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BRIEF REPORT

Non-severe COVID-19 is associated with endothelial damage and hypercoagulability despite pharmacological thromboprophylaxis

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Abstract

Background: Hypercoagulability and endothelial dysfunction are hallmarks of coronavirus disease 2019 (COVID-19) and appear to predict disease severity. A high incidence of thrombosis despite thromboprophylaxis is reported in patients with moderate to severe COVID-19. Recent randomized clinical trials suggest that therapeutic-intensity heparin confers a survival benefit in moderate-severity COVID-19 compared to standard-intensity heparin, potentially by harnessing heparin-mediated endothelial-stabilizing and anti-inflammatory effects.

Objective: We hypothesized that patients with moderate-severity COVID-19 exhibit enhanced hypercoagulability despite standard-intensity thromboprophylaxis with low molecular weight heparin (LMWH) compared to non-COVID-19 hospitalized patients.

Methods: Patients with moderate COVID-19 and a control group (severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]–negative hospitalized patients) receiving LMWH thromboprophylaxis were recruited. Markers of endothelial damage and plasma thrombin generation parameters were assessed.

Results: Tissue plasminogen activator levels were significantly increased in the COVID-19 group (8.3 ± 4.4 vs. 4.9 ± 2.4 ng/ml; $P = .02$) compared to non-COVID-19–hospitalized patients. Despite thromboprophylaxis, mean endogenous thrombin potential was significantly increased among COVID-19 patients (1929 ± 448 vs. 1528 ± 460.8 nM*min; $P = .04$) but lag time to thrombin generation was significantly

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prolonged (8.1 ± 1.8 vs. 6.2 ± 1.8 mins; $P = .02$). While tissue factor pathway inhibitor (TFPI) levels were similar in both groups, in the presence of an inhibitory anti-TFPI antibody, the difference in lag time between the groups was abrogated.

Conclusions: Collectively, these data demonstrate that COVID-19 of moderate severity is associated with increased plasma thrombin generation and endothelial damage, and that hypercoagulability persists despite standard LMWH thromboprophylaxis. These findings may be of clinical interest given recent clinical trial data which suggest escalated heparin dosing in non-severe COVID-19 may be associated with improved clinical outcomes.

KEYWORDS
COVID-19, endothelium, heparin, thrombosis, venous thromboembolism

1 | INTRODUCTION

Hypercoagulability has emerged as an important hallmark of coronavirus disease 2019 (COVID-19).[1] Increased rates of thrombosis have been observed among affected patients and the initial reports describing the complications of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection identified increased coagulation activation as an important marker of disease severity and mortality risk.[2–4] This observation has since become a major focus of clinical and scientific interest. Clinical trials have recently sought to evaluate the role of intensified pharmacological thromboprophylaxis in modulating the disease course in COVID-19.[5–7]

SARS-CoV-2 infection elicits a unique thromboinflammatory response.[1] Endothelial dysfunction, abnormal platelet activity, and procoagulant leukocyte dysfunction have been reported.[8–12] While other viral and bacterial infections have been reported to induce hemostatic derangements, the nature of the thromboinflammatory response in COVID-19 appears to be unique to SARS-CoV-2 infection.[12–16] Moreover, thromboinflammation and immunothrombosis may contribute to disease severity, as demonstrated by the pattern of localized coagulation activation and microthrombosis identified within the lung vasculature of patients who have died as a consequence of acute respiratory distress syndrome (ARDS) secondary to COVID-19.[8,17] Intriguingly, early administration of escalated heparin dosing (prior to requiring critical care–level support) may reduce the risk of progression to severe disease and death, independent of thrombosis risk.[5–7]

Admission to hospital with an acute inflammatory illness is associated with an increased risk of thrombosis. Pharmacological thromboprophylaxis is usually effective in reducing this risk.[18] Evidence of hypercoagulability, endothelial dysfunction, and increased thrombosis risk, despite pharmacological thromboprophylaxis, has been reported in severe COVID-19 (requiring critical care support).[4] The degree to which these derangements arise in patients with moderate disease (requiring hospital admission but not critical care–level management) and the degree to which this thrombosis risk is elevated in contrast to other hospitalized patients with acute inflammatory illness managed at general ward level is less clear.[2,19]

In this study, we aimed to characterize parameters of plasma thrombin generation and endothelial damage in a group of non-severe COVID-19 inpatients receiving low molecular weight heparin (LMWH) thromboprophylaxis compared to a group of matched hospitalized patients who had tested negative for SARS-CoV-2 and were receiving LMWH thromboprophylaxis.

Essentials

- Hypercoagulability and endothelial dysfunction are hallmarks of coronavirus disease 2019 (COVID-19).
- Plasma thrombin generation and markers of endothelial damage were assessed in patients with moderate-severity COVID-19 and a control group (severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]–negative hospitalized patients) receiving standard low molecular weight heparin (LMWH) thromboprophylaxis.
- Endogenous thrombin potential was significantly increased in patients with moderate-severity COVID-19. While levels of most markers of endothelial damage were similar between groups, elevated levels of plasma tissue plasminogen activator (t-PA) and increased sensitivity to the neutralization of plasma tissue factor pathway inhibitor (TFPI) activity in COVID-19 was seen.
- These data demonstrate that COVID-19 of moderate severity is associated with increased plasma thrombin generation and endothelial damage, and that hypercoagulability persists despite standard LMWH thromboprophylaxis.

2 | METHODS

2.1 | Patient recruitment

Ethical approval to proceed with this study was granted by the Institutional Review Board (IRB) of the Mater Misericordiae University Hospital, Dublin, Ireland (IRB reference 1/378/2077). Patients admitted to hospital for management of COVID-19 (with SARS-CoV-2 infection confirmed by real-time polymerase chain

reaction [RT-PCR] analysis of nasopharyngeal swab) and a control group, consisting of age-matched patients admitted with an acute infective illness but who had tested negative for SARS-CoV-2 by RT-PCR, were invited to participate. After receiving informed consent, samples of whole blood were obtained by direct venepuncture. Platelet-poor plasma (PPP) was obtained by centrifugation from citrated whole blood (2000 *g* for 10 min at room temperature) and frozen at −80°C prior to analysis. Recruitment was restricted to patients who were receiving appropriate LMWH thromboprophylaxis as per local and national guidelines.[18] The blood draw in each case was undertaken at 12 h following a LMWH dose, with analysis of plasma anti-factor X (FXa) activity levels carried out in parallel (ACL TOP 500 haematology analyser, HaemosIL liquid Anti-Xa reagent; Instrumentation Laboratory).

COVID-19 patients and controls were excluded if critical care–level support was required at any point during the hospital admission. Patients who had a pre-existing indication for intermediate or therapeutic dose anticoagulation were not recruited. Other exclusion criteria included severe chronic renal impairment (creatinine clearance <30 mls/min), liver disease associated with coagulopathy/cirrhosis (Child-Pugh B or C), extremes of body weight (<50kg or >100kg), active cancer, and prior history of unprovoked thrombosis or bleeding diathesis.

2.2 | Calibrated automated thrombography

Plasma thrombin generation was assessed by calibrated thrombography (CAT) using a Fluoroskan Ascent® Plate Reader (ThermoLab Systems®) in conjunction with Thrombinoscope™ software (Stago) as previously described.[20] Briefly, 80 μl aliquots of citrated PPP were incubated with 20 μl of a PPP reagent containing 4 μM phospholipids (composed of 60% phosphatidylcholine, 20% phosphatidylserine, and 20% phosphatidylethanolamine) and tissue factor (TF) (PPP-Low reagent, final TF concentration: 1 pM; PPP-reagent, final TF concentration: 5 pM; Stago). The activity of the activated protein C pathway was determined by assessing thrombin generation in the presence of soluble thrombomodulin (sTM; final concentration 6.25 nM; Haematologic Technologies) and TF-dependent thrombin generation was also assessed in the presence of a polyclonal inhibitory anti-tissue factor pathway inhibitor antibody (anti-TFPI; final concentration 100 μg/ml; Haematologic Technologies).

2.3 | Enzyme-linked immunosorbent assay

ELISAs for TFPI (DTFP10), tissue plasminogen activator (t-PA; DTPA00), thrombomodulin (DTHBD0), vascular cell adhesion molecule-1 (VCAM-1; DVC00), intercellular adhesion molecule-1 (ICAM-1; DCIM00), and E-selectin (DSLE00) were purchased from R&D Systems and a plasminogen activator inhibitor-1 (PAI-1; ab108891) ELISA was purchased from Abcam. All assays were performed according to the manufacturer's instructions.

2.4 | Statistical analysis

All experiments were performed at least in duplicate and data were tested for normal distribution using a Shapiro-Wilk test. Normally distributed continuous data were presented as mean ± standard deviation and assessed for statistical significance using an unpaired two-tailed Student's *t*-test. Non-parametric data was assessed using the Mann-Whitney *U* test. *P* values were adjusted for multiple comparisons using the Bonferroni correction. Statistical analysis was performed using the Prism™ software package (version 5.0; GraphPad Software Inc.).

3 | RESULTS AND DISCUSSION

Fourteen patients admitted to hospital with COVID-19 and 11 hospitalized controls (negative for SARS-CoV-2) were recruited. Baseline demographic and clinical characteristics are described in Table 1. There was no significant difference in age or body mass index (BMI) between groups. All patients were receiving pharmacological thromboprophylaxis with enoxaparin 40 mg once daily at the time of recruitment. Plasma anti-FXa activity levels were in the expected range in both groups (<0.1 IU/ml). All patients had been admitted to a general ward and none had required escalation to a critical care–level unit during the period of hospitalization. None of the COVID-19 patients required supplemental oxygen at the time of recruitment. No thrombotic events occurred in either group during the period of hospitalization.

Baseline clinical laboratory results were broadly similar in both groups, including D-dimer levels, C-reactive protein levels, and complete blood count parameters (Table 1). However, serum ferritin levels were markedly higher in the COVID-19 group in contrast to hospitalized controls (1277 ± 1284 vs. 153.9 ± 157.2 μg/ml; $P = .02$) and the activated partial thromboplastin time (APTT) was also more prolonged in the COVID-19 group (31.8 ± 0.9 vs. 27.8 ± 1; $P = .01$).

Markers of endothelial damage were measured in both groups (Figure 1). Levels of t-PA were significantly higher in the COVID-19 group (8.3 ± 4.4 vs. 4.9 ± 2.4 ng/ml; $P = .02$). Levels of sTM, E-selectin, VCAM-1, ICAM-1 and PAI-1 were similar in the COVID-19 patients and the hospitalized controls.

Parameters of TF-stimulated plasma thrombin generation are described in Table 2. In the presence of a 1pM TF stimulus, and despite pharmacological thromboprophylaxis (in samples taken at 12 h post-LMWH dosing), plasma endogenous thrombin potential (ETP) was significantly increased in the COVID-19 group compared to hospitalized controls (1929 ± 448 vs. 1528 ± 460.8 nM*min; $P = .04$). A trend to increased peak thrombin generation was also observed, although this difference did not reach statistical significance. The precise mechanism underlying this hypercoagulability remains to be determined. While t-PA levels were increased, which is suggestive of endothelial damage, elevated t-PA levels in isolation would not be expected to influence parameters of plasma thrombin generation in this assay.

TABLE 1 Clinical characteristics of hospitalized patients with non-severe COVID-19 compared with a group of hospitalized controls (confirmed negative for SARS-CoV-2 infection)

	COVID-19 ($n = 14$)	SARS-CoV-2–negative hospitalised patients ($n = 11$)	*p* Value
Age (years)	69.7 ± 16.9	61.6 ± 15.6	NS
Gender	Male = 7; Female = 7	Male= 3; Female= 8	NA
BMI (kg/m^2)	25.7 ± 4.3	22.7 ± 3.9	NS
Reason for hospital admission	COVID-19	Respiratory tract infection (SARS-CoV–2 PCR negative) $n = 10$; urinary tract infection, $n = 1$.	NA
Abnormal chest radiograph findings	Peripheral infiltrates only ($n = 7$); diffuse infiltrates ($n = 3$)	Peripheral/focal infiltrates ($n = 4$)	NA
Supplemental oxygen requirement at time of recruitment	None	2–4 L/min via nasal cannula ($n = 2$)	NA
Hemoglobin (g/dl)	12.1 ± 1.4	11.7 ± 2	NS
WCC (×10^9/L)	6.9 ± 3.6	9.2 ± 2.9	NS
Platelets (×10^9/L)	309.7 ± 177.1	325.3 ± 115.2	NS
Neutrophils (×10^9/L)	4.8 ± 3.7	6.3 ± 2.3	NS
Lymphocytes (×10^9/L)	1.4 ± 0.5	1.7 ± 0.7	NS
PT (s)	12 ± 0.9	12 ± 1.7	NS
APTT (s)	31.8 ± 0.9	27.8 ± 1	**.01**
Fibrinogen (g/L)	4.7 ± 1.3	4.3 ± 1.6	NS
D-dimer (mg/L)	1.8 ± 3.5	1.2 ± 1.2	NS
Ferritin (μg/L)	1277 ± 1284	153.9 ± 157.2	**.02**
CRP (mg/L)	36.1 ± 41.7	46.3 ± 65.4	NS
Anti-FXa activity (IU/ml)	0.06	0.04	NS

Note: Data expressed as mean ± SD. Bold values indicates $P < .05$ considered to represent statistical significance.

Abbreviations: APTT, activated partial thromboplastin time; BMI, body mass index; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; FXa, factor X; L/min, liters per minute; NA, not applicable; NS, not significant, $P > .05$; PCR, polymerase chain reaction; PT, prothrombin time; SARS-CoV-2, severe acute respiratory syndrome coronavirus disease 2; SD, standard deviation; WCC, white cell count.

Despite evidence of increased ETP in COVID-19 patients, conversely, the lag time to initiation of thrombin generation was significantly prolonged in the COVID-19 group (8.1±1.8 vs. 6.2 ± 1.8 min; $P = .02$). Interestingly, a recent study investigating the relationship between parameters of thrombin generation and clinical outcomes in severe COVID-19 (including death, ARDS, and intensive care unit admission) demonstrated a positive correlation between both ETP and increasing lag time with these negative clinical outcomes.[21] To determine if plasma thrombin generation in COVID-19 is modulated by TFPI activity, CAT was repeated in the presence of an inhibitory anti-TFPI antibody. Levels of plasma TFPI in both groups were similar; however, in the presence of the anti-TFPI antibody, lag time to initiation of thrombin generation became shortened and, in contrast to baseline thrombin generation parameters prior to inhibition of TFPI, no significant difference in lag time to initiation of thrombin generation was observed between groups (Figure 2).

TFPI is a physiological anticoagulant and is the primary inhibitor of the initiation phase of blood coagulation.[22] The primary functional pool of TFPI is expressed on endothelium but it is also found in platelets, leukocytes, and in plasma. The activity of plasma TFPI is modulated by co-factors, including protein S and factor V-short.[23] Increased plasma TFPI levels and increased TFPI activity have been reported in the setting of endothelial damage and in association with vascular complications, including myocardial infarction.[24,25] Plasma TFPI activity may not fully reflect physiological TFPI anticoagulant activity, as the latter is also mediated by TFPI expressed on the endothelial surface. Consequently, whether increased plasma TFPI anticoagulant activity following endothelial injury represents a protective mechanism in response to injury or merely an *in vitro* marker of injury remains to be determined.[22,26,27] Our group recently reported that early-onset preeclampsia (EOP) is also associated with increased plasma TFPI activity compared to healthy pregnant controls.[28] EOP is a severe form of preeclampsia which, similar to COVID-19, is characterized by marked endothelial injury and hemostatic dysfunction. Mirroring the results of the current study, plasma TFPI positively correlated with lag time to thrombin generation and inhibition of TFPI activity abrogated the difference in lag time between the EOP and control groups, suggesting a shared mechanism toward rebalanced hemostasis in

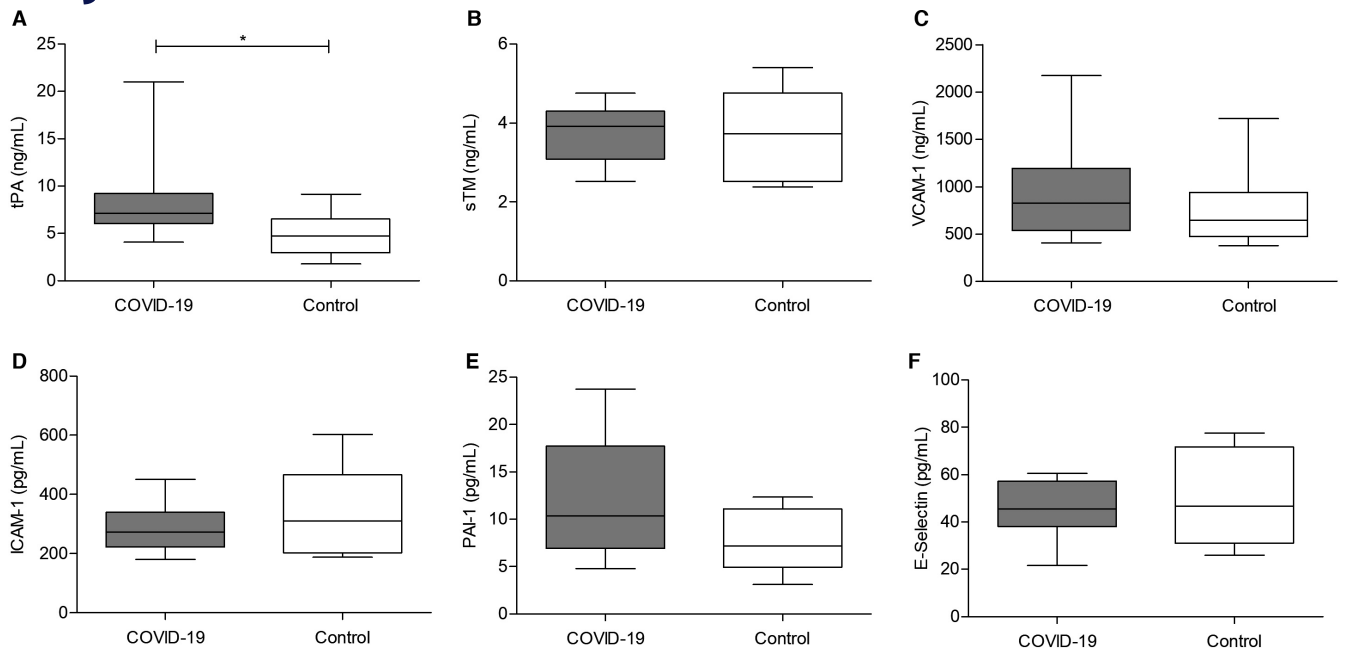

FIGURE 1 Plasma tissue plasminogen activator (t-PA) levels are significantly elevated in coronavirus disease 2019 (COVID-19). Plasma levels of t-PA (A) were significantly elevated in hospitalized patients with non-severe COVID-19 ($n = 14$) compared to a group of hospitalized patients ($n = 11$) who had screened negative for severe acute respiratory syndrome coronavirus 2 by real-time polymerase chain reaction. Levels of soluble thrombomodulin (sTM; B), vascular cell adhesion molecule-1 (VCAM-1; C), intercellular adhesion molecule-1 (ICAM-1; D), plasminogen activator inhibitor-1 (PAI-1; E) and E-selectin (F) were similar in both groups. All samples were assayed in technical duplicate. Data are presented as the mean ± standard deviation. Statistical analysis was performed using a two-tailed Student's *t* test. *$P < .05$

TABLE 2 Plasma thrombin generation in COVID-19 and in SARS-CoV-2–negative hospitalized controls

	1pM TF			5pM TF		
	COVID-19 ($n = 14$)	SARS-CoV-2 negative ($n = 11$)	*P* value	COVID-19 ($n = 14$)	SARS-CoV-2–negative ($n = 11$)	*p* Value
Lag time (min)	8.1 ± 1.8	6.2 ± 1.8	**.02**	5 ± 1.3	3.5 ± 0.8	**.004**
Time to peak (min)	11.7 ± 2.3	10.1 ± 2.1	NS	7.8 ± 0.5	6.2 ± 0.3	**.01**
ETP (nM*min)	1929 ± 448	1528 ± 460.8	**.04**	2043 ± 427.4	1756 ± 459.2	NS
Peak (nM)	267.3 ± 82.88	208.6 ± 59	NS	341 ± 81.7	301.3 ± 58.9	NS
Vel index (nM/min)	81.2 ± 35.3	58.5 ± 24.6	NS	130.1 ± 49.02	116.5 ± 34.63	NS
ETP-TM ratio	NA	NA	NA	0.28 ± 0.2	0.21 ± 0.1	NS

Bold values indicates $P < .05$ considered to represent statistical significance.

Abbreviations: COVID-19, coronavirus disease 2019; ETP, endogenous thrombin potential; NA, not assessed; NS, not significant, $P > .05$; SARS-CoV-2, severe acute respiratory syndrome coronavirus disease 2.

patients with disease states characterized by endothelial damage and hypercoagulability.

In conclusion, in this study we have demonstrated that despite pharmacological thromboprophylaxis, plasma thrombin generation is increased among patients with COVID-19 of moderate severity compared to a matched control group of hospitalized patients without COVID-19. The precise etiology of enhanced plasma thrombin generation in COVID-19 remains to be determined. We and others have previously reported evidence of increased platelet activity in COVID-19.[11,12] While the experiments described in this article were undertaken in PPP, it is possible that platelet or leukocyte-derived microparticles may have influenced our results. Several other investigators have demonstrated evidence of endothelial damage associated with SARS-CoV-2 infection, particularly among patients with severe disease.[8,17,29] Interestingly, t-PA was the only marker of endothelial damage found to be increased in our COVID-19 patients; this may reflect the non-severe nature of the underlying COVID-19 illness in our cohort. However, notwithstanding the similar levels of the other endothelial markers in both groups, we believe that elevated levels of t-PA and the increased sensitivity of the COVID-19 cohort to the neutralization of TFPI activity in the thrombin generation assay suggests that a degree of endothelial damage is present.

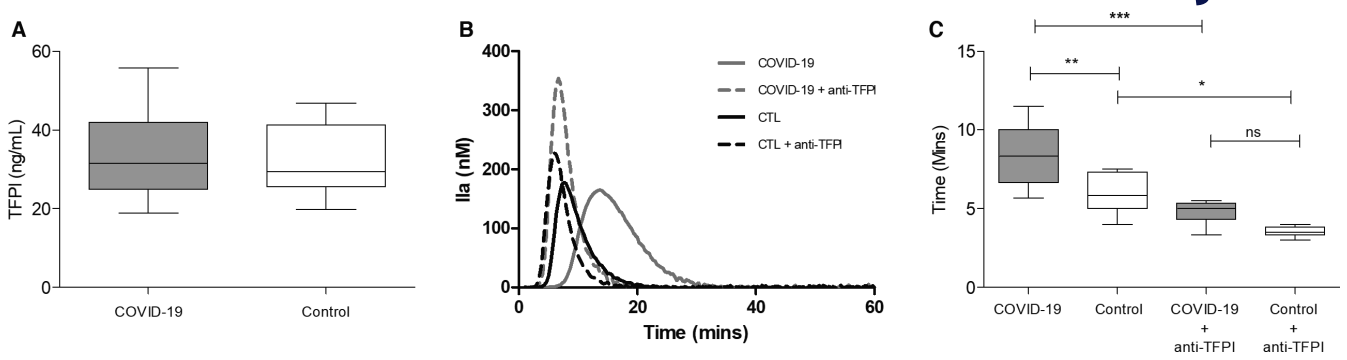

FIGURE 2 Plasma levels of tissue factor plasma inhibitor (TFPI) were similar in the group of patients with non-severe coronavirus disease 2019 (COVID-19; $n = 11$) and the group of hospitalized patients ($n = 7$) who had tested negative for severe acute respiratory syndrome coronavirus disease 2 (SARS-CoV-2) by real-time polymerase chain reaction (A). Representative thrombin generation curves from a patient with COVID-19 and a SARS-CoV-2–negative hospitalized control before and after incubation with an inhibitory antibody directed against TFPI are shown in (B). At baseline, the lag time to thrombin generation was prolonged in the COVID-19 group but in the presence of the antibody directed against TFPI, lag time became shortened and no significant difference was observed between the COVID-19 patients and hospitalized controls (C). All samples were assayed in technical duplicates. Data are presented as the mean ± standard deviation. Thrombin generation curves each describe the mean of duplicate replicates from an individual patient's plasma. Statistical analysis was performed using a two-tailed Student's *t* test and *P*-values were adjusted for multiple comparisons using a Bonferroni post hoc test. $^{*}P < .05$; $^{**}P < .01$; $^{***}P \leq .001$

While a causal relationship between these *in vitro* indicators of endothelial damage and hypercoagulability in COVID-19 cannot be confirmed based on these data, our observations do reflect findings from numerous other studies which also demonstrate evidence of endothelial injury and hypercoagulability following SARS-CoV-2 infection.[8,17,29,30]

Our findings are limited by our small sample size and the lack of a correlation with clinical outcomes. Consequently, the clinical implications of our observations are uncertain. It was also not possible to obtain plasma samples prior to initiation of LMWH thromboprophylaxis and consequently it is not clear to what extent LMWH attenuates baseline thrombin generation in COVID-19. However, we believe that comparing COVID-19 patients to a control group consisting of hospitalized medical patients receiving LMWH thromboprophylaxis (as opposed to healthy volunteers), is an important strength of this study, as is the precise timing of blood sampling (12 h post-LMWH dosing). Our observations may also be of interest in view of findings from recent randomized clinical trials which suggest that patients with non-severe COVID-19 may derive a survival benefit from escalated heparin dosing during hospitalization, a benefit which appears to be independent of a reduction in thrombosis risk.[6,7] Further analysis of the role of inflammation and endothelial/platelet activity associated with this infection may identify potential therapeutic opportunities.

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CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

All authors participated sufficiently in this work and made substantial contributions to this research. B. Kevane, F. Ní Áinle, P. B. Maguire, S. Gaine, K. A. M. O'Reilly, B. McCullagh, and J. Stack undertook study concept and design. S. Kelliher, S. Cullivan, E. O'Rourke, C. A. Murphy, and S. Toolan enrolled patients. S. Kelliher, L. Weiss, Á. Lennon, P. B. Szklanna, S. P. Comer, H. Macleod, A. Le Chevillier, and B. Kevane preformed laboratory experiments, data collection, and data analysis. S. Kelliher, L. Weiss, and B. Kevane drafted the manuscript. All authors contributed to literature review, draft revision and approval of the final manuscript.

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