Thrombin generation assay in COVID-19 patients shows a hypocoagulable pattern

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ABSTRACT

Patients with COVID-19 often exhibit coagulopathy, which can significantly impact prognosis. Therefore, investigating coagulation in this context is clinically relevant. The thrombin generation assay (TGA) provides comprehensive data on individual clotting patterns. In our study, we utilized a calibrated automated thrombogram to globally assess coagulation in COVID-19 patients. The study included 67 COVID-19 patients (40 hospitalized in the medical ward and 27 in intensive care units) and 45 blood donors for comparison. Our analysis revealed significant differences in TGA parameters (lag time, time-to-peak, thrombin peak, and endogenous thrombin potential) between patients and blood donors, suggesting a hypocoagulable state in the former. Specifically, COVID-19 patients exhibited prolonged lag time and time-to-peak values, as well as lower thrombin peak and endogenous thrombin potential compared to blood donors (Mann-Whitney test: $p<0.05$); notably, no significant differences in thrombin generation were observed based on the clinical setting. These findings suggest a reduced capacity for thrombin generation, indicating a consumptive coagulopathy in COVID-19 patients and that in this context, thrombosis is primarily attributable to localized effects in the lungs, platelet activation, and/or prothrombotic endothelial dysfunction. The thrombin generation assay is instrumental in defining coagulation patterns in COVID-19 and may also be applicable to other infectious diseases.

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Introduction

COVID-19 is characterized by a range of hematological abnormalities, including thrombocytopenia, elevated D-Dimer levels, and prolonged prothrombin time (PT), which resemble a unique form of consumptive coagulopathy that differs from classical disseminated intravascular coagulation (DIC).[1,2] We have previously documented the potential role of endothelial damage in the severe manifestations of COVID-19.[3] However, changes in coagulation are equally significant. A comprehensive assessment of coagulation could provide valuable insights for the clinical management of these patients. Thrombin generation assay (TGA) is one of the most reliable diagnostic tools for global evaluation of coagulation patterns, facilitating a detailed investigation of thrombin generation rates.[4] The aim of this study was to obtain comprehensive information regarding the coagulation patterns associated with COVID-19 patients.

Patients and Methods

Patients and controls

We evaluated TGA parameters in patients who tested positive for SARS-CoV-2 and were consecutively recruited at our

hospital between October 2020 and January 2021. The study was approved by the local Review Board (IRCSS Fondazione "Casa Sollievo della Sofferenza") and carried out in accordance with the Declaration of Helsinki. All patients provided informed consent to participate in the study. We excluded individuals with known pre-existing hemostatic and coagulation disorders (such as immune and congenital thrombocytopenia, factor deficiencies, and von Willebrand disease), pre-existing hematological diseases, and chronic liver disorders. Pregnant women were also excluded. Patients received recommended therapies for COVID-19, including remdesivir, antibiotics, and steroids. The antithrombotic regimen with low-molecular-weight heparin (LMWH) was determined by the attending physicians, with once-daily administration in the morning.

Plasma samples and analytical methods

Blood samples were collected within one week of hospitalization [median = 2 days (1-9)], using vacuum tubes containing 1/10 volume of trisodium citrate (0.109 M, Becton Dickinson). The samples were centrifuged twice for 10 minutes at 1620 g to obtain platelet-poor plasma (PPP), which was then frozen at -80°C until analysis. Patients were treated with prophylactic doses of LMWH at standard (4000 IU/day) or intermediate doses (6000 IU/day or 4000 IU twice daily for those with a body mass index >30), according to local protocols. Blood was drawn at trough levels (before the next dose). TGA was performed using calibrated automated thrombography (CAT, Stago, Asnieres sur Seine, France). PPP from patients and blood donors was tested with a commercial PPP reagent (Stago), featuring a mixture of 5 pM tissue factor and 4 µM phospholipids. All tests were conducted in triplicate to minimize inter-assay variability. TGA parameters included lag time (LT, min), time-to-peak (ttPeak, min), endogenous thrombin potential (ETP, nM/min), and thrombin peak (nM). TGA was assessed according to Hemker *et al.*[5] The results were recorded and analyzed using dedicated software (Thrombinoscope™ software, Thrombinoscope BV), which displays the thrombin concentration curve over time. Thrombin generation was measured using a fluorogenic substrate (Z-Gly-Gly-Arg-AMC HCl, Bachem) at a concentration of 617 µM with a fluorometer (Fluoroskan Ascent, Thermo Labsystems).

Clinical data

We collected data on in-hospital mortality, clinical manifestations, comorbidities, and therapeutic approaches. Additionally, we recorded blood counts, inflammatory markers such as the neutrophil-to-lymphocyte ratio (NLR), C-reactive protein (CRP), hemolysis markers, and clotting parameters. Information regarding LMWH administration was also compiled.

Statistical analysis

Categorical and continuous variables were described as counts (percentages) and medians (interquartile ranges), respectively. Comparisons between groups for continuous variables were performed using appropriate parametric and non-parametric tests. Association analyses were conducted using Pearson correlation or Spearman rank correlation tests, based on the distribution of the data. Statistical analyses were performed using GraphPad Prism version 8.0.0 for Windows.

Results

We investigated 67 COVID-19 patients and 45 blood donors. Median age of the cases was 49 years (IQR: 27-63), while the controls had a median age of 43 years (IQR: 33-61.5). As shown in Table 1, the majority of patients were male (62.2%) and primarily presented with hypertension, diabetes, and cardiovascular diseases. Pneumonia was diagnosed in 79% of patients (53/67), 19 of whom (35.8%) underwent tracheostomy. During the observation period, one-third of the patients (n=23) died, with most (n=16) non-survivors being hospitalized in the intensive care unit (ICU). Clinical information revealed a higher

Table 1. Demographic and clinical characteristics of patients (n=67) with COVID-19.

	Values
Variables	
Sex, males	41 (62.2)
Age, median (IQR)	66 (58-64)
Comorbidities	
Diabetes, n (%)	13 (19.4)
Hypertension, n (%)	26 (38.8)
History of cancer and/or active cancer, n (%)	7 (10.4)
Cerebrovascular disease, n (%)	2 (2.9)
Cardiovascular disease, n (%)	14 (20.8)
Chronic kidney disease, n (%)	15 (22.4)
None	6 (8.9)
Symptoms and vital signs	
Fever, n (%)	44 (65.6)
Cough, n (%)	45 (67.1)
Sore throat, n (%)	1 (1.5)
Dyspnea, n (%)	31 (46.2)
Pulse oximeter O_2 saturation, median (IQR)	91.5 (89-96)
Abnormal chest radiography	
Yes, n (%)	53 (79.1)
Anticoagulant/antiplatelets therapy	
Anticoagulant at admission, n (%)	5 (7.4)
Antiplatelets at admission, n (%)	14 (20.8)
COVID-19 treatment	
Remdesivir, n (%)	9 (13.4)
Antibiotics, n (%)	60 (89.5)
Steroids, n (%)	61 (91)
Oxygen therapy	
Conventional oxygen therapy, n (%)	20 (29.8)
❑ MW, n (%)	20 (100)
High-flow nasal oxygen, n (%)	16 (23.8)
❑ MW, n (%)	8 (50)
❑ ICU, n (%)	8 (50)
Tracheostomy, n (%)	19 (28.3)
LMWH treatment during hospital	
No prophylaxis	4 (6.0)
LMWH prophylaxis, n (%)	36 (53.7)
LMWH intermediate/therapeutic doses, n (%)	27 (40.3)
Deaths	
Overall, n (%)	23 (34.3)
❑ ICU, n (%)	16 (69.5)
❑ MW, n (%)	7 (30.5)

IQR, Interquartile range; MW, medical ward; ICU, intensive care unit; LMWH, low-molecular-weight heparin.

prevalence of diabetes and hypertension among medical ward (MW) patients, whereas ICU patients were predominantly affected by cardiovascular diseases (*Supplementary Table 1*). Furthermore, ICU patients exhibited significant differences in several blood parameters included in this study (*Supplementary Table 2*). Non-survivors (n=23) demonstrated significantly higher levels of LDH, D-Dimer, CRP, and NLR compared to survivors, even if no differences were found in terms of TG parameters (*Supplementary Table 3*). Additionally, patients requiring tracheostomy had significantly elevated LDH, D-Dimer, CRP, and NLR levels. Non-survivors hospitalized in ICU showed similar values of blood parameters in comparison to the non-survivors in MW (*Supplementary Table 4*). Overall, most patients (n=37) received LMWH prophylaxis, while the remainders were on intermediate dosages. The median anti-Xa level in the overall patient population was 0.12 U/ml (IQR: 0.10-0.16). Differences in blood parameters between patients and blood donors, as well as between those on LMWH prophylaxis and those on intermediate LMWH dosages, are presented in Table 2. COVID-19 patients exhibited lower thrombin generation compared to blood donors (Figure 1 A-D). These differences persisted when comparing clinical settings (MW or ICU) to blood donors (Figure 1 E-H). No significant differences were observed between ICU and MW patients (Figure 1 E-H). When patients under prophylaxis were compared to those under intermediate dosages, all TGA parameters showed comparable values. We found a positive correlation between lag time (LT) and CRP (Spearman r coefficient: 0.40; p=0.014), D-Dimer (Spearman r coefficient: 0.35; p=0.028), and creatinine (Spearman r coefficient: 0.25; p=0.049). We also assessed the relationship between TGA parameters and conventional clotting tests (PT-INR, aPTT), revealing an inverse association between ETP and prothrombin time (PT-INR) (Spearman coefficient = -0.31; p=0.02) (Figure 2). We further evaluated TG parameters in relation to clinical outcomes, including pneumonia and tracheostomy, finding that both did not significantly influence any of the TG parameters (*Supplementary Table 5*).

Discussion

Our study supports the hypothesis that COVID-19 patients experience a hypocoagulable state. The present results can be interpreted in light of evidence suggesting that thrombosis may result from localized effects in the lungs, platelet activation, or prothrombotic endothelial dysfunction, rather than reflecting global hypercoagulability of plasma.[6,7] Previous studies have shown that patients with similar clinical features exhibit throm-

Table 2. Baseline laboratory data and thrombin generation parameters of plasma samples from patients with COVID-19 and blood donors.

Variables	COVID-19 patients, n=67	Blood donors, n=45	p	COVID-19 patients$^{+LMWHproph.*}$, n=37	COVID-19 patients$^{+LMWH int.\ ^}$, n=30	p
RBC (x10^{12}/L)	4.3 (3.7-4.8)	5.0 (4.6-5.0)	<0.0001	4.4 (3.8-4.8)	4.5 (4.2-4.8)	ns
HCT (%)	38.0 (34.0-42.2)	43.0 (40.3-44.5)	<0.0001	36.7 (33.8-40.9)	39.8 (37.3-43.9)	0.04
MCV (fl)	89.5 (85.7-93)	89.6 (87.8-90.3)	ns	86.7 (83.6-91.5)	90.2 (88.4-93.1)	0.05
Haemoglobin (g/dL), median (IQR)	12.5 (11-13.8)	14.0 (13.3-15.1)	<0.0001	11.5 (10.8-13.7)	12.6 (12.3-13.9)	ns
Platelet count (x10^{9}/L), median (IQR)	243.0 (158.0-326.3)	241.0 (201.0-261.5)	ns	325.5 (169.0-324.0)	268.0 (175.8-346.0)	ns
White cell count (x10^{9}/L), median (IQR)	6.8 (4.8-10.9)	5.2 (4.2-6.4)	0.01	5.7 (3.9-10.3)	8.7 (6.7-13.5)	0.01
Neutrophil cell count (x10^{9}/L), median (IQR)	5.6(3.1-8.9)	2.9 (2.2-3.4)	<0.0001	4.0 (2.5-7.8)	7.3 (5.7-12.3)	0.005
Lymphocyte count (x10^{9}/L), median (IQR)	0.7 (0.5-1.2)	1.8 (1.5-2.2)	<0.0001	0.9 (0.6-1.4)	0.7 (0.4-0.8)	0.01
NLR, median (IQR)	7.0 (3.1-14.6)	1.3 (1.1-2.2)	<0.0001	4.1 (3.0-8.2)	14.5 (8.6-19.6)	0.0005
Prothrombin time (International Normalised Ratio), median (IQR)	1.1 (1.0-1.1)	1.0 (0.9-1.0)	<0.0001	1.0 (1.0-1.1)	1.1- (1.0-1.2)	ns
Activated partial thromboplastin time (s), median (IQR)	23.9 (21.5-26.7)	24.5 (22.3-26.5)	ns	23.2 (22.0-25.8)	23.1 (21.1-28.2)	ns
Fibrinogen (mg/dL), median (IQR)	427.0 (342.8-600.3)	247.5 (226.5-293.3)	<0.0001	427.0 (348.0-597.5))	414.0 (332.0-565.5)	
D-dimer (ng/mL), median (IQR)	1,161.0 (621.3-3,159)	201.1 (190.0-310.0)	<0.001	960.0 (458.7-2273.0)	2070.0 (1176.0-16756.0)	0.008
Aspartateaminotransferase, median (IQR)	31.0 (21.0-46.0)	17.0 (12.2-21.7)	<0.0001	28.0(20.0-44.0)	36.0 (24.5-46.3)	ns
Alanineaminotransferase, median (IQR)	42.0 (28.0-66.0)	21.5(15.5-30.0)	<0.0001	41.0 (28.0-63.0)	50.0 (29.5-72.8)	ns
Creatinine (mg/dL), median (IQR)	0.7 (0.6-1.0)	0.7 (0.5-0.9)	ns	0.8 (0.6-1.0)	0.7 (0.6-0.9)	ns
C-reactive protein (mg/L), median (IQR)	2.6 (0.8-9.4)	0.4 (0.3-0.5)	<0.01	2.0 (1.0-6.7)	14.0 (4.7-19.1)	0.01
TGA parameter [median (IQR)]						
Lag-time (min)	4.2 (3.2-5.4)	2.6 (2.5-2.8)	<0.0001	4.1 (3.1-4.7)	4.6 (3.8-6.0)	ns
ETP (nM/min)	991.2 (780.5-1,271)	1,676.0 (1,444.0-1,943.0)	<0.0001	1,007.6 (813.3-1,263.6)	901.7 (737.3-1,158.1)	ns
Peak (nM)	115.7 (62.3-165.7)	241 (210.5-291.4)	<0.0001	131.8 (77.25-175.79)	95.1 (60.4-147.6)	ns
ttPeak (min)	9.8 (7.3-12.1)	6.5 (5.7-7.3)	<0.0001	9.8 (7.6-11.7)	10.5 (8.3-12.8)	ns

Values are expressed as median and interquartile range; *4000 IU/die; ^6000 IU/die or 4000 IU/bid if body-mass-index >30; ns, not significant.

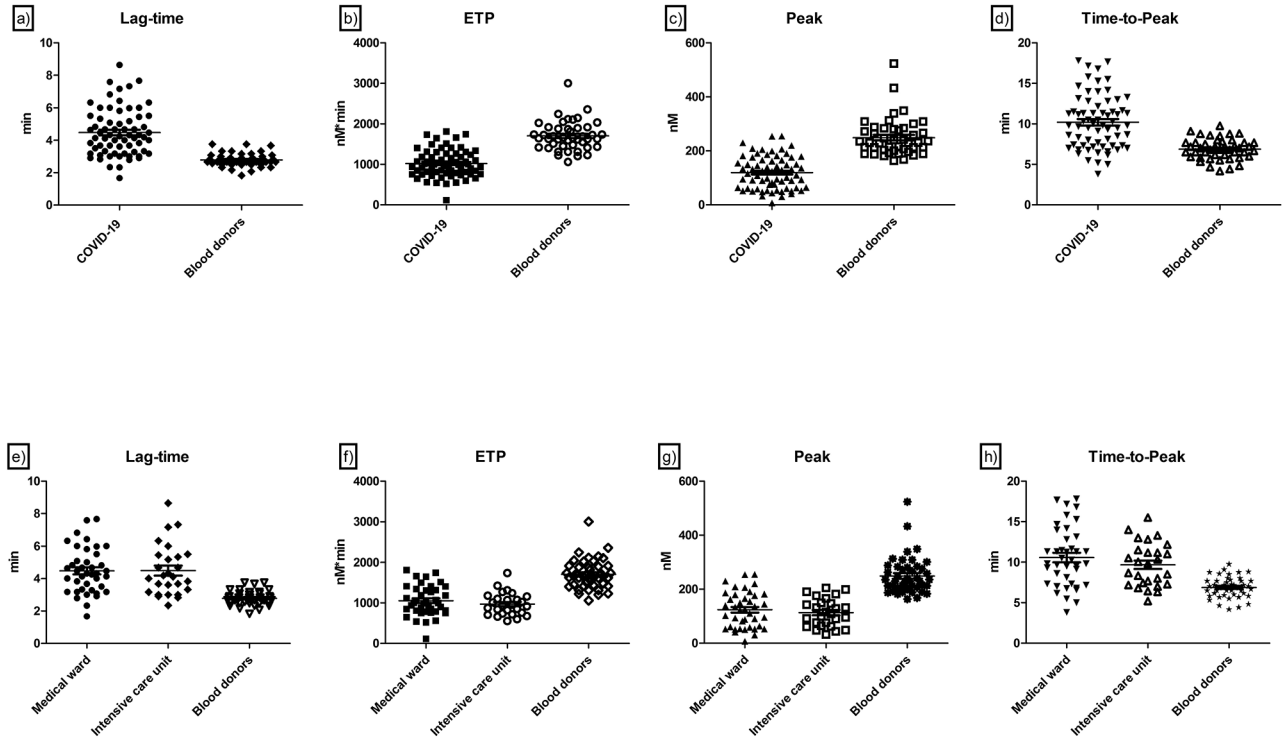

Figure 1. Thrombin generation assay in all the study populations. a) Lag time: COVID-19 patients *vs* blood donors (Mann-Whitney test, p<0.0001). b) ETP: COVID-19 patients *vs* blood donors (Mann-Whitney test, p<0.0001). c) Peak: COVID-19 patients *vs* blood donors (Mann-Whitney test, p<0.0001). d) Time-to-Peak: COVID-19 patients *vs* blood donors (Mann-Whitney test, p<0.0001). e) Lag time: comparisons between Medical Ward (MW) and Intensive Care Unit (ICU) patients and *vs* blood donors (Kruskal-Wallis test, p<0.05 MW or ICU patients *vs* blood donors. f) ETP: comparisons between MW and ICU patients and *vs* blood donors (Kruskal-Wallis test, p<0.05 MW or ICU patients *vs* blood donors. g) Peak: comparisons between MW and ICU patients and blood donors (Kruskal-Wallis test, p<0.05 MW or ICU patients *vs* blood donors. h) Time-to-peak: comparisons between MW and ICU patients and blood donors (Kruskal-Wallis test, p<0.05 MW or ICU patients *vs* blood donors. No significant difference observed between MW *vs* ICU patients.

bin generation (TG) patterns comparable to those described in this study.[6,8,9] For instance, White *et al*. found no significant difference in TG parameters between critical and non-critical patients. Additionally, in a cohort of 32 hospitalized COVID-19 patients from Israel, TG levels did not correlate with disease severity.[9] In that study, the authors found no correlation between TG and the Sequential Organ Failure Assessment (SOFA) score, mortality, or other clinical outcomes.

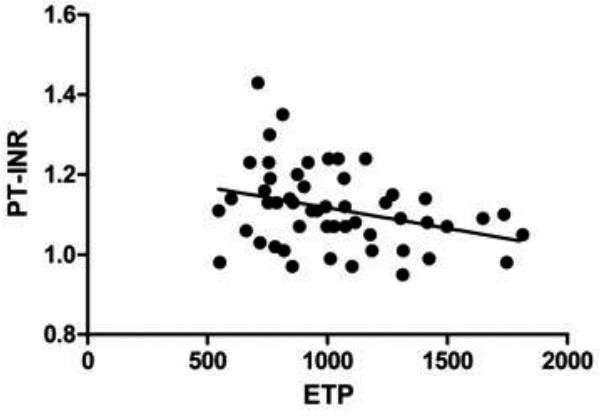

Figure 2. Correlation between ETP and PT-INR. ETP parameter shows a significant inverse relation with PT-INR (Spearman coefficient=-0.31; p=0.02).

Conversely, some researchers report an increased TG potential in COVID-19 patients, suggesting that the plasma pattern reflects a generalized hypercoagulable state.[10-13] However, some studies indicated coagulopathy in severely affected individuals, such as those in the ICU. Discrepancies among studies may be partially attributed to the high clinical heterogeneity regarding disease severity at recruitment. Additionally, different study designs can yield divergent results. For example, the prospective investigation by Gris *et al*. demonstrated a gradual modification in TG parameters as the disease progressed.[11] Consistent with similar studies, TG tests at admission did not predict early or late in-hospital mortality. In our cohort, as in other studies,[6,10,12] COVID-19 patients treated primarily with prophylactic doses of LMWH were able to maintain TG rates to some extent. It has also been shown that TG assays are not sufficiently sensitive to detect spike samples with low LMWH concentrations (0.1 U/ml).[14] Moreover, the potential for generating thrombin remains, even with high-dose heparin prophylaxis, although a benefit/risk balance is maintained.[15] In a study of healthy subjects,

plasma samples were spiked with LMWH (final concentration of 0.6 IU/mL), revealing moderate curves in experiments using our similar reagent.[16] Therefore, pre-analytical conditions, such as blood sampling timing relative to heparin dosing, and analytical conditions may vary across studies and impact results. However, we cannot entirely dismiss the possibility that LMWH influenced our findings, presenting a limitation to our research, even though blood samples in our investigation were collected at the LMWH trough point, where we would expect the lowest circulating drug levels. Coagulopathy can lead to intravascular coagulation hyperactivation and microvascular thrombosis, particularly in the context of severe disease and hyperinflammation. According to the model proposed by Gerber and Chaturvedi, an increase in D-dimer and fibrinogen, along with a decrease in platelet count and prothrombin time (PT), correlates with progressively greater disease severity and an increased risk of bleeding.[1] Our cohort primarily consisted of moderately severe patients, and we did not observe severe coagulopathy. Based on the model by Gerber and Chaturvedi, our patients can be classified as stages 1 or 2, showing no dramatic decreases in platelet count, prolonged PT, or increased fibrinogen levels, which are typically observed in the most severe patients (stage 3).[1] Our ICU and MW patients exhibited significant differences in CRP and D-dimer concentrations, as well as in pulse oximeter oxygen saturation. However, no statistically significant differences were noted in clotting parameters (PT and activated partial thromboplastin time, aPTT) between ICU and MW patients, reflecting similar results obtained with TG assays. No statistical differences were found in platelet count or fibrinogen levels between ICU and MW patients, although a slight non-significant increase in platelet count was observed in the former. It is expected that patients with sepsis would show reduced platelet counts or elevated fibrinogen levels.[17] Our findings suggest that, at least in part, our ICU patients parallel those in MW concerning coagulation and blood abnormalities as indicated by Gerber and Chaturvedi,[1] although inflammation was more pronounced in ICU patients. The lack of divergence between ICU and MW patients regarding severity biomarkers, such as platelet count or fibrinogen, and the unexpected higher platelet count in ICU patients may indicate that COVID-19 presents a spectrum of clinical manifestations that complicate the prediction of disease severity, potentially influenced by patient comorbidities. Additionally, platelets may play a role in COVID-19 through various mechanisms,[18] including inflammation-induced increases in thrombopoietin levels, which stimulate platelet production. The ISTH guidance on coagulopathy in COVID-19 indicates that platelet count is a less critical prognostic marker due to conflicting findings observed in the literature.[19] Despite statistical tests not showing significance for all TG parameters, the correlation between endogenous thrombin potential (ETP) and PT-INR provides some explanation for the coagulopathy observed in the patients described here. Nevertheless, the absence of correlation between all TG parameters and both PT-INR or aPTT may be attributed to methodological differences, such as variations in clotting activators and phospholipid concentrations.[4] Several limitations may have influenced our results. Firstly, LMWH may represent a source of error, although most patients were on prophylactic doses and blood samples were collected at through levels to minimize this issue. Secondly, the small sample size highlights the challenges of collecting data from patients with COVID-19. Despite these limitations, our findings suggest a reduced rate of thrombin generation in COVID-19 patients and emphasize the need for further data collection to determine the extent to which TG assays can assist clinicians in decision-making. The insights gained from COVID-19 may also be valuable for understanding other infectious diseases.

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Online supplementary material:

Supplementary Table 1.

Supplementary Table 2.

Supplementary Table 3.

Supplementary Table 4.

Supplementary Table 5.