Research Article

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Activity of protein C, protein S and antithrombin 3 in COVID-19 patients treated with different modalities of oxygen supplementation

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Abstract

Objectives: COVID-19 in it is more severe form is characterized by a hyperinflammatory condition, hypercoagulation state and the appearance of pulmonary microembolism. In this study we wanted to correlate levels of D-Dimer, protein C, protein S and antithrombin 3 with severity of disease and clinical outcome.

Methods: We included 134 of patients who were divided in 3 groups regarding oxygen support (high flow oxygen therapy, mechanical ventilation and oxygen supplementation with nasal cannula or mask).

Results: Concentration of D-Dimer, and activity of protein C and antithrombin 3 are presented as mean±SD and differed significantly between patients on mechanical ventilation (3.26 ± 1.15 mg/L, 86 ± 22.55 %, 81.21 ± 17.61 %)/HFNO (2.35 ± 1.68 mg/L, 109.6 ± 26.96 %, 94.67 ± 17.49 %)/BNC (1.37 ± 1.17 mg/L, 116.92 ± 28.16 %, 103.29 ± 15.63 %) with p<0.001 for all parameters. Mortality in oxygen group was 10.9 %, in HFNC group 40.7 % and in mechanical ventilated group 80 %.

Conclusions: determination of anticoagulant factors in COVID-19 patients may indicate which of them are at increased risk of developing severe disease, venous thromboembolism and fatal clinical outcome.

Keywords: COVID-19; protein C; protein S; antithrombin 3; D-Dimer

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Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spread rapidly all over the world in last 2.5 years with hundreds of million affected and millions of deaths. COVID-19 is mostly mild illness but 10–15 % of patients develop severe disease which requires hospitalization, and about 5 % become critically ill. It is characterized by acute respiratory distress syndrome (ARDS) with progression to multiorgan dysfunction syndrome (MODS) [https://covid19.who.int].

Although the symptoms of respiratory tract infection are predominant in COVID-19 patients, SARS-CoV2 may

predispose patients to development of thromboembolic events. Excessive activation of immune system leads to a cytokine storm and increased formation of acute phase proteins, especially IL-6, which can consequently modulate further inflammatory response. This phenomenon is known as thrombo-inflammation and has central place in pathophysiology of COVID associated coagulopathy (CAC) [1].

CAC is coagulopathy that has distinct features in comparison to other coagulopathies linked to critical illness, like disseminated intravascular coagulopathy (DIC), including preserved platelet count, normal or minimally prolonged coagulation time and predominance of thrombosis, rather than bleeding. Most common finding in CAC is markedly elevated D-Dimer level which has positive correlation with adverse events, including thrombosis [2]. In meta-analysis done by Nopp et al., with 28,173 patients, overall VTE prevalence was 14.1 % with screening and 9.5 % without screening. When comparing intensive care unit (ICU) and non-ICU patients, venous thromboembolism (VTE) prevalence was 22.7 and 7.9 % [3]. Another meta-analysis included 18,093 patients found 17 % incidence for VTE (12.1 % for deep venous thrombosis (DVT) and 7.1 % for pulmonary embolism (PE)) and 3.9 % incidence for major bleeding [4]. Autopsy studies have found that almost 58 % of patients died due to VTE and PE [5, 6]. Also, in one autopsy study, despite the use of anticoagulation, pulmonary microthrombi were found in 72 % of patients [7]. Despite many studies, correct estimation of VTE incidence is currently not available as of the high heterogeneity of different studies as well as high variability in reported event rates.

Level of procoagulants are increased in COVID-19 patients as well as activation of platelets, fibrin destruction and fibrinolysis [8, 9]. On the other hand, the level of anticoagulants (protein C, protein S, antithrombin 3) seems to be decreased [10, 11]. Excessive innate immune response activates the coagulation system with thrombin generation leading to increased inflammation [12]. Thrombin activity and thrombus formation are regulated by anticoagulant molecules such as protein C and protein S, antithrombin 3 and tissue factor pathway inhibitors [13, 14]. During steady state these mechanisms are protective, but in inflammation they become disrupted, as seen in COVID-19 patients, promoting the pro-inflammatory and procoagulant type of answer that can lead to the development of intravascular thrombosis and multiorgan failure. Overproduction of pro-inflammatory cytokines creates a cytokine storm, which results in platelet activation, hypercoagulability, leukocyte infiltration and increased vascular permeability [15]. This seems to be the mechanism for development of pulmonary edema and pulmonary embolism as seen in COVID-19 patients [9].

This study aims to investigate coagulation status by measuring levels of D-Dimer and activity of protein C, protein S and antithrombin 3 in patients with different severity of COVID-19 and their clinical outcome.

Materials and methods

Patients

From 1st of December 2020 up to 25th of March 2021 a cohort group of 145 COVID-19 patients who were treated at the University Hospital Dubrava, Croatia were randomly included in this prospective observational study. Inclusion of patients did not depend on the severity of the disease, changes of patient's clinical condition and changes of applying oxygen therapy, and it was performed randomly. Blood sampling was performed at the time of patient inclusion.

Within the mentioned cohort, 134 patients had their coagulation parameters measured at the time of inclusion, while 11 patients were excluded due to incomplete parameters of coagulation data. All patients were older than 18 years.

Patients with atrial fibrillation who were on chronic oral anticoagulant therapy with warfarin were not included in the study. Due to the influence of warfarin on coagulation parameters, the obtained results would not explain the impact of COVID-19 itself on coagulation parameters and would represent a significant bias. Given that the main goal of this research is to investigate the impact of COVID-19 itself on coagulation parameters, we tried to minimize the influence of other parameters that can affect coagulation parameters that are routinely determined in clinical practice. In the study we included only those patients who did not have signs of VTE until the time of blood sampling. In case the patients had a confirmed diagnosis of VTE (DVT or PE), they were not included in the study.

Prior to the data collection, each patient either signed the informed consent, or the consent was given by a family member in case of the patient's inability to sign due to sedation and/or mechanical ventilation at the time of inclusion in the research.

The severity of the disease was assessed through the level of oxygen support needed to maintain arterial PaO_2>60 mmHg, as levels below are considered as hypoxemia. The patients were stratified into three groups: the first group consisted of 25 patients who were mechanically ventilated (MV); the second group consisted of 54 patients who needed high flow oxygen therapy (HFNO) up to 60 L/min with FiO_2 100 %; the third group consisted of 55 patients who needed only oxygen supplementation via binasal cannula or a face mask up to 15 L/min. Basic stratification data are collected from each patient. The patient data are shown in Table 1. Blood samples were collected during routine morning sampling from each patient, and the serum levels of AST, ALT, GGT, ALP, conjugated bilirubin, total bilirubin, protein C, protein S, antithrombin 3, D-Dimer, fibrinogen and APTT were determined.

Given the almost similar outcomes in the HFOT group, we additionally analyzed whether there is a difference in certain stratification data and laboratory parameters in relation to the outcome of COVID-19 (death or survival).

Table 1: Basic demographic, clinical and laboratory characteristics of patients. Comparison between oxygen, high flow nasal catheter and mechanical ventilation group.

Characteristic	Oxygen supplementation by BNC or mask group	HFNO group	MV group	p-Value
Number[a]	55	54	25	–
Female, n (%)	24 (43.6)	11 (20.37)	5 (20.0)	0.17
Age, mean, years	64.29 ± 8.05	65.2 ± 8.51	63.24 ± 9.4	0.622
BMI, kg/m^2	28.74 ± 4.85	31.11 ± 5.69	31.24 ± 5.16	**0.037**
Day of illness (until sampling)	16.91 ± 8.56	14.76 ± 5.31	16.8 ± 8.04	0.264
Day of hospitalization (until sampling)	7.51 ± 6.67	6.8 ± 5	8.04 ± 6.57	0.665
Systolic BP, mmHg	130.18 ± 23.69	138.11 ± 22.08	132.56 ± 25.51	0.204
Mean arterial BP, mmHg	98.68 ± 16.43	103.54 ± 14.96	98.12 ± 18.65	0.215
Diastolic BP, mmHg	77.67 ± 13.22	80.5 ± 12.73	75.16 ± 15.72	0.239
Heart rate (1/min)	84.2 ± 15.31[c]	92.93 ± 17.23[b]	89.48 ± 16.99	0.023
Respiratory rate (1/min)	20.59 ± 5.55[c,d]	25.02 ± 6.17[b]	29 ± 7.58[b]	**<0.001**
Body temperature, °C	36.51 ± 0.57	36.7 ± 0.85	36.79 ± 0.85	0.233
Death, n (%)	6 (10.9)	22 (40.7)	20 (80)	**<0.001**
Diabetes mellitus, n (%)	17 (30.9)	22 (40.7)	12 (48.0)	0.321
Arterial hypertension, n (%)	33 (60)	35 (64.8)	17 (68.0)	0.339
Atrial fibrillation, n (%)	6 (10.9)	5 (9.3)	4 (16.0)	0.717
Ischemic heart disease, n (%)	6 (10.9)	10 (18.5)	1 (4.0)	0.182
Heart failure, n (%)	6 (10.9)	4 (7.4)	1 (4.0)	0.714
Dyslipidemia, n (%)	15 (27.3)	18 (33.3)	4 (16.0)	0.561
COPD, n (%)	3 (5.5)	6 (11.1)	2 (8.0)	0.552
Asthma, n (%)	2 (3.6)	0 (0)	0 (0)	0.667
Chronic kidney disease, n (%)	4 (7.3)	0 (0)	4 (16.0)	**0.011**
Hyperuricemia, n (%)	3 (5.5)	4 (7.4)	3 (12.0)	0.569
History of malignant disease, n (%)	14 (25.4)	8 (14.8)	2 (8.0)	0.740
Active malignant disease, n (%)	10 (18.18)	3 (5.55)	0 (0)	0.127
Rheumatic disease, n (%)	5 (9)	5 (9.26)	1 (4.0)	0.841
Outlive CVI, n (%)	1 (1.81)	6 (11.11)	1 (4.0)	0.087
Thyroid gland disorders, n (%)	9 (16.36)	2 (3.7)	2 (8.0)	0.073
Leukocytes, ×10^9/L	11.22 ± 8.3	12.51 ± 4.58	12.8 ± 4.83	0.467
Basophils, ×10^9/L	0.37 ± 0.3	0.41 ± 0.61	0.4 ± 0.4	0.903
Neutrophils, ×10^9/L	8.15 ± 4.08[c,d]	11.25 ± 4.22[b]	11.21 ± 4.49[b]	**<0.001**
Lymphocytes, ×10^9/L	0.93 (0.59–1.29)[c,d]	0.59 (0.42–1)[b,d]	0.36 (1–0.48)[b,c]	**<0.001**
Erythrocytes (total), ×10^{12}/L	4.32 ± 0.64[d]	4.42 ± 0.5[d]	3.95 ± 0.65[b,c]	**0.004**
Erythrocytes (males), ×10^{12}/L	4.53 ± 0.69[d]	4.46 ± 0.49[d]	3.98 ± 0.71[d]	**0.005**
Erythrocytes (females), ×10^{12}/L	4.05 ± 0.44	4.27 ± 0.54	3.81 ± 0.33	0.176
Hemoglobin (all patients), g/L	126.8 ± 18.7	128.7 ± 15.7[d]	118.0 ± 19.8[c]	**0.045**
Hemoglobin (males), g/L	131.8 ± 20.3	130.4 ± 15.0	120.9 ± 20.7	0.087
Hemoglobin (females), g/L	120.3 ± 14.5	121.6 ± 17.3	106.4 ± 10.3	0.144
Thrombocytes, ×10^9/L	302 ± 128.3[d]	328.9 ± 122.5[d]	226.9 ± 100.9[b,c]	**0.003**
MPV, fL (ref. 6.8–10.4)	9.17 ± 1.1	9.02 ± 1.3	9.11 ± 1.51	0.829
CRP, mg/L (ref. <5)	27.45 (8.1–78.3)[d]	54.5 (22.3–98.9)[d]	63.3 (98.9–99.6)[b,c]	**<0.001**
Urea, mmol/L (ref. 2.8–8.3)	9.1 ± 5.32[d]	9.2 ± 3.59[d]	12.83 ± 8.3[b,c]	**0.01**
Creatinine, µmol/L (ref. 64–104)	66 (53–90)	64.5 (50–77)	67 (40–95.5)	0.597
Uric acid, µmol/L (ref. 182–403)	278.6 ± 132.2	288.8 ± 141.8	227.9 ± 130.6	0.182
AST, U/L (ref. 11–38)	43.84 ± 29.2	49.56 ± 38.67	51.24 ± 28.99	0.554
ALT, U/L (ref. 12–48)	60 (31–98)	42 (30–70)	42 (24–64)	0.198
GGT, U/L (ref. 11–55)	95.33 ± 93.55	95.29 ± 85.02	97.28 ± 65.19	0.995
ALP, U/L (ref. 60–142)	84.58 ± 41.77	94.53 ± 43.55	99.2 ± 64.65	0.366
LDH, U/L (ref. <241)	361.2 ± 133.9[c,d]	557.9 ± 238.3[b]	579.5 ± 253.8[b]	**<0.001**
Total bilirubin, µmol/L (ref. 3–20)	11.08 ± 4.56[c]	13.99 ± 6.48[b]	13.91 ± 6.69	**0.021**
Conjugated bilirubin, µmol/L (ref. <5)	2.61 ± 1.75	3.19 ± 1.93	4.52 ± 3.22	0.005
D-Dimer, mg/L	1.37 ± 1.17[c,d]	2.35 ± 1.68[b,d]	3.26 ± 1.15[b,c]	**<0.001**
Fibrinogen, g/L (ref. 1.8–4.1)	5.86 ± 1.79	5.53 ± 1.33	5.93 ± 0.74	0.387
Protein C, % (ref. 70–140)	116.92 ± 28.16[d]	109.6 ± 26.96[d]	86 ± 22.55[b,c]	**<0.001**

Table 1: (continued)

Characteristic	Oxygen supplementation by BNC or mask group	HFNO group	MV group	p-Value
Protein S, % (ref. 60–130)	85.18 ± 20.04	83.29 ± 23.57	74 ± 14.78	0.176
Antithrombin 3, % (ref. 80–120)	103.29 ± 15.63[c,d]	94.67 ± 17.49[b,d]	81.21 ± 17.61[b,c]	**<0.001**
hsTnI, ng/L (ref. <19.8)	8.6 (5.9–13.8)[c,d]	13.8 (7.1–31.4)[b,d]	56.3 (20–124)[b,c]	**<0.001**
NT-proBNP, pg/mL (ref. <125)	467 (173–1,138)[d]	611 (294–1713)[d]	2,180 (561–4,258)[b,c]	**0.006**
IL-6, pg/mL (ref. <6.4)	7.4 (3.4–17.3)[c,d]	24.1 (11.9–66.6)[b,d]	94.5 (53.2–333)[b,c]	**<0.001**
Procalcitonin, ng/mL (ref. 0.065)	0.13 (0.06–0.22)[d]	0.19 (0.09–0.42)[d]	1.37 (0.21–2.23)[b,c]	**<0.001**

[a]With exclusion of patients treated with warfarin. [b]Significant difference in relation to oxygen group. [c]Significant difference in relation to HFNO group. [d]Significant difference in relation to MV group. BNC, binasal cannula; HFNO, high flow nasal oxygen; MV, mechanical ventilation; n, number; BMI, body mass index; BP, blood pressure; COPD, chronic obstructive pulmonary disease; CVI, cerebrovascular insult; SpO_2, peripheral oxygen saturation; CRP, C-reactive protein; AST, aspartate transaminase; ALT, alanine transaminase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; APTT, activated partial thromboplastin clotting time; hsTnI, high sensitive troponin I; NT-proBNP, N-terminal pro brain natriuretic peptide; IL-6, interleukin 6. Bold indicates the p-values less than 0.05 and were considered statistically significant.

Laboratory methods

Samples were collected during routine morning blood sampling to determine standard laboratory tests. All laboratory analyzes were performed at the Clinical Department for Laboratory Medicine at University Hospital Dubrava. Blood samples were processed immediately after arriving in the laboratory. The blood samples were centrifuged at 1,789×*g* for 10 min in a non-refrigerated centrifuge and analyzed on a BCS® XP (Siemens, Schwalbach, Germany) analyzer. Blood samples for protein S determination were centrifuged at 1,789×*g* for 20 min in a non-refrigerated centrifuge and immediately analyzed on a BCS® XP analyzer.

D-Dimer were determined by the immunoturbidimetric method on the analyzer BCS® XP (Siemens, Schwalbach, Germany) with the multicomponent reagent INNOVANCE® D-Dimer kit (Siemens). The upper limit of detection of D-Dimer was 4.45 mg/L. All concentration above this limit is listed as 4.45 mg/L value.

Quantitative determination of the functional activity of antithrombin 3 in plasma was determined by the chromogenic test on the BCS® XP analyzer (Siemens, Schwalbach, Germany) with the multicomponent reagent BC Antithrombin Test kit (Siemens).

Quantitative determination of functionally active protein C in plasma was determined by the chromogenic test on the BCS® XP analyzer (Siemens, Schwalbach, Germany) using the Berichrom® multicomponent reagent for protein C (Siemens).

The functional activity of protein S in plasma was determined by the immunoturbidimetric coagulometric test on the analyzer BCS® XP (Siemens, Schwalbach, Germany) and the INNOVANCE® multicomponent reagent protein S Ac (Siemens).

NT-proBNP was determined by the chemiluminescent immunochemical method chemiluminescent microparticle immunoassay (CMIA) on the Architect i1000SR analyzer.

C-reactive protein was determined by the immunoturbidimetric method using a test for quantitative determination of CRP in serum on Beckman Coulter analyzers DxC700 and AU 5800.

IL-6 was determined by the chemiluminescent immunochemical method CMIA on a Beckman Coulter DxI 800 analyzer.

Statistical analysis

For statistical analysis, the MedCalc® Statistical Software program was used. Continuous numeric variables are presented as mean±standard deviation in case of parametric distribution or as median with interquartile range for nonparametric.

Statistical analysis was performed for median with interquartile range values using Mann–Whitney test or Kruskal–Wallis test and Student's t-test, Tukey and ANOVA for *post hoc* comparison of mean±standard error values. Categorical data were compared using Chi-Squared test (in case of low frequency variables Fisher exact test was used). p-Values of less than 0.05 were considered statistically significant.

Results

The patient data are shown in Table 1.

There were 134 patients included in study who were stratified by the severity of the disease depending on the method of oxygen application at the time of blood sampling. There were 55 patients in the first group of patients in whom oxygen was applied to BNC or an oxygen mask. The second group consisted of 54 patients on high-flow nasal oxygen (HFNO) device, and there were 25 patients in the third group which included patients on mechanical ventilation.

The third, mechanically ventilated group had lower protein C (86 ± 22.55 %) and antithrombin 3 (81.21 ± 17.61 %) activity, and higher values of D-Dimer (3.26 ± 1.15 mg/L) compared to patients treated with HFNO (109.6 ± 26.96 %, 94.67 ± 17.49 % and 2.35 ± 1.68 mg/L for protein C, antithrombin 3 and D-Dimer) and patients treated with BNC or mask (116.92 ± 28.16 %, 103.29 ± 15.63 %, and 1.37 ± 1.17 mg/L for protein C, antithrombin 3 and D-Dimer). There was no significant difference in the activity of protein S and fibrinogen concentration among the mentioned groups. The data are shown in Table 1.

The concentrations of thrombocytes and erythrocytes also differed in the group of patients on mechanical ventilation compared to the other groups. Their lowest concentrations were in the group of patients on mechanical ventilation (226.9 ± 100.9×10^9/L for platelets and 3.95 ± 0.65×10^{12}/L for

erythrocytes), while their highest values were in the HFNO group ($328.9 \pm 122.5 \times 10^9$/L for thrombocytes and $4.42 \pm 0.5 \times 10^{12}$/L for erythrocytes).

In MV group, 5 patients (20 %) survive and are alive discharged from hospital. In HFNO group, 32 patients (59.3 %) are discharged from hospital alive. In first group (oxygen applay by BNC of mask) only 6 patients (10.9 %) died and 50 patients are alive discharged from hospital.

Additional subanalysis was done within the HFNO group due to the comparable number of patients outcomes. A total of 22 patients (40.74 %) died during hospitalization within the HFNO group. Antithrombin 3 activity was lower in the group of patients who died (87.77 ± 16.4 % vs. 100.3 ± 16.57 %, p=0.011). Activity of protein C and protein S and concentration of D-Dimer did not differ significantly between patients who died vs. patients who survived

Table 2: Comparison of group of patients who died and survived in high flow nasal oxygen (HFNO) group.

Group	Died	Survived	p-Value
Number, %[a]	22 (40.74)	32 (59.26)	–
Mean, age, year	67 ± 7.58	63.97 ± 9	0.201
BMI, kg/m^2	31.73 ± 6.98	30.68 ± 4.68	0.511
Mean arterial BP, mmHg	99.75 ± 14.27	106.15 ± 15.08	0.124
Body temperature, °C	36.81 ± 0.88	36.63 ± 0.84	0.442
SpO_2, %	81.36 ± 12.48	81.62 ± 10.99	0.936
Leukocyte, $\times 10^9$/L	12.01 ± 5.01	12.85 ± 4.31	0.513
Basophils, $\times 10^9$/L	0.44 ± 0.49	0.39 ± 0.69	0.769
Neutrophils, $\times 10^9$/L	11.26 ± 4.89	11.25 ± 3.78	0.994
Lymphocytes, $\times 10^9$/L	0.44 (0.39–0.74)	0.63 (0.52–1.1)	**0.017**
Erythrocytes, $\times 10^{12}$/L	4.3 ± 0.51	4.51 ± 0.48	0.133
Erythrocytes (males), $\times 10^{12}$/L	4.39 ± 0.52	4.52 ± 0.46	0.380
Erythrocytes (females), $\times 10^{12}$/L	3.92 ± 0.09	4.47 ± 0.60	0.105
Hemoglobin (all patients), g/L	125.6 ± 15.1	130.8 ± 16.0	0.24
Hemoglobin (males), g/L	128.2 ± 15.5	132.0 ± 14.8	0.417
Hemoglobin (females), g/L	113.8 ± 4.5	126.1 ± 20.6	0.275
Thrombocytes, $\times 10^9$/L	262.36 ± 109.47	374.56 ± 110.71	**<0.001**
MPV, fL (ref. 6.8–10.4)	9.25 ± 1.27	8.87 ± 1.33	0.296
CRP, mg/L (ref. <5)	67.15 (43.5–136.4)	43.4 (19.25–69.7)	**0.01**
Urea, mmol/L (ref. 2.8–8.3)	9.26 ± 4.19	9.16 ± 3.18	0.920
Creatinine, µmol/L (ref. 64–104)	69.5 (46–81)	61 (51.5–76.5)	0.812
Uric acid, µmol/L (ref. 182–403)	322.9 ± 181.55	266.47 ± 105.68	0.158
AST, U/L (ref. 11–38)	62.41 ± 49.62	40.72 ± 26.27	**0.042**
ALT, U/L (ref. 12–48)	45.5 (25–58)	42 (34–79.5)	0.603
GGT, U/L (ref. 11–55)	111.45 ± 82.25	83.03 ± 86.45	0.241
ALP, U/L (ref. 60–142)	119.48 ± 50.87	77.07 ± 26.82	**<0.001**
LDH, U/L (ref. <241)	694.64 ± 273.64	454.1 ± 139.14	**<0.001**
Total bilirubin, µmol/L (ref. 3–20)	16.03 ± 8.63	12.54 ± 3.94	0.053
Conjugated bilirubin, µmol/L (ref. <5)	3.61 ± 2.65	2.88 ± 1.15	0.238
D-Dimer, mg/L (ref. 0.17–0.5)	2.77 ± 1.66	2.07 ± 1.66	0.143
APTT, s (ref. 22–33)[a]	22.67 ± 4.00	21.77 ± 3.07	0.368
Fibrinogen, g/L (ref. 1.8–4.1)	5.34 ± 1.18	5.66 ± 1.42	0.396
Protein C, % (ref. 70–140)	102.62 ± 25.38	115.04 ± 27.36	0.114
Protein S, % (ref. 60–130)	89.94 ± 22.34	77.3 ± 23.58	0.099
Antithrombin 3, % (ref. 80–120)	87.77 ± 16.4	100.3 ± 16.57	**0.011**
hsTnI, ng/L (ref. <19.8)	18.1 (10.8–70.7)	11.8 (6–23.9)	0.065
NT-proBNP, pg/mL (ref. <125)	933.4 (612.6–2,124.7)	411.2 (249.6–1,023.9)	**0.012**
IL-6, pg/mL (ref. <6.4)	66.54 (35.15–112.4)	14.6 (8.09–24.88)	**<0.001**
Procalcitonin, ng/mL (ref. 0.065)	0.36 (0.23–0.66)	0.11 (0.07–0.27)	**0.001**

[a]With exclusion of patients treated with warfarin. BP, blood pressure; SpO_2, peripheral oxygen saturation; CRP, C-reactive protein; AST, aspartate transaminase; ALT, alanine transaminase; LDH, lactate dehydrogenase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; APTT, activated partial thromboplastin clotting time; hsTnI, high sensitive troponin I; NT-proBNP, N-terminal pro brain natriuretic peptide; IL-6, interleukin 6. Bold indicates the p-values less than 0.05 and were considered statistically significant.

(102.62 ± 25.38 % vs. 115.04 ± 27.36 % for protein C, p=0.114; 89.94 ± 22.34 % vs. 77.3 ± 23.58 % for protein S, p=0.099; 2.77 ± 1.66 mg/L vs. 2.07 ± 1.66 mg/L for D-Dimer, p=0.143). Data are shown in Table 2.

Discussion

The clinical course of COVID-19 can develop rapidly and cause severe and fatal complications. The inflammatory response leads to CAC which is linked with a high incidence of thromboembolic events, especially in the microvasculature [16]. Pulmonary microthrombosis has been previously reported during outbreaks of other coronaviruses, including SARS-CoV and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) as a consequence of severe ARDS [17, 18]. Several clinical studies were performed to investigate the incidence of venous thromboembolism as a marker of hypercoagulation and found it in up to one third of COVID-19 patients [5, 19, 20].

During the steady state when the endothelium becomes damaged, platelets adhere to the subendothelium, tissue factor is released and activation of the coagulation proteins in cascade system starts, which leads to production of fibrin and formation of fibrin mesh. As forementioned, to prevent excessive blood clotting formation, thrombin activity and thrombus formation are regulated by feedback mechanism of anticoagulants like antithrombin 3, protein C and its cofactor protein S, that limit further activation of coagulation cascade. As a final control mechanism, activation of fibrinolysis system occurs, which limits fibrin deposition and leads to cleavage of protein fragments from blood clot, known as D-Dimers, and their release into bloodstream. During inflammation these mechanism become disrupted with promotion of the proinflammatory and procoagulant type of answer, as seen in COVID-19 [15, 21].

The most frequent alteration of coagulation system in hospitalized COVID-19 patients is the elevation of D-Dimer, and has been connected with increased severity of disease and with higher mortality [22]. D-Dimer values are elevated in acute inflammations and the stronger the inflammatory reaction is, the higher the D-Dimer values are. Our research also showed that higher values of D-Dimer (p<0.001) are associated with a more severe form of disease. We found a difference in concentration of D-Dimer in the HFNO group due to survival but the difference was not statistically significant (p=0.143), see Table 2. Several studies have investigated the correlation between D-Dimer concentration and disease severity and clinical outcome. A study conducted on 343 patients concluded that D-Dimer are useful markers for predicting hospital mortality [23]. A systematic review published in 2020 found that COVID-19 patients with high D-Dimer levels had a higher risk of severe disease and mortality [24].

Protein C is found on endothelial cells, and in its activated form, in concert with protein S, acts as a strong inhibitor of the coagulation system, especially in the microvasculature. It also has anti-apoptotic and anti-inflammatory properties and can stabilize endothelium and epithelium [11, 25]. Two studies involving only COVID-19 patients requiring intensive care, reported lower levels of protein C activity. Abovementioned could suggest that an imbalance between procoagulants and anticoagulants may lead to hypercoagulable state in critically ill patients with COVID-19 [10, 26].

Hypoxia and hyperinflammation (as seen in severe forms of COVID-19 are the main mechanism of lowering protein S activity [1]. In patients with increased activation of the immune system (increased IL-6 values), the occurrence of venous thromboembolism increases [27]. Protein S has also been shown to be a prognostic marker in patients with COVID-19. Low protein S activity was associated with disease severity and the negative outcome [28]. Protein S has also been shown to be a prognostic marker in patients with COVID-19. In a study that measured the activity of protein S during hospital admission, its decreased activity indicated a higher possibility of death [27]. The mentioned research did not specify which day of the illness was the day of admission to the hospital. Patients with longer-lasting, more severe COVID-19 could also have lower values of protein S compared to those with a shorter duration of the disease.

In our study, a severe form of disease is associated with low activity of protein C (p<0.001) but it is not associated with low activity of protein S (p=0.176). In the HFNO group, patients who died had lower activity of protein C (p=0.114) and surprisingly higher activity of protein S (p=0.099) but these differences were not statistically significant. One of the possible pathophysiological mechanisms is that in severe forms of COVID-19 there is a low concentration of complement components in the blood (including the C4 component of complement) and therefore protein S is not found in a bound form with complement protein C4b-binding protein but as an independent substance, which increases its blood concentration and activity [29, 30].

We found that hospitalized patients with a severe clinical form of COVID-19 had lower activity of antithrombin 3 (p<0.001), and patients in HFNO group who died also had significantly lower activity of antithrombin 3 (p<0.011). Lower protein C and antithrombin 3 levels increase the risk for generation of critical symptoms and severe outcomes and their concentration are associated with severity of COVID-19 [31, 32]. Few studies observed antithrombin 3

deficiency in COVID-19 patients and found that antithrombin 3 is strongly associated with mortality in COVID-19 [32].

Although studies have shown that elevated levels of fibrinogen were associated with a higher risk of developing a critical illness and a higher risk of death in COVID-19 patients in our study fibrinogen values did not show a significant difference (p=0.387) in relation to the clinical severity of disease [33].

The increase potential for thromboembolic events seen in SARS-CoV-2 is mainly connected to severe inflammatory response along with thromboinflammation and endothelial damage [34]. Endothelial injury activates intrinsic and extrinsic coagulation pathways. It can occur through direct viral invasion of endothelial cells, where SARS-CoV2 spike protein binds to angiotensin-converting enzyme 2 receptor as well as through indirect inflammatory effects [35]. Damaged endothelium exposes the underlying matrix containing tissue factor and collagen and leads to activation of coagulation cascade, thrombin generation which cleaves fibrinogen, leading to stabilization of platelet aggregates and formation of thrombus. Also, expression of tissue factor on macrophages could also be induced by inflammatory cytokines. Exposed subendothelial matrix could lead to platelet activation and recruitment, as well. Endothelial dysfunction can cause the release of vWF and the impairment of eNOS, which subsequently promotes platelet activation and formation of thrombus [36].

All of the above indicates that hypercoagulability in COVID-19 is promoted by increased production of inflammatory reactants through hyperactivation of the immune system. In addition to the above, a secondary pathophysiological mechanism of hypercoagulability is also in the reduced activity of endogenous anticoagulants.

Selection of the ideal anticoagulant depends on various factors, such as the previous diseases which can contribute to formation of thrombus (for example atrial fibrillation), presence of other comorbidities such as renal or liver disease but also on individual bleeding risk [37]. D-Dimer level should not be used for anticoagulation dosage guidance, regardless the fact that its high levels are found to be predictor of poor outcome. A single-center randomized trial performed on critically ill mechanically ventilated patients with high D-Dimer level (n=20) compared the efficacy of prophylactic and therapeutic dose of anticoagulation and showed significant improvement in the oxygenation in the therapeutic anticoagulation group. Yet, there was no difference among both arms when in-hospital or 28-day mortality was compared [38].

Due to the influence on the coagulation parameters, we did not include patients who also take warfarin in their chronic therapy. Patients with clinically proven VTE were also not included in the study due to the influence on coagulation parameters. The presence of micro thrombosis cannot be confirmed clinically by existing routine diagnostic methods [39]. Disorders of coagulation parameters could be the first sign that could indicate the presence of microthrombi in the venous system or their increased possibility of occurrence and it can be one of the main parameters that could indicate the severity of the disease in COVID-19 patients. Given that clinically manifest VTEs represent one of the extremes of the coagulation cascade, they could represent a significant bias in the investigation of the coagulation effects of COVID-19 itself, and therefore these patients were not included in the research.

Small sample size is the main limitation of our study but our results are in accordance with other studies. Further investigations are required to determine which coagulation pathways mostly contribute to morbidity and mortality in COVID-19 infection. Better understanding of complex CAC could help in developing new treatments options for COVID-19 patients.

Conclusions

Concentrations of coagulation and anticoagulation factors and their activity in the blood of COVID-19 patients differ significantly depending on the severity of COVID-19. Through the aforementioned research, we have shown that COVID-19 affects the lowering of endogenous anticoagulation factors and their function, which is an additional factor in the hypercoagulable state and can have a significant impact in the pathogenesis of COVID-19 hypercoagulability. The mentioned mechanism could be the answer to the persistence of venous thromboembolism despite standard anticoagulant treatment. Lower blood values of endogenous anticoagulants and its activity may indicate a worse prognosis of a COVID-19 patient and may serve as an additional prognostic factor.

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Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.
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