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Decreased protein C activity, lower ADAMTS13 antigen and free protein S levels accompanied by unchanged thrombin generation potential in hospitalized COVID-19 patients

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ABSTRACT

Introduction: COVID-19 is associated with an increased thromboembolic risk. However, the mechanisms triggering clot formation in those patients remain unknown.

Patients and methods: In 118 adult Caucasian severe but non-critically ill COVID-19 patients (median age 58 years; 73 % men) and 46 controls, we analyzed in vitro plasma thrombin generation profile (calibrated automated thrombogram [CAT assay]) and investigated thrombophilia-related factors, such as protein C and antithrombin activity, free protein S level, presence of antiphospholipid antibodies and factor V Leiden R506Q and prothrombin G20210A mutations. We also measured circulating von Willebrand factor (vWF) antigen and a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) antigen and activity. In patients, blood samples were collected on admission to the hospital before starting any therapy, including heparin. Finally, we examined the relationship between observed alterations and disease follow-up, such as thromboembolic complications.

Results: COVID-19 patients showed 17 % lower protein C activity, 22 % decreased free protein S levels, and a higher prevalence of positive results for IgM anticardiolipin antibodies. They also had 151 % increased vWF, and 27 % decreased ADAMTS13 antigens compared with controls ($p < 0.001$, all). On the contrary, thrombin generation potential was similar to controls. In the follow-up, pulmonary embolism (PE) occurred in thirteen (11 %) patients. They were characterized by a 55 % elevated D-dimer ($p = 0.04$) and 2.7-fold higher troponin I ($p = 0.002$) during hospitalization and 29 % shorter time to thrombin peak in CAT assay ($p = 0.009$) compared to patients without PE.

Conclusions: In COVID-19, we documented prothrombotic abnormalities of peripheral blood. PE was characterized by more dynamic thrombin generation growth in CAT assay performed on admittance to the hospital.

1. Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome (SARS) coronavirus type 2 (CoV-2), is responsible for the worldwide pandemic associated with high morbidity and mortality rate. This disease may have a different clinical presentation, from an asymptomatic course to severe illness requiring ICU admission and aggressive, supportive treatment [1]. Currently, the number of severe

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cases constantly decreases due to the vaccination program and the global prevalence of the Omicron variant, related to the milder disease manifestation. However, COVID-19 still represents a significant public health problem in some countries. In addition, the further course of the pandemic, including the risk of new virus genetic variants, remains unknown.

It has been well documented that more severe COVID-19 cases, e.g., requiring hospitalization, were related to older age and male gender [2], accompanied by other internal medicine comorbidities, such as arterial hypertension, diabetes mellitus, obesity, and chronic lung diseases, with an overall mortality rate of about 6 % [1,3]. That severe illness form likely refers to the so-called "cytokine storm" with an uncontrolled massive release of various pro-inflammatory cytokines, followed by multiorgan failure and laboratory signs of disseminated intravascular coagulation. In the autopsy studies of COVID-19 patients who died, widespread microthrombi were detected in all pulmonary arteries, suggesting endothelial dysfunction possibly related to the SARS-CoV-2 binding to angiotensin-converting enzyme 2 receptors and the subsequent prothrombotic response of endothelial cells leading to local vascular thrombosis [4,5]. Deep venous thrombosis (DVT) and pulmonary embolism (PE) are indeed observed with high frequency in hospitalized COVID-19 patients, especially those with a severe course and requiring ICU admission despite thromboprophylaxis [6–9]. Moreover, COVID-19 may also be associated with an increased incidence of ischemic stroke [10]. It was suggested that such strokes might be related to the transient presence of antiphospholipid antibodies [11]. However, the exact mechanisms responsible for the widespread thrombus formation in that disease are still unknown and require further investigation, similar to the optimal thromboprophylaxis regimen in those patients.

Therefore, we decided to comprehensively investigate the prothrombotic properties of circulating blood in COVID-19 patients. We analyzed in vitro plasma thrombin generation potential by calibrated automated thrombogram (CAT) together with natural anticoagulant proteins, von Willebrand factor (vWF) antigen, and a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) antigen and activity. We also studied acquired antiphospholipid antibodies (APLA) and the presence of factor V Leiden R506Q and prothrombin G20210A mutations associated with thrombosis or fatal COVID-19 course.

2. Patients and methods

2.1. Participants

We studied symptomatic adult Caucasian COVID-19 patients who fulfilled study inclusion criteria, consecutively admitted to the Department of Pulmonology and Allergology, University Hospital, Krakow, Poland, from May 2020 to October 2021. The diagnosis was established based on typical clinical symptoms and positive results for SARS-CoV-2 in nasal swabs by RT-PCR test. On admission, patients were assessed for a severe pneumonia course by The Modified Early Warning Score (MEWS) and the pulmonary embolism using Wells and modified Geneva scores. In addition, in those who developed pulmonary embolism (PE) during hospitalization Pulmonary Embolism Severity Index (PESI) was assessed. All patients received from admission 0.4 mg (body weight 70 kg or less) or 0.6 mg (body weight higher than 70 kg) of low-molecular-weight heparin (LMWH) per day as standard therapy; however, blood samples for laboratory tests were taken on admission to the hospital and before the LMWH was introduced. After that, patients were followed up for the disease course until recovery and discharge. The clinical outcomes included thromboembolic events (myocardial infarction, stroke, transient ischemic attack (TIA), DVT, PE), ICU admission and mechanical ventilation requirement, and death.

Excluded criteria were: active cancer, congestive heart failure (WHO class III/IV), atrial fibrillation, and current anticoagulant therapy. Patients with liver injury and kidney insufficiency were eligible if diagnosed as related to the COVID-19 infection and not present in the preceding six months. Liver injury was defined as an increase in serum alanine transaminase more than twice the upper limit of the normal range. Kidney insufficiency was defined as an estimated glomerular filtration rate (eGFR) lower than 60 ml/min/1.73 m^2.

Recent stroke was defined according to the WHO criteria and demonstrated by brain imaging [12]. TIA was defined as a transient episode of neurological dysfunction caused by a focal brain, spinal cord, or retinal ischemia lasting <24 h. The diagnosis of the lower or upper limb DVT was based on typical symptoms and confirmed by color duplex sonography. PE was diagnosed based on the positive result of computed tomography pulmonary angiography.

Subjects who stopped smoking six months or more before enrollment were considered non-smokers.

The study received approval from the Bioethics Committee of Jagiellonian University Medical College (No: 1072.6120.333.2020). The study procedures were carried out under the ethical guidelines of the Declaration of Helsinki. All subjects were given a thorough description of the methodology and safety protocol before we obtained informed consent to participate in the study.

2.2. Laboratory analysis

Blood samples were drawn using a minimal tourniquet from the antecubital vein on admission to the hospital and before starting any medication, including LMWH, and at least 6 h after the last meal. Blood for hemostatic parameters was taken into tubes containing 0.109 mmol/l sodium citrate (vol/vol, 9:1) and immediately centrifuged 2000 ×*g* for 10 min at room temperature; plasma was frozen in aliquots and stored at −70 °C until tested.

2.2.1. Basic laboratory tests

Routine laboratory techniques were performed to investigate complete blood cell count (Sysmex XN, Japan), serum glucose, alanine and aspartate transaminase, gamma-glutamyl transferase, lactate dehydrogenase, bilirubin, urea, creatinine, N-terminal pro B-type natriuretic peptide (NT-proBNP), creatine kinase, myoglobin, troponin I, procalcitonin, C-reactive protein (CRP), and ferritin (Cobas, Roche; France). The eGFR was estimated by the MDRD formula. Circulating IL-6 was measured using electrochemiluminescent technique on COBAS E601 analyzer (Roche, France).

2.2.2. Coagulation tests

Prothrombin time [PT], activated partial thromboplastin time [aPTT], and fibrinogen were determined by routine laboratory assays (Siemens, Marburg, Germany). The activity of antithrombin and protein C were measured with chromogenic methods (Innovance Antithrombin, Berichrom Protein C; Siemens, Marburg, Germany). Free protein S and D-dimer were assessed by turbidimetric assay (Innovance Free protein S and Innovance D-dimer; Siemens, Marburg, Germany). Factor (F)VIII activity was analyzed by a coagulometric test (Siemens, Marburg, Germany), similarly to activated protein C (APC) resistance (ProCAcR Siemenes, Marburg, Germany).

Commercially available immunoenzymatic assays were used to determine vWF antigen (Asserachrom vWF:Ag, Diagnostica Stago, New York, USA) and ADAMTS-13 antigen and activity (Technozym ADAMTS13 antigen ELISA kit and Technozym ADAMTS13 activity kit; Technoclone, Vienna, Austria).

2.2.3. Assessment of antiphospholipid (APLA) antibodies

Lupus anticoagulant (LA) was determined in a three-step procedure according to the guidelines of The International Society of Thrombosis and Haemostasis, as described elsewhere [13]. Briefly, diluted Russell's viper venom time (dRVVT; LA1-screen; Siemens, Germany) and a sensitive aPTT (PTT LA; Diagnostica Stago, France) were used for screening, whereas LA2-confirm (Siemens, Germany) and Staclot LA (Diagnostica

Stago, USA) were run for the confirmation. Reference values for each test were established using 99th percentile of the healthy population. Commercially available immunoenzymatic assays were applied to determine anticardiolipin (aCL) and anti-β2-glycoprotein I (aβ2GPI) antibodies (QUANTA Lite® aCL and aβ2GPI (Inova Diagnostics, San Diego, USA)).

2.2.4. Genetic studies

Genotyping for factor V Leiden R506Q (mutation rs6025) (FVL) and prothrombin G20210A (mutation rs1799963) was done by TaqMan assays (Applied Biosystems), as reported previously [14,15].

2.2.5. Thrombin generation profile in Calibrated Automated Thrombogram

Calibrated Automated Thrombogram (CAT assay) was performed using commercial reagents (Thrombinoscope, BV, Maastricht, Netherlands) as described elsewhere [16,17]. Briefly, 80 μl of thawed platelet-poor plasma was mixed with 20 μl of a reagent containing recombinant relipidated tissue factor (TF) and phospholipids, with the final concentrations of 5 pmol/l and 4 μmol/l, respectively. The reaction was done in microtiter well (Thermo Electron, Denmark) after automatic addition of a fresh starting reagent containing a thrombin specific fluorogenic substrate (*Z*-Gly-Gly-Arg-AMC) (2.5 mmol/l) and calcium chloride (100 mmol/l) in HEPES buffer. The fluorescence intensity was analyzed by the Fluoroskan Ascent® microplate fluorometer (Thermo Fisher Scientific Oy, Vantaa, Finland) using the Thrombinoscope BV, version 3.0.0.29 software.

CAT assay evaluates thrombin generation, resulting from the action of both procoagulant and anticoagulant factors in plasma, assessing the balance between them. The maximum concentration of thrombin generated in them refers to the "thrombin peak," and the area under the thrombin generation curve characterizes the "endogenous thrombin potential" (ETP). Other relevant parameters of thrombin generation profile include the lag-time and the time to thrombin peak. The former variable is defined as a time from the beginning of the analysis until thrombin starts to be detected. The latter determines a time from the start of thrombin generation until the maximum thrombin concentration is reached. Higher values of ETP and thrombin peak, together with shorter lag-time and time to thrombin peak, indicate an enhanced and more rapid activation of blood coagulation in vitro, thus a prothrombotic state, which has been demonstrated as corresponding to the increased risk of thromboembolic events [17–19].

2.3. Statistical analysis

The results were obtained using STATISTICA Tibco 13.3 software. Data distribution was evaluated by the Shapiro-Wilk test. All continuous variables were non-normally distributed and thus were presented in the manuscript as a median with upper and lower quartiles and compared by Mann-Whitney *U* test, Kruskal-Wallis, or multiple repetition test, as appropriate. A one-way covariance analysis (ANCOVA) was performed to adjust for potential confounders, including age, sex, body mass index (BMI), and internal medicine comorbidities. Categorical variables were compared by the Chi^2 test. To evaluate the relationship between continuous variables, a Spearman rank correlation test or univariate linear regression model with adjustment for age, sex, and BMI was performed. Independent determinants of ETP and lag-time were established in multiple linear regression models. The R^2 was checked for a measure of the variance. To calculate the odds ratio (OR) with 95 % confidence interval (CI), the cut-off point of ETP and lag-time were calculated based on receiver operating characteristic (ROC) curves. Results that presented a p-value <0.05 were considered statistically significant.

3. Results

3.1. Patients' characteristics

We studied 118 hospitalized, severe but non-critically ill COVID-19-positive patients with a median age of 58 (range 22–76) years; 86 (73 %) were men. The control group consisted of 46 subjects recruited from the hospital personnel; they were matched for age and BMI, but a higher percentage of males represented the COVID-19 group. Clinical symptoms and radiographical lung abnormalities upon admission to the hospital are shown in Table 2. As expected, the most common were cough and fever reported in over 75 % of patients. In addition, dyspnea, tachypnoea, and musculoskeletal pain were recorded in 79 (67 %), 47 (40 %), and 46 (39 %) patients, respectively. Approximately 70 % ($n = 83$) of subjects required oxygen therapy at hospital admission. The MEWS scale score is shown in Table 2. Almost half of COVID-19 patients ($n = 57$) scored 0 or 1, while 31 (26 %) and 30 (25 %) individuals gathered 2 or 3 and more points, respectively. The risk of PE was low or moderate in all patients.

3.2. Laboratory investigations

Results of basic laboratory tests, thrombin generation profile, and thrombophilia-related factors in both studied groups are shown in Table 1.

As expected, in COVID-19 at admission to the hospital, we found higher acute phase reactants (CRP, fibrinogen, IL-6, ferritin, and FVIII) and moderate elevation of D-dimer (Table 1). Interestingly, CRP correlated positively with fibrinogen and D-dimer in COVID-19 group ($r = 0.50$, $p < 0.001$; $r = 0.38$, $p < 0.001$, respectively) and controls ($r = 0.51$, $p = 0.034$; $r = 0.62$, $p = 0.008$, respectively). The patient group also had decreased white blood cells, lymphocytes, platelet counts, and extended APTT (Table 1). Furthermore, they showed 151 % higher vWF and 27 % lower ADAMTS13 antigens but unchanged ADAMTS13 activity compared to controls.

Surprisingly, the thrombin generation curve in the COVID-19 revealed 33 % longer lag-time and 19 % extended time to peak ($p < 0.001$, $p = 0.003$, respectively, after adjustment for potential confounders), which might have suggested diminished thrombin generation potential in that disease (Table 1). However, the maximum concentration of thrombin formed and ETP, which describes best thrombin generation potential, did not differ between COVID-19 and controls.

Natural plasma anticoagulant analysis has shown 17 % lower protein C activity and 22 % decreased free protein S levels in the patient group ($p < 0.001$ for both, also after adjustment for potential confounders) (Table 1). Furthermore, in ten (8.5 %) COVID-19 patients, APC resistance was observed (APC ratio below the lower reference threshold of 1.8), and only four of them showed heterozygous FVL; the other four had significantly elevated APLA, while the decreased activity of protein C and lower free protein S level were documented in one subject each.

Positive LA was detected in two COVID-19 patients, and five/three patients were positive for the presence of IgM/IgG aβ2GPI antibodies, respectively. On the other hand, 52 (44.1 %) patients tested positively for aCL IgM (range from 2 to 54 MPL/ml) and 10 (8.5 %) for aCL IgG (range from 1 to 43 GPL/ml). In controls, the corresponding numbers were 4 (8 %) ($p < 0.001$) and 1 (2 %) ($p > 0.05$), respectively (Table 1). Therefore, COVID-19 infection was associated with a higher prevalence of aCL IgM antibodies in low titers on a single determination ($p < 0.001$).

3.3. Determinants of thrombin generation parameters in COVID-19

In the patient group in univariate analysis, ETP was associated with FVIII activity ($\beta = 0.30$ [95 % CI: 0.20 to 0.41]), C-reactive protein ($\beta = 0.30$ [95 % CI: 0.20 to 0.39]) and fibrinogen level ($\beta = 0.21$ [95 % CI: 0.11 to 0.31]), although presented associations were weaker than

Table 1
A summary of demographic, clinical, and laboratory characteristics of subjects studied.

	Patients n = 118	Controls n = 46	p-value
Demographic parameters			
Age, years	58 (47–64)	50 (41–66)	0.1
Sex, male, n (%)	86 (72.9 %)	21 (45.7 %)	0.001
Body mass index, kg/m^2	28.9 (26.7–32.7)	27.9 (24.9–30.1)	0.1
Clinical characteristics			
Hypertension, n (%)	57 (43.4 %)	5/27 (18.5 %)	0.003
Diabetes mellitus, n (%)	26 (22.0 %)	2/27 (7.4 %)	0.1
Hypercholesterolemia, n (%)	24 (20.3 %)	8/27 (29.6 %)	0.31
Smoking habit, n (%)	35 (29.7 %)	1/10 (10.0 %)	0.14
Basic laboratory tests			
Blood platelets, 10^3/μl	202 (168–270)	219 (209–262)	0.49
Hemoglobin, g/dl	13.8 (12.7–14.7)	14.1 (12.8–15.4)	0.33
White blood cells, 10^3/μl	6.58 (5.11–9.61)	5.72 (5.32–6.56)	0.026
Lymphocytes, 10^3/μl	1.00 (0.68–1.40)	1.69 (1.58–2.13)	<0.001
Glucose, mmol/l	6.6 (5.3–8.3)	5.0 (4.7–5.4)	0.001
Creatinine, mmol/l	73.3 (63.1–88.1)	79.9 (72.2–90.0)	0.24
Urea, mmol/l	5.3 (3.9–7.2)	4.6 (4.1–5.7)	0.19
Alanine transaminase, U/l	42 (25–68)	21 (12–29)	<0.001
Bilirubin, μmo/l	7.1 (5.5–9.7)	7.3 (6.4–15.3)	0.59
C-reactive protein, mg/dl	86.8 (29.9–168)	1.0 (1.0–2.0)	<0.001
Procalcitonin, ng/ml	0.06 (0.02–0.19)	reference values <0.05	n.a.
Interleukin 6, pg/ml	31.5 (15.7–90.1)	1.5 (0.8–2.3)	<0.001
International normalized ratio	0.96 (0.90–1.04)	1.03 (1.02–1.04)	0.12
Activated partial thromboplastin time, s	30.9 (28.1–34.0)	23.6 (22.2–25.0)	<0.001
D-dimer, mg/ml	1.21 (0.71–3.22)	0.21 (0.18–0.37)	<0.001
Factor VIII, %	192.5 (150.8–226.3)	113.4 (92.9–144.3)	<0.001
Fibrinogen, g/l	5.6 (4.4–6.6)	2.4 (2.1–3.3)	<0.001
von Willebrand factor:Ag, %	259.1 (219.8–293.8)	103.2 (90.0–115.6)	<0.001
ADAMTS13 activity, IU/ml	0.85 (0.78–0.93)	0.85 (0.73–0.94)	0.84
ADAMTS13 antigen, IU/ml	0.67 (0.58–0.74)	0.85 (0.80–0.88)	<0.001
Thrombin generation profile in Calibrated Automated Thrombogram			
Lag-time, min	4.0 (3.3–4.7)	3.0 (2.7–3.7)	<0.001
Time to thrombin peak, min	6.3 (5.7–7.8)	5.3 (5.1–6.7)	<0.001
Thrombin peak, nmol/l	306 (195–352)	304 (257–341)	0.50
Endogenous thrombin potential, nmol/l thrombin × min	1565 (1326–1753)	1601 (1395–1724)	0.78
Anticoagulant proteins			
Antithrombin, %	103.3 (91.3–114.4)	107.0 (100.5–113.9)	0.10
Protein C, %	102.1 (87.9–120.2)	123.3 (112.3–142.8)	<0.001
Activated protein C resistance, ratio	2.8 (2.4–3.2)	Reference ratio > 1.8	n.a.
Free protein S, %	80.4 (67.8–91.0)	102.7 (86.9–115.1)	<0.001
Antiphospholipid antibodies (number of subjects with values above the reference)			
Anticardiolipin antibodies IgM >12 MPL, n(%)	52 (44.1 %)	4 (8.3 %)	<0.001
Anticardiolipin antibodies IgG >15 GPL, n(%)	10 (8.5 %)	1 (2.1 %)	0.29
Anti-β2 glycoprotein I antibodies IgM >20 SMU, n(%)	5 (4.2 %)	0 (0 %)	0.33
Anti-β2 glycoprotein I antibodies IgG >20 SGU, n(%)	3 (2.5 %)	0 (0 %)	0.57

Categorical variables are presented as numbers with percentages, continuous variables as median and interquartile (25 %–75 %) values. Abbreviations: ADAMTS13 - a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, Ig – immunoglobulin.

Table 2
Symptomatic and clinical characteristics of COVID-19 patients.

Patients, n = 118	
Symptoms on admission to the hospital	
Fever, n (%)	94 (79.7 %)
Sore throat, n (%)	11 (9.3 %)
Dyspnea, n (%)	79 (66.9 %)
Cough, n (%)	91 (77.1 %)
Pleural pain, n (%)	19 (16.1 %)
Tachypnoea, n (%)	47 (39.8 %)
Oxygen therapy, n (%)	83 (70.3 %)
Musculoskeletal pain, n (%)	46 (39.0 %)
Diarrhea and stomach pain, n (%)	27 (22.9 %)
Smell and taste disturbances, n (%)	37 (31.4 %)
Imaging lung findings on admission (chest X-ray or computed tomography)	
Normal lungs, n (%)	20 (16.9 %)
Interstitial changes, n (%)	63 (53.4 %)
Pleural fluid, n (%)	12 (10.2 %)
Consolidations, n (%)	53 (44.9 %)
Risk assessment (severe disease or thromboembolic complications)	
The Modified Early Warning Score, number of points	1 (0–5)
Wells criteria for pulmonary embolism, number of points	0 (0–1)
Geneva score (revised) for pulmonary embolism, number of points	3 (3–5)
Unfavorable outcomes of the disease	
Pulmonary embolism, n (%)	13 (11.0 %)
Sepsis, n (%)	11 (9.3 %)
Invasive mechanical ventilation, n (%)	19 (16.1 %)
Death, n (%)	14 (11.9 %)
Acute renal failure, n (%)	19 (16.1 %)

Categorical variables are presented as numbers with percentages, continuous variables as median and interquartile (25 %–75 %) values.

expected.

In a multiple regression model, elevated FVIII, lower serum creatinine, and surprisingly, increased aβ2GPI IgG antibody titer predicted greater ETP values, but their impact was relatively weak (Table 3).

Lag-time, which was longer in COVID-19 and correlated inversely with peak thrombin generation (β = −0.63 [95 % CI: −0.71 to −0.56]), was associated with glucose (β = 0.21 [95 % CI: 0.11 to 0.31]) and vWF (β = 0.32 [95 % CI: 0.18 to 0.47]). Furthermore, in a multiple regression model, extended lag-time was predicted by increased procalcitonin, creatinine, fibrinogen, and lower CRP. However, those variables explained only 19 % of the lag-time variability.

3.4. Adverse events during follow-up

The median follow-up time from the hospital admission and discharge was 16 (10–23) days. Chest imaging remained normal during hospitalization in only 20 (16.9 %) subjects. In other cases, lung disease developed and/or progressed with characteristic interstitial lung changes finally documented in 63 (53 %) patients and/or consolidations reported in 53 (45 %) of them. Additionally, pleural effusion was noticed in 12 (10 %) subjects.

Table 3
Multiple linear regression models for a relative increase of endogenous thrombin potential (ETP) and lag time in COVID-19 patients. Presented variables have been documented as independent determinants of both studied parameters, explaining 25 % and 19 % of ETP and lag time variability, respectively.

	β (95 % CI)	R^2
Endogenous thrombin potential, nmol/l thrombin × min		
Creatinine, mmol/l	−0.20 (−0.30 to −0.09)	0.25
Creatine kinase, U/l	0.23 (0.13 to 0.33)	
Factor VIII, %	0.25 (0.15 to 0.35)	
Anti-β2-glycoprotein I antibodies IgG, SGU	0.23 (0.13 to 0.33)	
Adjustment statistics	F = 5.83, p = 0.0004	
Lag-time, min		
Procalcitonin, ng/ml	0.18 (0.08 to 0.28)	0.19
Fibrinogen, g/l	0.17 (0.08 to 0.28)	
Creatinine, mmol/l	0.14 (0.05 to 0.23)	
C-reactive protein, mg/dl	−0.21 (−0.32 to −0.1)	
Adjustment statistics	F = 2.7, p = 0.04	

The resulting standardized regression coefficient (β) with 95 % confidence interval (95%CI) for a factor (independent variable) indicates the increase/decrease in standard deviations (SDs) of dependent variable (ETP or lag time), when that particular factor increases with 1 SD and all other variables in the model remain unchanged.

There were no strokes or DVT in our patient group. On the other hand, we recorded fourteen deaths. Surprisingly, the mortality was not associated with demographic variables, such as age, BMI and gender, or internal comorbidities, comparing the remaining patients. In turn, those who died had higher CRP (175.5 [125.0–217.0] vs. 77.6 [23.0–154.0] mg/dl, p = 0.006), lactate dehydrogenase (553.0 [416.0–693.0] vs. 353.5 [262.0–484.0] U/l, p = 0.007), urea (10.8 [7.2–12.5] vs. 5.0 [3.8–6.5] mmol/l, p = 0.015), NT-proBNP (1014.0 [275.0–2320.0] vs. 145.0 [85.0–287.0] pg/ml, p = 0.048) and neutrophil count (7.14 [5.87–8.30] vs. 4.58 [3.27–7.32] k/μl, p = 0.015) analyzed during the whole hospitalization period. Finally, death in COVID-19 was observed 2.5-times more often [95 % CI: 1.36–4.48] in those with troponin levels above the cut-off point of 11 μg/l in follow-up. On the other hand, it was not linked to any of the studied coagulable parameters, including lower ADAMTS13 or higher vWF antigen or increased vWF antigen/ADAMTS13 activity ratio.

PE was recorded in 13 patients (8 males and 5 females). All but three had at least one cardiovascular comorbidity, with hypertension presented the most often (9 individuals), following obesity and hypercholesterolemia diagnosed in 7 and 4 PE patients, respectively. Only one PE patient had diabetes, and neither were current smokers. PE patients did not differ in activities of protein C and antithrombin or free protein S levels. In turn, as expected, they were referred to as elevated concentrations of D-dimer (4.9 [1.8–36.5] vs. 2.7 [1.2–5.1] mg/ml, p = 0.043) and troponin I (11.1 [6.3–22.8] vs. 4.1 [2.5–6.3] μg/l, p = 0.002) analyzed anytime during the hospitalization and had a shorter time to thrombin peak in CAT assay determined at admittance to the hospital, before starting therapy with any medication, including LMWH (6.1 [5.2–7.0] vs. 7.9 [6.9–9.0] min, p = 0.009).

The analyzed genetic variants were not related to the PE episodes. Four (31 %) PE patients tested positive for any APLA, and 2 (15 %) had confirmed APC resistance. Thus, the prevalences of all analyzed thrombophilia-related factors were similar to those observed in non-PE COVID-19 patients. PE was also not associated with lower ADAMTS13 antigen, higher vWF antigen, or decreased vWF/ADAMTS13 activity ratio.

Comorbidities, age, sex, BMI, and initial lung changes had no impact on the prevalence of PE episodes.

4. Discussion

The present study demonstrates that symptomatic COVID-19 may be related to lower protein C activity and decreased free protein S levels, accompanied by increased vWF and decreased ADAMTS13 antigens. Moreover, those patients had a higher prevalence of APLA, particularly IgM aCL. On the contrary, thrombin generation capacity was similar to controls.

A significant decline has been mainly observed in the free protein S levels, which was noticed below the reference value in 20 % of patients. Therefore, COVID-19 might be related to the acquired natural anticoagulant deficiency. Inherited protein C and protein S deficiency has been associated with an increased risk of thrombosis, mainly venous thromboembolism, at a young age [20]. Thus, their decreased activity might partially explain the hypercoagulable state of COVID-19 [6]; however, we did not find such a relationship with the reported PE cases, making our findings uncertain. There are also several reports on the role of proteins C and S in COVID-19; nevertheless, the results are ambiguous. Interestingly, protein C was suggested as a marker of COVID-19 worsening and high mortality rate [21]. Moreover, a study by Stoichitoiu et al. [22] revealed a correlation between the activity of protein S and COVID-19 patients' survival and disease severity. Thus, one can speculate that both those proteins might serve as biomarkers of thrombotic manifestations in COVID-19. However, other reports demonstrate similar protein C and S levels or activity in on-admission COVID-19 patients and controls [23,24]. It is worth highlighting that some studies evaluated free protein S levels, whereas others total protein S according to its activity or concentration. Unfortunately, there are confounding factors associated with the assessment of protein S, including the high activity of factor VIII and APLA presence, which are both frequent in COVID-19, and enhanced inflammatory response or thrombosis. Therefore, the possible use of these molecules as biomarkers in COVID-19, especially protein S, should be taken with caution and needs further investigation.

In our study, ETP, a parameter that best describes thrombin generation capacity, was similar in the COVID-19 group and controls. This finding follows an observation published by Nougier et al. [25], in which thrombin generation potential in COVID-19 remained unchanged despite therapeutic LMWH doses but was associated with impaired fibrinolysis. Likewise, we have documented unchanged ETP before the LMWH introduction, which is a surprising finding. COVID-19 is an airway and lung disease related to cytokine oversecretion and severe systemic inflammatory response. At the same time, it has been shown that chronic inflammatory airway diseases such as asthma [26] or chronic obstructive pulmonary disease [27] are related to higher thrombin generation capacity. Thus, the opposite result in the COVID-19 group was a surprise, particularly since Sim et al. [28] recently reported that the standard thrombin generation assay, as used in our study, is insensitive to proteins C and S deficiency, documented in our study. Another unexpected finding regarding CAT assay is the longer lag-time and extended time to peak in COVID-19, which may paradoxically indicate hypocoagulability. However, the published data on that subject are inconsistent. For example, prolonged lag-time in COVID-19 was reported by White D et al. [29], but not in data documented by de la Morena-Bario et al. [30]. The discrepancy might be related to the clinical characteristics of the subjects studied. In our data, longer lag-time correlated with CRP and procalcitonin, which is also a surprising finding. Inflammation leads to a prothrombotic condition. It has been demonstrated that CRP stimulates TF expression in blood monocytes [31]. Thus, it might be consistent with the positive correlation of CRP with ETP, also found in our study. Furthermore, our data stay in line with those reported by de la Morrena-Bario et al. [30], which documented prolonged lag time and lowered ETP in COVID-19 patients compared to non-SARS-CoV-2 pneumonia, and both those parameters correlated with inflammatory biomarkers (IL-6, CRP, fibrinogen) and D-dimer. In summary, it seems that the increase in CRP and procalcitonin reflects the inflammation caused by SARS-CoV-2, but also the consumption of coagulation factors in the microcirculation on the endothelial lining, which leads to the rise in the lag-time and, finally

unchanged ETP [32].

Interestingly, in 10 COVID-19 patients, infection was associated with APC resistance demonstrated at admission to the hospital. In only four of them, it was explained by heterozygosity for FVL. Acquired APC resistance has been shown as linked to lower protein S and elevated FVIII [33] and the presence of APLA, all observed in our patients. Furthermore, interestingly, it has been reported that cytokine release during inflammation might result in thrombomodulin and protein C receptor decrease on endothelial cells, resulting in further in vivo APC resistance [34]. Both those phenomena might lead to the increased thromboembolic risk in COVID-19. In individual cases, a marked APC resistance might potentially serve therapeutic purposes, including recombinant APC use; however, experience in those with sepsis is not encouraging [35].

Previously viral and bacterial infections were demonstrated as an APLA trigger, particularly if transient and of the IgM class [36,37]. However, their prevalence was generally not associated with thrombotic events, similar to our study. Nevertheless, the occurrence of APLA in COVID-19 patients is common in several reports, and in some cases, it results in thrombotic complications [38].

The last issue that merits comment is the lower ADAMTS13 antigen accompanied by its unchanged activity in the COVID-19 group. Similar data were also reported by Doevelaar et al. [39]. Lower antigen level is likely related to its higher consumption in forming clots, reflected best by an increase in D-dimer. In turn, retained activity might be referred to as weakening inhibitory mechanisms. Three factors are known to regulate ADAMTS13 activity: (1) shear stress in the microcirculation, which allows vWF to unfold and expose its A2 domain for ADAMTS13 binding; (2) factor VIII, which enhances the ADAMTS13 proteolytic activity; and (3) platelet glycoprotein 1bα (GP1bα) [40]. In our data, but also in other publications [23,39], vWF was significantly increased in the COVID-19 group; and at least two first previously mentioned conditions were fulfilled, leading to the depletion of inhibitory mechanisms. Thus COVID-19 is related to the significantly higher vWF antigen/ADAMTS13 activity ratio, which recently has been found to predict mortality [41] or severe disease course [42]. However, in our cohort, such association was not observed, although, for those requiring mechanical ventilation in follow-up, the relationship tended to be significant ($p = 0.07$). The explanation might be related to the fact that blood samples were taken only once at the beginning of hospitalization when patients were generally in good clinical condition. Thus, that predictive index value may refer only to those who clinically worsen.

Finally, our study again confirmed the critical role of internal medicine comorbidities in COVID-19 complications. For example, in PE patients, the most frequent was arterial hypertension, which stays in line with other extensive observational studies [1,2]. Noteworthy, all PE episodes occurred despite the standard LMWH thromboprophylaxis, and those patients had a shorter time to thrombin peak, indicating increased thrombin generation potential. This “breakthrough VTE” might suggest that in some COVID-19 cases, higher doses of antithrombotic drugs may be warranted [32,43]. In addition, a large group of asymptomatic or managed as outpatients COVID-19 patients is left without thromboprophylaxis, while we are presently witnessing case reports indicating acute venous thromboembolism in some of them [44]. Large studies are needed to verify whether thromboprophylaxis beyond the hospital wards and with higher drug doses will benefit COVID-19 patients. Currently, however, such an approach is not recommended.

4.1. Study limitations

First, all coagulatory variables were measured once at the beginning of hospitalization, and we cannot exclude their changes over time. Patients and control groups differed in gender and hypertension prevalence; however, all statistical analyses were performed with adjustment for those confounders, and they likely have no impact on our results. Additionally, subgroup analysis needs to be interpreted with caution as, in some cases, it may be incidental and not represent cause-and-effect relationships.

4.2. Conclusions

In summary, we have documented that hospitalized, severe, but non-critically ill COVID-19 patients showed decreased levels of natural plasma anticoagulants, higher prevalence of antiphospholipid antibodies, and unchanged thrombin generation capacity compared to controls. That potentially unfavorable prothrombotic balance shift might contribute to the increased risk of thromboembolic events reported previously in this disease. Large controlled clinical studies are needed to verify this hypothesis.

CRediT authorship contribution statement

Study conception and design: KW, SBS, MS, JM, KS, TI. Acquisition of data: all authors. Analysis and interpretation of data: KW, SBS, LZ, RD, AG, KS, TI. Drafting of manuscript: KW, SBS, KS, TI. Critical revision: all authors. All authors have approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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