 (100%)			
Influenza		1 (5.2%)		
Bacterial		7 (36.8%)		
Fungal		4 (21.1%)		
Mixed (bacterial and fungal)		7 (36.8%)		
Multiple Organ Dysfunction Score	4 (3–5.5)	6 (5–10)	0.0196	
Sequential Organ Failure Assessment	4.5 (2–9.25)	9 (6–12)	< 0.0001	
Mean arterial pressure	84 (72.75–97.5)	73.5 (68.3–78.8)	0.1266	
PaO <sub>2</sub> /Fio <sub>2</sub> ratio	107 (65.5–161.675)	147 (87–200)	0.3922	
WBC	8.45 (6.9–16.075)			
Lymphocytes	0.7 (0.55–1)	10.3 (1.5–20.1)	0.0025	
Neutrophils	7.3 (5.6–12.55)	11 (3.8–24.2)	0.2671	
Lactate	1.5 (1–2)	Three missing values		
Platelets	206 (133.5–293.75)	180 (92–247)	0.3155	
Hemoglobin	121.5 (101.5–134.5)	90 (86.5–100.5)	< 0.0001	
Creatinine	81.5 (57.5–187)	95 (62–204)	0.3566	
International normalized ratio	1.2 (1.1–1.3)	1.6 (1.2–2.0)	0.0674	
Partial thromboplastin time	28 (25–31)			
Treatments				
Antibiotics	14 (100%)	19 (100%)	1	
Antivirals	3 (21.4%)	5 (26.3%)	0.7554	
Steroids	3 (21.4%)	9 (47.4%)	0.1338	
Vasoactive medications	11 (78.6%)	14 (73.7%)	0.7554	
Renal replacement therapy	2 (14.3%)	0 (0%)	0.0945	
Antiplatelet agent	5 (35.7%)			
Prophylactic anticoagulation	13 (92.9%)	4 (21.1%)	< 0.0001	

(Continued)

**TABLE 1. (Continued).**

**Baseline Characteristics of Coronavirus Disease 2019 Patients, Noncoronavirus Disease Septic Patients With Pneumonia, and Sex- and Age-Matched Healthy Volunteers**

Patient Characteristics	COVID-19 Patients ( <i>n</i> = 14)	Non-COVID Septic Patients With Pneumonia ( <i>n</i> = 19)	<i>p</i>	Healthy Controls ( <i>n</i> = 14)
Respiratory support				
High-flow nasal oxygen and/or noninvasive mechanical ventilation	4 (28.6%)			
Invasive ventilation	10 (71.4%)	19 (100%)	0.0119	
Died	7 (50.0%)	6 (31.6%)	0.2992	

COVID-19 = coronavirus disease 2019.

Median (interquartile range) and *n* (%).

Paired Wilcoxon rank-sum tests and Fisher exact tests.

**TABLE 2.**

**Receiver Operating Characteristic Analysis to Distinguish 14 Coronavirus Disease 2019 Patients From 14 Healthy Volunteers and From 19 Noncoronavirus Disease Septic Patients With Pneumonia**

Biomarker	Intercept	Coefficient	Area Under the Curve	Cutoff Value of Biomarker	Sensitivity	Specificity	Sensitivity + Specificity
Reference group: 14 healthy volunteers							
cfDNA (µg/mL)	-58.787	24.427	1.00	2.4	1.00	1.00	2.00
D-dimer (µg/mL)	-19.859	15.467	1.00	1.3	1.00	1.00	2.00
sEPCR (ng/mL)	7.970	-0.023	0.95	340	0.93	1.00	1.93
Protein C (U/mL)	14.095	-0.177	0.95	80	0.86	1.00	1.86
Soluble thrombomodulin (ng/mL)	-8.595	2.194	0.89	4.0	0.79	0.92	1.71
Fibrinogen (mg/mL)	-6.544	0.777	0.86	8.0	0.79	0.92	1.71
Citrullinated histones (µg/mL)	-1.960	4.274	0.81	0.31	0.35	0.58	1.58
Thrombin-antithrombin (nM)	-0.731	0.001	0.81	375	0.79	0.78	1.57
Reference group: 19 noncoronavirus disease septic patients with pneumonia							
Fibrinogen (mg/mL)	-9.544	1.256	0.95	8.0	0.79	1.00	1.79
sEPCR (ng/mL)	3.059	-0.013	0.89	190	0.64	1.00	1.64
Antithrombin (U/mL)	-6.011	0.083	0.89	75	0.79	0.84	1.63
cfDNA (µg/mL)	4.218	-1.039	0.83	4.58	0.86	0.79	1.65

cfDNA = cell-free DNA, sEPCR = soluble endothelial protein C receptor.

The day 1 values of the biomarkers were used in a binomial logit model. The cutoff value of the biomarker is defined as the value that maximizes the sum of the sensitivity and specificity. All biomarkers shown in this table have the lower limit of the 95% CI of the area under the curve being greater than 0.5. The positive coefficients of the biomarkers indicate that coronavirus disease 2019 (COVID-19) patients have higher values compared with either healthy volunteers or non-COVID patients with sepsis from pneumonia. In contrast, negative coefficients indicate that the COVID-19 patients have lower values compared with the other two groups. The positive coefficients of biomarkers indicate that the COVID-19 patients tended to have higher values compared with healthy volunteers.

from both healthy volunteers and non-COVID septic pneumonia patients. Based on Table 2, fibrinogen and sEPCR were selected for this purpose since they are the only two biomarkers that appear in the upper and lower panels of Table 2 and have consistent signs. We pooled the data of all three groups into one dataset and used these two biomarkers in a logit model with the probability of having COVID-19 as the dependent variable. As shown in **Supplemental Table 2** (<http://links.lww.com/CCX/A861>), this combination has a high distinguishing power (AUC, 0.99; 95% CI, 0.96–1.00).

### Identification of Biomarkers for Predicting ICU Mortality in COVID-19 Patients and in Non-COVID Septic Patients With Pneumonia

Using single-marker binomial logit models, we found that sTM (AUC, 0.90; 95% CI, 0.71–1.00) and H3-Cit (AUC, 0.88; 95% CI, 0.67–1.00) have significant powers for predicting ICU mortality in COVID-19 patients (**Table 3**, left panel; **Supplemental Fig. 1**, <http://links.lww.com/CCX/A861>). Nonsurvivors tend to have higher levels of sTM and H3-Cit levels compared with survivors. With a two-marker model, we found that the combination of sTM and H3-Cit yields an AUC value of 1.00 for predicting ICU mortality. In contrast, sTM and H3-Cit (either individually or in combination) do not have significant powers to predict ICU mortality

in the 19 patients with sepsis from pneumonia (as reflected by the lower limits of the 95% CI for AUC being less than 0.5; right panel of Table 3).

Next, we investigated if any of the biomarkers are significant predictors of mortality in ICU non-COVID patients with sepsis from pneumonia. As shown in **Table 4** (left panel), PC is a significant predictor of ICU mortality in these patients (AUC, 0.85; 95% CI, 0.62–1.00). The combination of PC and cfDNA improves the prediction of ICU mortality (AUC, 0.87; 95% CI, 0.68–1.00). In contrast, neither PC (AUC, 0.65; 95% CI, 0.32–0.89) nor cfDNA (AUC, 0.71; 95% CI, 0.37–1.00) has a significant power in predicting ICU mortality in the COVID-19 patients. Taken together, the results suggest that the main predictors of ICU mortality differ between the two patient groups: sTM and H3-Cit for COVID-19 patients, and PC and cfDNA for non-COVID patients with sepsis from pneumonia. We also demonstrated that use of prophylactic anticoagulation or invasive ventilation did not have confounding effects (**Supplemental Text 4** and **Supplemental Tables 11** and **12**, <http://links.lww.com/CCX/A861>).

Finally, we examined longitudinal changes in the biomarkers in both patient groups (**Fig. 1**). Using analysis of variance, we observed that there are significant differences in the following biomarkers

**TABLE 3.**  
**Estimation Results of Predicting the Probabilities of Dying in the ICU by Soluble Thrombomodulin and Histones via a Binomial Logit Model: Contrast Between 14 Coronavirus Disease 2019 Patients and 19 Noncoronavirus Disease Septic Patients With Pneumonia**

Predictive Biomarker	Specification 1	Specification 2	Specification 3	Specification 4	Specification 5	Specification 6
	For 14 Coronavirus Disease 2019 Patients			For 19 Septic Patients With Pneumonia		
Intercept	-4.418	-1.499	-66.318	-1.712	-0.759	-1.650
Thrombomodulin	0.477 (t = 1.95) (p = 0.0506)	– – –	5.537 (t = 1.07) (p = 0.2839)	0.114 (t = 1.20) (p = 0.2317)	– – –	0.122 (t = 1.22) (p = 0.2207)
Citrullinated histones	– – –	1.230 (t = 1.32) (p = 0.1884)	15.270 (t = 1.03) (p = 0.3010)	– – –	-0.002 (t = -0.06) (p = 0.9557)	-0.023 (t = -0.37) (p = 0.7148)
AUC	0.90	0.88	1.00	0.56	0.33	0.56
AUC (95% CI)	0.71–1.00	0.67–1.00	1.00–1.00	0.17–0.96	0.01–0.65	0.17–0.96

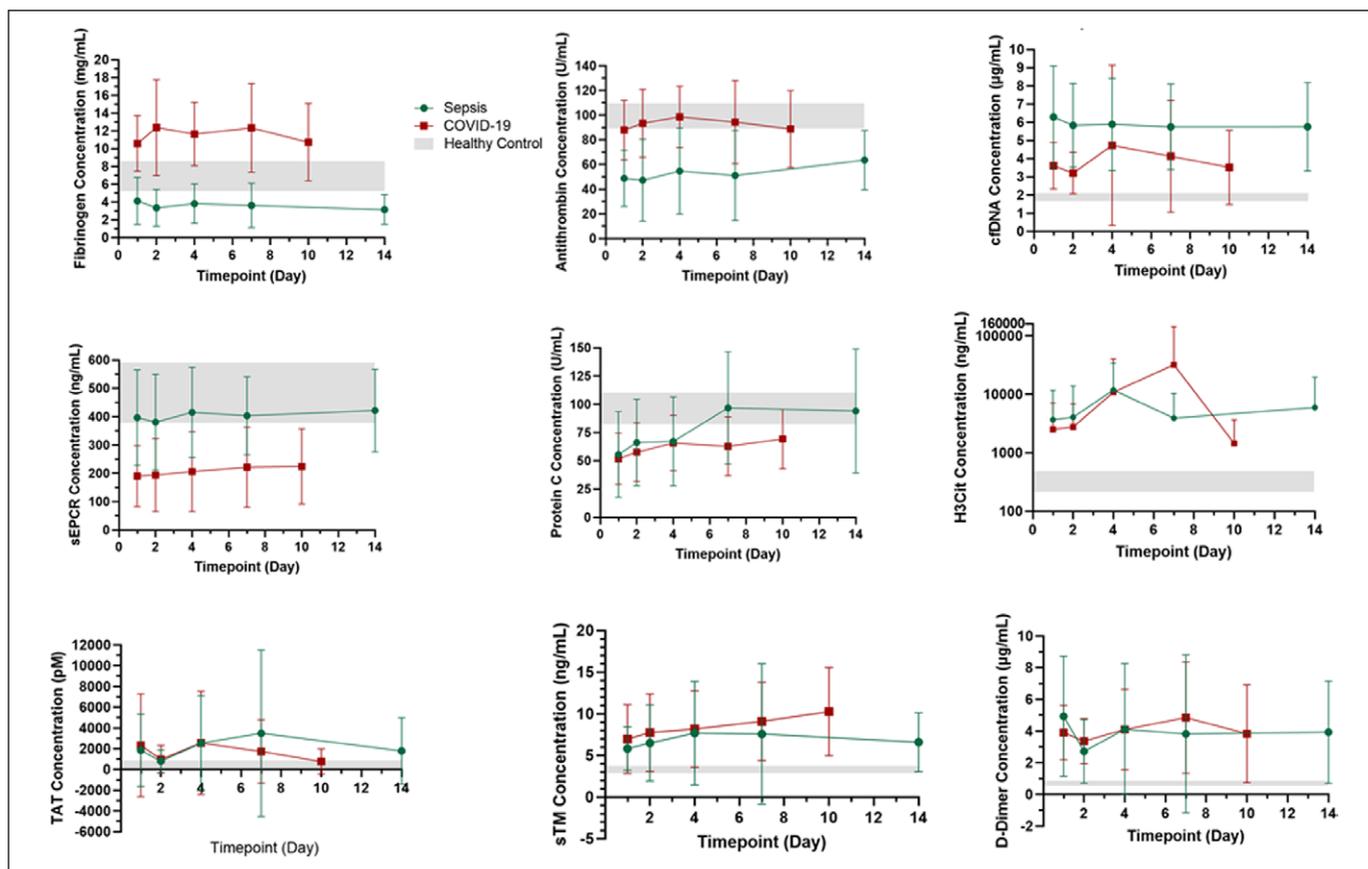
AUC = area under the curve.  
Dashes refers to not applicable.

**TABLE 4.**  
**Estimation Results of Predicting the Probabilities of Dying in the ICU by Protein C and Cell-Free DNA via a Binomial Logit Model: Contrast Between 14 Coronavirus Disease 2019 Patients and 19 Noncoronavirus Disease Septic Patients With Pneumonia**

Predictive Biomarker	Specification 1	Specification 2	Specification 3	Specification 4	Specification 5	Specification 6
	For 19 Septic Patients With Pneumonia			For 14 Coronavirus Disease 2019 Patients		
Intercept	2.105	-2.530	0.084	-1.571	-2.559	-7.727
Protein C	-0.038 (t = -2.09) (p = 0.0367)	-	-0.039 (t = -2.11) (p = 0.0349)	0.022 (t = 0.95) (p = 0.3443)	-	0.048 (t = 1.29) (p = 0.1969)
Cell-free DNA	-	0.317 (t = 1.26) (p = 0.2084)	0.378 (t = 1.25) (p = 0.2116)	-	0.826 (t = 1.22) (p = 0.2243)	1.499 (t = 1.33) (p = 0.1845)
AUC	0.85	0.69	0.87	0.65	0.71	0.84
AUC (95% CI)	0.62–1.00	0.41–0.97	0.68–1.00	0.32–0.98	0.37–1.00	0.59–1.00

AUC = area under the curve.

As shown in the left panel, protein C is a significant predictor of ICU mortality in noncoronavirus disease patients with sepsis from pneumonia (AUC, 0.85; 95% CI, 0.62–1.00). The combination of PC and cell-free DNA improves the prediction of ICU mortality in these patients (AUC, 0.87; 95% CI, 0.68–1.00). Dashes refers to not applicable.



**Figure 2.** Longitudinal measurements of biomarker levels in 14 coronavirus disease 2019 (COVID-19) patients (red squares) and 19 non-COVID septic patients with pneumonia (green circles). The data are shown as mean ± SD. The biomarker levels in age- and sex-matched healthy controls (n = 14) are shown in the shaded gray boxes that represent the upper and lower limits of 1 SD. cfDNA = cell-free DNA, H3-Cit = citrullinated histones, sEPCR = soluble endothelial protein C receptor.

between the two patient groups: fibrinogen, anti-thrombin, sEPCR, and cfDNA ( $p < 0.001$ ). We also investigated if the most recent biomarker values are better than the day 1 values for predicting ICU mortality. As shown in **Supplemental Table 3** (<http://links.lww.com/CCX/A861>), the predictive powers of sTM (for the COVID-19 patients) and PC (for the non-COVID septic pneumonia patients) are greater when using the most recent biomarker values compared with the day 1 values. These findings are consistent with our previous longitudinal study that showed that for most biomarkers, the change variables are more powerful than the day 1 variables in predicting ICU mortality (27).

## DISCUSSION

COVID-19 is associated with a prothrombotic state although the pathophysiologic mechanisms remain unclear. In this hypothesis-generating study, we identified eight immunothrombosis biomarkers that distinguished COVID-19 patients from healthy individuals: cfDNA, D-dimer, PC, sEPCR, sTM, fibrinogen, H3-Cit, and TAT complexes (Table 2). We also identified four biomarkers that differentiate COVID-19 patients from non-COVID septic pneumonia patients: fibrinogen, antithrombin, sEPCR, and cfDNA (Table 2). In addition, our data suggest that the predictors of ICU mortality differ between the two patient groups: sTM and H3-Cit for COVID-19 patients, and PC and cfDNA for non-COVID septic patients with pneumonia (Tables 3 and 4). This suggests that endothelial dysfunction and NETosis (as reflected by increases in sTM and H3-Cit, respectively) contribute to immunothrombosis and poor outcome in COVID-19 patients. In contrast, immunothrombosis in non-COVID patients with sepsis from pneumonia may reflect ongoing microvascular coagulation (as reflected by consumption of PC, a natural anticoagulant) and release of procoagulant cfDNA from injured or dying cells.

With respect to coagulation biomarkers, a recent single-center study of 46 COVID-19 patients and 53 non-COVID sepsis patients demonstrated that COVID-19 patients had increased thrombin generation potential despite prophylactic anticoagulation, whereas septic patients did not (32). It should be noted that the study included a heterogeneous group of septic patients (pneumonia, urosepsis, abdominal sepsis, and skin/soft-tissue

infections) (32). In the current study, the sepsis patients are limited to those with severe respiratory failure (pneumonia) that may provide an opportunity to identify COVID-specific immunothrombosis mechanisms.

Compared with healthy volunteers, D-dimer and fibrinogen levels are markedly elevated in COVID-19 patients (**Fig. 2**; and Supplemental Table 1, <http://links.lww.com/CCX/A861>), a finding consistent with previous studies (2, 7). The persistently high fibrinogen levels in COVID-19 patients observed in this study (up to day 10) may reflect a sustained acute phase response by the liver, possibly to protect the host (33). In addition to its prothrombotic role, fibrinogen plays a protective role in the host response to pathogens (33). For example, fibrinogen binds to Mac-1 (CD11b/CD18) on neutrophils, thereby inhibiting neutrophil-endothelium interactions (34). In contrast, fibrinogen levels in non-COVID pneumonia patients are persistently lower than in COVID-19 patients (Fig. 2) that suggests consumptive coagulopathy, a hallmark of disseminated intravascular coagulation (DIC). Using the International Society on Thrombosis and Haemostasis criteria for DIC diagnosis (35), we found that the frequency of overt DIC in the non-COVID pneumonia patients is higher than that in the COVID-19 patients (72% vs 50%) (**Supplemental Table 4**, <http://links.lww.com/CCX/A861>).

Another biomarker that differs between COVID-19 patients and non-COVID patients with sepsis from pneumonia is antithrombin (Fig. 2). In the non-COVID patients, TAT levels are persistently elevated (which reflects ongoing thrombin generation) and antithrombin levels are persistently decreased. Decreased levels of antithrombin may reflect excessive thrombin generation and/or impaired liver synthesis as a negative acute phase response (36). Although TAT levels are also persistently elevated in COVID-19 patients, antithrombin levels remain in the normal range (Fig. 2). A recent cross-sectional study of 48 COVID-19 patients reported that heparanase levels are elevated in COVID-19 infections that would presumably lead to increased degradation of heparan sulfate. Heparan sulfate, the most abundant glycosaminoglycan in the endothelial glycocalyx, exerts anticoagulant, anti-inflammatory, and barrier protective functions (37). Since heparan sulfate catalyzes antithrombin-mediated inactivation of coagulation enzymes, loss of heparan sulfate in COVID-19 may impair the anticoagulant function

of antithrombin. Glycocalyx degradation has been shown to contribute to COVID-19-associated endothelial dysfunction as evidenced by increased levels of syndecan-1 and hyaluronic acid, particularly on ICU day 3 and thereafter (15). Incubation of human pulmonary microvascular endothelial cells with plasma from COVID-19 patients induces endothelial dysfunction (38). Recent studies suggest that COVID-19 patients have sustained endothelial dysfunction at 4 months after hospital discharge as evidenced by persistent elevations in coagulation factor VIII and plasminogen activator inhibitor (39).

Thrombomodulin, an endothelial glycoprotein, also maintains the endothelium in an anticoagulant and anti-inflammatory state (40). Increased plasma levels of sTM have been described in various vascular disease states including COVID-19 infections, sepsis, and DIC (32, 41, 42). The shedding of TM occurs via proteolytic cleavage by neutrophil elastase, cathepsin G, proteinase 3, and metalloproteases (43, 44). A recent multiplex immunoprofiling study of 10 COVID-positive and 10 COVID-negative patients reported that levels of neutrophil elastase were persistently elevated in COVID-positive patients on ICU days 2 to 7 (11). In this study, we found that plasma levels of sTM are higher in COVID-19 patients who died, suggesting that endothelial dysfunction contributes to poor outcome. These findings are consistent with a single-center study of hospitalized COVID-19 patients that showed that elevated levels of sTM predicts in-hospital mortality (42, 45). We also found that the predictive power of sTM is greater when using the most recent ICU plasma samples compared with the day 1 samples (Supplemental Table 3, <http://links.lww.com/CCX/A861>). In contrast, day 1 values of inflammatory cytokines (e.g., heat shock protein 70) are associated with mortality in COVID-19 patients (11), suggesting that endothelial dysfunction and immunothrombosis manifest at the later stages of disease pathophysiology in COVID-19.

We also found that H3-Cit levels are higher in COVID-19 nonsurvivors compared with survivors, suggesting excessive NET release from activated neutrophils. Recently, a study of 40 COVID-19 ICU patients and nine COVID-19 non-ICU patients identified neutrophil markers that identify patients who are at risk of becoming critically ill (46). Specifically, granulocyte colony-stimulating factor and interleukin-8

(which drive neutrophil activation) distinguish patients at risk of future clinical decompensation (46). More broadly, transcriptomic analyses of COVID-19 versus non-COVID viral infections identified a “COVID-19 specific gene signature” that is consistent with high neutrophil-lymphocyte ratio in COVID-19 patients (47).

Although sTM and H3-Cit are elevated in non-COVID septic patients with pneumonia compared with healthy controls, our results suggest that these biomarkers are not useful for predicting ICU mortality. Instead, elevations in cfDNA and consumption of PC are significant predictors of ICU mortality in non-COVID septic patients with pneumonia (Table 4 and Supplemental Fig. 1, <http://links.lww.com/CCX/A861>). Based on tissue-specific methylation profile analysis, circulating cfDNA in sepsis are mainly derived from neutrophils and hepatocytes, with hepatocyte cfDNA strongly correlating with alanine aminotransferase that is a marker of hepatocyte damage (48).

A strength of this study is the availability of longitudinal plasma samples from a well-characterized cohort of non-COVID ICU patients with sepsis from pneumonia recruited from a multicenter prospective observational study (25). The major limitation of this study is that it is a small single-center study of COVID-19 patients. A larger sample size would allow us to explore additional biomarkers such as vWF that has been shown to have prognostic utility in a small pilot study of COVID-19 patients (49). Also, the patients were recruited relatively early during the COVID-19 pandemic when ICU care was not yet standardized, which may explain the high mortality rate in this cohort. Thus, the findings of this hypothesis-generating pilot study should be validated prospectively.

## CONCLUSIONS

Our results suggest that four biomarkers (fibrinogen, sEPCR, antithrombin, and cfDNA) have significant powers for distinguishing COVID-19 patients from non-COVID patients with sepsis from pneumonia. Our data also suggest that the predictors of ICU mortality differ between the two patient groups: sTM and H3-Cit for COVID-19 patients, and PC and cfDNA for non-COVID septic patients with pneumonia. These findings suggest that there are pathophysiological differences in the mechanisms of immunothrombosis between the two patient groups.

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