ORIGINAL ARTICLE

Assessment of the Natural Anticoagulants (Protein C, Protein S, and Antithrombin III) for COVID-19 Patients

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ABSTRACT

Keyword: Coronavirus, COVID-19, Anticoagulants.

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Background: COVID-19 was declared a pandemic at the end of 2019. The clinical manifestations diversified from minor non-specific symptoms to respiratory failure and even death. Infection leads to micro-thrombosis, hypercoagulability, and in some patients, large vessel thrombosis. **Objective:** To estimate natural anti-coagulants (protein S, antithrombin antithrombin III (ATIII), and protein C) in COVID-19 patients at the time of diagnosis and correlate our findings with the hypercoagulable state and prognosis of patients. **Methodology:** This study was performed on 100 patients were divided into 2 groups: 75 confirmed COVID-19 patients, and 25 pulmonary embolism (PE) patients. Patients were undergone some laboratory tests like PCR, Hemostatic status evaluation, serum ferritin, C reactive protein (CRP), LDH, and Complete Blood Count. **Results:** In the PE group the values of D-dimer, total leucocytic count, PT, and fibrinogen were significantly higher than COVID-19 group ($P < 0.05$). While in the COVID-19 group the values of protein C, and LDH were significantly higher than PE group ($P < 0.05$) and there was an insignificant difference between both groups regarding values of neutrophils, monocytes, platelets, CRP, ferritin, aPTT, ATIII, or protein S. **Conclusion:** ATIII, protein S, and protein C lower levels significantly affect the COVID-19 patients' severity and mortality.

INTRODUCTION:

COVID-19 was declared a pandemic at the end of 2019. The disease's clinical manifestations are diverse, from minor nonspecific symptoms (fever, dry cough, and diarrhea) to severe pneumonia, respiratory failure, and death [1]. Acute severe respiratory syndrome A multisystem disease caused by coronavirus-2 (SARS-CoV-2) infection is distinct from the typical viral pneumonia. Infection with SARS-CoV-2 results in thrombosis of large vessels, micro-thrombosis, and hypercoagulability, which manifest as ischemic stroke, myocardial infarction, or digital ischemia [2]. Endothelial cells can be infected by SARS-CoV-2, resulting in the development of endothelialitis. The proliferation of proinflammatory and hypercoagulable responses by infected endothelial cells can result in thrombosis of both small and large vessels [3].

The manifestation of pulmonary embolism (PE) is exceedingly diverse and is caused by elevated pulmonary pressures brought about by complete or partial pulmonary vasculature obstruction. Dyspnea at rest or during exertion, pleuritic pain, cough, orthopnea, calf or thigh pain and/or swelling, wheezing, and hemoptysis are frequently reported symptoms. In general, males exhibit

a greater incidence in comparison to females (56 versus 48 per 100,000, respectively). The prevalence increases as age increases, specifically among women [4, 5]. Laboratory tests, while not diagnostic in nature, can modify the clinical suspicion for PE; leukocytosis, high serum lactate dehydrogenase (LDH), and aspartate aminotransferase (AST) [6].

COVID-19 infection is associated with various coagulation complications, including arterial thromboembolism (ATE), PE, deep venous thrombosis (DVT), venous thromboembolism (VTE) and disseminated intravascular coagulopathy (DIC). DVT and VTE are frequently clinically asymptomatic; in many instances, a sudden fatal PE is the initial indication. Systematic screening has been recommended for COVID-19 patients due to the high incidence of VTE and DVT. Obstruction caused by arterial thromboembolism transpires in SARS-CoV-2-associated hyperinflammation presence. A worsening of the disease and the occurrence of septic shock, which can cause endothelial damage and coagulation issues (increased platelet count and coagulability), may culminate in DIC [7-9]. There have been reports of abnormalities in anticoagulants; protein S deficiency results in elevated levels of factor Va, which induces thrombophilia; thus, protein S is associated with the inhibition of Va factor activity. Deficiencies in protein C elevate the microvascular thrombosis and DIC risk. Furthermore, PC diminishes its anticoagulant properties. Antithrombin-III deficiency has been documented in approximately 50% of VTE patients. Consequently, AT deficiency need to be considered to prevent thrombus-related fatalities among COVID-19 patients [10-12].

SUBJECT AND METHODS

This prospective case-control study was conducted at the Clinical Pathology Department in Aswan University Hospital in the period from January 2022 to December 2022 including one hundred patients selected regarding our inclusion criteria as follows:

1. COVID-19 cases PCR positive admitted in intensive care unit (ICU) who have ARDS or non-ICU who not suffering from ARDS.
2. Those diagnosed with PE PCR negative with no restriction on the sex of the patients.

Patients are excluded if they are under 18 year's old, pregnant women, and have liver cell failure, renal failure, cancer or malignancy.

The cases were categorized into two groups: group A evolves 75 cases hospitalized with COVID-19 PCR positive. Group B (control group) evolved 25 patients with PE PCR negative. Patients were subjected to some laboratory tests such as Polymerase Chain Reaction (PCR), LDH, hemostatic status evaluation, C-reactive protein (CRP), Serum ferritin level, Complete Blood Count (CBC), ATII, protein C, and protein S on admission.

PCR was done by Dacron nasopharyngeal swab in viral transport media and extracted by QIAamp DSP Virus Spin Kit. Detection was done by genesing® Real-Time PCR COVID-19 CE IVD kit using a Rotor-Gene Q thermal cycler.

The hemostatic status evaluation was performed on Sysmex CS1600 (HQ: Kobe, Japan; Chairman and CEO: Hisashi Ietsugu) (fully automated hemostasis analyzer) as follows:

-Protein C by SIEMENS Berichrom® Protein C(REF OUVV15) For the quantitative determination of functionally active protein C using a chromogenic substrate as an aid in the diagnosis of inherited and acquired deficiencies. Protein C is activated in the patient sample by an activator derived from snake venom. In a kinetic test, the Ca content of the resulting protein is determined by observing the increase in absorbance at 405 nm; typical values range from 70 to 140 %.

-Protein S by SIEMENS INNOVANCE® (United States; Siemens,REF OPGL03) Free PS Ag, Particles of polystyrene were imbued with two distinct monoclonal antibodies, each of which was specific for free protein S. Mixing the latex reagent that is produced with samples that contain free protein S causes aggregation. As measured by the increase in turbidity, the aggregation degree is directly proportional to the free protein S concentration in the sample. Constant values: 67.5 to 139.0 % male 60% to 113.6 % are female.

-Anti-thrombin III: by SIEMENS Berichrom® (United States; Siemens REF OWWR17) antithrombin III (ATIII) (A), Heparin transforms the ATIII present in the sample into an immediate inhibitor, thereby causing the thrombin to become inactive. By measuring the increase in absorbance at 405 nm during a kinetic test, the residual thrombin content can be ascertained. The change in absorbance is inversely proportional to the ATIII activity present in the sample. Typical ranges from 79.4% to 112%.

-Fibrinogen: by SIEMENS Dade® Thrombin (United States; Siemens REF B4233-25) Reagent, the soluble plasma protein fibrinogen is converted to its fibrin, insoluble polymer, by the enzyme thrombin. In diluted plasma, the clotting time is inversely proportional to the concentration of fibrinogen. Clauss devised a straightforward methodology for quantifying fibrinogen by employing this principle-assessing the diluted plasma clotting time after thrombin addition. Following this, the measured clotting time is compared to that of a standardised fibrinogen formulation, whose values range from 180 to 350 mg/dL.

-PT (Prothrombin time): by SIEMENS Thromborel® S, (United States; Siemens) the coagulation process is initiated when plasma is incubated with thromboplastin and calcium in the ideal proportions. Then, the time required for a fibrin clot to form is determined; the reference interval is 10.4 to 12.6 seconds.

-aPTT (activated partial thromboplastin time): by SIEMENS Pathromtin (United States; Siemens) * SL, A surface activator combined with the ideal number of phospholipids to incubate plasma induces intrinsic coagulation system factors activation. The coagulation process is initiated by the introduction of calcium ions; the duration until a fibrin clot form is quantified. The reference interval was 26.4 to 37.5 seconds

-D-dimer: by VIDAS D-Dimer Exclusion II TM (DEX2) (United States; VIDAS) , combining a two-step sandwich enzyme immunoassay method with a final fluorescent recognition constitutes the assay principle. Standard < 500 ng/ml.

-Serum ferritin level: Immuno-unsymmetric Assay method by TOSOH AIA-360 Normal range Male:24 to 336 microgram/L Female: 11 to 307

-CRP (US; BBI solution): Immunoturbidimetric assay by BECKMAN COULTER AU 480 (Japan; Beckman Coulter) Normal < 10 mg/dl

-LDH: kinetic method by BECKMAN COULTER AU 480 Normal range 105 to 333 IU/L

-Complete Blood Count: by Sysmex XN-1000 (HQ: Kobe, Japan; President: Hisashi Ietsugu) automated 5-part differential hematology analyzer.

Ethical consideration:

The ethical committee of the faculty of medicine at Aswan University has ensured that this research adheres to all procedures in the period from January 2022 to December 2022 Approval code:603/2/22). Patient signature is obtained on an informed consent form containing all pertinent details regarding this study. Participants were exclusively recruited for the study upon providing informed consent by signing the consent form. At any time and without explanation, participants may withdraw from this research at their discretion.

Statistical analysis:

The data were analyzed on an IBM-ompatible computer utilizing SPSS (statistical package for social science) version 26.0 (SPSS Inc., Chicago, IL, USA). The quantitative and percentage-based descriptions of the qualitative data were accompanied by a Chi-square analysis. The Shapiro-Wilks test utilized to assess the data normality of quantitative data under the assumption that $P > 0.05$. The means and standard deviations of quantitative data were calculated utilizing the Mann–Whitney U test and the Wilcoxon signed-rank test, respectively. A ROC curve analysis was performed to evaluate the accuracy of COVID-19 severity predictions based on the levels of anticoagulant. Logistic regression was also used to estimate the relationship between a dependent variable and one (univariate) or more independent variables (multivariate). In this study, the established level of significance was 0.05.

RESULTS:

This study was conducted on 100 cases and divided into 2 groups: 75 confirmed COVID-19 patients with positive PCR (ICU patients and non-ICU patients), and 25 PE patients. The total patient's age ranged from 20 – 90 years, with a mean ± SD of 61.3 ± 16.9. The mean age of the COVID-19 group was 61.7 ± 16.7, while the PE group's mean age was 59.8 ± 17.7 years. The studied patients included 41 males and 59 females; including 40 (53.3%) females and 35 (46.7%) males in the COVID-19 group and 6 (24%) males and 19 (76%) females in the PE group. 36% of the COVID-19 patients were diabetics, 44% were hypertensive, 2.7% had asthma, 14.7% had chronic cardiac diseases, and 2.7% were smokers. Regarding the PE group, none of them had diabetes, asthma, or chronic cardiac diseases, 16% were hypertensive, and 4% were smokers **(Table 1)**.

Table (1): Sociodemographic and baseline clinical characteristics of the studied group (N=100)

		COVID-19 (n =75)	**PE (n = 25)**
Age (Years)	**Mean ± SD**	61.7 ± 16.7	59.8 ± 17.7
Gender	**Male**	35 (46.7 %)	6 (24 %)
	Female	40 (53.3 %)	19 (76 %)
Diabetes mellitus	**Yes**	27 (36 %)	0
Hypertension	**Yes**	33 (44 %)	4 (16 %)
Asthma	**Yes**	2 (2.7 %)	0
Chronic cardiac disease	**Yes**	11 (14.7 %)	0
Smoking	**Smoker**	2 (2.7%)	1 (4 %)
	Non-smoker	73 (97.3 %)	24 (96%)

Data are presented as mean ± SD or number. Chi-square test, Mann–Whitney U test and the Wilcoxon signed-rank test

Patients' WBCs and lymphocytes mean values were significantly higher in the PE group. No statistically significant disparity was observed between the two groups in terms of platelets, neutrophils, or monocytes **(Table 2)**. **Table (2): CBC characteristics of the studied group (N=100)**

		COVID-19 (n =75)	**PE (n = 25)**	**P value**
White blood cells (10^3/ul)	**Mean ± SD**	15.0 ± 5.9	18.1 ± 7.1	0.040
Neutrophils %	**Mean ± SD**	80.5 ± 6.6	77.8 ± 9.2	0.765
Lymphocytes %	**Mean ± SD**	9.5 ± 4.3	16.0 ± 10.0	0.011
Monocytes %	**Mean ± SD**	4.1 ± 3.6	3.5 ± 1.1	0.364
Platelets (10^3/ul)	**Mean ± SD**	254.2 ± 99.0	231.2 ± 72.7	0.491

Data are presented as mean ± SD. Mann–Whitney U test and the Wilcoxon signed-rank test

D-dimer, PT, and fibrinogen mean values for cases were significantly elevated in the PE group. In the COVID-19 group, however, the mean values of the patients' protein C were considerably higher. Instatistically significant distinctions were detected between the two groups in terms of aPTT, ATIII, or protein S **(Table 3)**.

Table (3): Coagulation characteristics of the studied group (N=100).

		COVID-19 (n =75)	PE (n = 25)	P value
D-dimer(ng/ml)	**Mean ± SD**	2799.3 ± 2358.3	3634.2 ± 1872.1	0.008
PT (Seconds)	**Mean ± SD**	12.6 ± 1.6	14.1 ± 1.3	<0.0001
aPTT (Seconds)	**Mean ± SD**	39.8 ± 11.5	41.3 ± 5.6	0.075
Antithrombin III (%)	**Mean ± SD**	76.0 ± 21.0	72.0 ± 22.1	0.355
Protein C (%)	**Mean ± SD**	99.0 ± 30.4	76.1 ± 31.9	0.002
Protein S (%)	**Mean ± SD**	72.2 ± 19.5	66.1 ± 20.0	0.135
Fibrinogen (mg/dL)	**Mean ± SD**	399.3 ± 146.2	482.6 ± 136.1	0.015

Data are presented as mean ± SD. PT: Prothrombin time, aPTT: activated partial thromboplastin time, Mann–Whitney U test and the Wilcoxon signed-rank test

ICU COVID-19 cases had significantly decreased ATIII, protein C, and protein S levels than the non-ICU cases **(Table 4)**.

Table (4): Relationship between natural anticoagulant and the cases severity of COVID-19 (n=75)

		Severity		
		ICU patients (n = 44)	**Non-ICU patients (n= 31)**	**P value**
Antithrombin III	**Mean ± SD**	68.4 ± 20.3	86.8 ± 17.3	<0.0001
Protein C	**Mean ± SD**	98.5 ± 33.1	112.4 ± 19.9	0.004
Protein S	**Mean ± SD**	66.0 ± 20.2	81.0 ± 14.8	0.001

Data are presented as mean ± SD. ICU: intensive care unit. Mann–Whitney U test and the Wilcoxon signed-rank test

The dead COVID-19 cases had significantly decreased levels of protein C, ATIII, and protein S than the recovery cases **(Table 5)**.

Table (5): Relationship between natural anticoagulants and the cases mortality of COVID-19 patients (n=75)

		Outcome		
		Recovery (n=35)	**Death (n=40)**	**P value**
Antithrombin III	**Mean ± SD**	86.7 ± 16.8	66.7 ± 20.0	<0.0001
Protein C	**Mean ± SD**	112.5 ± 19.4	87.1 ± 33.4	0.001
Protein S	**Mean ± SD**	78.9 ± 15.3	66.4 ± 21.1	0.005

Data are presented as mean ± SD. Mann–Whitney U test and the Wilcoxon signed-rank test

Severe PE cases had significantly lower levels of protein S than the non-severe cases. At the same time, there is no significant difference between the not severe and severe PE cases regarding ATIII or protein C **(Table 6)**.

Table (6): Relationship between natural anticoagulants and the cases severity of PE patients (n=25)

		Severity		
		Severe (n = 20)	**Not (n= 5)**	**P value**
Antithrombin III	**Mean ± SD**	67.5 ± 21.4	87.1 ± 17.1	0.073
Protein C	**Mean ± SD**	70.2 ± 31.9	98.8 ± 20.8	0.053
Protein S	**Mean ± SD**	60.9 ± 18.6	83.5 ± 14.8	0.019

Data are presented as mean ± SD. Mann–Whitney U test and the Wilcoxon signed-rank test

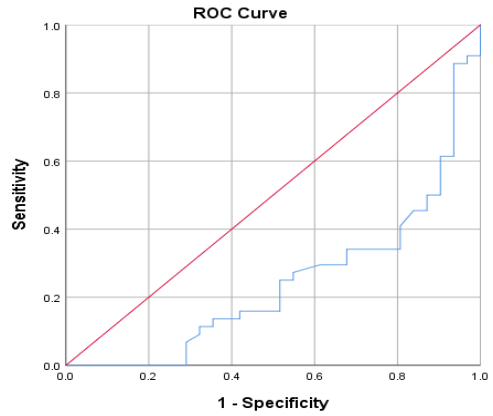
The dead PE cases had significantly decreased levels of protein S and protein C, then–the recovery cases. At the same time, there is an insignificant difference between the dead ~~a~~nd recovery PE regarding ATIII **(Table 7)**.

Table (7): Relationship between natural anticoagulant and the cases mortality of PE patients (n=25).

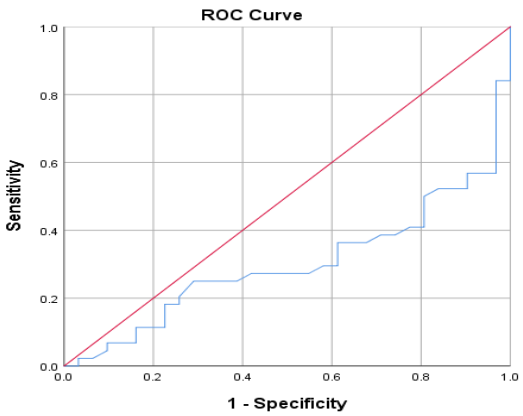
		Outcome		
		Recovery (n=10)	**Death (n=15)**	**P value**
Antithrombin III	**Mean ± SD**	77.3 ± 18.5	67.5 ± 23.6	0.280
Protein C	**Mean ± SD**	103.7 ± 25.9	59.6 ± 22.4	0.001
Protein S	**Mean ± SD**	76.7 ± 16.2	57.8 ± 18.8	0.016

Data are presented as mean ± SD. Mann–Whitney U test and the Wilcoxon signed-rank test

Among COVID-19 patients' estimation for prediction of severity was done as follows: at a cut-off point of 50.1 %, the specificity and sensitivity of ATIII are 6.5% and 88.6% respectively. At a cut-off point of 136.7 %, the sensitivity and specificity of protein C are 6.8% and 90.3% respectively. At a cut-off point of 101.6 %, the sensitivity and specificity of protein S are 6.8% and 96.8% respectively. For the prediction of mortality, estimation was done as follows: at a cut-off point of 50.1 %, the sensitivity and specificity of ATIII are 87.5% and 5.7% respectively. At a cut-off point of 142.1 %, the sensitivity and specificity of protein C are 2.5% and 97.1% respectively. At a cut-off point of 101.6 %, the sensitivity and specificity of protein S are 7.5% and 97.1 respectively. **Figure 1**



A



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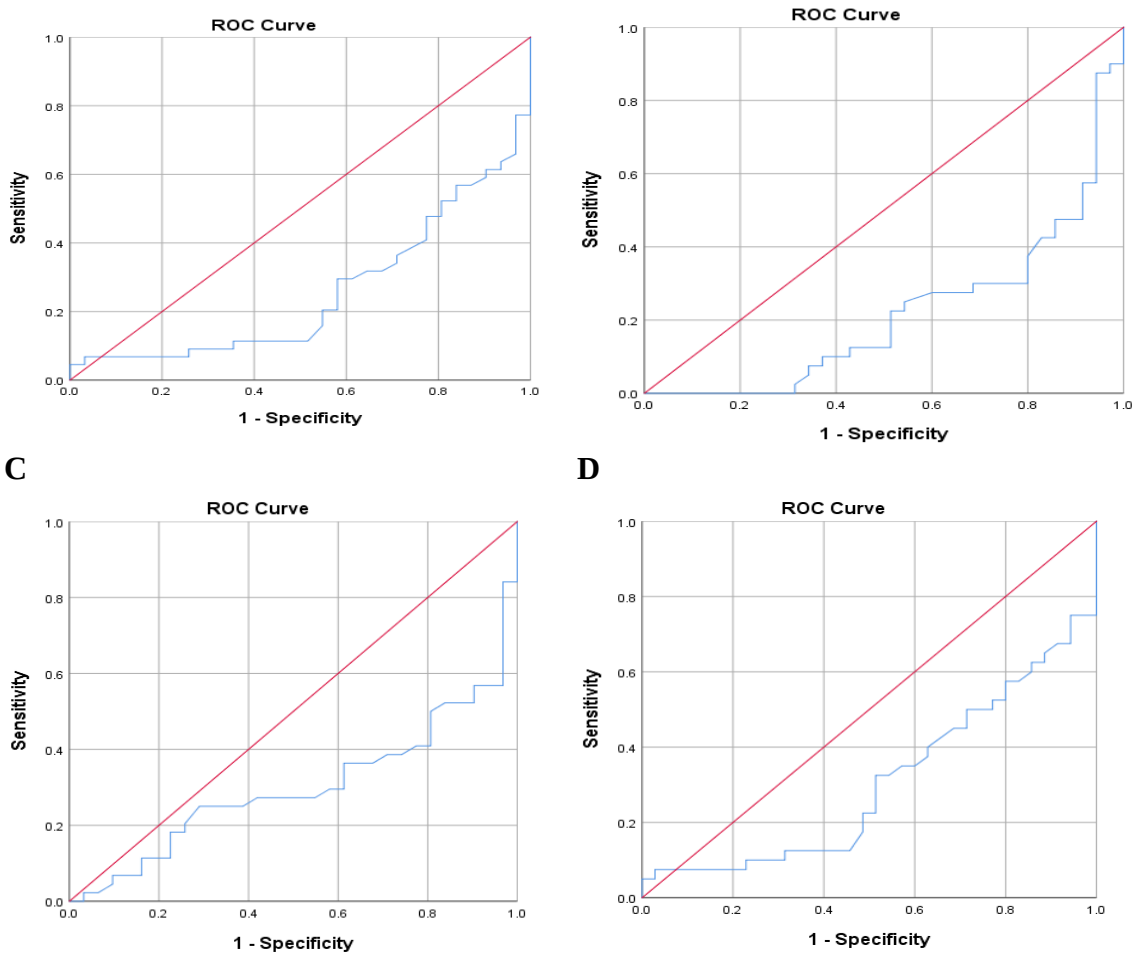

Figure 1: ROC curve for the accuracy of (A) antithrombin III, (B) protein C, (C) protein S, to predict severity, (D) antithrombin III, (E) protein C, (F) protein S to predict mortality in COVID-19 patients.

Among PE patients' estimation for prediction of severity was done as follows: at a cut-off point of 103.1 %, the sensitivity and specificity of ATIII are 5% and 80% respectively. At a cut-off point of 107.5 %, the sensitivity and specificity of protein C are 15.8% and 80% respectively. At a cut-off point of 93.9 %, the sensitivity and specificity of protein S are 5% and 80% respectively. For the prediction of mortality, estimation was done as follows: at a cut-off point of 93.9 %, the sensitivity and specificity of ATIII are 6.7% and 90% respectively. At a cut-off point of 107.5 %, the sensitivity and specificity of protein C are 6.7% and 66.7% respectively. At a cut-off point of 93.8 %, the sensitivity and specificity of protein S are 26.7% and 80% respectively.

DISCUSSION:

COVID-19 is an infectious pandemic that has a widespread global impact. It is characterized by its rapid transmission, significant morbidity and mortality, and a diverse range of clinical manifestations. It exhibits a spectrum of manifestations, including mild upper respiratory tract disorder, asymptomatic conditions, and severe pneumonia accompanied by acute respiratory distress syndrome. 15% of hospitalized cases are diagnosed with progressive respiratory failure, which is among the leading causes of mortality [13].

Post-mortem research by Carsana *et al.* [14], who examined lung tissue samples from acute lung injury and platelet-fibrin thrombi in small arterial vessels, identified thromboembolic manifestations, which range from venous to arterial events, as one of the most prominent defining features of the disease. Patients with COVID-19 had a ninefold higher alveolar capillary

microthrombi incidence contrast to those with influenza, as demonstrated by Ackermann *et al*. [3].

Protein S, which is widely recognized for its role as a cofactor for activated protein C and as an anticoagulant, also plays a crucial role in stimulating the immunosuppressive TAM receptors (Tyro3, Axl, and Mer). This is a critical function in preventing acute lung injury, which is characterized by hyperinflammation [15].

ATIII, which is essential for heparin to maintain its clinical effectiveness, is the most potent endogenous anticoagulant. A severe inflammatory and hypercoagulable state induced by COVID-19 may lead to a reduction in ATIII levels and render heparin treatment ineffective, ultimately resulting in an elevated risk of mortality [16]. ATIII is the principal endogenous thrombin inhibitor and a potent anti-inflammatory agent also, influencing the extrinsic, intrinsic, and general coagulation cascade pathways. Coagulation disorders are associated with decreased levels of ATIII as a result of accelerated uptake after the formation of the AT complex, as well as diminished synthesis and heightened neutrophil clearance [16, 17], heparin treatment also results in a reduction in ATIII levels [18].

In the current study, in terms of coagulation characteristics, the mean protein C levels in the COVID-19 group were significantly more than those in the PE group. In contrast, protein S and ATIII levels did not differ significantly between the two groups. Lower concentrations of protein C, protein S, and ATIII were also observed to be associated with COVID-19 mortality and severity in ICU patients. Disagree with our results, published literature studying COVID-19 coagulopathy in COVID-19 patients and comparing them with non-COVID-19 found statistically insignificant difference in the protein C and ATIII levels [19]. Also, Gerotziafas *et al*. [20] reported statistically significant lower values of Protein C and S as well as anti-thrombin in ICU-admitted COVID-19 patients than in normal control. In 65% of the COVID-19 patients, Protein S activity was diminished, according to Stoichitoiu *et al*. [21] A lower activity of protein S was associated with mortality, and this association persisted even after controlling for variables such as age, inflammation markers, and alanine aminotransferase (ALAT) El Menshawy *et al*. discovered, along the same lines, that severe cases exhibited a substantial decrease in protein Cand S compared to moderate cases [22, 23], as well as reports about ATIII have shown decreased levels in COVID-19 cases [16, 24]. Siregar and Ihsan observed significant differences in the protein C and ATIII level in both groups distributed regarding severity and concluded that lower ATIII and protein C levels increase the risk of developing critical symptoms [25].

Mean concentrations of D-dimer, prolonged PT, and fibrinogen were significantly elevated in the PE group in our study. Concerning aPTT, however, no significant difference existed between the two groups. In contrast to those results, a recent cohort study by Vaughn *et al*. detected a noteworthy elevation in the PT and aPTT, which were associated with a substantial rise in mortality rates among COVID-19 patients in ICU as opposed to general care [26]. Zhou *et al*. observed that critically ill patients exhibited a prolonged PT time.According to Li *et al*. [27] reported that the PT of critically ill patients is approximately 1.9 seconds longer in comparison to nonfatal and critical patients. When fatal cases comprise 48% of cases during the late stages of the disease, the PT prolongation exceeds 6 seconds. As a result, PT has clinical utility in determining the prognosis of critically ill patients. So, this can explain the difference in both groups as those with PE may be more critically ill than those with COVID-19, So they had more prolonged PT than COVID-19 patients [28]. Zhang *et al*. found a significantly higher D-dimer level that was detected in severe COVID-19 cases. Also, Tang *et al*. demonstrated that the death rate was found to be correlated with high D-dimer levels in cases admitted with COVID-19 [29, 30].

The differences between previously mentioned studies and our study may contributed to the differences in COVID-19 severity in cases and the used control, as our control was PE patients

who were considered as high coagulopathy state and considered as critically ill patients not healthy controls.

Strength of this study according to our knowledge, it is the first investigation that compares Covid-19 and PE cases.

Limitations of our study are Objective measures, including blood oxygen saturation and inflammatory biomarkers, were not obtained from admitted COVID-19 patients. These parameters are crucial in assessing the severity of cases and the extent to which a cytokine storm and inflammatory response develop, both of which are recognized as mechanisms that impact all body cells because of the COVID-19 infection. In addition, lack of adequate control cases. As a result of the inclusion of only a subset of the population, the results lack generalizability to the entire population.

CONCLUSION

Lower levels of protein S, protein C, and ATIII significantly reflect the severity and COVID-19 patients' mortality than PE group. We advised conducting further studies with a higher sample size, in a multicenter distributing the COVID-19 patients into groups regarding their severity and adding a healthy control to the study to illustrate the difference in a clear view.

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