Türk Klinik Biyokimya Derg 2023; 21(2): 75-88

Araştırma Makalesi / *Research Article*

https://doi.org/10.56615/tkbd.2023.10

Relationship of Semaphorin Proteins with Blood Markers in Patients with COVID-19

COVID-19 Hastalarında Semaphorin Proteinlerinin Kan Belirteçleri ile İlişkisi

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Başvuru Tarihi / *Received:* 19 Mart 2023 Kabul Tarihi / *Accepted:* 22 Haziran 2023

ABSTRACT

Aim: The COVID outbreak is a serious health problem affecting socio-economic life and healthcare systems worldwide. Although the role of Semaphorins in some diseases is relatively known, the association of these molecules with the pathogenesis of COVID-19 remains unclear. Therefore, we aimed to investigate the relationship of Semaphorins (Sema3A, Sema4A, Sema4D and Sema7A) with biochemical and inflammatory alterations and their roles in predicting the presence of disease and disease severity in COVID-19 patients.

Material and Method: A total of 144 COVID-19 patients and 20 healthy individuals were enrolled in the current study. Serum Semaphorins were analyzed using Enzyme-linked Immunosorbent Assay. Other laboratory parameters were measured using routine laboratory techniques.

Results: Sema3A concentrations were elevated in both patients with severe and non-severe COVID-19 groups compared with healthy controls ($p<0.0001$). Sema4A levels were significantly decreased in patients with the severe COVID-19 group ($p=0.002$). Sema3A was negatively correlated with routine hematological markers such as EOS, RBC, HGB, HCT and MCV. Further, Sema3A was positively correlated with coagulation markers such as D-dimer and fibrinogen and the inflammatory markers, such as ESR, CRP, PCT and ferritin and biochemical markers such as ALT, AST, BUN, CK and LDH. Sema4A was negatively correlated with WBC, while it was positively correlated with LYM and HCT. Sema3A levels over 3.03 ng/mL and Sema4A concentrations of less than 11.8 ng/mL may predict the presence of COVID-19 ($p<0.0001$, $p=0.02$, respectively).

Conclusion: Our data presented here suggest that Sema3A and Sema4A could be diagnostic markers for COVID-19 and may have importance in the clinical management of the disease.

Keywords: COVID-19; Semaphorins; SARS-CoV-2; inflammation; Sema3A; Sema4A; Sema4D; Sema7A; Biomarker

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Etik onay: Hatay Mustafa Kemal Üniversitesi, Etik Kurulu 03.06.2021 tarihi ve 2021/03 sayılı kurul kararı

ÖZET

Amaç: COVID salgını dünya çapında sosyo-ekonomik yaşamı ve sağlık sistemlerini etkileyen ciddi bir sağlık sorunudur. Semaforinlerin bazı hastalıklardaki rolü nispeten bilinmesine rağmen, bu moleküllerin COVID-19'un patogenezi ile ilişkisi belirsizliğini koruyor. Bu nedenle, Semaforinlerin (Sema3A, Sema4A, Sema4D ve Sema7A) biyokimyasal ve inflamatuar değişikliklerle ilişkisini ve COVID-19 hastalarında hastalık varlığını ve hastalık şiddetini öngörmedeki rollerini araştırmayı amaçladık.

Gereç ve Yöntem: Bu çalışmaya toplam 144 COVID-19 hastası ve 20 sağlıklı birey dahil edildi. Serum Semaforinleri, Enzime bağlı İmmünosorbent Tahlili kullanılarak analiz edildi. Diğer laboratuvar parametreleri, rutin laboratuvar teknikleri kullanılarak ölçüldü.

Bulgular: Sema3A konsantrasyonları, sağlıklı kontrollerle karşılaştırıldığında hem şiddetli hem de şiddetli olmayan COVID-19 grubundaki hastalarda yüksekti ($p<0.0001$). Şiddetli COVID-19 grubundaki hastalarda Sema4A seviyeleri anlamlı olarak azaldı ($p=0.002$). Sema3A, EOS, RBC, HGB, HCT ve MCV gibi rutin hematolojik belirteçlerle negatif korelasyon gösterdi. Ayrıca Sema3A, D-dimer ve fibrinojen gibi pıhtılaşma belirteçleri ve ESR, CRP, PCT ve ferritin gibi inflamatuar belirteçler ve ALT, AST, BUN, CK ve LDH gibi biyokimyasal belirteçler ile pozitif korelasyon göstermiştir. Sema4A, WBC ile negatif korelasyon gösterirken, LYM ve HCT ile pozitif korelasyon gösterdi. 3,03 ng/mL'nin üzerindeki Sema3A seviyeleri ve 11,8ng/mL'nin altındaki Sema4A konsantrasyonları COVID-19'un varlığını öngörebilir (sırasıyla $p<0,0001$, $p=0,02$).

Sonuç: Burada sunulan verilerimiz, Sema3A ve Sema4A'nın COVID-19 için tanısal belirteçler olabileceğini ve hastalığın klinik yönetiminde önemi olabileceğini düşündürmektedir.

Anahtar Kelimeler : COVID-19; Semaforinler; SARS-CoV-2; inflamasyon; Sema3A; Sema4A; Sema4D; Sema7A; Biyobelirteç

INTRODUCTION

Coronavirus disease (COVID-19) is a highly transmissible disease associated with many disorders that mainly affect the respiratory, immune, and cardiovascular systems, and the devastating pandemic of this disease is responsible for increased mortality and morbidity worldwide (1, 2). Most patients who suffer from COVID-19 have mild symptoms, while approximately %15-20 patients experience some severe symptoms such as dyspnea and multi-organ dysfunction (1). The mortality rate of the disease is increased with comorbidity and age. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a contagious single-strand RNA virus that becomes apparent in China at the end of 2019 with a genome size of approximately 30 kb, is responsible for the ongoing COVID-19 outbreak (1). SARS-CoV-2 enters cells by interacting with the receptor known as angiotensin-converting enzyme II, which is mainly expressed on the surface of cells in the respiratory system (3). The SARS-CoV-2 infection triggers various pathophysiological events such as fibrosis, injury and inflammation (4, 5). Subsequently, damaged alveolar cells stimulate the synthesis and release of cytokine, interferons and other mediators. Consequently, activation of the innate and adaptive immune response induced by a viral infection leads to a cytokine storm resulting in multi-organ damage accompanied by severe clinical conditions (4, 6). Furthermore, the long-term clinical impacts of SARS-CoV-2 infection are alarming. SARS-COV-2 infection sustains various tissue and organ injuries such as fibrotic disorder and neuronal injury among healed people (7, 8). Recent studies aimed at eliminating the adverse effects of this devastating disease focus on developing specific vaccines, diagnostic markers and therapeutic approaches (1, 3, 9). Studies providing more details about the pathogenesis of SARS-CoV-2 infection are needed for the prevention and treatment of COVID-19 disease (1, 3, 10, 11). Therefore, understanding the molecular mechanism of SARS-CoV-2 infection is of great significance for developing practical treatment approaches and vaccine designs for COVID-19.

Semaphorins are a group of secreted and membrane-bound proteins expressed in the

immune and respiratory systems (12). Semaphorins play crucial roles in the various pathophysiological events such as regulation of inflammatory response, apoptosis and angiogenesis (12). Semaphorin 3A (Sema3A), synthesized in many immune cells, is a potent modulator in the inflammatory response. Sema3A has been reported to regulate T cell function and cytokine synthesis. Semaphorin 4A (Sema4A), a transmembrane protein expressed in lung and kidney tissues, plays a crucial role in pulmonary fibrosis and allergic asthma (13, 14). Furthermore, Sema4A receptors are highly expressed in various immune cells, including macrophages and lymphocytes during lung inflammation (15). Semaphorin 4D (Sema4D), mainly expressed in T and B immune cells, is another transmembrane protein that regulates macrophage activity by stimulating cytokine synthesis (12, 16-18). In addition, Sema4D induces inflammation by enhancing B cell activation (19). Semaphorin 7A (Sema7A) is a conserved immune protein that plays a vital role in the initiation of the immune response and cytokine release, and it is abundantly expressed in lung and thymus tissues (20). Existing evidence has shown that Semaphorins act as critical mediators in the diseases of the immune and respiratory systems. Although the role of Semaphorins in some conditions is relatively elucidated, the association of these molecules with altered biochemistry in COVID-19 remains unclear. Therefore, we aim to investigate the relationship of Semaphorins with biochemical and inflammatory alterations and their roles in predicting the presence of disease and disease severity in COVID-19 patients.

MATERIAL AND METHODS

Study Design and Population

Hatay Mustafa Kemal University's clinical ethics committee reviewed and approved the present study with protocol number: 2021/92. We carried out the study in accordance with the Declaration of Helsinki in Hatay Mustafa Kemal University Hospital between August 08, 2021 and November 25, 2021. We included 144 COVID-19 patients treated at the Department of Infectious Diseases and Clinical Microbiology and 20 healthy individuals in the present study and acquired informed consent forms from all participants at the beginning of the study. Considering the World Health Organization interim guidance for COVID- 19, all patients were diagnosed by a physician, and we confirmed the diagnosis in nasal and pharyngeal swab specimens collected from all patients by utilizing the quantitative polymerase chain reaction method (qPCR) and evaluated only verified COVID-19 patients in the present study. In addition, we assessed the demographic and clinical data in the current study.

Clinical Classification of COVID-19 Patients

Considering the World Health Organization (WHO) classification criteria for COVID-19 (21, 22), we classified all patients into four groups: 1) the mild group, patients who have COVID-19 symptoms with no findings of viral pneumonia or hypoxia; 2) the moderate group, patients with clinical findings of non-severe pneumonia and with oxygen saturation (SpO2) above 90% on room air; 3) severe group, patients with clinical symptoms of severe pneumonia and a ventilation frequency greater than 30 breaths/min and/or SpO2 lower than 90% on room air; and 4) critically ill group, patients with acute respiratory distress syndrome (ARDS) and/or septic shock and/or multiple organ dysfunction, patients in this group need mechanical ventilation and intensive care unit admission.

Sample Collection and Processing

The vacuum tube method was used to obtain venous blood samples from all participants, and blood samples were transferred into appropriate laboratory tubes (22). We allowed the blood samples to clot for approximately 20-30 minutes at room

temperature to obtain serum samples. Subsequently, we centrifuged all blood samples at 1500 ×g for 10 min +4 °C and stored serum samples at -80 °C until further Enzyme-linked Immunosorbent Assay (ELISA).

Biochemical Analysis

We conducted all laboratory analyses in Hatay Mustafa Kemal University Hospital Central Laboratory. As we previously stated (22), hematological parameters and coagulation parameters were measured by BC6800 hematology analyzer (Mindray, China) and STA Compact Max (Stago, USA), respectively. We determined the Erythrocyte sedimentation rate (ESR) with Vision-C and measured the C-reactive protein (CRP) levels with BNII Nephelometer (Siemens, Germany). Other biochemical markers were measured by Advia 1800 autoanalyzer (Siemens, Germany). Pro-calcitonin (PCT) and ferritin levels were analyzed by Architect i2000 immunoassay analyzer (Abbott, USA) and Advia Centaur XP immunoassay analyzer (Siemens, Germany), respectively.

Semaphorin Proteins Measurements

We utilized the ELISA kits to analyze the Sema3A, Sema4A, Sema4D and Sema7A concentrations in serum samples of COVID-19 patients. We presented the assay ranges, sensitivity and intra- and interassay coefficients of variance (CV %) for each specific protein in Table 1.

Statistical Analysis

We first tested the normality of the data utilizing the Kolmogorov-Smirnov test and then used the Kruskal Wallis H test and the Mann-Whitney U test to compare the difference between the groups and presented the continuous data as medians (interquartile range). We analyzed the categorical data using the chi-square and presented the data as percentages and numbers. We performed Spearman's rho correlation analysis for association analyses of Semaphorins and biochemical markers. To assess the diagnostic value of Semaphorins for COVID-19, we conducted a receiver operating characteristic (ROC) analysis. R Studio version 0.92.382 and "corrplot version 0.92" package were employed to create the correlogram that presents the correlation analysis. We utilized the SPSS (version 23.0) and GraphPad Prism (version 8.0) for all analyses and graphic presentations of the data, and p values <0.05 were considered statistically significant.

Table 1. The assay range, sensitivity and intra-assay and inter-assay coefficients of variance (CV %) for each specific protein

Parameters	Assay range	Sensitivity	Intra-assay coefficients of variance (CV %)	Inter-assay coefficients of variance (CV %)
Sema3A	0.16-10 ng/mL	0.1 ng/mL	<10%	<10%
Sema4A	0.1-40 ng/mL	0.046 ng/mL	<8%	<10%
Sema4D	0.31-20 ng/mL	0.19 ng/mL	<10%	<10%
Sema7A	0.16-10 ng/mL	0.1 ng/mL	<10%	<10%

Sema3A: Semaphorin 3A, Sema4A: Semaphorin 4A, Sema4D: Semaphorin 4D, Sema7A: Semaphorin 7A.

Table 2. Demographic and clinic characteristics of COVID-19 patients and healthy controls.

Variables		Healthy Control Group (n=20)	All COVID-19 patients			*p*-value
			Non-severe group (n=76)	Severe group (n=68)	All patients (n=144)	
Age		42 (39.25-44)	50.5 (41-57.5)	58.5 (53.25-69)	55 (45-62)	**<0.001**
Gender (n, %)	Male	11 (55)	43 (56.6)	48 (70.6)	91 (63.2)	0.174
	Female	9 (45)	33 (43.4)	20 (29.4)	53 (36.8)	
Hospitalization (Day)			9.29±3.11	18.01±8.2	14±7.71	**<0.001**
Comorbidity (n, %)						
Hypertension	Yes		21 (27.6)	30 (44.1)	51 (35.4)	**0.001**
	No		55 (72.4)	38 (55.9)	93 (64.6)	
Diabetes mellitus	Yes		9 (11.8)	31 (45.6)	40 (27.8)	**0.001**
	No		67 (88.2)	37 (54.4)	104 (72.2)	
Chronic kidney disease	Yes		2 (2.6)	13 (19.1)	15 (10.4)	**0.001**
	No		74 (97.4)	55 (80.9)	129 (89.6)	
Malignancy	Yes		3 (3.9)	5 (7.4)	8 (5.6)	0.356
	No		73 (96.1)	63 (92.6)	136 (94.4)	
Cerebrovascular disease	Yes		2 (2.6)	3 (4.4)	5 (3.5)	0.577
	No		74 (97.4)	65 (95.6)	139 (96.5)	
Pulmonary disease	Yes		4 (5.3)	6 (8.8)	10 (6.9)	0.321
	No		72 (94.7)	62 (91.2)	134 (93.1)	
Thyroid disease	Yes		5 (6.6)	5 (7.4)	10 (6.9)	0.468
	No		71 (93.4)	63 (92.6)	134 (93.1)	
Clinical symptoms (n, %)						
Fever	Yes		24 (31.6)	16 (23.5)	40 (27.8)	**0.014**
	No		52 (68.4)	52 (76.5)	104 (72.2)	
Cough	Yes		40 (52.6)	29 (42.6)	69 (47.9)	**<0.001**
	No		36 (47.4)	39 (57.4)	75 (52.1)	
Loss of appetite	Yes		0 (0)	6 (8.8)	6 (4.2)	**0.012**
	No		76 (100)	62 (91.2)	138 (95.8)	
Expectoration	Yes		6 (7.9)	0 (0)	6 (4.2)	**0.027**
	No		70 (92.1)	68 (100)	138 (95.8)	
Dyspnea	Yes		6 (7.9)	40 (58.8)	46 (31.9)	**<0.001**
	No		70 (92.1)	28 (41.2)	98 (68.1)	
Chest tightness	Yes		0 (0)	7 (10.3)	7 (4.9)	**0.006**
	No		76 (100)	61 (89.7)	137 (95.1)	
Fatigue	Yes		25 (32.9)	21 (30.9)	46 (31.9)	**0.011**
	No		51 (67.1)	47 (69.1)	98 (68.1)	
Myalgia	Yes		3 (3.9)	9 (13.2)	12 (8.3)	**0.042**
	No		73 (96.1)	59 (86.8)	132 (91.7)	
Sore throat	Yes		6 (7.9)	0 (0)	6 (4.2)	**0.027**
	No		70 (92.1)	68 (100)	138 (95.8)	
Nausea or vomiting	Yes		0 (0)	3 (4.4)	3 (2.1)	0.116
	No		76 (100)	65 (95.6)	141 (97.9)	
Deaths (n, %)	Yes		0 (0)	15 (22.1)	15 (10.4)	**<0.001**
	No		76 (100)	53 (77.9)	129 (89.6)	

Continuous data were analyzed using the *Kruskal Wallis H* test followed by the *Mann-Whitney U* test and presented as mean ± standard deviation or medians (interquartile range). Categorical data were analyzed using the *chi-square* test and were shown as numbers (percentages). *p* values less than 0.05 were considered statistically significant and presented as bold.

RESULTS

Demographic and clinical data of COVID-19 patients and healthy controls

A total of 144 clinically diagnosed and laboratory-confirmed COVID-19 patients and 20 healthy individuals were enrolled in the current study, and we presented their demographic and clinical data in Table 2. We divided COVID-19 patients into two groups as Non-severe (18 Mild + 58 Moderate) and Severe (38 Severe and 30 Critically ill) after evaluating the clinical severity of all patients. Of these patients, 91 (63.2%) were male, while 53 (36.8%) were female, and we did not find a considerable difference in gender between all groups, but there was a significant difference in age among the groups. Fever, cough, fatigue and dyspnea are the most common symptoms that affect a large proportion of all patients enrolled in the present study. Cough is the most common symptom in the non-severe group, while dyspnea is the most common symptom in the severe group. A large proportion of clinically severe COVID-19 patients had some comorbidities. As seen in Table 2, hypertension, diabetes mellitus and chronic kidney disease are the most frequent comorbidities in the severe group ($p<0.001$). We also observed a high mortality rate in the severe group, and fifteen patients in the severe group died ($p<0.001$).

The laboratory results of COVID-19 patients and healthy controls

Hematological, coagulation, inflammatory and biochemical markers were analyzed in COVID-19 patients and healthy controls. We observed numerous differences in all laboratory markers in COVID-19 patients and showed the findings in Table 3. Our hematological analyses revealed that severe COVID-19 patients had higher WBC levels than non-severe COVID-19 patients and healthy controls ($p<0.001$). Further, our findings unveiled that LYM, EOS, HGB and HCT levels were decreased in severe and non-severe COVID-19 patients ($p<0.001$). Moreover, we found that severe COVID-19 patients had reduced RBC levels compared with healthy controls and non-severe COVID-19 patients. MON and MCV did not differ between the groups ($p>0.05$). In addition, we assessed the D-dimer and fibrinogen, which are coagulation markers in all groups, and we found that severe and non-severe COVID-19 patients had elevated D-dimer and Fibrinogen concentrations ($p<0.0001$). We also analyzed the inflammatory markers, such as ESR, CRP, Ferritin and PCT. We observed that inflammatory markers were significantly increased in severe and non-severe COVID-19 patients compared with healthy controls ($p<0.0001$). Our biochemical analyses showed that some parameters, such as LDH, ALT, AST, CREA, BUN and CK, increased in severe and non-severe COVID-19 patients ($p<0.05$).

Serum Semaphorin protein concentrations in COVID-19 patients and healthy controls

We analyzed serum Sema3A, Sema4A, Sema4D and Sema7A concentrations with ELISA to determine whether COVID-19 pathophysiology was associated with alterations of Semaphorin protein levels and presented the results in Figure 1. Our results revealed that serum Sema3A concentrations were elevated in patients with severe and non-severe COVID-19 groups compared with healthy controls ($p<0.0001$, Figure 1a). Moreover, we observed significant increases in Sema3A in mild, moderate, severe and critically ill groups, suggesting that the alterations of Sema3A levels may reflect the presence of COVID-19. However, there were no significant differences among all patient groups ($p=0.30$, Figure 1e). We found that Sema4A levels were significantly decreased in patients with the severe COVID-19 group ($p=0.002$, Figure 1b). Still, there were no significant differences between the non-severe group and the healthy controls ($p=0.168$, Figure 1b). We observed that Sema4A levels were significantly reduced in the severe group compared to healthy controls ($p=0.007$, Figure 1f). Sema 4D levels were diminished considerably in the non-severe group compared with healthy controls ($p=0.03$, Figure 1c). In subgroups, we observed markedly decreases in Sema4D in the moderate group ($p=0.001$, Figure 1g). In addition, our data revealed no considerable differences in Sema7A among all groups ($p=0.14$, $p=0.17$ Figure 1d and 1h).

Table 3. Comparison of laboratory test results between COVID-19 patients and healthy controls.

Parameters	Healthy controls (n=20)	COVID-19 patients			*p*-value
		Non-severe group (n=76)	Severe group (n=68)	All patients (n=144)	
Sema3A, ng/mL	2.04±0.75	4.21±1.04	4.22±0.59	4.21±0.85	**<0.0001[a,b,c]**
Sema4A, ng/mL	17.83±12.08	12.64±10.83	9.61±11.08	11.21±11.02	**<0.0001[a] =0.002[c]**
Sema4A, ng/mL	4.41±6.42	1.66±2.62	2.65±3.31	2.13±3.0	**<0.0001[a,] =0.03[b]**
Sema7A, ng/mL	3.23±3.88	3.16±3.25	3.81±3.23	3.47±3.25	**=0.017 [a,b,c,d]**
WBC, $10^3/\mu L$	6.45 (6.1-6.91)	5.70 (4.41-7.41)	10.89 (7.25-15.68)	7.25 (5.06-11.3)	**<0.0001 [a,c,d],** =0.102[b]
LYM, $10^3/\mu L$	2.12 (1.65-2.45)	1.38 (.96-1.85)	0.75 (0.55-1.29)	1.13 (0.72-1.61)	**<0.0001[a,b,c,d]**
MON, $10^3/\mu L$	0.425 (0.40-49)	0.43 (0.27-0.55)	0.47 (0.34-0.70)	0.44 (0.31-0.63)	=0.046[a],=0.330[b], =0.378[c],=0.015[d]
EOS, $10^3/\mu L$	0.16 (0.13-0.27)	0.02 (0.01-0.08)	0.01 (0.0-0.04)	0.01 (0-05)	**<0.0001[a,b,c],=0.005[d]**
RBC, $10^6/\mu L$	4.47 (4.24-4.89)	4.79 (4.46-5.18)	4.04 (3.52-4.41)	4.47 (3.94-4.89)	**<0.0001[a,d], =0.039[b],=0.001[c]**
HGB, g/dL	14.4 (13.1-15.45)	13.25 (12.42-14.4)	11.45 (9.3-12.77)	12.7 (11.3-13.77)	**<0.0001[a,c,d],=0.016[b]**
HCT, %	42.9 (39-46.05)	39.01 (37.32-43.2)	35 (28.91-38.47)	38.2 (34.3-40.1)	**<0.0001[a,c,d],=0.023[b]**
MCV, fL	87.75 (82.05-91.02)	84.4 (80.67-87.8)	86.45 (80.04-89.07)	84.95 (80.67-88.52)	=0.147[a],=0.055[b], 0.157[c] =0.411[d]
D-dimer, ng/mL	203 (146.75-211.5)	462 (270-679)	1080 (730-2820)	657 (390-1748)	**<0.0001[a,b,c,d]**
FIB, mg/dL	207 (194.5-226.5)	364 (301.5-471.25)	526 (450-617)	450 (330-556)	**<0.0001[a,b,c,d]**
ESR, mm/h	7.5 (5.25-9)	17 (11.25-25.75)	37 (21-50)	23 (14-43)	**<0.0001[a,b,c,d]**
CRP, mg/L	1.47 (1.12-1.77)	22.7 (6.18-52.97)	49 (24.1-102)	36.5 (13.8-63.1)	**<0.0001[a,b,c,d]**
PCT, ng/mL	.012 (.01-.014)	0.03 (0.01-0.05)	0.15 (0.05-0.39)	0.05 (0.02-0.21)	**<0.0001[a,b,c,d]**
Ferritin, ng/mL	40.5 (20-64.8)	126.75 (57.67-317.25)	558.7 (219-1098)	296.9 (89-690.4)	**<0.0001[a,b,c,d]**
LDH, U/L	130 (122.5-136.5)	240 (201-318.5)	310 (245-428)	279.5 (204-367.75)	**<0.0001[a,b,c], 0.005[d]**
ALT, U/L	20.5 (16.5-22.75)	29 (22-40.75)	33.5 (20-67)	31.5 (21-52)	**<0.0001 [a,b], 0.001[c] .097[d]**
AST, U/L	21 (18-24.75)	33 (25-44)	35.5 (29-56)	34 (27.25-48)	**<0.0001 [a,b,c], 0.097[d]**
CREA, mg/dL	0.75 (0.65-0.77)	0.81 (0.65-1)	0.94 (0.72-1.25)	0.89 (0.69-1.09)	**<0.0001 [a,c], <0.021[b], =0.003[d]**
BUN, mg/dL	8.5 (8-9)	13 (11-17.75)	23.5 (15-34.5)	16 (12.25-25)	**<0.0001[a,b,c,d]**
CK, U/L	62.5 (50.5-82)	95 (76-172.25)	201 (115.5-285)	124 (89-244.75)	**<0.0001[a,c], 0.001[b,d]**

Continuous data were analyzed using the *Kruskal Wallis H* test followed by the *Mann-Whitney U* test and presented as mean ± standard deviation or medians (interquartile range). Categorical data were analyzed using the *chi-square* test and were shown as numbers (percentages). *p* values less than 0.05 were considered statistically significant and presented as bold. Abbreviations: Sema3A: Semaphorin 3A, Sema4A: Semaphorin 4A, Sema4D: Semaphorin 4D, Sema7A: Semaphorin 7A, WBC: White blood cells, LYM: Lymphocyte, MON: Monocytes, EOS: Eosinophil, RBC: Red blood cells, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, FIB: Fibrinogen, ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, PCT: Procalcitonin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, CREA: Creatinine, BUN: Blood urea nitrogen, CK: Creatine kinase, LDH: Lactate dehydrogenase.

[a]Comparison among all groups.

[b]Comparison between Healthy controls and non-severe group.

[c]Comparison between Healthy controls and severe group.

[d]Comparison between Non-severe group and severe group.

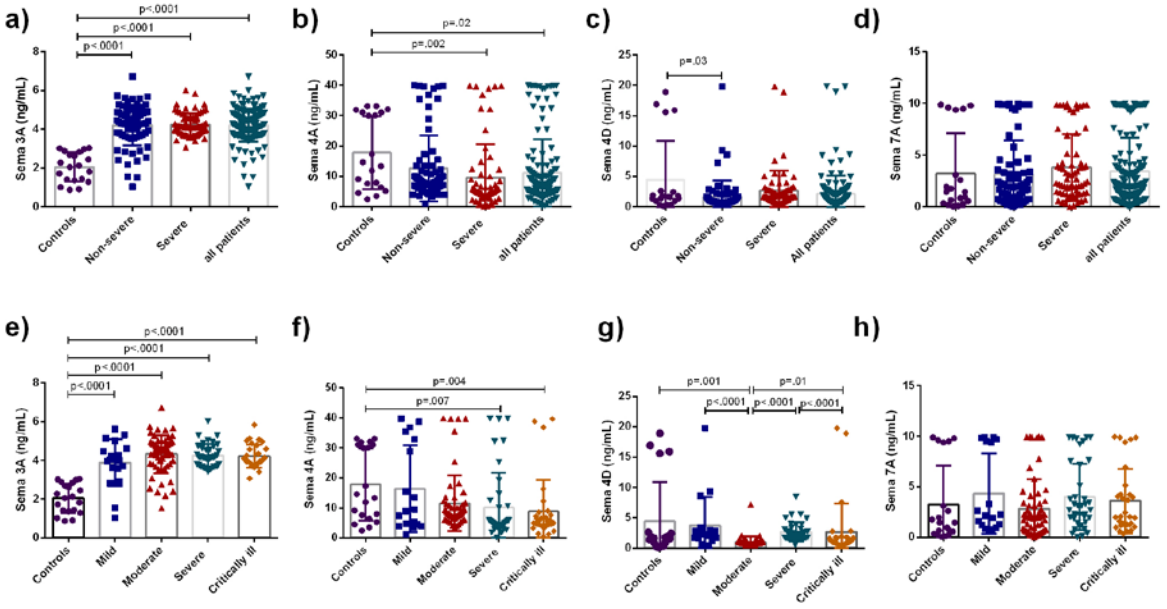

Figure 1. The Sema3A, Sema4A, Sema4D and Sema7A protein levels in patients with COVID-19 and healthy controls.

Comparison of Sema3A (a), Sema4A (b), Sema4D (c) and Sema7A (d) protein expression in COVID-19 patients according to the classification of Non-severe (mild, moderate) and severe (severe and critically ill). Comparison of Sema3A (e), Sema4A (f), Sema4D (g) and Sema7A (h) protein expressions in COVID-19 patients according to the classification of mild, moderate, severe and critically ill disease. Abbreviations: Sema3A: Semaphorin 3A, Sema4A: Semaphorin 4A, Sema4D: Semaphorin 4D, Sema7A: Semaphorin 7A. All data were presented as mean ± SD. $p < 0.05$ values were considered as significant.

The correlations of serum Semaphorins levels with other laboratory parameters in COVID-19 patients

We conducted a *Spearman rank* correlation analysis to clarify the relationship of Sema3A, Sema4A, Sema4D and Sema7A with hematological, coagulation, inflammatory and biochemical markers in patients with COVID-19 and presented the results in Figure 2. We revealed that serum Sema3A was negatively correlated with routine hematological markers such as EOS, HGB, HCT and MCV (r=0.286, p<0.0001, r=0.257, p<0.001, r=0.245, p=0.002 and r= 0.159, p=0.042, respectively). Further, our data showed that Sema3A was positively correlated with coagulation markers such as D-dimer and fibrinogen (r=0.205, p=0.008 and r=0.333, p<0.0001, respectively) and the inflammatory markers, such as ESR, CRP, PCT and ferritin (r=0.161, p=0.039, r=0.208, p=0.008, r=0.184, p=0.019 and r=0.236, p=0.002, respectively) and biochemical markers such as ALT, AST, BUN, CK, LDH (r=0.157, p=0.045, r=0.236, p=0.002, r=0.186, p<0.017, r=0.241, p=0.002 and r=0.303, p<0.0001, respectively). Our analyses showed that Sema4A was negatively correlated with WBC (r=0.217, p=0.005) while it was positively correlated with LYM and HCT (r=0.213, p=0.006 and r=0.160, p=0.04). Similar to Sema3A, Sema4A was significantly correlated with all coagulation markers such as D-dimer and fibrinogen (r=0.289, p<0.0001 and r=0.262, p=0.001, respectively) and inflammatory factors such as ESR, CRP, PCR and ferritin (r=0.229, p=0.003, r=0.180, p=0.021, r=0.231, p=0.003 and r=0.192, p=0.014, respectively). Sema4A was significantly correlated with biochemical parameters such as BUN and CK (r=0.253, p=0.001 and r=0.158, p=0.043, respectively). Moreover, we did not find any correlations between Sema4D and Sema7A and other laboratory markers in COVID-19 patients.

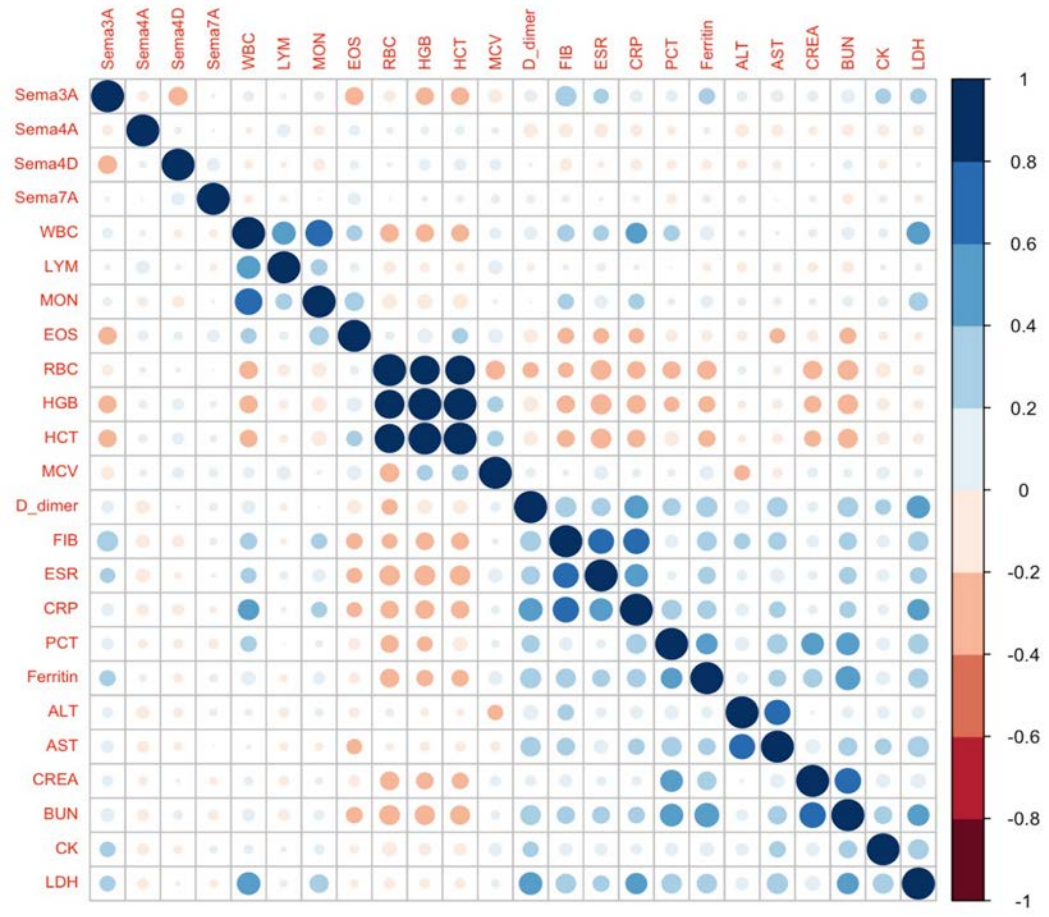

Figure 2. Correlogram graph showing the associations between Semaphorin proteins and other laboratory parameters.

Spearman's rho test was used. Blue represents positive correlation, and red represents negative correlation, and the darker the colour shows the more potent the correlation. The numerical values presented represent the correlation coefficients.

Sema3A: Semaphorin 3A, Sema4A: Semaphorin 4A, Sema4D: Semaphorin 4D, Sema7A: Semaphorin 7A, WBC: White blood cells, LYM: Lymphocyte, EOS: Eosinophil, RBC: Red blood cells, HGB: Hemoglobin, HCT: Hematocrit, FIB: Fibrinogen, ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, PCT: Procalcitonin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, BUN: Blood urea nitrogen, CK: Creatine kinase, LDH: Lactate dehydrogenase.

Diagnostic values of Semaphorins levels for COVID-19 patients

We performed ROC curve analyses to determine whether alterations of Semaphorin proteins and other blood markers could be used as biomarkers to predict the presence of COVID-19 and presented all results in Table 4 and Figure 3. Our ROC curve analyses revealed that serum Sema3A levels have an excellent ability to distinguish COVID-19 patients from healthy controls ($p<0.0001$). The optimal cut-off value of Sema3A for predicting the presence of COVID-19 was 3.03 ng/mL, and its sensitivity and specificity were 92.4% and 95%, respectively. Moreover, Sema4A concentrations of less than 11.8 ng/mL may predict the presence of COVID-19 ($p=0.02$), and the sensitivity and specificity for Sema4A were 76.4% and 60%, respectively. Our data here suggest that Sema3A and Sema4A could be diagnostic markers for COVID-19 and may have importance in the clinical management of the disease.

Table 4. The receiver operating characteristic curve analyse results and optimal cut-off levels of Sema3A, Sema4A, Sema4D and Sema7A, CRP, Ferritin, D-dimer and Fibrinogen in patients with COVID-19.

Variables	AUC (95%CI)	Sensitivity %	Specificity %	Cut-off	p-value
Sema3A	0.9682 (0.9442-0.9923)	92.4	95.0	3.03	<0.0001
Sema4A	0.6594 (0.5304-0.7883)	76.4	60.0	11.8	=0.02
Sema4D	0.5561 (0.4049-0.7073)	45.1	45.0	1.50	>0.05
Sema7A	0.6000 (0.4522-0.7478)	59.0	60.0	1.93	>0.05
CRP	1 (1000-1000)	99.3	100	3.045	<0.0001
Ferritin	0. 9512 (0.9179-0.9845)	89.6	90.0	47	<0.0001
D-dimer	0.9474(0.9147-0.9801)	88.9	90.0	255	<0.0001
FIB	0.9679 (0.9435-0.9922)	91.7	90.0	242.5	<0.0001

AUC: Area under the receiver operating characteristic curve, CI: confidence intervals, Sema3A: Semaphorin 3A, Sema4A: Semaphorin 4A, Sema4D: Semaphorin 4D, Sema7A: Semaphorin 7A, CRP: C-reactive protein.

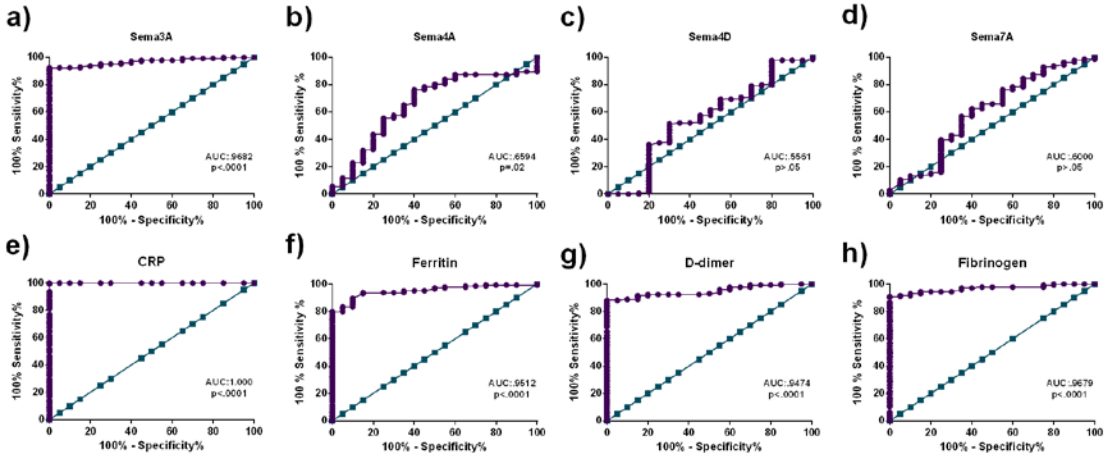

Figure 3. The evaluation of potential predictive values of Sema3A (a), Sema4A (b), Sema4D (c), Sema7A (d), CRP (e), Ferritin (f), D-dimer (g) and Fibrinogen (h) in COVID-19 patients using ROC curve analyses.

Sema3A: Semaphorin 3A, Sema4A: Semaphorin 4A, Sema4D: Semaphorin 4D, Sema7A: Semaphorin 7A, CRP: C-reactive Protein.

DISCUSSION

COVID-19 is a serious health problem affecting socio-economic life and healthcare systems worldwide. Recent studies mainly focus on understanding the molecular pathogenesis of COVID-19 and developing therapeutic and diagnostic approaches to prevent the adverse effects on general populations and improve the clinical conditions of the disease (1, 3, 22, 23). However, the pathogenesis of the disease remains unclear, and current clinical management is not yet adequate for alleviating the clinical outcomes of COVID-19 (23). Although some immunological properties of Semaphorins are relatively known, there are no studies investigating the possible roles of these proteins in the pathogenesis of COVID-19 (16, 24). Herein, we first investigated whether Semaphorin proteins (Sema3A, Sema4A, Sema4D and Sema7A) are associated with hematological, coagulation, inflammation and biochemical markers in patients with COVID-19.

In the lung and inflammatory disorders context, Sema3A plays a crucial role in apoptosis, cell migration, angiogenesis and inflammatory events (25, 26). *Cozacov et al.* reported that serum Sema3A levels were diminished in Asthma and Sema3A acts as a

potent regulatory in inflammatory events (27). Moreover, *Sawaki et al.* revealed that Sema3A levels were decreased in an animal model of Allergic Rhinitis and administration exogenously of recombinant Sema3A may alleviate symptoms of the disease (28). On the other hand, increased expressions of Sema3A and its receptor have been reported in patients with asthma and the animal model of asthma (29). In another study, *Ji et al.* revealed that differentiated macrophages and activated T cells have high expressions of Sema3A and its receptors (24). The same study showed that Sema3A could regulate inflammatory conditions by stimulating apoptosis in macrophages (24). The discrepancy in Sema3A expressions could be explained by the differences in cell activation status, type of disease, examined biological tissue and inflammatory conditions. More importantly, a recent study investigating the possible role of Sema3A in an animal model of bronchial asthma has shown that Sema3A acts as a suppressor against excess inflammation, suggesting that Sema3A might be a novel therapeutic molecule for lung-related diseases (30). Our results revealed that all COVID-19 patients had significantly higher serum Sema3A levels than healthy controls. Taken together, we may speculate that elevated Sema3A plays a role in the negative regulation of the immune system to prevent the overactivation of inflammatory events in patients with COVID-19, although experimental evidence for that is still needed. We know little about the possible role of Sema3A in the molecular pathogenesis of COVID-19 and therefore merit further investigations. Previous studies have shown that Sema3A concentrations are related to the pathogenesis of inflammatory diseases, including Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA) (31, 32). Vadasz et al. unveiled that serum Sema3A levels correlate with SLE disease activity (31). Another study showed that Sema3A mRNA expressions were associated with the inflammation score and histopathological changes in RA synovial tissues (32). In the present study, our correlation analyses revealed that Sema3A was correlated with hematological, coagulation, inflammation and biochemical markers reflecting the presence of COVID-19. Further, our ROC analyses showed that Sema3A could be a potential diagnostic marker detectable in the peripheral blood of COVID-19 patients.

Sema4A, expressed in various types of cells, including macrophages and T cells, plays a crucial role in immune response, carcinogenesis, fibrosis and angiogenesis (33, 34). The data on the serum Sema4A concentrations in inflammatory diseases are minimal and its role in peripheral blood remains unclear. A recent study has shown that Sema4A can stimulate inflammation and fibrosis in systemic sclerosis, an inflammatory disease (35). Furthermore, it was reported that Sema4A is a suppressor of inflammation in a mouse model of allergic asthma (36). In another study, *Eiza et al. reported* that serum Sema4A levels were correlated with the severity of multiple sclerosis (37). Our study found that serum Sema4A expressions were decreased in only severe and critically ill patients. Our analyses showed that serum Sema4A was correlated with hematological, coagulation, inflammation and biochemical markers in COVID-19 patients. In addition, our ROC analysis revealed that Sema4A might predict the presence of COVID-19.

Sema4D is a protein involved in immune response and has diagnostic potential for some diseases such as Heart failure and RA (38, 39). Considering these potential features of Sema4D, we evaluated serum Sema4D concentrations in COVID-19 patients, and our data revealed that Sema4D levels were reduced only in the moderate group compared to the healthy controls. Moreover, we did not find any correlation between Sema4D and other routine biomarkers reflecting the COVID-19 pathogenesis.

Sema7A acts as a potent modulator in immunological events such as inflammation, cytokine synthesis and cell interactions.

Although some studies investigating lung injury and airway inflammation have revealed the essential role of Sema7A in pulmonary inflammation, its role in COVID-19 remains unclear. In the present research, we examined the serum Sema7A expressions and evaluated the association of blood markers involved in the pathogenesis of COVID-19. We found that there was no significant difference in Sema7a expressions between the groups. Our findings suggest that serum Sema7A levels may not be associated with the pathogenesis of COVID-19. Nevertheless, more study investigating the action mechanism of Sema7A in COVID-19 is needed.

Here, we examined the Sema3A, Sema4A, Sema4D and Sema7A proteins and our results showed that Sema3A and Sema4A, but not other Semaphorins, may play a role in the molecular pathophysiology of COVID-19. Moreover, we may suggest that Sema3A is one of the most active Semaphorins in inflammatory events, consistent with previous studies (38). Further, our findings propose that it is unclear whether other Semaphorins (Sema4D and Sema7A) are actively involved in inflammatory events in COVID19.

Our study is not without any limitations, and the findings provided here should be confirmed with multi-center and large-scale studies since the population size in the study was relatively small. The COVID-19 patients enrolled in the study were receiving treatment during sample collection, and we do not know the effects of this therapy on the expressions of Semaphorin proteins. Moreover, some patients had comorbidities such as cardiovascular disease and diabetes mellitus, and the effects of these comorbidities on the concentrations of Semaphorin proteins remain unclear. Further, functional studies focusing on the possible role of Semaphorins in inflammatory processes in SARS-COV-2 infection will contribute to elucidating the comprehensive molecular mechanisms of COVID-19.

CONCLUSION

Our research has shown that SARS-Cov2 infection can boost Sema3A, a critical factor in inflammatory events to maintain physiological homeostasis. Our study provides evidence that Sema3A has clinical importance in predicting the presence of COVID-19. Moreover, we report here for the first time that Sema3A is correlated with hematological, inflammatory and biochemical markers in patients with COVID-19. Furthermore, the data presented here improve our understanding of the molecular pathophysiology of COVID-19. These findings may contribute to identifying potential therapeutic targets and diagnostic markers for preventing the morbidity and mortality of COVID-19. However, further functional studies are required to elucidate the detailed molecular mechanism of Semaphorins in inflammation-related pathologies, including COVID-19.

ACKNOWLEDGEMENTS

The authors thank the Hatay Mustafa Kemal University for funding this Project. (Project Number: 031-2021).

FINANCIAL DISCLOSURE

The present study was supported by the Scientific Research Projects Coordination Unit of Sakarya University of Applied Sciences. Project Number: 031-2021

COMPETING INTERESTS

No potential conflict of interest was reported by the author(s)

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article or uploaded as supplemental information.

AUTHOR CONTRIBUTIONS

Study concept and design (HMO), Acquisition of data (SD, TB and MC), Analysis and interpretation of data (HMO, TB and SD),

Drafting of the manuscript (HMO, SD), Critical revision of the manuscript for important intellectual content (HMO, SD, TB and MC) Statistical analysis (HMO, SD and TB), GuarantoR (HMO).

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