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

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
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/m², 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 [26]. The STG®-ThromboScreen kit contains a mixture of phospholipid vesicles (4 μM) 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 fluorescent 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%6. 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 inter-quartile 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 thromboprophylaxis (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/m² 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    | MD       | ICU patients |
|---------------------------|----------|----------|--------------|
|                           | no. 89   | no. 59   | no. 30       | p-Value   |
| Age, years                | 60.4±16.1| 56.6±17.2| 68.4±9.7     | 0.001     |
| Gender, F, %              | 21 (23.6)| 14 (23.7)| 7 (23.3)     | n.s.      |
| BMI, kg/m²                | 28.1±4.9 | 32.0±7.4 | 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, % | 26 (29.2) | 15 (25.4) | 11 (16.9) | 0.012     |
| Thrombophrophylaxis       |          |          |              |           |
| No                        | 24 (26.9)| 24 (40.7) | –            |           |
| Standard dose             | 45 (50.6) | 29 (49.1) | 16 (53.3)    | 0.012     |
| Intermediate dose         | 20 (22.5)| 6 (10.2)  | 14 (46.7)    |           |

*One tocilizumab; one tocilizumab + plasma; eight remdesivir; two remdesivir + plasma; three plasma. bNine tocilizumab; two tocilizumab and remdesivir. c29 enoxaparin and 16 fondaparinux. d12 enoxaparin 4,000 IU OD; one enoxaparin 6,000 IU OD; 16 fondaparinux 2.5 mg OD. e11 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 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 - × 10³/L         | 6.18±3.57    | 5.84±3.84 | 6.57±3.25           | n.s.    |
| Platelet count - × 10³/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±82.5            | n.s.    |
| Protein C, %          | 118±22.4     | 116.3±18.0| 122.8±21.1          | n.s.    |
| CRP, mg/L             | 81 [64–152.5] | 56 [23.25–122.5] | 120 [68–190]      | 0.0014  |
| Anti-Xa activity LMWH, IU/mL | 0.36 [0.30–0.44] | 0.30 [0.26–0.40] | 0.38 [0.33–0.47] | 0.052   |
| Anti-Xa activity fondaparinux, μg/mL | –          | 0.25 [0.18–0.32] | –                  | –       |

Data are shown as mean ± standard deviation or median and range interquartile, as appropriate. p is calculated between ICU and MD cohorts. *Detected in 49 patients undergoing LMWH; †detected in 19 patients undergoing LMWH; ‡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. 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 | 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]   | 2.45 [1.92–3.36] | 0.00001 |
| Peak height, %       | 69.8 [54.4–85.6]      | 64.9 [58.6–83.7] | ns                | 74.8 [56.9–94.4] | 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] | 1.91 [1.62–2.45] | 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] | 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] | 0.003   |

Data are shown as median and range interquartile. p is 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: †p<0.01, ‡p<0.001, ‡‡p<0.0001. 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 increased in patients without prophylaxis (73.1 [62.3–82.3]% vs. 57.2 [46.6–66.5]%; p=0.002) (Figure 1).

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 thromboprophylaxis 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 anti-coagulant factors. TM is a transmembrane protein expressed on the surface of endothelial cells that binds to thrombin, thus switching its function from pro- to anti-coagulant [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).

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

|                  | Healthy no. 54 | No prophylaxis no. 24 | Yes prophylaxis no. 65 | p-Value for No vs. Yes |
|------------------|----------------|-----------------------|-----------------------|-----------------------|
| Lag time, ratio  | 1.11           | 1.27                  | 1.91                  | <0.0001               |
| Peak height, %   | [58.6–83.7]    | [78–102]              | [45–75]               | <0.0001               |
| Time to peak, ratio | 1.19           | 1.16                  | 1.75                  | <0.0001               |
| ETP, nM*min      | [1,028–1,253]  | [1,161–905–1,312]    | 1,148                 | 0.009                 |
| ETP, %           | 82.45          | 92.2                  | 82.6                  | 0.003                 |
| Start tail, ratio | [75.8–93.7]    | [85.5–11.2]           | [63.2–93.6]           | 0.004                 |
| ETP + TM, nM*min | [397.4–620.5]  | [513–884]            | [286–630]            | 0.0003                |
| ETP inhibition, % | [46.6–64.1]    | [34.2–55.6]           | [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 are calculated between No prophylaxis and Yes. Superscript letters indicates p vs. healthy controls: 'p<0.01, 'p<0.001, 'p<0.0001. ETP, endogenous thrombin potential; TM, thrombomodulin.
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 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.

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**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.

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