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ORIGINAL RESEARCH

Procoagulant Microvesicles in COVID-19 Patients: Possible Modulators of Inflammation and Prothrombotic Tendency

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Background: The hypercoagulability and thrombotic tendency in coronavirus disease 2019 (COVID-19) is multifactorial, driven mainly by inflammation, and endothelial dysfunction. Elevated levels of procoagulant microvesicles (MVs) and tissue factor–bearing microvesicles (TF-bearing MVs) have been observed in many diseases with thrombotic tendency. The current study aimed to measure the levels of procoagulant MVs and TF-bearing MVs in patients with COVID-19 and healthy controls and to correlate their levels with platelet counts, D-Dimer levels, and other proposed calculated inflammatory markers.

Materials and Methods: Forty ICU-admitted patients with COVID-19 and 37 healthy controls were recruited in the study. Levels of procoagulant MVs and TF-bearing MVs in the plasma of the study population were measured using enzyme linked immunosorbent assay.

Results: COVID-19 patients had significantly elevated levels of procoagulant MVs and TF-bearing MVs as compared with healthy controls ($P<0.001$). Procoagulant MVs significantly correlated with TF-bearing MVs, D-dimer levels, and platelet count, but not with calculated inflammatory markers (neutrophil/lymphocyte ratio, platelet/lymphocyte ratio, and platelet/neutrophil ratio).

Conclusion: Elevated levels of procoagulant MVs and TF-bearing MVs in patients with COVID-19 are suggested to be (i) early potential markers to predict the severity of COVID-19 (ii) a novel circulatory biomarker to evaluate the procoagulant activity and severity of COVID-19.

Keywords: microvesicles, tissue factor, procoagulant, D-dimer, NLR, PLR, PNR, COVID-19

Introduction

At the end of 2019, novel coronavirus disease 2019 (COVID-19) emerged, causing severe acute respiratory syndrome, and affecting the whole world till date, as well as paralyzing all life activities and placing devastating burdens on the economies and healthcare systems of many countries. COVID-19 is one of the most contagious viral infection, associated with a high percentage of morbidity and mortality in a short time.[1,2] Apart from the severe acute respiratory syndrome, COVID-19 also involves other pathophysiological processes, including inflammatory response, endothelial damage, and coagulopathy associated with systemic organ involvement, disseminated intravascular coagulopathy (DIC),[2] and cardio- and cerebrovascular complications.[3] COVID-19′s pathogenesis and endothelial dysfunction drive hypercoagulability and thrombotic tendency, which are hallmarks of this disease.[3,4] In addition, inflammatory responses, vascular damage, and oxidative stress play key roles in driving the microthrombogenic status, in which microvesicles (MVs) have a crucial role. MVs are involved in microthrombi in cerebrovascular compilations.[3]

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Microvesicles are small membrane fragments that can be released from activated platelets or cells undergoing apoptosis. Moreover, they are found in blood, saliva, semen, urine, and tissues,[5–9] and are released from almost all types of cells, including megakaryocytes, endothelial cells, leukocytes, erythrocytes, stem cells, vascular smooth muscle cells, mast cells, and even in vitro in culture cell lines. MVs are submicron in size (from 10 nm to 1000 nm) and heterogeneous in contents.[5,6] MVs are a key player in driving the thrombotic tendency in many diseases. Elevated levels of procoagulant MVs have been reported in physiological states[10–12] and pathological[13–16] conditions, including viral infection including dengue infection[17] and COVID-19 infection.[18–24] Furthermore, MVs have been shown to be associated with thrombosis.[15,16,18,19] MVs are procoagulant in nature because of the expression of phosphatidylserine (PS) on their surfaces. PS is a negatively charged phospholipid that is generated through the flip-flop mechanism from activated platelets or cells undergoing apoptosis.[25] The procoagulant activity of MVs increases in the presence of tissue factor (TF), and PS on the surface of the procoagulant MVs enhances TF activity.[26] MVs have the ability to support the binding of coagulation complexes and the acceleration of thrombin formation have been shown in vivo and in vitro.[27,28] The aims of the current study were to evaluate the levels of procoagulant MVs and TF-bearing MVs in patients with COVID-19 and to correlates their levels with D-Dimer levels and other inflammatory markers such as neutrophils-lymphocytes ratio (NLR), mean platelet ratio to mean platelet count (MPR) and mean platelet count to mean neutrophil count ratio (PNR).

Materials and Methods

This case-control study involved a total of 77 adult participants, including intensive care unit (ICU)-admitted patients with COVID-19 (n=40; 22 males and 18 females) and healthy controls (n=37; 23 males and 14 females). The patient group consisted of diagnosed COVID-19 patients ICU-admitted patients to Baish General Hospital, Baish, Jazan region, Saudi Arabia. The patient groups were diagnosed with RT-polymerase chain reaction (RT-PCR). Healthy controls were blood donors, who donated blood in the blood center in Baish General Hospital with normal hematological parameters and negative status of transfusion transmitted infections.

Sample Collection

Venous blood samples were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) and sodium citrate anticoagulants from patients and controls. The EDTA anticoagulated blood samples were used for complete blood count (CBC) analysis, whereas the sodium citrated samples were used for the evaluation of coagulation profiles, D-dimer levels and procoagulant MVs, and TF-bearing MV analysis.

Plasma Preparation and Microvesicles Isolation

Citrated whole blood was centrifuged at 3000 rpm for 30 minutes at 22 °C, followed by a second centrifugation step at 13,000 rpm for 2 minutes to obtain platelet-free plasma (PFP). The supernatant (PFP) was separated and distributed into two aliquots; one was used freshly for the evaluation of the coagulation profile and D-dimer test, whereas the other was stored at −80 °C for MVs and TF-bearing MVs analysis.

Complete Blood Count

CBC from EDTA anticoagulant tubes was performed on a Sysmex XN-550 Hematology Analyzer (Sysmex, Kobe, Japan).

Coagulation Profile and D-Dimer Testing

A Stago compact analyzer (Diagnostica Stago, Asnieres sur Seine, France) was used to measure the coagulation profile including prothrombin time; PT and activated partial thromboplastin; aPTT and D-Dimer levels. The D-dimer levels was evaluated in patients with COVID-19 using immune-turbidimetric Assay (Diagnostica Stago, Asnieres sur Seine, France).

Complete Blood Count–Derived Parameters

The complete blood count-derived parameters were neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), and platelet/neutrophil ratio (PNR). NLR obtained by dividing neutrophil count/lymphocyte count), PLR obtained by dividing platelet count/lymphocyte count), and PNR obtained by platelet by neutrophil count).[21]

Detection of Microvesicles and Tissue Factor–Bearing Microvesicles

The analyses of MVs and TF-bearing MVs in the plasma were conducted according to the manufacturer procedure. Procoagulant MV and TF-bearing MV levels were measured using a Zymuphen MP-Activity Kit and a Zymuphen MP-TF ELISA Kit (Aniara Diagnostica, OH, USA), respectively.

Statistical Analysis

GraphPad Prism (version 8.0) was used for statistical analysis. Unless otherwise stated, the data in the current study are shown as mean ± standard deviation (SD). Independent unpaired *t*-test was used to test for the differences between patients and controls, and Chi-square test was used for the demographic data analysis. Single-sample *t*-test was used for the evaluation of correlation coefficients. Significant statistical differences were considered when P-values were less than 0.05.

Results

Demographic Data

Forty COVID-19 patients (22 males and 18 females; patient group) and 37 healthy controls (23 males and 14 females; control group) were evaluated in both groups. No significant differences were seen between the genders of the study groups. The age range of the patient group was 56–65 years (60.2±2.5), and that of the control group was 40–63 years (51.1±3.9) (Table 1).

Hematological Parameters

The hematological values of both groups are shown in Table 1. The results showed statistically significant differences among all CBC parameters except mean cell hemoglobin, red cell distribution width, and absolute monocyte count. A significant higher white blood cell (WBC) count including neutrophils was observed in the patient group as compared to the control group ($P < 0.0001$). Conversaly, significantly low platelet count ($P < 0.0001$), absolute lymphocyte count ($P < 0.0001$), absolute eosinophil count ($P < 0.0001$) and absolute basophil count ($P < 0.05$) were observed in the patient group as compared with the control group, whereas a higher absolute monocyte count was observed in patients as compared with controls, however, the difference was not statistically significant.

Complete Blood Count–Derived Parameters

The NLR, and PLR, were higher and lower PNR in the patient group as compared with the control group ($P < 0.0.5$; Table 2).

Coagulation and D-Dimer Tests

The PT and aPTT results were significantly higher in the patient group as compared with the control group ($P < 0.0001$). The mean values of D-dimers, in the patient group, were 3.2±3.5 ng/mL (Table 3) which were considered high as compared with the preestablished interlaboratory reference range).

Procoagulant Microvesicles and Tissue Factor-Bearing Microvesicles

Table 4 and Figure 1A show that the level of MVs in the patient group was ~5.6-folds higher than the control group (21.73±12.5 nM vs 3.9±1.8 nM; $P < 0.0001$). Similarly, increased levels of TF-bearing MVs (~3.6-folds) were also observed in the patient group as compared to control group (1.9±0.7 pg/mL vs 0.5±0.3 pg/mL; $P < 0.0001$) (Table 4 and Figure 1B).

Table 1 Comparison of Demographic Data and Hematological Parameters Between COVID-19 Patient Group and Control Group. The Statistical Analysis Between Groups Was Performed Using Chi-Squared Test for Age and Independent Unpaired *t*-test for Hematological Parameters. Date is Shown as Mean±SD

Variables	COVID-19 (n = 40)	Control (n = 37)	P
Male/Female (n)	22/18	23/14	> 0.05
Age	60.2±2.5	51.1±3.6	< 0.0001
WBC (10^9/L)	11.8±6.9	6.2±19.6	< 0.0001
RBC ($\times10^{12}$/L)	3.7±0.9	5.1±0.5	< 0.0001
Hb (g/dl)	10.0±1.9	13.5±1.6	< 0.0001
HCT (%)	31.7±6.3	41.4±4.6	< 0.0001
MCV (fl)	84.9±9.6	81.4±4.6	0.0456
MCH (pg)	26.7±3.4	26.5±3.1	0.7837
MCHC (g/dl)	31.5±2.0	31.4±1.0	0.0144
RDW (%)	17.0±3.1	17.9±3.4	0.2251
Platelet (10^9/L)	184±114	362±71	< 0.0001
ANC (10^9/L)	10.9±1.9	4.7±1.4	<0.0001
ALC (10^9/L)	1.1±1.0	3.2±0.76	< 0.0001
AMC (10^9/L)	0.5±0.4	0.4±0.2	0.0639
AEC (10^9/L)	0.001±0.0	0.15±0.14	< 0.0001
ABC (10^9/L)	0.008±0.018	0.018±0.013	0.0162

Note: P < 0.05 considered statistically significant.
Abbreviations: WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red blood cell distribution width; N, neutrophils; L, lymphocytes; M, monocytes; E, eosinophils; B, basophils; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; AMC, absolute monocyte count; AEC, absolute eosinophil count; EBC, absolute basophil count.

Table 2 Comparison of Inflammatory Parameters' Values Between COVID-19 Patients and Control Group. The Statistical Analysis Between Groups Was Performed Using Independent Unpaired *t*-test for Hematological Parameters. Date is Shown as Mean±SD

Parameters	COVID-19 (n = 40)	Control (n = 37)	P
NLR	19.5±28.0	1.8±0.9	0.0003
PLR	327±408	148±107	0.0125
PNR	24±28	82±33	< 0.0001

Note: P < 0.05 considered statistically significant.
Abbreviations: NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; PNR, platelet to neutrophil ratio.

Correlation Studies

Statistically significant positive correlation was observed between MVs and TF-bearing MVs levels among the COVID-19 patient group (r = 0.4703, R squared = 0.2212; P = 0.0022), and D-dimer levels (r = 0.516, R squared = 0.2663; P =0.0011) (Figure 2; Table 5). In addition, statistically significant negative correlation was observed between MVs and platelets (r= - 0.3347, R squared = 0.1120; P = 0.0429). On the other hand, no significant correlation was observed between MVs levels and NLR, PLR, or PNR (Table 5).

Table 3 Comparison of Coagulation Profile Parameters and D-Dimers Values Between COVID-19 Patient Group and Healthy Control Group. The Statistical Analysis Between Groups Was Performed Using Independent Unpaired *t*-test for Hematological Parameters. Date is Shown as Mean±SD

Parameters	COVID-19 (n = 40)	Control (n = 37)	P
PT (seconds)	18.4±8.1	14.7±1.10	0.007
aPTT (seconds)	44.6±12.3	33.7±2.1	< 0.0001
D-Dimer (ng/mL)	3.2±3.5	NT	NA

Note: P < 0.05 considered statistically significant.
Abbreviations: NA, not available; PT, prothrombin time; aPTT, activated partial thromboplastin; NT, not tested.

Table 4 Comparison of Microvesicles and Tissue Factor-Bearing Microvesicles Levels Between COVID-19 Patient Group and Healthy Control Group. The Statistical Analysis Between Groups Was Performed Using Independent Unpaired *t*-test for Hematological Parameters. Date is Shown as Mean±SD

Parameters	COVID-19 (n = 40)	Control (n = 37)	P
MVs (nM)	21.7±12.5	3.9±1.8	< 0.0001
TF-MVs (pg/mL))	1.9±0.7	0.5±0.3	< 0.0001

Note: P < 0.05 considered statistically significant.
Abbreviations: MVs, microvesicles; TF-MVs, tissue factor-bearing microvesicles.

Discussion

Our findings showed abnormalities in the hematological parameters, including increased WBC count (leukocytosis), neutropenia and lymphopenia, low red blood cell (RBC) count, low hemoglobin concentration, and low platelet count. These results are consistent with previous reports in COVID-19 patients elsewhere.[21,29,30]

In addition, like previous reports,[20,21] the current study reports elevated levels of D-dimer in COVID-19 patients. Elevated D-dimer levels are associated with increased mortality in these patients.[19,31] Moreover, PT and aPTT were found to be prolonged in our patient group as compared with the control group. A recent study demonstrated longer PT and aPTT among COVID-19 non-survivors compared to survivors on admission.[31] Thrombocytopenia, high D-dimer, and prolonged PT have been proposed to contribute to the hypercoagulability in COVID-19 patients,[21] which may result in DIC;[31] coagulopathy in COVID-19 is accompanied with vascular/endothelial damage.[32]

The current study shows elevated levels of procoagulant MVs in the patient group as compared to healthy controls. Elevated MVs have been reported in COVID-19 infection[18–23] lung diseases,[33] other viral diseases,[17] and inflammatory

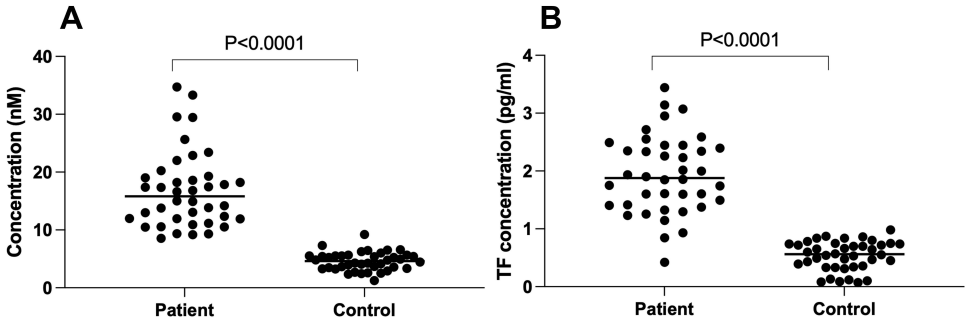

Figure 1 Levels of microvesicles (**A**) and tissue factor-bearing microvesicles (**B**) in COVID-19 patient compared to healthy controls. (**A**) Levels of microvesicles in the plasma of the study cohort. (**B**) Levels of tissue factor-bearing microvesicles in the plasma of the study cohort. Individual data of patients (n=40) and controls (n=37) are shown with mean line. The statistical analysis between groups was performed using independent unpaired *t*-test between the two groups.

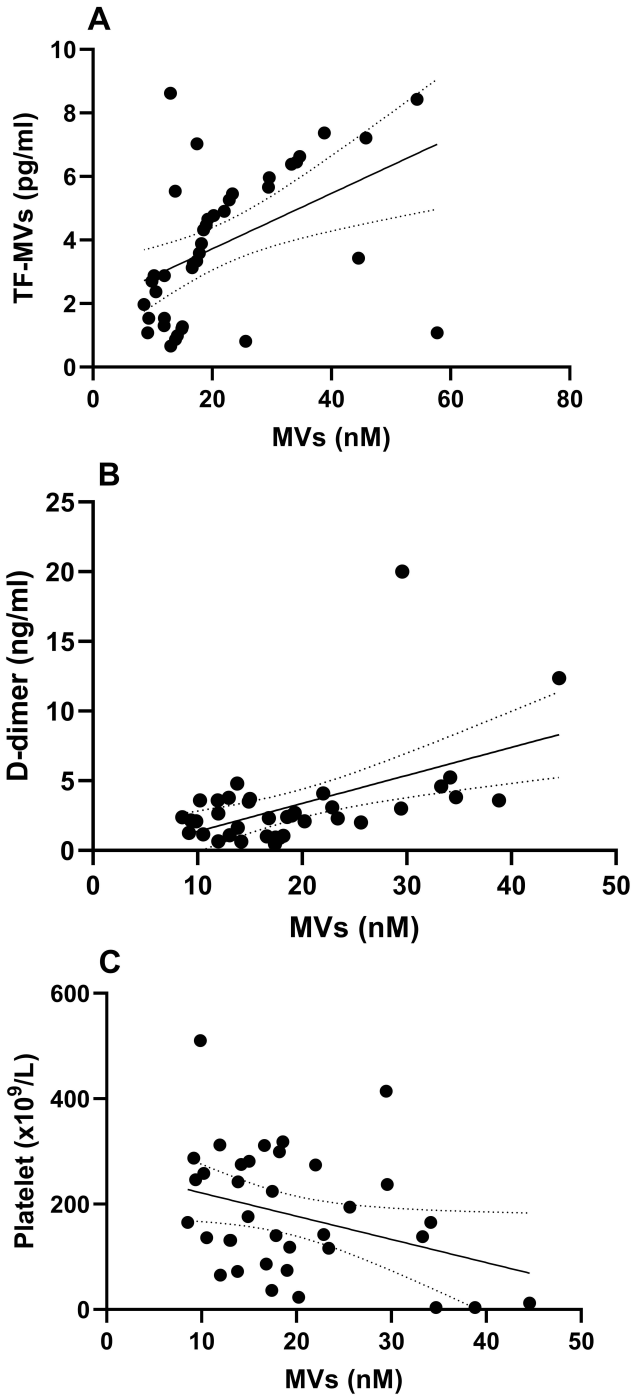

Figure 2 Scatter plot between microvesicles and (**A**) tissue factor-bearing microvesicles, (**B**) D-dimer levels and (**C**) platelet count.

conditions,[13] which have been associated with tendency for thrombosis. Interestingly, elevated levels of MVs derived from endothelial cells and platelets have been reported in COVID-19 with malignancy as compared to healthy controls.[21] Elevated levels of MVs have been reported elsewhere to contribute to COVID-19 severity and mortality.[18,23]

Because of their procoagulant nature, MVs can assemble coagulation complexes and support thrombin generation.[34,35] The prothrombotic changes in patients with COVID-19 are driven by many factors including increased thrombin generation and decreased fibrinolytic activity.[36] The procoagulant surface of MVs is similar to the surface of activated platelets, which supports the assembly of coagulation cascade in healthy individuals.[28] Furthermore, the surface of MVs are more

Table 5 Correlation of Microvesicles with the Studied Parameters in COVID-19 Patient Group. Statistical Analysis Was Performed Using the Single-Sample *t*-test for Correlation Coefficients Between the Microvesicles with Each Parameter in the Table. P < 0.05 Considered Statistically Significant

Parameter	COVID-19 (n=40)		
	r	R Squared	P
MVs and TF-MVs	0.4703	0.2212	0.0022
MVs and D-dimer	0.516	0.2663	0.0011
MVs and Platelets	−0.3347	0.1120	0.0429
MVs and PLR	−0.0911	0.0083	0.5918
MVs and PNR	−0.1419	0.02013	0.4022
MVs and NLR	0.03450	0.001191	0.8464

procoagulant (50–100-fold) than the surface of activated platelets.[27] MVs transfer active mediators and are considered a reliable marker of vascular damage. In addition, MVs modulate inflammatory responses by inducing endothelial cells to release TF, interleukin-6 (IL-6), cytokines, and other inflammatory modulators, which lead to attract and increase other cells' adhesiveness to the damaged endothelium.[37–40] The level of plasma endothelial-derived MVs has been suggested to be an early marker for lung diseases.[41,42] Endothelial-derived MVs have been shown to carry angiotensin-converting enzyme (ACE),[43,44] including in cases of lung injury,[44] which has been linked to thrombosis in COVID-19.[45]

Tissue factor is the principal activator of the coagulation cascade, which is another procoagulant player.[46,47] Although TF is normally found in the sub-endothelium matrix and TF is sequestrated from the circulation, some studies have demonstrated that TF-bearing MVs[13,17] or soluble TF[48,49] can be found in the blood circulation in many inflammatory diseases. Elevated levels of TF and increased TF patient with COVID-19 compared to non-COVID-19 patients.[18,50] The current study found significantly increased levels of TF-bearing MVs in the circulation of patient group, which is similar to previously published findings related to COVID-19[18,21] and viral infections.[17] In COVID-19, TF-bearing MVs have been suggested to play a role in thrombosis and even the severity and mortality.[18]

There is a growing recognition of MVs as key players in hemostasis, thrombosis, inflammation, and other pathological mechanisms because of their procoagulant nature (PS and TF), by which they contain and express biologically active mediators. Thus, understanding the underlying pathophysiology of many diseases could be a new discovery for potentially promising new therapeutic strategies.[15,51,52] The PLR, NLR, and PNR have been proposed to be associated with inflammation and the severity of many diseases, including COVID-19.[53–59] In the current study, the PLR and NLR were higher in the patient group as compared with the control group; this finding is similar to previously reported findings.[21] An elevated NLR has been reported to be associated with poor outcomes and mortality in COVID-19.[60–64] In the current study, we could not find any correlation between MVs or TF-bearing MVs and NLR, PLR, and PNR. In contrast, we found a significant positive correlation of MVs with TF-bearing MVs and D-dimer levels. This is similar to the result of a recently published reports.[18,19,21] At the same time, we found a significant negative correlation with platelet count; Zahran et al also reported a negative correlation between MVs derived from platelets and MVs derived from endothelial cells with platelet count.[21] Elevated levels of MVs, TF and D-dimer have been suggested to be associated with thrombosis, severity and mortality in COVID-19.[18,22]

As similar to many other studies, this research has some limitations. The study did not consider any thrombotic data and the comorbidities of the patients, and it did not follow up with the patient outcomes. In addition, we used only one study application (functional assay) to evaluate the procoagulant activity of MVs and TF-bearing MVs. Combining the functional assay with other applications is highly recommended, such as by utilizing flow cytometry for antigenic features and nanoparticle tracing analysis for precise size determination; this represents a future research direction.

Although the sample size was relatively small, the data generated in the current study are in line with the literature on viral infection and add to the growing literature on the procoagulant activity of COVID-19.

Conclusions

The findings of the current study showed significantly elevated levels of MVs and TF-bearing MVs in patients with COVID-19; these high levels are associated with increased procoagulant activity of patients with COVID-19 and therefore lead to increased risk of hypercoagulability in COVID-19 and subsequently increase the risk of prothrombotic tendency. Although the current study showed the significant procoagulant activity of the MVs and TF-bearing MVs in COVID-19, further research is needed to explain the exact mechanism of hypercoagulability in relation to other factors, such as endothelial damage markers and the origin of MVs. MVs role in driving procoagulant activity in COVID-19 may represent a novel and potentially key role as early marker in predicting inflammatory response and thrombotic events.

Ethical Considerations

The current study was approved by the Jazan Health Ethics Committee, Ministry of Health, and was carried out according to the Declaration of Helsinki. Informed consent was waived off by the Ethics committee on special request to avoid close contact with the COVID-19 patients. Personal identification and bioinformation of study subjects were neither collected nor disclosed and the data were kept anonymously and confidentiality.

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Author Contributions

All authors have made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have read the journal's policy and declare no conflict of interest.

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