Elena Campello, Cristiana Bulato, Luca Spiezia, Annalisa Boscolo, Francesco Poletto, Marco Cola, Sabrina Gavasso, Chiara Simion, Claudia Maria Radu, Annamaria Cattelan, Ivo Tiberio, Roberto Vettor, Paolo Navalesi and Paolo Simioni*

Thrombin generation in patients with COVID-19 with and without thromboprophylaxis

https://doi.org/10.1515/cclm-2021-0108
Received December 10, 2020; accepted January 25, 2021; published online February 4, 2021

Abstract

Objectives: Thrombin generation (TG) with and without thrombomodulin (TM) was evaluated in COVID-19 patients with different disease severity and thromboprophylaxis regimen, in order to understand the prothrombotic profile.
Methods: We enrolled consecutive patients with confirmed diagnosis of COVID-19 admitted to Medical Departments (MD) or Intensive Care Units (ICU), and 54 healthy controls.
Results: Eighty-nine patients were included (mean age 60.4±16.1 years, 68.5% male); 33.7% admitted to ICU. Twenty-four patients (26.9%) were enrolled before thromboprophylaxis administration; 45 patients (50.6%) received standard and 20 (22.5%) intermediate sub-therapeutic dose thromboprophylaxis. Overall, patients with COVID-19 showed a TG profile comparable to that of healthy subjects (i.e. comparable peak height, endogenous thrombin potential [ETP] with and without TM). The only exception was lag time and time to peak, prolonged in COVID-19 patients vs. controls. MD patients showed a similar TG profile to healthy controls, and ICU patients showed significantly decrease ETP (p=0.030) compared to MD. As for thromboprophylaxis, TG profile was significantly increased in COVID-19 patients without thromboprophylaxis vs. controls and vs. those with thromboprophylaxis. In this latter group, ETP inhibition was significantly decreased (p=0.0003) and positively correlated with anti-Xa activity (r=0.49, p=0.0017). However, patients with thromboprophylaxis had similar TG profile vs. controls. Intermediate dose thromboprophylaxis more effectively inhibited TG in severe COVID-19 patients by increasing ETP inhibition via ETP with TM reduction vs. standard dose.
Conclusions: COVID-19 patients showed increased TG at diagnosis. Standard thromboprophylaxis reduced TG to levels of healthy controls. Intermediate sub-therapeutic thromboprophylaxis more effectively inhibited TG by decreasing ETP with TM.

Keywords: coagulopathy; hypercoagulability; inflammation; SARS-CoV-2; venous thromboembolism.

Introduction

The typical symptoms of pandemic SARS-CoV-2 infection (COVID-19) are fever, cough, dyspnea, enteric disorders; they can develop rapidly into interstitial pneumonia with severe respiratory distress requiring hospitalization, including mechanical ventilation in intensive care units (ICU) [1–4]. In addition to respiratory complications, high incidence rates of venous thromboembolism (VTE) have been reported in critically ill COVID-19 patients [5–7]. Indeed, patients with severe acute respiratory syndrome have shown different changes in the coagulation processes such as elevated D-dimer and fibrinogen, and mild prolongation of prothrombin time [8–10] at admission; as the disease progresses, widespread microvascular thrombosis may develop, resulting in lung and multiple organ failure [11, 12]. The haemostatic disturbance observed in COVID-19 is rather peculiar and it has been recently coined "COVID-associated coagulopathy" (CAC) [11]. A severe hypercoagulable profile has been detected also by Rotational Thromboelastometry (ROTEM®) in COVID-19 patients and the hypercoagulability – i.e. increased maximum clot firmness (MCF) in FIBTEM – was significantly

***Corresponding author: Paolo Simioni**, Department of Medicine, General Medicine and Thrombotic and Haemorrhagic Diseases Unit, Padova University Hospital, Via Giustiniani 2, 35128, Padova, Italy, Phone: +39 0498212667, Fax: +39 0498212665, E-mail: paolo.simioni@unipd.it
Elena Campello, Cristiana Bulato, Luca Spiezia, Francesco Poletto, Marco Cola, Sabrina Gavasso, Chiara Simion and Claudia Maria Radu, Department of Medicine, General Medicine and Thrombotic and Haemorrhagic Diseases Unit, Padova University Hospital, Padova, Italy
Annalisa Boscolo and Paolo Navalesi, Department of Medicine, Anaesthesia and Intensive Care Unit, Padova University Hospital, Padova, Italy
Annamaria Cattelan, Division of Infectious and Tropical Diseases, Padova University Hospital, Padova, Italy
Ivo Tiberio, Intensive Care Central Unit, Padova University Hospital, Padova, Italy
Roberto Vettor, Department of Medicine, Third Chair of Internal Medicine, Padova University Hospital, Padova, Italy

more pronounced in intensive care unit (ICU) patients with severe COVID-19 [13–15].

In addition, VTE complications appears to occur despite standard dose thromboprophylaxis [6] and anticoagulation appears to be associated with lower mortality and better prognosis [16]. Peyvandi et al. recently described the pro-coagulant imbalance in COVID-19 in the context of an inflammation-linked coagulopathy [17]. However, data on the overall thrombin generation in CAC are scanty. Thrombin generation assay (TGA), performed in the presence of thrombomodulin (TM) – the cofactor in the thrombin-induced activation of the anticoagulant protein C pathway – is increasingly recognized as a valuable tool to assess the haemostatic balance in platelet-poor plasma, for its ability to concomitantly evaluate the effect of plasma pro- and anticoagulant factors [18–20]. In patients with complex haemostatic disorders, TM-modified TGA provides more information than traditional coagulation tests such as prothrombin time or partial thromboplastin time which are sensitive to procoagulant proteins only [21–25]. Thus, TGA has the potential to better describe a COVID-19 patient's coagulation status.

The aim of this prospective observational study was to evaluate thrombin generation profiles in patients with COVID-19 with different disease severity and thromboprophylaxis regimen.

Materials and methods

Patients

We enrolled consecutive patients aged ≥18 years with confirmed COVID-19 admitted to Padova University Hospital between 28th February to 28th April 2020. COVID-19 was confirmed by high throughput sequencing or real-time reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay on nasopharyngeal swab. Patients admitted to Internal Medicine Departments (COVID Internal Medicine and Infectious Diseases Division) for a mild disease, or to ICU for severe to critical disease were included. Exclusion criteria were: pregnancy, known pre-existing congenital bleeding disorders and/or pre-existing coagulopathies, Child's C liver disease, severe chronic kidney disease, pre-existing haematological disorders and ongoing anticoagulant therapy. Patients who received platelets, fresh frozen plasma or other procoagulant substances during the 24 h preceding the enrollment were also excluded. The protocol was approved by the local Institutional Ethical Committee (Ref: 3419/2015-652/AO/19). The study was conducted in compliance with the Declaration of Helsinki.

Patients received standard treatment for COVID-19 according to available international recommendations (hydroxychloroquine, lopinavir/ritonavir, azithromycin, steroids). The antithrombotic regimen (i.e. standard dose or intermediate sub-therapeutic thromboprophylaxis) was selected by the attending physician, who decided to use prophylactic or higher doses based on the perceived thromboembolic risk. The cohort of patients reported in this study has been partially described in previous studies [13, 14]. Reference TGA ranges were determined by concomitantly testing 54 healthy adults of both sexes (M:F=1:1), mean age 42.4±18.1 years, mean BMI 26.5±3.2 kg/m^2, with no personal or family history of thrombosis or bleeding disorders who were taking no medications, including oral contraceptives.

Laboratory tests

Venous samples were obtained within five days of admission and collected at 7 am (at the trough level of heparin administration). Blood was drawn by venipuncture directly into BD Vacutainer® Citrate Tubes with 0.109 M (3.2%) sodium citrate (9:1 blood to anticoagulant ratio) without corn trypsin inhibitor. Platelet poor plasma (PPP) was prepared within 1 h of blood collection by double centrifugation at 1,500 × *g* for 10 min at room temperature. Aliquots (1 mL) were immediately frozen and stored at −80 °C until analysis.

Traditional coagulation parameters such as platelet count, prothrombin time (PT), PT ratio, activated partial thromboplastin time (aPTT), fibrinogen, antithrombin, D-dimer, and C-reactive protein were obtained for all enrolled patients using standardized laboratory methods. Protein C coagulometric activity (PC) was measured using a commercial kit: Protein C Reagent (Siemens Healthcare Diagnostics). PC and FVIII activities were performed on a BCS XP coagulation analyzer (Siemens Healthcare Diagnostics). Anti-Xa activity was measured by STA®-Liquid Anti-Xa assay from Stago (Asnières-sur-Seine, France) with the STA®-Multi-Hep and STA®-Fondaparinux Calibrators (Stago) and the STA®-Quality LMWH and STA®-Fondaparinux Control (Stago) on the STA-Compact Max® (Stago).

Thrombin generation assay

Thrombin generation (TG) was measured in PPP samples thawed in a 37 °C water bath for 2–3 min, using the automated and standardized ST Genesia® analyzer and the STG®-ThromboScreen kit (Stago) following the manufacturer's instructions [24]. The STG®-ThromboScreen kit contains a mixture of phospholipid vesicles (4 uM) and a medium picomolar concentration of human recombinant tissue factor (TF) with and without rabbit lung thrombomodulin (TM) as activator of the coagulation system. TM concentration is that required to decreased endogenous thrombin potential (ETP) by 50% in normal pool plasma. The test allows the activation of the protein C anticoagulant pathway, and thus the assessment of both arms of haemostasis. The kit also includes three levels of quality control (for low, normal and high TM resistance) and a reference plasma to normalize the results and minimize inter-assay variability. The calibration curve (generated by STG®-ThrombiCal) was run in parallel with STG®-FluoSet allowing a maximal fluorescence level determination and the calculation of a correction factor according to the optical characteristics of each aliquot of plasma assayed. Calibration curve, quality controls and reference plasma were run on each day of testing. TG was initiated by dispensing a solution of a thrombin fluorogenic substrate and calcium chloride (STG®-FluoStart). Analyses with and without TM were performed in duplicate. The following TG parameters with and without TM were measured and analyzed: lag time, peak height, time to peak, start tail, ETP, and ETP inhibition mediated by TM. The latter was calculated by the software as [(ETP without TM – ETP with TM)]/(ETP

without TM)%. TG parameters are relative to reference plasma and expressed as ratios (temporal parameters) or percentages (thrombin concentration-related parameters).

Outcomes

The first endpoint was the evaluation of TGA in overall COVID-19 cohort vs. healthy controls and according to COVID-19 severity (MD vs. ICU). The second endpoint was the comparison of TG profiles in COVID-19 patients without and with thromboprophylaxis and according to thromboprophylaxis regimen.

Statistical analysis

Given our previous findings on TG [23] and most recent findings [26], considering an alpha of 0.05 and a power of 80% in a two-sided test, we estimated that a minimum sample size of 41 patients in each group (cases vs. healthy subjects) was required to detect a difference of ETP of at least 200 nM*min, with an expected standard deviation of 300 nM*min.

Continuous variables were expressed as median and interquartile ranges (IQR) or mean and standard deviation (SD), as appropriate. The normality assumption was assessed by Shapiro-Wilk normality test. Categorical variables were summarized as counts and percentages. One-way ANOVA or Kruskal-Wallis test with Dunn correction were performed for comparison of continuous variables, and Fisher's exact test for categorical variables. Correlations and partial correlations between parameters were analyzed by Pearson's test. All statistical analyses were performed by GraphPad Prism 7 (GraphPad Software Inc., CA, USA) and MedCalc statistical software for Windows.

Results

Characteristics of the study population

After the exclusion of 16 patients (oral anticoagulant treatment no. 4; full dosage heparin no. 10; pre-existing haematological diseases no. 2), 89 consecutive patients admitted to our hospital for COVID-19 were enrolled (mean age 60.4±16.1; male 61 [68.5%]).

Among enrolled patients, 30 (33.7%) had severe COVID-19 and were admitted to ICU, whereas the remaining 59 (66.3%) were admitted to Internal Medical Departments (MD) for mild COVID-19 (Table 1). Particularly, 24 COVID-19 MD patients (26.9%) were enrolled before heparin administration; 45 patients (50.6%) received standard dose thromboprohylaxis (LMWH 4,000 IU o.d. or fondaparinux 2.5 mg o.d.) and 20 (22.5%) received intermediate sub-therapeutic dose enoxaparin (0.5 mg/kg b.i.d.). Patients enrolled before heparin administration had mean age 59.4±19 years, mean BMI was 26.9±3.9 kg/m^2 and 10 (22%) were female; these characteristics were similar to those of patients enrolled after heparin administration. Patients admitted to ICU more often received thromboprophylaxis with intermediate sub-therapeutic dose enoxaparin (p=0.012), namely 4,000 IU b.i.d. or 6,000 IU b.i.d. (Table 1).

Twenty-one (23.5%) patients experienced VTE (4 in MD and 17 in ICU, p<0.0001). Overall 11 bleeding events (12.3%) occurred (6 MD vs. 5 ICU; p=n.s.). Particularly, 3 (27%) were classified as major bleeding events (1 MD vs. 2 ICU; p=n.s.) and 6 (54.4%) were clinically relevant non-major bleedings (4 MD vs. 2 ICU; p=n.s.) (see Supplementary Table 1).

Laboratory findings

ICU patients vs. MD showed significantly lower levels of antithrombin (p=0.011), though both groups remained within the normal range. In addition, ICU patients vs. MD showed significantly higher levels of C-reactive protein (p=0.0014) (Table 2). No differences were detected between groups as regards other coagulation parameters (platelet count, PT/INR, aPTT, D-dimer, fibrinogen, factor VIII and protein C).

Median anti-Xa activity was 0.36 [0.30–0.44] IU/mL in patients treated with LMWH and 0.25 [0.18–0.32] μg/mL in patients treated with fondaparinux (Table 2).

Table 1: Main characteristics of the study population.

	Total no. 89	MD no. 59	ICU patients no. 30	p-Value
Age, years	60.4±16.1	56.6±17.2	68.4±9.7	0.001
Gender, F, n (%)	21 (23.6)	14 (23.7)	7 (23.3)	n.s.
BMI, kg/m^2	28.1±4.9	27.05±4.4	29.2±5.1	0.068
SOFA score, n	–	–	5±2.9	–
Cancer, n (%)	7 (11)	4 (11.4)	3 (10)	n.s.
COVID-19 therapeutic protocols, n (%)	26 (29.2)	15 (25.4)[a]	11 (36.6)[b]	n.s.
Thromboprophylaxis, n (%)				
No	24 (26.9)	24 (40.7)	–	
Standard dose	45 (50.6)[c]	29 (49.1)[d]	16 (53.3)[e]	0.012
Intermediate dose	20 (22.5)	6 (10.2)	14 (46.7)	

[a]One tocilizumab; one tocilizumab + plasma; eight remdesivir; two remdesivir + plasma; three plasma. [b]Nine tocilizumab; two tocilizumab and remdesivir. [c]29 enoxaparin and 16 fondaparinux. [d]12 enoxaparin 4,000 IU OD; one enoxaparin 6,000 IU OD; 16 fondaparinux 2.5 mg OD. [e]11 enoxaparin 4,000 IU OD; five enoxaparin 6,000 IU OD. Data are shown as mean ± standard deviation or number and frequency, as appropriate. p-Values are calculated between ICU and MD cohorts. ICU, intensive care unit; MD, medical departments; BMI, body mass index; SOFA, sequential organ failure assessment.

Table 2: Laboratory findings in the study population.

	Total no. 89	MD no. 59	ICU patients no. 30	p-Value
WBC, 10^3/L	6.18±3.57	5.84±3.84	6.57±3.25	n.s.
Platelet count, $\times 10^9$/L	208.9±82.2	205.4±74.9	213.1±89.1	n.s.
PT, %	90.9±16.1	92±17	89.7±16.2	n.s.
PT ratio	1.14±0.31	1.15±0.33	1.16±0.35	n.s.
aPTT, s	26.8±9.1	25.6±4.5	28.1±12.3	n.s.
D-dimer, ng/mL	200.5 [150–550]	191.5 [150–453]	243 [150–759]	n.s.
Antithrombin, %	97.4±16.9	103.2±15.8	91.9±16.3	**0.011**
Fibrinogen, g/L	5.10±1.55	4.7 [3.55–5.4]	5.05 [4.50–6.17]	n.s.
Factor VIII, %	259.5±78.8	247±77.9	278.4±82.5	n.s.
Protein C, %	118±22.4	116.3±18.0	122.8±21.1	n.s.
CRP, mg/L	81 [44–152.5]	56 [23.25–122.5]	120 [68–190]	**0.0014**
Anti-Xa activity LMWH, IU/mL	0.36 [0.30–0.44][a]	0.30 [0.26–0.40][b]	0.38 [0.33–0.47]	0.052
Anti-Xa activity fondaparinux, µg/mL	–	0.25 [0.18–0.32][c]	–	–

Data are shown as mean ± standard deviation or median and range interquartile, as appropriate. p-Values are calculated between ICU and MD cohorts. [a]Detected in 49 patients undergoing LMWH; [b]detected in 19 patients undergoing LMWH; [c]detected in 16 patients undergoing fondaparinux. Reference ranges: WBC 4.40–11.00; platelet count 150–450; PT 75–112; PT ratio 0.9–1.20; aPTT 22–32; D-dimer 0–350; antithrombin 80–120; fibrinogen 1.50–4.50; factor VIII 60–160; protein C 80–120; CRP 0–6.0. p-Values in bold: statistically significant. aPTT, activated partial thromboplastin time; CRP, C-reactive protein; ICU, intensive care unit; LMWH, low molecular weight heparin; MD, medical departments; PT, prothrombin time; WBC, white blood cells.

Thrombin generation in the overall COVID-19 population and according to disease severity

Compared to TG values measured in the healthy subjects, COVID-19 patients showed significantly prolonged lag time (p<0.0001), time to peak (p<0.0001) and start tail (p<0.01). All the other TG parameters remained within the normal ranges (Table 3).

Considering the two COVID-19 groups separately, MD patients showed a similar TG profile to healthy controls, except for significantly prolonged lag time (p<0.0001). On the other hand, ICU patients showed significantly prolonged lag time (p<0.0001), decreased peak height (p=0.010), prolonged time to peak (p=0.00004) and start tail (p=0.008) and decrease ETP (p=0.030) compared to MD patients. Although ETP with TM was not significantly reduced, it nonetheless resulted in a significantly increased ETP inhibition (p=0.003) in ICU vs. MD patients (Table 3).

Thrombin generation according to presence and regimen of thromboprophylaxis

Compared to TG values measured in the healthy subjects, COVID-19 patients without thromboprophylaxis showed significantly increased peak height, ETP and ETP with TM (p<0.01, 0.01 and 0.0001, respectively).

Considering TG parameters according to the presence of thromboprophylaxis, we found that peak height, ETP and

Table 3: Thrombin generation results in the study population.

	Total COVID-19 no. 89	Healthy no. 54	p-Value	MD COVID-19 no. 59	ICU COVID-19 no. 30	p-Value
Lag time, ratio	1.67 [1.41–2.29]	1.11 [1.01–1.24]	**<0.0001**	1.5 [1.32–1.85][c]	2.45 [1.92–3.36][c]	**0.00001**
Peak height, %	69.8 [54.4–85.6]	64.9 [58.6–83.7]	ns	74.8 [56–94.4]	62.3 [36.4–70.9]	**0.010**
Time to peak, ratio	1.54 [1.28–1.85]	1.19 [1.10–1.35]	**<0.0001**	1.42 [1.21–1.70][c]	1.91 [1.62–2.45][c]	**0.00004**
ETP, nM*min	1,188 [995–1,339]	1,120 [1,028–1,253]	ns	1,215 [1,067–1,360]	1,078 [791–1,272]	**0.030**
ETP, %	84.4 [69.2–95.4]	82.45 [75.8–93.7]	ns	88.2 [78.6–97.7]	74.3 [54.6–90.5]	**0.012**
Start tail, ratio	1.28 [1.04–1.54]	1.11 [1.01–1.31]	**<0.01**	1.22 [1.02–1.47]	1.42 [1.24–1.73][c]	**0.008**
ETP + TM, nM*min	497 [333–717]	493.9 [397.4–620.5]	ns	561 [374–779]	363 [290–443]	0.06
ETP inhibition, %	56.7 [43.7–71.2]	57.8 [46.6–64.1]	ns	53.6 [40.8–63.4]	70.6 [62.3–75.9][a]	**0.003**

Data are shown as median and range interquartile. p are calculated between total COVID-19 and healthy subjects and between ICU and MD cohorts. TG parameters are relative to reference plasma and expressed as ratios (temporal parameters) or percentages (thrombin concentration-related parameters). ETP was also reported as absolute value. Superscript letters indicates p vs. healthy controls: [a]p<0.01, [b]p<0.001, [c]p<0.0001. p-Values in bold: statistically significant. ETP, endogenous thrombin potential; ICU, intensive care unit; MD, medical departments; TM, thrombomodulin.

ETP with TM were significantly increased in patients without thromboprophylaxis compared to those with thromboprophylaxis (p<0.001, 0.009 and 0.0003, respectively); ETP inhibition was significantly decreased (p=0.0003) (Table 4). However, patients with thromboprophylaxis had similar values of peak height, ETP and ETP with TM compared to controls (Table 4).

Considering thrombin generation parameters according to LMWH regimen, we found that lag time was not significantly increased in patients receiving intermediate dose heparin (2.22 [1.68–3.04] ratio) vs. standard dose (1.77 [1.44–2.41] ratio; p=0.09); peak height was not significantly reduced in patients receiving intermediate (64.4 [50.8–71.4]%) vs. standard dose (69.1 [46.2–87.4]%; p=0.46) and similarly for ETP (1,100 [857–1,271] nM*min vs. 1,188 [948–1,374] nM*min; p=0.38). On the other hand, ETP with TM was significantly lower in patients receiving intermediate (310 [160–427] nM*min) vs. standard dose thromboprophylaxis (532 [334–757] nM*min; p=0.002), resulting in a significantly increased ETP inhibition in the former (73.1 [62.3–82.3]% vs. 57.2 [46.6–66.5]%; p=0.002) (Figure 1).

Table 4: Thrombin generation results in patients with and without thromboprophylaxis.

	Healthy no. 54	No no. 24	Yes no. 65	p-Value No vs. Yes
Lag time, ratio	1.11 [1.01–1.24]	1.27 [1.12–1.45][b]	1.91 [1.49–2.98][c]	**<0.0001**
Peak height, %	64.9 [58.6–83.7]	85 [78–102][a]	63 [45–75]	**<0.001**
Time to peak, ratio	1.19 [1.10–1.35]	1.16 [1.13–1.39]	1.75 [1.42–1.95][c]	**<0.0001**
ETP, nM*min	1,120 [1,028–1,253]	1,265 [1,161–1,530][a]	1,148 [905–1,312]	**0.009**
ETP, %	82.45 [75.8–93.7]	92.2 [85.5–11.2]	82.6 [63.2–93.6]	**0.003**
Start tail, ratio	1.11 [1.01–1.31]	1.07 [0.98–1.3]	1.22 [1.02–1.47][b]	**0.004**
ETP + TM, nM*min	493.9 [397.4–620.5]	705 [513–884][c]	432 [286–630]	**0.0003**
ETP inhibition, %	57.8 [46.6–64.1]	44 [34.2–55.6][b]	62.3 [49.9–73.6]	**0.0003**

Data are shown as median and range interquartile. TG parameters are relative to reference plasma and expressed as ratios (temporal parameters) or percentages (thrombin concentration-related parameters). ETP was also reported as absolute value. p-Values are calculated between No prophylaxis and Yes. Superscript letters indicates p vs. healthy controls: [a]p<0.01, [b]p<0.001, [c]p<0.0001. p-Values in bold: statistically significant. ETP, endogenous thrombin potential; TM, thrombomodulin.

Lag time positively correlated with anti-Xa LMWH activity (r=0.44, p=0.0020); ETP, ETP with TM and peak height negatively correlated with anti-Xa LMWH activity (r=0.54, p=0.0001 and r=0.46, p=0.0042 and r=0.44, p=0.0022, respectively). ETP inhibition positively correlated with anti-Xa LMWH activity (r=0.49, p=0.0017). Conversely, anti-Xa fondaparinux activity did not correlate with TG parameters. ETP with TM and ETP inhibition significantly correlated also with the presence of intermediate dose thromboprophylaxis (r=0.40, p=0.003 and r=0.44, p<0.001, respectively).

The trend of TG parameters according to each thromboprophylaxis dosage (standard with LMWH; standard with fondaparinux or intermediate) is shown in Supplementary Figures 1 and 2.

Other coagulation parameters were not associated with thromboprohylaxis regimen (data not shown). Moreover, COVID-19 therapeutic protocols were not associated with significant changes in coagulation and TG parameters (data not shown).Finally, no differences in TG parameters were detected in COVID-19 patients with and without VTE complications (data not shown).

Discussion

COVID-19 is characterized by an abnormal immune response and an exaggerated pro-inflammatory state, which ultimately compound to foster the development of a profound haemostasis disturbance - mainly hypercoagulability – resulting in VTE complications and poor outcomes [11–15, 17, 27, 28].

TG test allows to evaluate the overall haemostatic balance by concomitantly measuring both pro and anticoagulant factors. TM is a transmembrane protein expressed on the surface of endothelial cells that binds to thrombin, thus switching its function from pro- to anticoagulant [29]. The thrombin-TM complex activates protein C, which in turn downregulates coagulation by cleavage of activated factors V (FVa) and VIII (FVIIIa) [30]. It can also activate thrombin activatable fibrinolysis inhibitor (TAFI) to attenuate fibrinolysis [31]. The addition of TM enables TGA to assess these aspects of the coagulation system in patients with complex haemostatic alterations [20–25, 32].

We evaluated TG with and without TM in a cohort of patients with COVID-19 with different degrees of disease severity (admitted to ICU or MD) and different thromboprophylaxis regimen. Our main findings were that, overall, patients with COVID-19 showed a TG profile comparable to that of healthy subjects (i.e. comparable peak height, comparable ETP with and without TM and ETP inhibition).

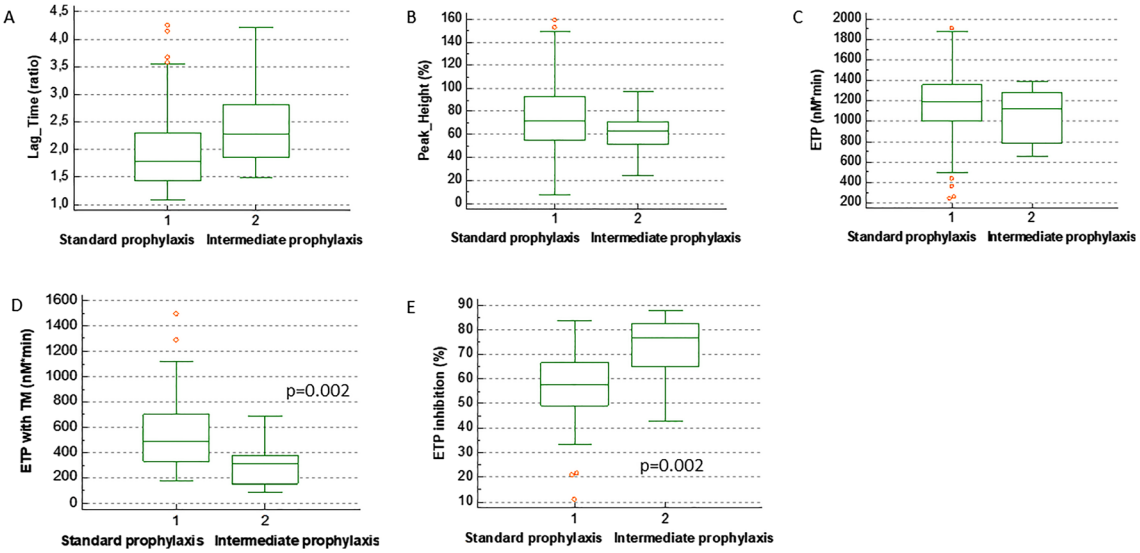

Figure 1: Trend of thrombin generation parameters according to standard or intermediate antithrombotic prophylaxis.
(A) Lag time; (B) peak height; (C) endogenous thrombin potential (ETP); (D) ETP with thrombomodulin (TM); (E) ETP inhibition.

The only significant exception was lag time and time to peak, which were prolonged in COVID-19 patients vs. healthy controls, irrespective of disease severity. This may be attributable to the thromboprophylaxis regimen (i.e. standard or intermediate dose) administered to the majority of COVID-19 cohort.

Our results are perfectly in line with three very recent findings by Blasi A. et al. [26], Nougier C. et al. [33], as well as White et al. [34] who measured TG on ST Genesia®. All these studies demonstrated normal TG profile in COVID-19 under thromboprophylaxis. Additionally, a recent small observation by Benati M. et al. [35] on ST Genesia® as well, confirmed the presence of prolonged lag time and time to peak in COVID-19 patients vs. controls.

Furthermore, we considered a cohort of COVID-19 patients before the administration of any thromboprophylaxis, and found that TG profile was significantly enhanced compared to both healthy controls and COVID-19 patients undergoing thromboprophylaxis (i.e. higher peak height, higher ETP and ETP with TM, and reduced ETP inhibition). Our study has definitely shown an increased TG in patients with COVID-19 before the administration of thromboprophylaxis.

As for possible mechanistic insight underlying the enhanced thrombin generation in COVID-19 patients, it appears that particularly TM-modified thrombin generation is different between patients and controls; in fact, ETP with TM was significantly increased in COVID-19 without thromboprophylaxis and controls. This would point to a role for protein C deficiency (or another defect in TM-mediated anticoagulation pathway due to endothelial injury) in the enhanced thrombin generating capacity of COVID-19 plasma. Another factor that may contribute are the elevated FV levels that have been reported in COVID-19 patients [36], which may lead to enhanced thrombin generation either directly or indirectly via an activated protein C resistance-type mechanism. High fibrinogen levels may contribute to the to the hypercoagulable state of COVID-19 patients by protecting thrombin from inactivation by antithrombin [37].

When patients admitted to ICU or MD were considered separately, we observed that patients in the latter cohort showed a rather normal TG profile, whereas those in the former showed a reduced TG pattern characterized by prolonged lag time, decreased peak height, increased time to peak and start tail. Another peculiarity was decreased ETP with TM, resulting in increased ETP inhibition.

The normal (reduced) TG profile observed in patients with (severe) COVID-19 was explained by the administration of (intermediate dose) thromboprophylaxis. In fact, TG parameters did significantly correlate with anti-FXa LMWH activity, and LMWH dosage was significantly associated with reduced ETP with TM and increased ETP inhibition.

Therefore, our study confirmed that standard dose LMWH thromboprophylaxis could reduce TG at levels of healthy controls. Intermediate sub-therapeutic LMWH dose more effectively inhibited TG in patients with severe COVID-19 by decreasing ETP with TM and increasing ETP inhibition. However, though intermediate dose LMWH was effective in inhibiting TG in patients with severe COVID-19, they nonetheless remained at significantly increased risk to develop VTE, suggesting that ICU patients may benefit to

a further reduction of TG to levels substantially lower than controls. We must nonetheless bear in mind a major downside of increased thromboprophylaxis, namely bleeding complications. In fact, 12.3% of patients in our cohort suffered bleeding complications, independently of disease severity. The possible clinical significance of our findings may lie in the need of an additional/different antithrombotic strategy (besides standard thromboprophylaxis) in order to overall control the hypercoagulable profile of these patients.

Our study has some main limitations. Firstly, the small size of our COVID-19 cohort, though plasma samples were consecutively collected according to pre-analytic and analytic conditions for TGA [38], and exclusion criteria were very strict. Only consecutive patients who underwent proper venous sampling and processing were enrolled. Moreover, although different TG working sessions were performed, in every working session (of 20 samples each), we included patients with different prophylaxis regimen and healthy controls in order to limit methodological variability. Secondly, we did not perform the analysis of procoagulant, anticoagulant (i.e. factors II, V, and protein S) and fibrinolytic factors. Thirdly, for clinical reasons we could not enroll ICU COVID-19 patients without thromboprophylaxis. Lastly, the study was not powered to assess association between TG parameters and clinical outcomes.

In conclusion, our study showed that patients with COVID-19 had increased TG at diagnosis and confirmed that standard dose thromboprophylaxis could at most reduce TG to the levels of healthy controls. Intermediate sub-therapeutic LMWH dose more effectively inhibited TG in patients with severe COVID-19 by increasing ETP inhibition via ETP with TM reduction.

Research funding: The study was funded by a GRANT by Cariparo for COVID-19 to the Department of Medicine (R.V.).

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The protocol was approved by the local Institutional Ethical Committee (Ref: 3419/2015-652/AO/19). The study was conducted in compliance with the Declaration of Helsinki.

References

1. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med 2020;382:1708–20.
2. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. JAMA Intern Med 2020;180:1–11.
3. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. N Engl J Med 2020;382:1199–207.
4. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72,314 cases from the Chinese center for disease control and prevention. J Am Med Assoc 2020;323:1239–42.
5. Di Minno A, Ambrosino P, Calcaterra I, Di Minno MND. COVID-19 and venous thromboembolism: a meta-analysis of literature studies. Semin Thromb Hemost 2020;46:763–71.
6. Avruscio G, Camporese G, Campello E, Bernardi E, Persona P, Passarella C, et al. COVID-19 and venous thromboembolism in intensive care or medical ward. Clin Transl Sci 2020;13:1108–14.
7. Al-Samkari H, Karp Leaf RS, Dzik WH, Carlson JCT, Fogerty AE, Waheed A, et al. COVID-19 and coagulation: bleeding and thrombotic manifestations of SARS-CoV-2 infection. Blood 2020;136:489–500.
8. Lippi G, Favaloro EJ. D-Dimer is associated with severity of coronavirus disease 2019: a pooled analysis. Thromb Haemostasis 2020;120:876–8.
9. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. J Thromb Haemostasis 2020;18:844–7.
10. Han H, Yang L, Liu R, Liu F, Wu KL, Li J, et al. Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. Clin Chem Lab Med 2020;58:1116–20.
11. Iba T, Levy JH, Connors JM, Warkentin TE, Thachil J, Levi M. The unique characteristics of COVID-19 coagulopathy. Crit Care 2020;24:360.
12. Colling ME, Kanthi Y. COVID-19-associated coagulopathy: an exploration of mechanisms. Vasc Med 2020;25:471–8.
13. Spiezia L, Boscolo A, Poletto F, Cerruti L, Tiberio I, Campello E, et al. COVID-19 -related severe hypercoagulability in patients admitted to intensive care unit for acute respiratory failure. Thromb Haemostasis 2020;120:998–1000.
14. Boscolo A, Spiezia L, Correale C, Sella N, Pesenti E, Beghetto L, et al. Different hypercoagulable profiles in patients with COVID-19 admitted to the internal medicine ward and the intensive care unit. Thromb Haemostasis 2020;120:1474–7.
15. Panigada M, Bottino N, Tagliabue P, Grasselli G, Novembrino C, Chantarangkul V, et al. Hypercoagulability of COVID-19 patients in intensive care unit: a report of thromboelastography findings and other parameters of hemostasis. J Thromb Haemostasis 2020;18:1738–42.

16. Tang N, Bai H, Chen X, Gong J, Li D, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. J Thromb Haemostasis 2020;18:1094–9.
17. Peyvandi F, Artoni A, Novembrino C, Aliberti S, Panigada M, Boscarino M, et al. Hemostatic alterations in COVID-19. Haematologica 2020 Aug 27. https://doi.org/10.3324/haematol.2020.262634 [Epub ahead of print].
18. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemostasis Thrombosis 2003;33:4–15.
19. Tripodi A. Thrombin generation assay and its application in the clinical laboratory. Clin Chem 2016;62:699–707.
20. Tripodi A. Detection of procoagulant imbalance. Modified endogenous thrombin potential with results expressed as ratio of values with-to-without thrombomodulin. Thromb Haemostasis 2017;117:830–6.
21. Dielis AW, Balliël WM, van Oerle R, Hermens WT, Spronk HM, Ten Cate H, et al. Thrombomodulin-modified thrombin generation after in vivo recombinant factor VIII treatment in severe hemophilia A. Haematologica 2008;93:1351–7.
22. Tripodi A, Primignani M, Chantarangkul V, Dell'Era A, Clerici M, de Franchis R, et al. An imbalance of pro- vs. anti-coagulation factors in plasma from patients with cirrhosis. Gastroenterology 2009;137:2105–11.
23. Russo FP, Zanetto A, Campello E, Bulato C, Shalaby S, Spiezia L, et al. Reversal of hypercoagulability in patients with HCV-related cirrhosis after treatment with direct-acting antivirals. Liver Int 2018;38:2210–8.
24. Morrow GB, Beavis J, Harper S, Baker P, Desborough MJR, Curry N, et al. Coagulation status of critically ill patients with and without liver disease assessed using a novel thrombin generation analyzer. J Thromb Haemostasis 2020;18:1576–85.
25. Campello E, Marobin M, Barbot M, Radu CM, Voltan G, Spiezia L, et al. The haemostatic system in acromegaly: a single-centre case-control study. J Endocrinol Invest 2020;43:1009–18.
26. Blasi A, von Meijenfeldt FA, Adelmeijer J, Calvo A, Ibañez C, Perdomo J, et al. In vitro hypercoagulability and ongoing in vivo activation of coagulation and fibrinolysis in COVID-19 patients on anticoagulation. J Thromb Haemostasis 2020;18:2646–53.
27. Connors JM, Levy JH. COVID-19 and its implications for thrombosis and anticoagulation. Blood 2020;135:2033–40.
28. Ranucci M, Ballotta A, Di Dedda U, Bayshnikova E, Dei Poli M, Resta M, et al. The procoagulant pattern of patients with COVID-19 acute respiratory distress syndrome. J Thromb Haemostasis 2020;18:1747–51.
29. Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. J Biol Chem 1989;264:4743–6.
30. Esmon CT. The protein C pathway. Chest 2003;124(3 Suppl): 26S–32S.
31. Nesheim M, Wang W, Boffa M, Nagashima M, Morser J, Bajzar L. Thrombin, thrombomodulin and TAFI in the molecular link between coagulation and fibrinolysis. Thromb Haemostasis 1997;78:386–91.
32. Bulato C, Radu CM, Campello E, Gavasso S, Spiezia L, Tormene D, et al. New prothrombin mutation (Arg596Trp, prothrombin padua 2) associated with venous thromboembolism. Arterioscler Thromb Vasc Biol 2016;36:1022–9.
33. Nougier C, Benoit R, Simon M, Desmurs-Clavel H, Marcotte G, Argaud L, et al. Hypofibrinolytic state and high thrombin generation may play a major role in SARS-COV2 associated thrombosis. J Thromb Haemostasis 2020;18:2215–9.
34. White D, MacDonald S, Edwards T, Bridgeman C, Hayman M, Sharp M, et al. Evaluation of COVID-19 coagulopathy; laboratory characterization using thrombin generation and nonconventional haemostasis assays. Int J Lab Hematol 2020; 43:123–30.
35. Benati M, Salvagno GL, Nitto S, Gelati M, Lavorgna B, Fava C, et al. Thrombin generation in patients with coronavirus disease 2019. Semin Thromb Hemost 2021 Jan 22. https://doi.org/10.1055/s-0041-1722844 [Epub ahead of print].
36. Stefely JA, Christensen BB, Gogakos T, Cone Sullivan JK, Montgomery GG, Barranco JP, et al. Marked factor V activity elevation in severe COVID-19 is associated with venous thromboembolism. Am J Hematol 2020;95:1522–30.
37. Dielis AW, Castoldi E, Spronk HM, van Oerle R, Hamulyák K, Ten Cate H, et al. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. J Thromb Haemostasis 2008;6:125–31.
38. Dargaud Y, Wolberg AS, Gray E, Negrier C, Hemker HC. For the subcommittee on factor VIII, factor IX, and rare coagulation disorders. Proposal for standardized preanalytical and analytical conditions for measuring thrombin generation in hemophilia: communication from the SSC of the ISTH. J Thromb Haemostasis 2017;15:1704–7.

Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/cclm-2021-0108).