Investigation of the Molecular Mechanism of Coagulopathy in Severe and Critical Patients With COVID-19

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Coagulopathy is a frequently reported finding in the pathology of coronavirus disease 2019 (COVID-19); however, the molecular mechanism, the involved coagulation factors, and the role of regulatory proteins in homeostasis are not fully investigated. We explored the dynamic changes of nine coagulation tests in patients and controls to propose a molecular mechanism for COVID-19-associated coagulopathy. Coagulation tests including prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen (FIB), lupus anticoagulant (LAC), proteins C and S, antithrombin III (ATIII), D-dimer, and fibrin degradation products (FDPs) were performed on plasma collected from 105 individuals (35 critical patients, 35 severe patients, and 35 healthy controls). There was a statically significant difference when the results of the critical (CRT) and/or severe (SVR) group for the following tests were compared to the control (CRL) group: PTCRT (15.014) and PTSVR (13.846) (PT CRL = 13.383, \( p < 0.001 \)), PPTCRT (42.923) and PTT SVR (37.8) (PTT CRL = 36.494, \( p < 0.001 \)), LACCRT (49.414) and LAC SVR (47.046) (LAC CRL = 40.763, \( p < 0.001 \)), FIBCRT (537.66) and FIBSVR (480.29) (FIB CRL = 283.57, \( p < 0.001 \)), ProCCRT (85.57%) and ProCSSVR (99.34%) (ProC CRL = 94.31%, \( p = 0.04 \)), ProSSCRT (92.91%) and ProSSSVR (65.06%) (ProS CRL = 75.03%, \( p < 0.001 \)), D-dimer (\( p < 0.0001 \), \( \chi^2 = 34.812 \)), and FDP (\( p < 0.002 \), \( \chi^2 = 15.205 \)). No significant association was found in the ATIII results in groups (ATIII CRT = 95.71% and ATIII SVR = 99.63%);
INTRODUCTION

Coagulation is a dynamic process that is driven by the regulated proteolytic activation of zymogens (commonly known as coagulation factors) in injured vessels. Coagulation factors, except for FVIII, which is produced by liver sinusoidal endothelial cells and lymphatic tissue, are all produced by hepatocytes (1). The main mechanisms (pathways) that trigger blood clotting include intrinsic and extrinsic pathways, each including a set of coagulation proteins in which factors I, II, IX, X, XI, and XII are the main factors in the intrinsic pathway and factors I, II, VII, and X are the factors described in the extrinsic pathway. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) tests primarily measure the activity of the factors involved in the intrinsic and extrinsic pathways, respectively (2, 3). Moreover, the common pathway is composed of factors I, II, V, VIII, and X (4). The proper proteolytic activation of coagulation factors controlled by a variety of regulatory proteins results in the conversion of soluble fibrinogen to insoluble fibrin strands (5). Fibrinogen, a 340-kDa glycoprotein, is an acute-phase protein consisting of three polypeptide chains, Aα, Bβ, and γ, and becomes upregulated in response to injury and inflammation (5). The term lupus anticoagulant (LAC) is used to determine heterogeneous immunoglobulins, their function resulting in the inhibition of phospholipid-dependent coagulation reactions (6). Moreover, LACs can prolong the PT test; therefore, a LAC test is used to evaluate prolonged PT (7). Coagulation regulatory proteins such as antithrombin III (ATIII), protein C, and D-dimer are involved in the normal function and homeostasis of the coagulation system. ATIII, a crucial anticoagulant molecule in mammalian blood, benefits from its cofactor, heparin, to inhibit the coagulation proteases, mainly thrombin and factor Xa (8). Proteins C and S are vitamin K-dependent glycoproteins. Protein S, the cofactor for protein C, supports the activated protein C in the presence of phospholipids and calcium in the inactivation of membrane-bound factors V (FVa) and FVIIIa (9). The mechanistic pathways through which protein C exerts its effects on the coagulation cascades include degrading factors V/Va and VIII/VIIIa, releasing a tissue-type plasminogen activator, and stimulating fibrinolyis by interacting with the plasminogen activator inhibitor (10). Fibrinolysis is an essential step in homeostasis that is finely controlled by a set of cofactors and inhibitors. Plasmin acts as the primary fibrinolyisin and is activated from plasminogen in the presence of a tissue plasminogen activator (tPA) or urokinase (uPA) (11). Plasmin, after being produced, lyses the cross-linked fibrin polymers and consequently forms fibrin degradation products (FDPs) such as D-dimer, which is widely used as a specific marker for thrombosis and physiological fibrinolysis (12) (Figure 1). The coagulopathy and abnormal results in coagulation tests have become common features reported in patients with COVID-19 from the very early days of the emergence of the new coronavirus strain. We listed both the common coagulation tests, including PT, aPTT, fibrinogen, and D-dimer, and those rarely investigated, such as regulatory proteins C and S as well as ATIII, in patients with COVID-19 along with the main results in Table 1. COVID-19-dependent coagulopathy gained attention when PT, aPTT, fibrinogen, and D-dimer tests were recommended by researchers to evaluate the proper homeostasis of the system associated with the prognosis of patients. Moreover, the prophylactic use of anticoagulants was proven to be effective in lowering the mortality rate and highlighted the role of the coagulation system in COVID-19 (31). The link between thrombosis and COVID-19 as an inflammatory disease has been investigated (32, 33). In the present study, we used a coagulation panel of nine coagulation tests to assess the coagulation pathways in 105 included individuals to determine the molecular mechanism through which COVID-19 disrupts the homeostasis of the coagulation system.

MATERIALS AND METHODS

Inclusion/Exclusion Criteria

We followed the guidelines for Corona Virus Disease 2019 edited by the Iranian National Health Commission (similar to the WHO guidelines and the New Coronavirus Pneumonia Prevention and Control Program, 7th edition, published by the National Health Commission of China) to classify the patients into critical and severe groups (34, 35). The criteria used for the inclusion of individuals into each group are summarized in Table 2. All 70 included patients had a positive result of the nucleic acid test of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by RT-PCR using primers targeting the RNA-dependent RNA polymerase (RdRP) and either nucleocapsid (N), envelope (E), or spike (S) genes. A negative result (using the same probes) was used as the main inclusion criterion for the control (CRL) group. All patients were tested for lung involvement by CT imaging. Moreover, individuals in the CRL group had no physical features of

Abbreviations: COVID-19, coronavirus disease 2019; PT, prothrombin time; aPTT, activated partial thromboplastin time; ATIII, antithrombin III; LAC, lupus anticoagulant; ISI, international sensitivity index; INR, international normalized ratio; ICU, intensive care unit; CRL group, control group; SVR group, severe group; CTL group, critical group.
FIGURE 1 | Depiction of the intrinsic, extrinsic, and fibrinolysis pathways in green, blue, and yellow, respectively. The involved coagulation factors for each pathway are shown. Regulatory proteins and their target molecules are shown in red.
| Test (unit) | Target population or center/country | Consistency (with our results) | Included patients/main findings | Reference |
|------------|-----------------------------------|-------------------------------|-------------------------------|-----------|
| PT (s)     | Tianyou Hospital, Wuhan, China    | ✓                             | • 115 patients were included.  |
|            |                                   |                               | • The mean ± SD results for the PT test in critical and severe groups were 13.70 ± 3.38 and 12.14 ± 1.16 s, respectively. | (13)      |
|            | Jinyintan Hospital and Wuhan Pulmonary Hospital, China | ✓                             | • 191 patients [survivors (n = 137), non-survivors (n = 54)] were studied. |
|            |                                   |                               | • PT_{Survivors} = 11.4 s, PT_{Non-Survivors} = 12.1 s (p = 0.0004) |
|            | Chongqing, China                  | ✓                             | • 7 (13%) of expired patients had PT ≥ 16, while only 4 (3%) of patients who survived had PT ≥ 16. |
| aPTT (s)   | Tongji Hospital, Wuhan, China     | ✓                             | • 147 patients were enrolled.  |
|            |                                   |                               | • The mean aPTT results were 42.4 in expired patients (N = 35) and 40.6 in survivors (N = 112, p = 0.256) |
|            | Tianyou Hospital, Wuhan, China    | ✓                             | • 115 patients were included.  |
|            |                                   |                               | • Critical group had higher aPTT results when compared with the severe and mild groups (36.98 ± 8.60, 36.47 ± 9.29, and 34.9 ± 9.17 s, respectively). |
|            | Chongqing, China                  | ✓                             | • 135 patients were recruited. |
|            |                                   |                               | • The mean aPTT values for the critical and severe groups were 29.7 and 26.6 s, respectively (p = 0.011). |
| Fibrinogen | Renmin Hospital of Wuhan University | ✓                             | • 94 patients were studied.    |
|            |                                   |                               | • 502 ± 153 mg/ml in COVID-19 patients compared to 290 ± 53 mg/ml in the control group (p < 0.001) |
|            | Suzhou Hospital                   | ✓                             | • 75 patients were enrolled.   |
|            |                                   |                               | • While the average fibrinogen levels in controls were 200–400 mg/ml, patients were reported to have significantly higher levels (430 ± 119 mg/ml, p < 0.03). |
|            | Fuyang Second People’s Hospital  | ✓                             | • 43 patients were studied.    |
|            |                                   |                               | • The average fibrinogen levels in the severe group was 384 ± 100 mg/ml and in the mild group was 311 ± 083 mg/ml (p = 0.14). |
| Anti-lupus coagulant (s) | Hospitals in Liechtenstein and Switzerland | N/A                           | • 64 patients were studied.    |
|            | Lariboisière Hospital, Paris      | ✓                             | • Higher total IgA and IgA anti-phospholipid antibodies were found in severe patients (p < 0.001). |
|            |                                   |                               | • 74 consecutive mechanically ventilated patients were enrolled. |
|            |                                   |                               | • LAC was positive in 63 patients (85%). |
|            |                                   |                               | • 23 out of 26 patients with thrombotic complications were positive. |
|            | Tan Tock Seng Hospital, Singapore | ✓                             | • 12 ICU patients with severe COVID-19 pneumonia were included. |
|            |                                   |                               | • Lupus anticoagulants were present in 50% of patients. |
| Protein C (%) | R Adams Cowley Shock Trauma Center, Maryland, USA | ✓                             | • 10 critically ill patients were included, who were using mechanical lung ventilation. |
|            | Tenon University Hospital, Paris, France | ✓                             | • The mean protein C activity was 104 ± 40 (normal = 83%–168%). |
|            |                                   |                               | • 430 patients were included. |
|            |                                   |                               | • Protein C activity was higher in conventional (mild) patients (97%, 79–113) than the worsening disease group (88%, 71–100). |
|            | Tan Tock Seng Hospital, Singapore | ✓                             | • 12 ICU patients with severe COVID-19 pneumonia were included. |
|            | Colentina University Hospital, Bucharest, Romania | ✓                             | • The average activity of protein C was 77.5%. |
|            |                                   |                               | • 91 patients were enrolled, of whom 21 (23.3%) died. |
|            | Tan Tock Seng Hospital, Singapore | ✓                             | • 65% of the patients were reported to have decreased protein S activity. |
|            | Gregorio Maranon Hospital, Madrid, Spain | N/A                           | • Death cases had lower protein S activity (median = 42% vs. 58%, p < 0.001). |
| Protein S (%) |                                   |                               | • 12 ICU patients with severe COVID-19 pneumonia were included. |
|            |                                   |                               | • The average activity of protein S was 65.2%. |
|            |                                   |                               | • 206 patients were enrolled. |
|            |                                   |                               | • The average protein S activity in COVID-19 patients with thrombosis was 60.9 (46.3–69.4), while the mean activity in patients without thrombosis was 53.2 (42.1–66.9, p = 0.429). |
| ATIII (%)  | Milan, Italy                      | ✓                             | • 24 intubated patients were included. |
|            |                                   |                               | • The mean antithrombin activity was slightly decreased [74 U/dl, reference range mean = 102 (82–122)] |
|            | Tan Tock Seng Hospital, Singapore | ✓                             | • 12 ICU patients with severe COVID-19 pneumonia were included. |
|            | R Adams Cowley Shock Trauma Center, Maryland, USA | ✓                             | • The average activity of ATIII was 84.4%. |
|            |                                   |                               | • 10 critically ill patients were included, who were using mechanical lung ventilation. |
|            |                                   |                               | • The mean ATIII activity was 84 (normal = 75%–135%). |

(Continued)
Severe b, d  disease, and 2 with pulmonary disease. Patients in this group were hospitalized in either Urmia General Hospital or Urmia Taleghani Hospital. after admission before any other therapeutic and medical interventions. In this group, 9 patients had cardiovascular disease, 15 had hypertension, 5 were found with diabetes, 1 with kidney coagulation factors.

Elevated D-dimer levels >5.15 µg/ml was shown to increase mortality nearly 3 times.

The D-dimer levels of the expired patients were notably higher than those of surviving individuals.

D-dimer levels of COVID-19 patients with DIC were higher when compared to those without DIC.

TABLE 1 | Continued

| Test (unit) | Target population or center/country | Consistency (with our results) | Included patients/main findings | Reference |
|------------|-------------------------------------|--------------------------------|--------------------------------|-----------|
| D-dimer (µg/ml) | Tianyou Hospital of Wuhan, China | N/A | Classified 115 patients into four groups according to the disease severity. 18 out of 22 deceased patients had increased levels of D-dimer in the first lab test (3.47 ± 7.41 mg/l in expired patients compared to 0.87 ± 1.73 mg/l in discharged patients). | (13) |
| | | | The change in CT imaging was in correlation with the increase of the D-dimer levels. | |
| | | | 41 patients were included (ICU patients: n = 13; no ICU care: n = 28). | |
| | | | D-dimer levels on admission were higher in ICU patients (2.4 mg/l) than those in non-ICU patients (0.5 mg/l). | (27) |
| | | | Elevated D-dimer levels >5.15 µg/ml was shown to increase mortality nearly 3 times. | (28) |
| | | | The value 2.025 mg/L was determined as the optimal probability cutoff to predict death. | (29) |
| | | | The D-dimer levels of the expired patients were notably higher than those of surviving individuals. | |
| | | | D-dimer levels of COVID-19 patients with DIC were higher when compared to those without DIC. | |
| FDP | Tongji Hospital, Wuhan | ✓ | 147 patients were enrolled. | (16) |
| | | | The mean FDP levels were 70.8 in expired patients (N = 35) and 4.8 g/L in survivors (N = 112, p < 0.001). | |
| | | | 94 patients were studied. | (17) |
| | | | 33.83 ± 82.28 mg/L in COVID-19 patients compared to 1.55 ± 1.09 mg/L in healthy controls (p < 0.001) | |
| | | | Significant increase in FDP levels between survivors and non-survivors [4.0 (4.0–4.3) vs. 7.6 g/µL (4.0–23.4)] | (30) |

TABLE 2 | Inclusion criteria for the recruitment of individuals into the control (CRL), severe (SVR), and critical (CTL) groups.

| Group | Criteria |
|-------|----------|
| Control a | • Having a negative result for severe acute respiratory coronavirus 2 (SARS-CoV-2) by RT-PCR during the last 48 h (to exclude the chance of infection even at the earlier stage among enrolled healthy controls) | |
| | • No history of abnormal liver function tests (both direct and total bilirubin, SGOT, SGPT, ALKP) (considering that the majority of coagulation factors are produced in the liver, applying this criterion ensures no healthy control has a liver disease) | |
| | • No history of COVID-19 positivity reported from any immediate family member (to minimize the chance of getting infected from immediate family members during the time between PCR test and the time of blood collection. Additionally, it helps minimize the chance of being an asymptomatic carrier) | |
| | • Having no signs of fever, coughing, or other physical features of COVID-19 | |
| | • Having no history of hemorrhagic diseases in the past and present (any individuals with a history of recent hemorrhagic events such as a recent operation or menstruation in females were excluded to avoid impacts on the coagulation hemostasis) | |
| | • No history of heparin, low-molecular-weight heparins (LMWHs), and warfarin therapy | |
| | • Having respiratory distress (respiratory rate ≥30 times/min) | |
| | • Oxygen saturation ≥93% | |
| | • Progression of lesion >50% within 24–48 h in lung CT imaging | |
| Severe b, d | • Having respiratory failure and requiring mechanical ventilation | |
| | • Shock | |
| | • Organ failure | |
| | • Requiring ICU treatment | |
| Critical c, d | • Having underlying diseases who were using methotrexin, glibenclamide, captopril, or losartan to control their chronic diseases. These drugs are not reported to have effects on coagulation factors. | |

SGOT, aspartate aminotransferase; SGPT, alanine aminotransferase; ALKP, alkaline phosphatase; COVID-19, coronavirus disease 2019.

aThirty-five healthy controls were recruited [15 females (42.9%) and 20 males (57.1%)]. The average age was 50.34 ± 20.84 years.

bThirty-five severely ill hospitalized individuals were recruited [15 females (42.9%) and 20 males (57.1%)]. The average age was 50.91 ± 16.42 years. Blood samples were collected immediately after admission before any other therapeutic and medical interventions. In this group, 3 patients had cardiovascular disease, 9 had hypertension, 7 were found with diabetes, and 1 with pulmonary disease. Patients in this group were hospitalized in either Urmia General Hospital or Urmia Taleghani Hospital.

cThirty-five critically ill individuals in ICU were recruited [15 females (42.9%) and 20 males (57.1%)]. The average age was 52.03 ± 15.06 years. Blood samples were collected immediately after admission before any other therapeutic and medical interventions. In this group, 9 patients had cardiovascular disease, 15 had hypertension, 5 were found with diabetes, 1 with kidney disease, and 2 with pulmonary disease. Patients in this group were hospitalized in either Urmia General Hospital or Urmia Taleghani Hospital.

dPatients with underlying diseases who were using methotrexin, glibenclamide, captopril, or losartan to control their chronic diseases. These drugs are not reported to have effects on coagulation factors.
COVID-19 such as fever or coughing and never had a positive RT-PCR result before. We also checked immediate family history to exclude those who have and/or had a family member with a positive PCR test to exclude the possibility of including asymptomatic carriers as healthy controls. Considering that almost all coagulation factors are produced in the liver, any functional disorder in the organ may result in abnormal plasma levels of the factors; therefore, we performed liver functional tests (LFTs) for all 105 included individuals.

**Demographic Features of Patients**

In this case–control study, 105 individuals were included and classified into three groups (critical, severe, and control), each consisting of 35 individuals [20 males (57.1%) and 15 females (42.9%)]. The demographic data are presented in Table 3. The mean ages in the three groups were 52.03 (SD = 15.06), 50.91 (SD = 16.42), and 50.34 years (SD = 20.84), respectively. In the critical (CTL) group, the mean weight and height were 72.51 ± 14.75 kg and 163.26 ± 13.88 cm (BMI = 27.86 ± 9.35), while the same parameters in the severe group were measured at 79.14 ± 9.95 kg and 171.80 ± 7.50 cm (BMI = 26.83 ± 3.09), respectively. In the severe (SVR) group, 3 patients had cardiovascular disease, 9 had hypertension, 7 were found with diabetes, and one had pulmonary disease. Furthermore, in the CTL group, 9 had cardiovascular disease, 15 had hypertension, 5 were found with diabetes, one with kidney disease, and two with pulmonary disease. These patients were using metformin, glibenclamide, captopril, or losartan to control their chronic diseases, which have no effects on the coagulation factors. Due to the prophylactic guidelines for the administration of anticoagulation drugs to patients with poor health conditions, we collected the samples at admission before any medical intervention. Moreover, we checked for history of any drug use that could potentially interfere with our results by referring to medical insurance records and through collecting information using an enrollment form. Assuming an α value set at 0.05 (type I error), β at 0.10 (type II errors), a dropout rate of 5%, and a peak of the fifth wave of the disease in Iran in order to include as many patients as possible. This strategy helped us collect all the required samples rapidly and to perform the coagulation tests quickly without freezing the plasma samples. All tests were run within 3 h after sample collection. According to the partially low stability of D-dimer in plasma (40), and using semi-quantitative kits to measure D-dimer and FDPs, we performed these tests before the other tests.

**Materials**

The PT (NeoPTimal), aPTT (C.K. PREST), CaCl2 (0.025 M), fibrinogen (STA-Liquid Fib), anti-lupus coagulant, fibrinogen, protein C (STAACL), protein S (STAACL), antithrombin III (STACHROM), Owren−Koller, Desorb-U, D-dimer, and FDP kits were purchased from Stago Co., Asnières sur seine, France. All tests, except for the D-dimer and FDPs, were performed using the fully automated STA Compact® System (Diagnostica Stago, Asnières sur seine, France). We used semi-quantitative D-dimer and FDP kits (benefiting from latex particles coated with monoclonal antibodies to D-dimer or FDP, respectively). Moreover, Toshiba Alexion 16-slice (Toshiba, Japan) and GE BrightSpeed Elite 16 Slice (Chicago, IL, USA) were used for CT scans of the patient groups, and miPCR Biomolecular Systems (Upper Coomera, Australia) was used for the PCR testing of all individuals.

**Performing Coagulation Tests**

After obtaining the samples, we checked them twice before and after putting them on the mixer to exclude any samples with visible signs of clotting or micro-clots. After 5 min of mixing the samples, they were centrifuged and the obtained plasma samples were analyzed for D-dimer and FDPs; then, the plasma samples were poured into conventional plastic tubes and loaded into the autoanalyzer. To perform semi-quantitative D-dimer and FDP tests, we used the glycine buffer to dilute each plasma sample in plastic test tubes. For the D-dimer test, we added 20 ml of reagent 1 (including ready-to-use latex particles coated with mouse anti-human D-dimer monoclonal antibody) to 20 ml of undiluted/diluted plasma samples of each individual, mixed gently, and assessed the agglutination. The interpretation of the results was done using the protocol provided in Table 4. A similar protocol was used for the FDP test, but with only two diluted concentrations/titers (1:2 and 1:8) assessed for agglutination according to the instruction of the manufacturer. We performed the rest of the tests using a fully automated STA

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**TABLE 3** | Demographic features of the patients in control (CRL), severe (SVR), and critical (CTL) groups.

| Parameter | Group | No. | Mean | SD  | p-value |
|-----------|-------|-----|------|-----|---------|
| Age (years) | CRL 35 | 50.34 | 20.84 | 0.92 |
|           | SVR 35 | 50.91 | 16.42 |     |
|           | CTL 35 | 52.03 | 15.06 |     |
| Weight    | CRL  0  | 79.14 | 9.95  | 0.031|
|           | SVR 35 | 79.14 | 9.95  |     |
|           | CTL 35 | 72.51 | 14.75 |     |
| Height    | CRL 0  | –    | –     | 0.002|
|           | SVR 35 | 171.80| 7.50  |     |
|           | CTL 35 | 163.26| 13.88 |     |
| BMI (kg/m²)| CRL 0  | –    | –     | 0.539|
|           | SVR 35 | 26.83 | 3.09  |     |
|           | CTL 35 | 27.86 | 9.35  |     |
TABLE 4 | Approximate diluted/undiluted concentrations and the titers for the D-dimer and fibrin degradation product (FDP) test.

| Test sample/titers | Levels |
|--------------------|--------|
|                    | Undiluted | 1:2 | 1:4 | 1:8 | 1:16 |
| D-dimer            | (-)       | <0.5 <1.0 |      |      |      |
| (+)                | ≥0.5      |      |      |      |      |
| (-)                | ≥1.0      |      |      |      |      |
| (+)                | ≥2.0      |      |      |      |      |
| (-)                | ≥4.0      |      |      |      |      |
| (+)                | ≥8.0      |      |      |      |      |
| FDP                | -         |      |      |      |      |
| 1:2                | -         |      |      |      |      |
| 1:8                | -         |      |      |      |      |
| (-)                | -         | <5.0 |      |      |      |
| (+)                | ≥5.0      | <20  |      |      |      |

(+): presence of agglutination; (-): no agglutination. FEU, fibrinogen equivalent unit.

Statistical Analysis

The data obtained from the autoanalyzer for PT, PTT, fibrinogen, lupus anticoagulant, proteins C and S, and ATIII (quantitative tests) and for D-dimer and FDPs (semi-quantitative tests) were reported. Statistical analysis was performed using SPSS (ver. 21; IBM, Armonk, NY, USA). To compare the groups, we used one-way ANOVA, Fisher’s exact test, and chi-square tests. A p-value <0.05 was considered to be statistically significant.

RESULTS

Analysis of the Results of the Three Studied Groups

According to the results, there was no association between the age, weight, and BMI of individuals (p = 0.92, 0.03, 0.54, respectively). The elevated PT test results have been frequently reported in previous investigations worldwide. Table 5 summarizes the statistical analysis of the data obtained from the STA Compact® system. Our results showed that while the average PT result in the CRL group was 13.38 ± 0.73 (international sensitivity index (ISI) of the kit was 1.05; international normalized ratio (INR) = 1.03 ± 0.06), it was significantly elevated in both patient groups, in which the mean PT in the SVR group was 13.85 ± 1.12s (INR = 1.07 ± 0.09) and that in the CTL group was 15.01 ± 1.68s (INR = 1.17 ± 0.14, p < 0.001). Notably, the one-way ANOVA results showed a significant difference in the mean PT results between the patient groups (p < 0.001). The general trend for the results of the PTT test was similar to that of the PT test, in which PT, prothrombin time; INR, international normalized ratio; PTT, partial thromboplastin time; ATIII, antithrombin III; CTL, control group; SVR, severe group; CRL, critical group.

aSignificance of the difference with the CTL group, (indicates a P-value of 0.05 or less between the mentioned group and the control group).

bSignificance of the difference with the SVR group, (indicates a P-value of 0.05 or less between the mentioned group and the severe group).

TABLE 5 | Results of the quantitative tests performed using the STA Compact® system.

| Variables                  | Groups | N  | Mean   | SD    | p-value   |
|----------------------------|--------|----|--------|-------|-----------|
| PT                         | CTL    | 35 | 13.38  | 0.73  | <0.001    |
|                            | SVR    | 35 | 13.85  | 1.12  | a (CTL/CRT) < 0.001 |
|                            | CRL    | 35 | 15.01a, b | 1.68  | b (CRL/SVR) < 0.001 |
| INR                        | CTL    | 35 | 1.03   | 0.06  | <0.001    |
|                            | SVR    | 35 | 1.07   | 0.09  | b < 0.001 |
|                            | CRL    | 35 | 1.17a, b | 0.14  | b < 0.001 |
| PTT                        | CTL    | 35 | 36.50  | 2.64  | <0.001    |
|                            | SVR    | 35 | 37.80  | 3.73  | a < 0.001 |
|                            | CRL    | 35 | 42.92a, b | 6.62  | b < 0.001 |
| Lupus anticoagulant        | CTL    | 35 | 40.76  | 3.48  | <0.001    |
|                            | SVR    | 35 | 47.05a | 8.25  | a (SVR/CRL) < 0.002 |
|                            | CRL    | 35 | 49.41a | 9.24  | a (CTL/CRL) < 0.001 |
| Fibrinogen                 | CTL    | 35 | 283.57 | 70.51 | <0.001    |
|                            | SVR    | 35 | 480.29a | 129.60 | a (SVR/CRL) < 0.001 |
|                            | CRL    | 35 | 537.86a | 142.68 | a (CTL/CRL) < 0.001 |
| ATIII                      | CTL    | 35 | 98.74  | 10.40 | 0.321     |
|                            | SVR    | 35 | 99.63  | 11.56 |           |
|                            | CRL    | 35 | 95.71  | 11.96 |           |
| Protein C                  | CTL    | 35 | 94.31  | 17.07 | 0.04      |
|                            | SVR    | 35 | 99.34  | 31.60 | a (CTL/CRL) = 0.032 |
|                            | CRL    | 35 | 85.57a | 15.79 |            |
| Protein S                  | CTL    | 35 | 75.03  | 9.39  | <0.001    |
|                            | SVR    | 35 | 65.06a | 12.76 | a (SVR/CRL) < 0.001 |
|                            | CRL    | 35 | 62.91a | 12.32 | a (SVR/CRL) = 0.001 |

One-way ANOVA, Fisher’s exact test, and chi-square test were used for statistical analysis. A p < 0.05 was considered to be statistically significant.

PT, prothrombin time; INR, international normalized ratio; PTT, partial thromboplastin time; ATIII, antithrombin III; CTL, control group; SVR, severe group; CRL, critical group.

aSignificance of the difference with the CTL group, (indicates a P-value of 0.05 or less between the mentioned group and the control group).

bSignificance of the difference with the SVR group, (indicates a P-value of 0.05 or less between the mentioned group and the severe group).
the mean results for the test (expressed in seconds) in the CRL group was 36.50 ± 2.64, while it was 37.80 ± 3.73 in the SVR group and 42.92 ± 6.62 in the CTL group. Interestingly, the difference between the two patient groups was statistically significant \((p < 0.001)\). For a better presentation of the obtained data, we showed the results of each patient independently. According to Figures 2A–C, the PT test results showed an increasing trend from the CRL to the SVR and CTL groups. Non-survivors have been marked by black circles. The INR results for each included individual are presented in Figures 2D, F. The PTT test results followed a similar trend (minimum in the CRL group and maximum in the CRL group) (Figures 2G–I). Analysis of the results for the anti-lupus coagulant test revealed an increasing trend among the groups, in which the average for the controls was 40.76 ± 3.48 s; however, it increased to 47.05 ± 8.25 s in the SVR group and to 49.41 ± 9.24 s in the CTL group. The results for the fibrinogen test provided solid evidence that the fibrinogen levels vary between patients and controls significantly. It is clear that, while the average level of fibrinogen was 283.57 ± 70.51 mg/dl in the CRL group, it went up to 537.66 ± 142.68 mg/dl in the CTL group \((p < 0.001)\). According to the results of the ATIII test, there was no significant difference among the three studied groups \((p = 0.321)\), where the average activity of ATIII in the CRL group was 98.74 ± 10.40%, in SVR group was 99.63 ± 11.56%, and in the CTL group was 95.71 ± 11.96%. The results for each individual for the anti-lupus coagulant test are represented in Figures 3A–C. The CRL group had the lowest levels, while the CTL group had the highest levels. Investigation of the fibrinogen levels showed that the CRL group had the lowest levels. The fibrinogen levels were significantly increased in the SVR group; however, the CTL group had the highest levels among all groups (Figures 3D–F). The results for ATIII, unlike other tests, revealed that there was no

![FIGURE 2](image-url)
significant difference among the three studied groups (Figures 3G–I). Additionally, analysis of the results for protein C showed that the activity of this regulatory protein was $94.31 \pm 17.07\%$ in the CRL group, while it increased to $99.34 \pm 31.6\%$ in the SVR group. The activity levels of this protein were found to be lower in the CTL group ($85.57 \pm 15.79\%, p = 0.04$). The ANOVA results showed that the difference between the activity levels of protein C between the CTL and CRL groups was statistically significant, but not to that of the previous tests ($p = 0.032$). Moreover, our result showed that protein S, the cofactor of protein C, had lower activity in the patient groups compared to the CRL group, in which the highest activity was reported in the CRL group ($75.03 \pm 9.39\%$), while its activity dropped to $65.06 \pm 12.76\%$ in the SVR group. The lowest activity of protein S was observed in the CTL group ($62.91 \pm 12.32\%, p < 0.001$). According to Figures 4A–C, the minimum activity of protein C was observed in the CTL group. A similar trend was observed when we investigated the protein S levels (Figures 4D–F). According to the results regarding the D-dimer test, of the 35 individuals in the CRL group, 31 (88.6%) had D-dimer levels <0.5 $\mu$g/ml and 4 (11.4%) had D-dimer levels between 0.5 and 1.0 $\mu$g/ml. Twenty-four (68.6%) patients in the SVR group were found to have D-dimer levels below 0.5 $\mu$g/ml, and 11 (31.4%) had levels between 0.5 and 1.0 $\mu$g/ml. In the CTL group, 25 patients had D-dimer levels below 1.0 $\mu$g/ml, whereas, only 2 (5.7%) patients had D-dimer levels of 2–4 $\mu$g/ml. The same numbers were found to have D-dimer levels over 8 $\mu$g/ml [$\chi^2 (8) = 34.81, p = 0.0001$] (Table 6). Data regarding the D-Dimer test results for each individual are represented in Figures 5A–C. There was a significant difference among the three studied groups, in which the CTL group had the highest levels of D-dimer, whereas the CRL group had the lowest levels. The results for

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FDP also revealed that the majority of healthy controls (34 out of 35) had FDP levels below 5 mg/ml, while only 1 was found to have FDP levels between 5 and 20 mg/ml. In the SVR group, 31 (88.6%) patients had FDP levels below 5 mg/ml and 4 (11.4%) had FDP levels between 5 and 20 mg/ml. In contrast, only 23 patients in the CTL group had FDP levels below 5 mg/ml, and 9 (25.7%) were found to have levels between 5 and 20 mg/ml. There were 3 (8.6%) patients with FDP levels over 20 mg/ml. According to Figures 5D–F, the results for each group showed that patients in the CTL group had the highest levels of FDP, while healthy controls had the lowest levels.

**Analysis of the Results of Deceased Individuals in the Critical and Severe Groups**

We analyzed the data for the deceased individuals (11 out of 35 in the CTL group) and compared them with those of survivors in the same group in order to obtain a better understanding of the impact of abnormal coagulation test results on the fate of the patients. We marked these patients with black bullet points in Figures 2–5. According to Figures 2C, F, I and Table 7, the expired individuals in the CTL group had higher mean values for PT, INR, and PTT when compared to all individuals in the CTL group. Additionally, according to Figures 3C, F, I and Table 7, the expired individuals in the CTL group had higher mean values for LAC, but lower values for the ATIII test. They had slightly lower mean values in the fibrinogen test than the rest of the CTL group. Moreover, according to Figures 4C, F, the expired individuals had lower mean values of protein C, but higher protein S, when compared to the mean values in the CTL group. Finally, according to Figures 5C, F, individuals who expired were among those with the highest values both in the D-dimer test [≥8 (n = 1), 2 < to ≥4 (n = 2)] and in the FDP test [≥20 (n = 2), ≥5 to <20 (n = 1)].

**LIMITATIONS AND RECOMMENDATIONS**

In this section, we provide recommendations for further investigations (Table 8) and address our limitations. We included 35 individuals in each group. Recruiting more patients will provide more accurate results in prospective studies. The enrollment of more patients provides the opportunity to determine cutoffs and design an alarm panel to be used in ICUs. We also applied a single-sampling strategy; however, monitoring the results by obtaining at least 2–3
### TABLE 6 | Results of the semi-quantitative tests including D-dimer and fibrin degradation products (FDPs).

|          | CRL   | SVR   | CTL   | Total |
|----------|-------|-------|-------|-------|
| **D-dimer** |       |       |       |       |
| <0.5     | 31    | 24    | 11    | 66    |
| ≥0.5 to <1| 4     | 11    | 14    | 29    |
| ≥1 to <2 | 0     | 0     | 6     | 6     |
| ≥2 to <4 | 0     | 0     | 2     | 2     |
| ≥4 to <8 | 0     | 0     | 0     | 0     |
| ≥8       | 0     | 0     | 2     | 2     |
| **Total**| 35    | 35    | 35    | 105   |
| **FDP**  |       |       |       |       |
| <5       | 34    | 31    | 23    | 88    |
| ≥5 to <20| 1     | 4     | 9     | 14    |
| ≥20      | 0     | 0     | 3     | 3     |
| **Total**| 35    | 35    | 35    | 105   |

χ²(8) = 34.81, p = 0.0001

χ²(4) = 15.205, p = 0.004

The measurement unit for both tests was micrograms per milliliter.

CTL, control group; SVR, severe group; CRL, critical group.

*Five cells (60.0%) have expected count less than 5. The minimum expected count is 0.67.

*Six cells (66.7%) have expected count less than 5. The minimum expected count is 1.00.

**FIGURE 5 | (A–C) D-dimer test results in the control (CRL, green), severe (SVR, yellow), and critical (CTL, red) groups. (D–F) Fibrin degradation product (FDP) test results in the CRL, SVR, and CTL groups. Expired individuals in the SVR and CTL groups are shown with a black bullet point. Bullet points in red, blue, purple, green, and yellow indicate cardiovascular disease, hypertension, diabetes, pulmonary disease, and kidney disease, respectively, in the SVR and CTL groups.**
samples in the CTL and SVR groups could more effectively monitor the test results and their association with the outcomes. One limitation of this study was the use of latex-based semi-quantitative kits to assess D-dimer and FDPs. The results will be more reliable when both tests are performed using fully automated methods. Although the biofunctions of proteins S and C as biological regulators of factors V and VIII are well documented, we did not assess these two factors.

**DISCUSSION**

In the present study, we investigated the dynamic changes in 9 coagulation tests on 105 individuals classified into CTL, SVR, and CRL groups. Our study revealed significant aberrant coagulation changes among the studied groups in 4 aspects: extrinsic and intrinsic pathways, fibrinolysis, and the regulatory factors. Our results were consistent with those of the majority of

| Test | CTL (expired) | CTL (all) |
|------|---------------|-----------|
| PT   | 15.53 (median = 15.4, SD = 1.38) | 15.01 |
| INR  | 1.21 (median = 1.2, SD = 0.11) | 1.17 |
| PTT  | 46.85 (median = 48.4, SD = 7.34) | 42.92 |
| LAC  | 53.21 (median = 50.8, SD = 10.30) | 49.41 |
| Fibrinogen | 533.27 (median = 587, SD = 162.71) | 537.66 |
| ATIII | 92.63 (median = 94, SD = 10.55) | 95.71 |
| Protein C | 82.36 (median = 80, SD = 13.32) | 85.57 |
| Protein S | 66.45 (median = 62, SD = 12.36) | 62.91 |
| D-dimer | <0.5 (n = 5), 0.5 < to ≥ 1 (n = 3) | <2 to ≥4 (n = 2), ≥8 (n = 1) |
| FDP  | <5 (n = 8), ≥5 to <20 (n = 1), ≥20 (n = 2) | |

PT, prothrombin time; INR, international normalized ratio; PTT, partial thromboplastin time; LAC, lupus anticoagulant; ATIII, antithrombin III; FDP, fibrin degradation product.
previously published papers. A brief literature review for all tests with consistency levels is represented in Table 1. The CTL group had higher PT test (therefore INR) results when compared to the SVR and CRL groups, indicating a disruption in the extrinsic coagulation pathway. In addition, the prolonged PTT results in the CTL group and also similar results in the LAC test showed that not only the extrinsic pathway but even the intrinsic pathway was dysregulated. It should be considered that, in critically ill patients, lupus anticoagulant could be positive. An elevated fibrinogen level was one of the main findings in COVID-19-associated coagulopathy. We showed that there was a significant difference in the fibrinogen levels among the three groups and that the CTL group had the highest levels. It can be used as a common biomarker to predict the severity of the disease; however, the analysis of fibrinogen levels in deceased patients in the CTL group with the whole group showed that it had no significance in predicting death. Investigation of ATIII revealed that its activity was not significantly interrupted in COVID-19 patients \((p = 0.321)\). However, proteins C and S, the other regulatory proteins, showed a significant decrease in their activity levels \((p = 0.04\) and \(p < 0.001\), respectively). The difference between the reported \(p\)-values for these proteins was probably due to the low number of individuals recruited in each group; increasing the sample size will provide more accurate data. Considering that proteins C and S regulate the conversion of factors V and VIII to their active forms, we conclude that the disruption of homeostasis in protein C (and S) regulating the conversion of factors V and VIII to their active form could be a mechanism for COVID-19-associated coagulopathy. The fibrinolysis pathway was also affected in the presence of SARS-COV-2, in which the production of FDPs, mainly D-dimer, was accelerated, and according to our results, deceased patients were found to have significantly higher FDP and D-dimer levels when compared to survivors. The majority of coagulation factors are produced in the liver; to prevent the effects of hepatopathy on the levels of the coagulation factors and the corresponding tests, we enrolled normal controls and patients whose liver function tests were normal. Interestingly, factors including FVIII and vWF (which act as markers of endothelial activation) \((53)\) were produced in the endothelial cells. Investigation of the levels of these factors in COVID-19 patients revealed that their levels increased and may correlate with poorer prognosis \((54–56)\). We showed that D-dimer, fibrinogen, PT, PTT, LAC, protein S, FDPs, and protein C (ordered according to their \(p\)-values) could effectively be used in the prognosis of the severity of the disease and that disruptions in proteins C and S regulating the conversion of factors V and VIII to their active form may interfere the homeostasis of the coagulation system.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by the Ethics Committee of Urmia Medical University (IR.UMSU.REC.1399.264). The patients/participants provided written informed consent to participate in this study.

**AUTHOR CONTRIBUTIONS**

DEAK designed the study, performed the tests, generated the figures, and prepared the manuscript. YR contributed to performing semiquantitative tests. RA and RJ supervised the recruitment of patients and the control group. JR analyzed the data. MM performed sample preparation. AA supervised quality control. RN and FR collected clinical data. VS-I supervised the research progression. All authors contributed to the article and approved the submitted version.

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