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International Immunopharmacology 110 (2022) 108939

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Elevated serum midkine levels in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected patients

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ARTICLE INFO

Keywords:
Midkine
Serum levels
SARS-CoV-2
COVID-19

ABSTRACT

Background: The coronavirus disease-2019 (COVID-19) pandemic has caused important health, economic, social, and cultural problems worldwide. Recent findings demonstrate an excessive cytokine release during the disease development, especially in the seriously life-threatening form of COVID-19. Among other chemokines and cytokines that are released in high amounts at the infection site of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), midkine (MK), which is a potent pro-inflammatory growth factor/ cytokine, can be also over-expressed and contribute to the pathophysiological process in patients infected with SARS-CoV-2.
Materials and method: Serum was collected from 87 intensive care unit (ICU) patients that are COVID-19 positive and 50 healthy volunteers in the control group with a negative PCR test and without disease symptoms. Circulating MK concentration was measured by enzyme-linked immunosorbent assay (ELISA).
Results: COVID-19 patients had a significantly higher serum MK concentration compared to non-COVID-19 control subjects (1892.8 ± 1615.8 pg/mL versus 680.7 ± 907.6 pg/mL, respectively; $P < 0.001$). The cut-off MK concentration was 716.7 pg/ mL, with the sensitivity and specificity of 75.9 % and 76.0 %, respectively. The area under the receiver operating characteristic (ROC) curve of MK was = 0.827. Our findings showed that circulating MK levels are significantly increased in SARS-CoV-2 infected patients.
Conclusion: We suggest that MK is involved in the pathogenesis of COVID-19 and may be a part of hyper-cytokinaemia. Therefore, MK may serve as a supporting biomarker in the diagnosis of COVID-19, and blocking MK actions or its targets may attenuate the inflammatory process and the severity of the disease.

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), by causing systemic inflammation, multi-organ damage, and even death, is responsible for coronavirus disease 2019 (COVID-19), which initiate many terrible problems worldwide for more than two years [1]. As described for other SARS infections that occurred in the near past, the primary pathophysiologic infection process begins with the binding of SARS-CoV-2 to angiotensin-converting enzyme 2 (ACE2) that serves as a receptor for the virus, followed by the internalization of the virus-ACE2 complex into the cell and continued with the replication of SARS-CoV-2, which in turn triggers the pathological reactions related to COVID-19 [2–4]. The symptomatology of patients infected with SARS-CoV-2 varies widely, ranging from asymptomatic or mild symptom states to life-threatening conditions such as pneumonia, pulmonary edema, respiratory failure, vascular hyper-permeability, acute respiratory distress syndrome (ARDS), septic shock, multiple organ failure and even death [5,6]. In such serious pathologies, the production of pro-inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukin (IL) 1 beta (IL-1β), IL-6, and interferon-gamma (IFNΥ), are highly increased. Consequently, this "cytokine storm" generated COVID-19 symptoms (fever, headaches, dizziness, etc.) and caused diffuse alveolar damage [5,7–9]. Some of these mediators are also involved in the development of life-threatening pathologies such as cardiomyopathy, lung injury, and septic shock [10,11]. Furthermore, other factors such as hypoxia, oxidative stress, and neutrophil extracellular traps (NETs) released from neutrophils also contribute to cytokine storm-related events [12–14].

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https://doi.org/10.1016/j.intimp.2022.108939
Received 16 March 2022; Received in revised form 6 June 2022; Accepted 7 June 2022
Available online 9 June 2022

Midkine (MK), a heparin-binding growth factor and cytokine, is highly expressed during embryogenesis, regresses to relatively low levels in healthy adults, and overexpressed once again in certain pathologies, including inflammatory diseases and malignancies [15–18]. It contributes to the growth and repair, survival, reproduction, and differentiation of various cells and has chemotactic activity as well as pro-inflammatory actions [19,20]. MK promotes inflammatory processes in autoimmune and inflammatory diseases via its chemotactic action, resulting in the accumulation of inflammatory cells, which in turn aggravate the pathological inflammatory response [21]. Several studies showed a close interaction between MK and other chemokines and cytokines, which are key mediators in SARS-CoV-2 infection [5,7–9,22]. For example, the expression of MK is induced by TNFα, an important component of cytokine storm in COVID-19, and vice versa [23]. In addition, there is growing evidence that MK is potentially involved in the development of pulmonary fibrosis, which is one of the serious outcomes in SARS-CoV-2 infection. MK may modulate or regulate the inflammatory cell migration and expression of some mediators in this condition [24,25]. Considering all these data, in a recent article, we tried to explain the possible mechanisms by which MK may be involved in the pathogenesis of SARS-CoV-2 infection [26]. Therefore, to find out whether MK is involved in the inflammatory and other pathophysiological processes associated with SARS-CoV-2 infection, we measured circulating MK concentration in hospitalized COVID-19 patients and compared it to non-infected subjects.

2. Materials and methods

2.1. Study population

Blood serum samples were collected from COVID-19 patients (n = 87) who were hospitalized in the intensive care unit (ICU) of Istanbul Atlas University Medicine Hospital between 20 February 2021 and 12 April 2021. A patient flow diagram showing the selection of the COVID-19 patient group is presented in Fig. 1. As a control group, 50 healthy volunteers with negative COVID-19 PCR test and no symptoms of disease were included into the study. COVID-19 patients with liver dysfunction, rheumatoid arthritis, and other inflammatory diseases were evaluated in terms of concomitant disease. Informed consent was obtained from all patients and healthy volunteers. This study was performed in accordance with the Declaration of Helsinki and was approved by The Republic of Turkey Ministry of Health (Number: 2020-10-23T23_59_00) and Local Ethics Committee of Istanbul Atlas University (Number: E-22686390-050.01.04-4187). All participants underwent a standard clinical evaluation that included medical history and physical examination. Routine biochemical tests were performed in all ICU patients. Healthy volunteers were followed for 14 days to rule out the possibility of later contracting COVID-19 disease. None of them had a positive PCR test and no signs of COVID-19 after this period.

2.2. Analytical methods

Serum was obtained from 5 mL venous blood samples of COVID-19 patients and healthy controls. Serum samples were taken and stored at −80 °C until measuring MK levels using the Enzyme-Linked Immuno-Sorbent Assay (ELISA) assay. To determine the serum concentration of MK in the study population, we used a commercially available human MK ELISA kit (Boster CA, USA) according to the manufacturer's instructions. Serum samples obtained from patients were incubated with anti-human MK antibodies and absorption of the solution was analyzed photometrically at 450 nm (OD450) wavelengths in the microplate reader (Dynatech MR 500). To measure the exact concentration of the analysts in the liquid phase, a calibration curve was plotted based on samples with known concentrations. MK concentration is given in pg/mL.

2.3. Statistical analysis

All data were analyzed by IBM SPSS Statistics for Windows 10, version 25.0. The non-normality of the data distribution was determined by the Sahpiro-Wilk test, Kolmogorov-Smirnov and histograms. Parametric values of the patients were expressed as numbers and percentages and categorical values were analyzed with a Chi-square test. MK concentration values of patients and controls were presented as mean and

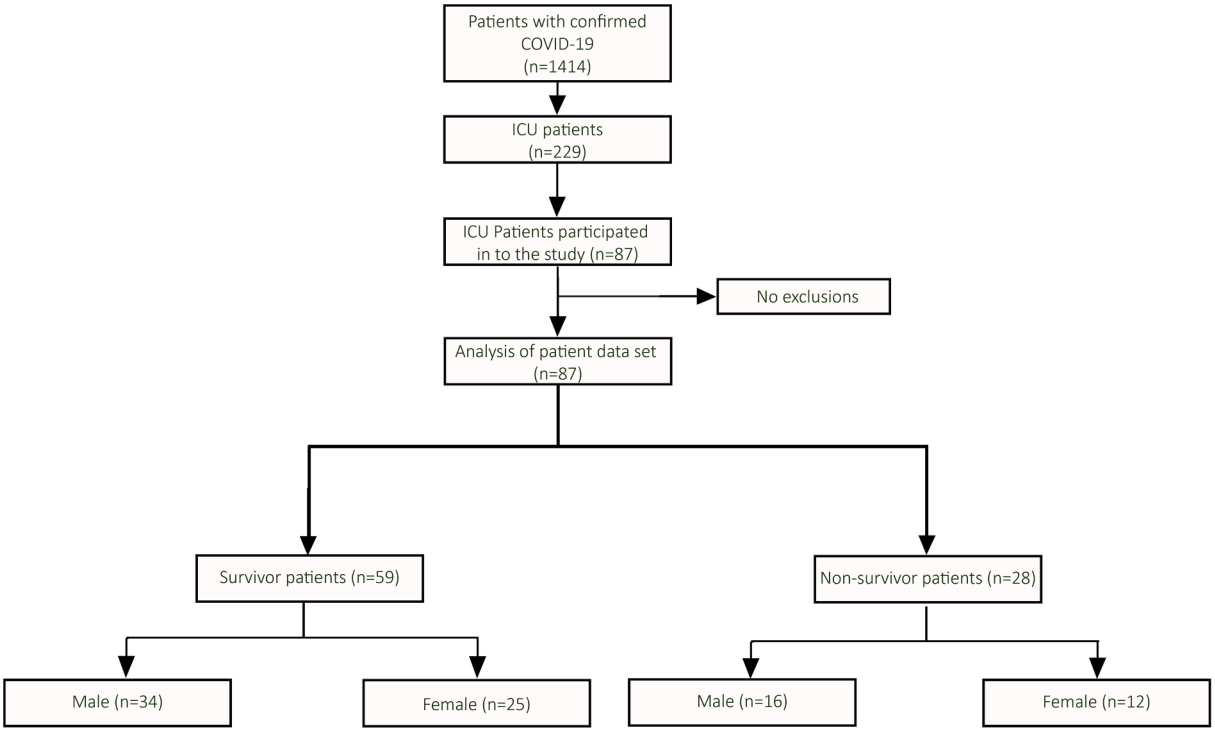

Fig. 1. Patient flow diagram.

standard deviation (SD) or median values and an interquartile range of 25–75%. Mann-Whitney *U* test was used to test non-parametric values. In COVID-19 patients, to observe the effect of MK concentration values, to determine the distinction between severe and mild COVID-19 groups, and to see the effect of comorbidity on the MK levels of patients, the increase in the MK level in patients with varying thresholds was calculated. For this calculation, the receiver-operating characteristic curve (ROC) was generated and the area under the curve (AUC) was calculated. Multivariable regression analysis was performed to identify two or more independent variables that explain variations in the dependent variable. Where appropriate, 95% Cl were calculated and two-tailed *P*-value ($P < 0,05$) was considered statistically significant.

3. Results

From the 87 COVID-19 patients and 50 non-COVID-19 subjects, 37 (42.5%) and 37 (74%) were female and 50 (57.5%) and 13 (26%) were male, respectively. While 16 of 50 males and 12 of 37 female patients hospitalized in the ICU died, 34 male and 25 female patients were discharged. The mean age of deceased and discharged COVID-19 patients was 71.3 ± 12.1 and 57.15 ± 11.2, respectively, and the difference was as expected statistically significant ($P < 0.001$). The mean duration of hospital stay in the ICU was 9.1 ± 6.3 days who were died and 10.2 ± 6.6 days who were discharged ($P > 0.05$). Twenty-six of the deceased (93%) and 9 (15%) of the discharged patient had at least 2 comorbidities (Table 1). Out of 87 patients, 42 had one, and 35 had two or more concomitant diseases. The remaining 10 COVID-19 patients had no accompanying pathologies. The mean serum MK concentration was 1892.8 ± 1615.8 pg/mL versus 680.7 ± 907.6 pg/ mL in COVID-19 patients and non-COVID-19 controls, respectively (Table 2, Fig. 2). Serum MK concentration was approximately 2.9 times higher in COVID-19 patients than that in non-COVID controls ($P < 0.001$). The area under the ROC curve (AUC_{ROC}) of MK was 0.827 with the 95% confidence interval (CI) 0.751–0.904 ($P < 0.001$). The cut-off concentration of MK was 716.7 pg/ mL. The sensitivity and specificity of serum MK concentration in predicting COVID-19 were 75.9% and 76.0%, respectively (Fig. 3). Seventy-seven% of all patients with COVID-19 and 13% of the control group were above the cut-off point and vice versa. No statistically significant difference was detected between deceased patients and discharged patients in regard of serum MK concentration (1913.9 ± 1588.1 versus 2014.9 ± 1629.8).

A multivariable regression analysis was performed using serum levels of MK, comorbidity, gender, age, smoking status, and non-survival or survival status, which was statistically insignificant ($P = 0.22$). The values of the main prognostic biomarkers that we have analyzed were elevated in all of COVID-19 patients. Furthermore, the mean levels of glucose, CRP, NLR, d-dimer, procalcitonin, ferritin and IL-6 were significantly higher in deceased patients compared to discharged patients (Table 1).

Table 1
Demographic and biologic characteristic of the COVID-19 patients.

	Total (n = 87)	Survivor (n = 59)	Non-survivor (n = 28)	*P*
Age, years*	64.2 ± 11.5	57.15 ± 11	71.3 ± 12	< 0.01
Sex, female/male	37/50	25/34	12/16	0.002
Stay in the ICU, days*	9.1 ± 6.3	9.0 ± 6.2	9.2 ± 6.6	0.018
More than one co-morbidity	35	9	26	0.001
Glucose (mg/dl)	70	48	26	
>126	(80%)17	(81%)11	(92%)2	< 0.01
<126	(20%)	(19%)	(8%)	
CRP (mg/L)	84	56	28	
>5	(96%)3	(94%)3	(100%)	< 0.01
<5	(4%)	(6%)	0	
NLR (cells/μl)	62	50	25	
>4.75	(71%)15	(84%)9	(89%)3	< 0.01
<4.75	(29%)	(16%)	(11%)	
D-dimer (mg/L)	77	50	27	
>0.55	(88%)10	(84%)9	(96%)1	< 0.01
<0.55	(12%)	(16%)	(4%)	
PCT (ng/ml)	74	49	22	
>0.5	(85%)13	(83%)10	(78%)6	< 0.01
<0.5	(15%)	(17%)	(22%)	

* Data are presented as mean ± SD.
CRP, C-reactive protein; NLR, neutrophil/ lymphocyte ratio; PCT, procalcitonin; FER, ferritin; IL-6, Interleukin 6.

Table 2
Comparison of serum MK concentration between COVID-19 patients and non-COVID-19 controls.

	Cases (n = 87)	Controls (n = 50)	*P* value
Mean serum MK concentration (pg/mL ± SD)	1892.8 ± 1615.8 (392.6–5595.4)	680.7 ± 907.6 (12.2–3414.1)	< 0.001
Median (pg/mL)	1231.4	336.6	< 0.001

4. Discussion

To the best of our knowledge, this is the first investigation that analyzed the relationship between MK and COVID-19. Our findings showed that serum MK concentration was approximately 2.9 times higher in SARS-CoV-2 infected patients than non-infected volunteers. Several pathophysiological changes may be responsible for this elevation of circulating MK levels in SARS-CoV-2 infected COVID-19 patients which will be discussed below.

The binding of SARS-CoV-2 to pulmonary angiotensin-converting enzyme 2 (ACE2) has some major consequences. One of them is the recruitment of macrophages and neutrophils into the alveolar space, resulting in the secretion of pro-inflammatory cytokines and chemokines that augment capillary permeability and pulmonary edema. Furthermore, it has been demonstrated that downregulated expression of ACE2 by SARS-CoV-2 caused over-activation of renin-angiotensin-system (RAS) that resulted in increased pulmonary vasoconstriction, edema, hypoxia, and lung damage in COVID-19 patients [3,4]. In addition to releasing pro-inflammatory mediators, the neutrophil invasion caused to release of neutrophil extracellular traps (NETs). Several studies showed that this process, called NETosis, is closely associated with COVID-19 [14,27,28]. The excessive neutrophil degranulation precipitated in lung injury and damage of the alveolar-capillary barrier. Also, NETosis is associated with increased levels of intracellular reactive oxygen species (ROS) of neutrophils. Excessive ROS production could lead to alveolar damage and red blood dysfunction that contributed to hypoxic respiratory failure in COVID-19 [29].

Although some studies proposed a protective effect of MK, such as promoting repair of the nervous system after injury and protective action against ischemia/reperfusion injury in the heart, it was known in some others as harmful including, carcinogenesis, chemo-resistance, and promoting inflammation [18,20,30,31]. MK has strong pro-inflammatory characteristics, causing macrophage and neutrophil recruitment to the inflamed region and interacting with other chemokines and cytokines, particularly TNFα and ILs, that are closely associated with the development of SARS-CoV-2 infection [32]. Furthermore, MK played an important role in mediating and generating enhancement of fibrinolytic activity, which is crucial in the initial stage of inflammatory responses of various pathologies [18,33]. Recently, Weckbach et al. showed that during myocarditis MK promotes, via the low-density lipoprotein receptor-related protein 1, infiltration of polymorphonuclear neutrophils into the myocardium and contributes to NETosis, which is detrimental for patients with chronic inflammatory cardiomyopathy. Therefore, they suggested that inhibition of MK or NETs could have beneficial effects in treating myocarditis [34]. It has

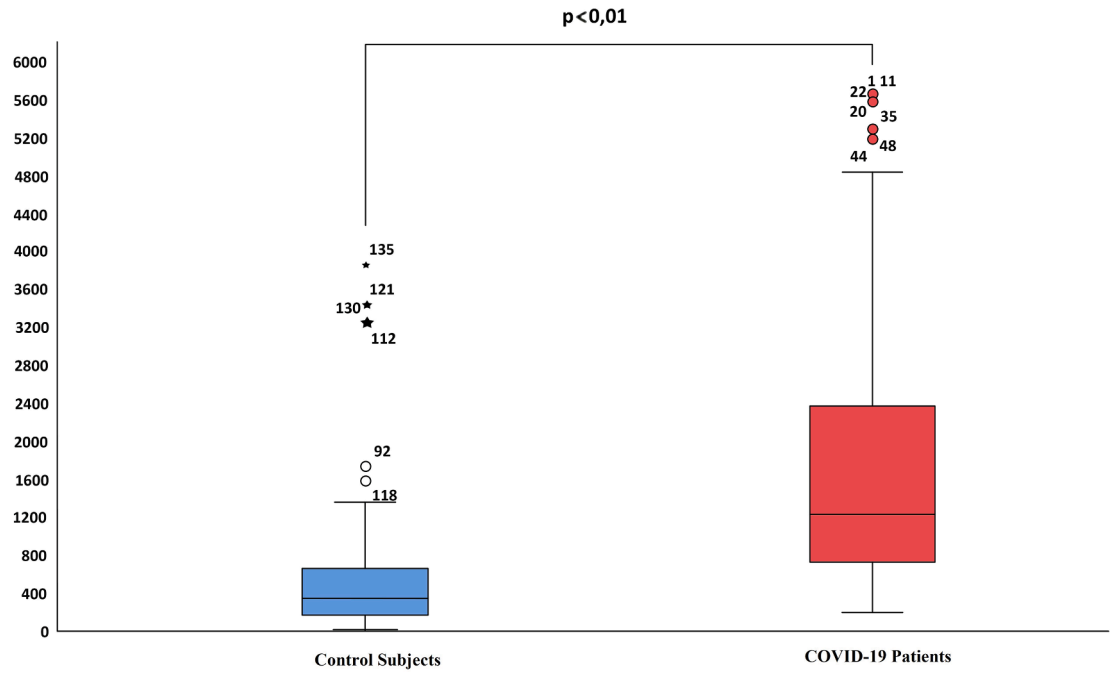

Fig. 2. Serum MK concentrations in control subjects and COVID-19 patients.

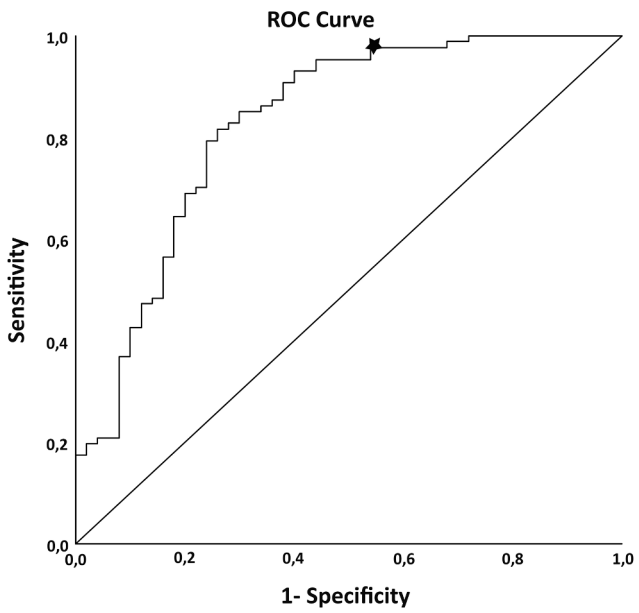

Fig. 3. ROC curve demonstrating AUC (0.827; 95% CI: 0.751–0.904; $P < 0.001$) for MK. The Youden's index was calculated as 0.519 and * indicates the highest J value in the figure.

been demonstrated that MK influences the RAS as well [35]. In an animal study, Hobo et al. showed that the expression of MK was induced in the lung endothelium of micro-vessels and alveolar-capillary endothelial cells by oxidative stress and upregulated by ACE, which generated Ang II from Ang I, followed by an induction of NADPH oxidase (Nox) expression by Ang II that in turn initiated ROS production, and subsequently MK and ACE expression. In the context of COVID-19, which used the ACE2 receptor for its clinical manifestations, MK activated ACE, leading to higher concentrations of Ang II that impaired functions of the alveoli [36]. On the other hand, SARS-CoV-2 invasion itself caused fluid accumulation in the alveoli and the effectiveness of air exchange decreased dramatically, resulting in hypoxia, hypoxemia, and subsequently ARDS. Hypoxia, a powerful inflammatory stimulant, is induced in inflammatory conditions, leading to cytokine storm, inflammation, and other molecular events that are important components of SARS-CoV-2 infection [37]. One of the pathophysiological processes that are triggered by hypoxia is the induction of hypoxia-inducible factor-1α (HIF-1α). Under normal pressure of oxygen in bloodstream, the expression of HIF-1α caused by phagocytic cells, such as neutrophils and macrophages, is low. However, in infection sites, including SARS-CoV-2, high HIF-1α expression stimulated the expression of several pro-inflammatory cytokines [38]. Owing to its pro-inflammatory properties, it has been suggested that inhibition of HIF-1α activity can reduce the SARS-CoV-2 related inflammation and relieve the severity of COVID-19 [39,40]. In a mice model, it has been shown that MK expression was markedly increased in the lungs during exposure to hypoxia via binding of HIF1-α to hypoxia-responsive elements located in the MK promoter [41]. Considering the interplay between MK, hypoxia and HIF1-α in pulmonary tissue, it seems possible that they reinforce each other's pathophysiological actions, as well as in SARS-CoV-2 infection.

Teuwen et al. suggested that endothelial cells are pivotal contributors to the initiation and development of COVID-19 severity [42]. Therefore, endothelial dysfunction, one of the important determinant factors, is responsible for clinical manifestations observed in COVID-19 patients such as hypertension, diabetes mellitus, kidney disease, neurologic disorders, cerebrovascular events and thrombosis [43–46]. In addition, endothelial cells have played a central role in the pathogenesis of ARDS and multiple organ damage in patients with COVID-19 [47]. An important case of SARS-CoV-2 infection is the development of ARDS, which can cause severe lung injury. Some studies demonstrated that MK is closely involved in the pathogenesis of ARDS. Zhang et al. showed that exposure to a mechanical stretch of lung epithelial cells led to an epithelial-mesenchymal transition profile associated with increased expression of ACE, which was attenuated by silencing MK in mice [23]. Furthermore, they found that the plasma concentration of MK was significantly elevated in patients with ARDS. Similarly, in idiopathic pulmonary fibrosis patients, the serum MK level was found higher compared to healthy subjects, supporting the role of MK in the development of ARDS. In the same study, it has been proposed that MK may participate in the progression of pulmonary fibrosis, mainly by regulating inflammatory cell migration into the lung and augmenting

transforming growth factor β (TGF- β) expression, which may be primarily responsible for the lung fibrosis at the end stage of COVID-19 [24,48].

In conclusion, the results of our study demonstrated an approximately 3-fold higher serum concentration of MK in a cohort of COVID-19 ICU patients compared to non-infected subjects. MK, like other pro-inflammatory cytokines aggravate the inflammatory process and cytokine storm in COVID-19. Since MK is significantly upregulated upon exposure to various harmful stimuli such as inflammation, it is likely to accompany the cytokine attack that occurs in SARS-CoV-2 infection. Thus, among treatment strategies targeting cytokines (e.g. inhibition of TNFα, TGF- β or IL-6), drugs that suppress additionally the generation or action of MK may also contribute to COVID-19 treatment.

Ethical approval

This study was approved by The Republic of Turkey Ministry of Health (Number: 2020-10-23T23_59_00) and the Local Ethics Committee of Istanbul Atlas University (Number: E-22686390-050.01.04-4187). The authors obtained informed consent from all patients.

Author contributions

SK and AŞA designed the study. MUK and BD enrolled the patients and collected the data. RD performed the statistical analysis of this study. SK and AŞA wrote the manuscript. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

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

Acknowledgements

None.

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