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Evaluation of the relationship between serum netrin-1 level and patient characteristics in rheumatoid arthritis

Romatoid artritte serum netrin-1 düzeyi ile hasta özellikleri arasındaki ilişkinin değerlendirilmesi

Gizem Özan[1], Şükran Erten[2], Serdar Can Güven[3], Ahmet Kor[4], Funda Eren[5], Pınar Akyüz Dağlı[3], Yüksel Maraş[6], İsmail Doğan[2], Kevser Orhan[3], Orhan Küçükşahin[2], Salim Neşelioğlu[7], Özcan Erel[7]

[1]*Ankara Yıldırım Beyazıt University Faculty of Medicine, Department of Internal Medicine, Ankara City Hospital, Clinic of Internal Medicine, Ankara, Turkiye*
[2]*Ankara Yıldırım Beyazıt University Faculty of Medicine, Department of Rheumatology, Ankara City Hospital, Clinic of Rheumatology, Ankara, Turkiye*
[3]*Ankara City Hospital, Clinic of Rheumatology, Ankara, Turkiye*
[4]*Department of Rheumatology, Aksaray Education and Research Hospital, Aksaray, Turkiye*
[5]*Ankara City Hospital, Clinic of Biochemistry, Ankara, Turkiye*
[6]*University of Health Sciences Turkiye, Department of Rheumatology, Ankara City Hospital, Clinic of Rheumatology, Ankara, Turkiye*
[7]*Ankara Yıldırım Beyazıt University Faculty of Medicine, Department of Biochemistry, Ankara City Hospital, Clinic of Biochemistry, Ankara, Turkiye*

Abstract

Objective: Patients with rheumatoid arthritis (RA) are generally seropositive, but 15-20% of patients with RA may be seronegative. Diagnosing patients with RA sometimes requires long and challenging research, and new diagnostic biomarkers are needed. This study aimed to investigate the use of netrin-1 as a biomarker in the differential diagnosis of RA from healthy controls and the relationships between netrin-1 and RA's disease activity scores and joint erosions.

Methods: The study group included 60 RA patients, and the control group included 41 healthy volunteers. Serum netrin-1 was measured using an ELISA kit with the quantitative sandwich enzyme immunoassay method. The RA Disease Impact (RAID) score was used to determine changes in quality of life, and the disease activity score-28 (DAS-28) and Clinical Disease Activity Index (CDAI) were used to assess disease activity.

Results: In the comparison of netrin-1 serum levels, netrin-1 levels were found to be similar between RA and healthy controls [64.4 (35.8-551.4) and 65 (35.8-436.6), p=0.786, respectively]. No significant correlation was found between netrin-1 levels and disease indices (CDAI, DAS-28 ESR, RAID, and DAS-28 CRP) (p>0.05).

Conclusion: Netrin-1 is not higher in the serum of RA patients compared to healthy controls, and its levels have no correlation with disease indices of RA.

Keywords: Rheumatoid arthritis, netrin-1, disease activity, fibrosis

Öz

Amaç: Romatoid artrit (RA) hastalık aktivitesini öngörmek için pek çok belirleyici öne sürülmesine rağmen, bu belirteçlerin çoğu RA'ya özgü değildir ve düzeyleri birçok başka faktörden etkilenebilir. RA'da hastalık aktivitesini tahmin etmek için ideal belirteçlere olan ihtiyaç devam etmektedir. Netrin-1'in RA hastalık aktivitesi ile ilişkisi daha önce değerlendirilmemiştir. Bu çalışmada RA hastalarında netrin-1 molekülünün plazma düzeylerinin ve bunun RA hastalık aktivitesi ile ilişkisinin araştırılması amaçlandı.

Yöntem: Bu çalışmaya 60 RA hastası ile yaş ve cinsiyet uyumlu 41 sağlıklı gönüllü birey dahil edilmiştir. Kantitatif sandviç enzim immünoassay yöntemi ile analiz yapan bir ELISA kiti netrin-1 değerlerinin hesaplanmasında kullanıldı. Hastalık aktivitesini ölçmek için hastalık aktivite skoru-28 (DAS-28) ve Klinik Hastalık Aktivite İndeksi (CDAI), hastalığa bağlı yaşam kalitesinde bozulmayı ölçmek için Romatoid Artrit Hastalığın Etkisi (RAID) skoru hesaplandı.

Bulgular: Netrin-1 serum düzeyleri RA ve sağlıklı kontrollerde benzer bulunmuştur [sırasıyla 64,4 (35,8-551,4), 65 (35,8-436,6), p=0,786]. Netrin-1 ile düzeyleri DAS-28-CRP, DAS-28-ESR, CDAI ve RAID arasında anlamlı bir korelasyon ilişkisi tespit edilmemiştir (p>0,05).

Sonuç: Netrin-1 RA hasta serumlarında yüksek değildir ve düzeylerinin RA hastalık aktive skorları ile korelasyon ilişkisi yoktur.

Anahtar Kelimeler: Romatoid artrit, netrin-1, hastalık aktivitesi, fibrozis

İletişim / Correspondence: Ahmet Kor MD, Department of Rheumatology, Aksaray Education and Research Hospital, Aksaray, Turkiye
Phone: +90 532 646 86 61 **E-mail:** ahmetkor_61@hotmail.com **ORCID ID:** orcid.org/0000-0002-5794-6951
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Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory, autoimmune disease of unknown etiology that causes synovial inflammation and proliferation, resulting in joint cartilage destruction and juxta-articular bone erosion, and can affect many systems.[1] RA is generally seropositive, but 15-20% of patients with RA may be seronegative. Current research has tested many biomarkers that can predict RA disease activity, but the majority of these biomarkers are not specific for RA, and their levels can be affected by many physiological or pathophysiological factors.[2] Diagnosing RA patients sometimes requires long and challenging investigations, and new diagnostic biomarkers are needed both for the diagnosis of RA and for the prediction of disease activity.

Netrin-1, a member of the axonal guidance protein family, has a laminin-like matrix protein structure. Netrin-1 essentially functions as a chemorepulsant and inhibits lymphocyte, monocyte, and neutrophil migration through activation of its receptors adenosine-A and Unc5b, thus preventing macrophage exit from the inflammatory environment. These properties suggest that netrin-1 may play a role in the pathogenesis of many inflammatory diseases.[3] Netrin-1 can change the inflammatory response by acting in inflammatory cascades, reducing tissue damage due to hypoxia, and suppressing apoptosis.[4,5] Netrin-1 may play a pathogenic role in atherosclerosis developing in obesity,[6,7] in osteoporosis by stimulating osteoclast differentiation,[8] in the onset of sepsis/inflammation,[9] in the development of inflammatory arthritis,[10] and the development of systemic sclerosis.[11]

It has been shown that netrin-1 can limit the inflammatory response with the participation of inflammatory cascades, reduce tissue damage due to hypoxia, and simultaneously suppress apoptosis. It has been stated that netrin-1 can be used as a biomarker in various situations due to its immune regulatory effects. Considering these findings, whether netrin-1 can have utility as a novel marker in rheumatic conditions is also a matter of interest. Therefore, there is a continuing need for inexpensive, practical, and ideal biomarkers that can predict RA disease activity.

In this study, we investigated the usability of netrin-1 as a biomarker in the differential diagnosis of RA from healthy controls and the relationships between netrin-1 and RA disease activity scores and joint erosions. To the best of our knowledge, this is the first study to evaluate the potential of netrin-1 in RA patients.

Materials and Methods

Study Design

This study was planned as a cross-sectional case-control study. The study was conducted in Ankara City Hospital, Clinic of Rheumatology, between November 2021 and February 2022. Sixty individuals with RA, between the ages of 18 and 70, who met the American College of Rheumatology 2010 RA diagnostic criteria[12] were included in the patient group. Individuals with additional systemic diseases other than RA, chronic drug use, pregnancy, malignancy, and active infection were excluded from the study due to their possible effects on serum netrin-1 levels. A control group was formed from 41 healthy volunteers with similar demographics who attended Ankara City Hospital, Internal Diseases outpatient clinic.

To minimize the effect of other confounders on serum netrin-1 levels between the RA and control groups, individuals in the control group were selected to have similar age, gender, body mass index, and smoking rate as the RA group. Clinical, demographic, radiologic, and laboratory data were obtained from patients and hospital medical records upon acceptance. Laboratory parameters of the last visit were recorded. Disease activity was evaluated with disease activity score-28 (DAS-28) C-reactive protein (CRP), DAS-28 erythrocyte sedimentation rate (ESR), and Clinical Disease Activity Index (CDAI). The RA Impact of Disease (RAID) score was used to assess disease-related impairment in quality of life.[13-15]

DAS-28 is calculated by the pain visual analog scale, ESR, CRP values, and the number of tender and swollen joints. High disease activity is considered to be DAS-28 >5.1, moderate disease activity is considered to be DAS-28 between 3.2 and 5.1, low disease activity is considered to be between 2.6 and 3.2, and remission is considered to be DAS-28 <2.6. CDAI is calculated by the number of tender and swollen joints, and patient and evaluator global assessments. High disease activity is CDAI >22, moderate disease activity is considered to be CDAI between 22 and 10, low disease activity is considered to be between 2.8 and 10, and remission is considered to be DAS-28 <2.8.

In RA patients, the presence of interstitial lung disease (ILD) and lung nodules was detected by high-resolution tomography or lung tomography; bone erosions of the hand and wrist were detected by hand ultrasonography, hand radiography or hand magnetic resonance imaging; and hand deformities were detected by physical examination. Those with ulnar deviation, radial deviation, interosseous atrophy, swan neck deformity, boutonniere deformity, and thumb Z deformity on physical examination were considered positive for the presence of RA hand deformity.

The study was approved by the Ankara City Hospital Ethics Committee IRB no: E1/2087/2021, date: 03.11.2021.

Biochemical Method for Netrin-1

Venous blood samples were taken into vacuum tubes with a volume of 10 mL, were centrifuged at 1300 x g for 10 minutes, and kept at -80 °C until analysis. All biochemical tests were performed on venous blood samples obtained from the last visits of the individuals in the study. Serum samples were analyzed approximately 4 months later. Analysis of serum netrin-1 levels was performed with an ELISA kit using the quantitative immunoassay method (Catalog number: E/EL/H2328-elabscience-lot number GZWTKZ5SWK-Texas-USA). For serum Netrin-1 analyses, blood samples and standards were incubated with specific antibodies in micro-ELISA plate wells for 1.5 hours (37 °C) and biotin-enriched Avidin-Horseradish Peroxidase (HRP), and human netrin-1 specific detection antibodies were added. This mixture was then incubated for 30 minutes (37 °C). Then, the free components were mixed with the washing process, and the substrate material was added to the wells. After this process, a blue color was formed only in the human netrin-1 wells, Avidin-HRP conjugate, and biotin-enriched detection antibodies. Finally, the enzyme-substrate reaction was terminated with stop solution, and a yellow color was formed in the solution. Optical densities, which are considered as an indirect netrin-1 level determination, were performed with spectrophotometric microplate readers with a wavelength of 450 nm. Optical density standard curves were used to calculate human netrin-1 concentrations. The sensitivity of this test for netrin-1 was detected at concentrations in the range of 31.25-2000 pg/mL. The intra-assay and inter-assay sensitivity for the test used for netrin-1 levels at all levels was less than 10%.

Statistical Analysis

Statistical analyses of this study were performed using IBM SPSS Statistics for Windows 26.0 (IBM Corp.-Armonk-NY-USA). Shapiro-Wilk test was used to determine the normal distribution of numerical variables. Numerical variables with normal distribution were shown as mean ± standard deviation, and numerical variables without normal distribution were shown as median (minimum-maximum). Number (n) and percentage (%) were used to show categorical variables. Pairwise comparisons were made with Student's t test for normally distributed variables and with Mann-Whitney U test for non-normally distributed groups. Comparative analyses of multiple groups were performed using ANOVA test (Post-hoc: Bonferroni test) or Kruskal-Wallis H test (Post-hoc: Dunn test). Analyses of categorical parameters were performed using chi-square test, Yates correction, and Fisher's exact test. The correlation relationship between netrin level and numerical variables was performed using Spearman correlation analysis. For all statistical analyses, the upper limit of $p \leq 0.05$ was considered statistically significant.

Results

A total of 60 patients with RA and 41 healthy volunteers were enrolled in the study. The mean (standard deviation) age at RA diagnosis was 40.8 (12.4) years. Of the control group, 29 (69%) were female, 13 (31%) were male, 42 (70%) of the patients were female and 18 (30%) were male. The mean age and gender of the patients were found to be similar when compared with the control group. Demographics of all participants and clinical characteristics of RA patients are presented in Table 1.

Rheumatoid Arthritis and Serum Netrin-1 Levels

The median (minimum-maximum) serum netrin-1 level was similar between RA and the control group [64.4 (35.8-1832.9) vs. 65 (35.8-436.6), p=0.786] (Table 1). When RA patients were divided into subgroups according to disease activity, no significant differences in netrin-1 levels were observed among patients with remission, low, moderate, or high disease activity (Table 2). No differences in netrin-1 levels were observed when patients were subgrouped according to treatment condition [treatment naive vs. conventional disease-modifying antirheumatic drugs (cDMARD) alone vs. biologic DMARD (bDMARD) with or without bDMARD] (Table 2).

Netrin-1 levels were similar between female and male RA patients, and between smokers and non-smokers (Table 3). When patients with and without seropositivity [positive RF and/or anti-citrullinated peptide (CCP)], family history, and lung nodules were compared, netrin-1 levels were found similar between groups (Table 3). The difference between netrin-1 levels and the presence of ILD was, however, close to clinical significance (p=0.083).

When the relationship between netrin-1 levels and demographics of RA patients was investigated, no significant correlations were observed between age, body mass index, symptom and diagnosis age, disease duration, and serum netrin-1 levels (Table 4). Netrin-1 levels also did not correlate significantly with disease activity scores and RAID scores (Table 4). When laboratory parameters at the time of the evaluation were considered, netrin-1 levels did not significantly correlate with kidney function tests, transaminases, hemoglobin and white blood count, acute phase reactants (ESR and CRP), rheumatoid factor, and anti-CCP titers (Table 4).

Table 1. Demographic characteristics, laboratory results and medical treatment data of RA and control groups

	RA patients (n=60)	Controls (n=41)	p
Age, years, mean ± SD	48.6±12.1	44.6±10.9	0.521
Gender, female, n (%)	42 (70.0)	29 (69.0)	0.874
BMI, kg/m^2, mean ± SD	22.4±3.8	24.5±5.4	0.456
Active smokers, n (%)	2 (3.3)	4 (9.7)	0.296
Serum Netrin-1 value, (pg/mL), median (min-max)	64.4 (35.8-551.4)	65 (35.8-436.6)	0.786
ESR (mm/h), median (min-max)	14.5 (9.6-55.8)	10.3 (6.2-38.6)	0.13
CRP (mg/L), median (min-max)	4.9 (0.5-26)	2.8 (0.4-16)	0.084
Age at the onset of RA symptoms, years, mean ± SD	39.3±12.8		
Family history of RA, n	22		
Age at the diagnosis of RA, years, mean ± SD	40.8±12.4		
Disease duration, years, median (min-max)	6 (1-49)		
Morning stiffness time, minute, mean ± SD	20.1±6.2		
Patients with ILD, n	4		
Hand deformity, n	21		
DAS-28-CRP, median (min-max)	2.2 (0.96-8.4)		
DAS-28-ESR, median (min-max)	2.5 (0.45-8.9)		
RAID score, median (min-max)	5 (1.2-9.4)		
CDAI, median (min-max)	8 (0-65)		
RF positivity, n	29		
Anti-CCP positivity, n	15		
Patients with active treatment, n			
Corticosteroids	42		
cDMARDs	41		
bDMARDs	12		
tsDMARDs	0		

Anti-CCP: Anti-citrullinated peptide, bDMARD: Biologic disease/modifying rheumatic drug, BMI: Body mass index, CDAI: Clinical Disease Activity Index, cDMARD: Conventional disease/modifying rheumatic drug, CRP: C-reactive protein, DAS-28-CRP: Disease activity score-28-CRP, ESR: Erythrocyte sedimentation rate, ILD: Interstitial lung disease, Min: Minimum, Max: Maximum, NSAID: Non-steroidal anti-inflammatory drugs, RA: Rheumatoid arthritis, RAID: Rheumatoid arthritis impact of disease, RF: Rheumatoid factor; SD: Standard deviation, tsDMARD: Target specific disease/modifying rheumatic drug

Discussion

This study aimed to investigate the use of netrin-1 as a biomarker in the differential diagnosis of RA from healthy controls and the relationships between netrin-1 and RA's disease activity scores and joint erosions. In the present study, netrin-1 levels were found to be similar between RA patients and healthy subjects. There was no association between netrin-1 levels and disease characteristics, demographics, disease activity, joint erosions or treatment agents, and no correlations with laboratory parameters. There was a difference between patients with and without ILD, yet it did not reach statistical significance.

Netrin-1 has been previously associated with different mechanisms through its receptors in angiogenesis,[16] kidney damage,[17] multiple myeloma,[18] lymphomas,[5] solid tumors,[19] wound healing,[20] and atherosclerosis.[21] There are limited studies in the literature evaluating the relationship of netrin-1 with rheumatological diseases. Mediero et al.[22] investigated the usability of the netrin-1 blockade in the treatment of inflammatory arthritis. They found that bone erosion was reduced in mouse models of RA treated with an anti-netrin-1 antibody, and there was a significant reduction in osteoclasts on histological examination. In addition, Maruyama et al.[23] measured netrin-1 levels in the joint fluid of RA patients and found high levels of netrin-1. At the same time, it was observed in this study that netrin-1 inhibited osteoclast formation and was effective in increasing bone mass by stimulating osteoblasts. In another study evaluating serum netrin-1 levels between familial Mediterranean fever (FMF) patients (n=42) and healthy controls (n=44), serum netrin-1 levels were found to be similar between the two groups (p=0.19).[24] In another study, netrin-1 serum levels were compared in scleroderma and systemic sclerosis (SSc) patients (n=56) and healthy controls (n=58), and netrin-1 levels were found to be significantly higher in SSc patient serum [respectively, 268.8 (82-8-1006.6) pg/mL and 108.6 (21.02-351.5) pg/mL, p<0.0001].[11]

In this study, we investigated the utility of netrin-1 as a biomarker in the diagnosis of RA and disease activity. Our results demonstrated that netrin-1 levels were similar in the serum of RA patients compared to healthy controls. We

Table 2. The relationship between serum Netrin-1 values, disease activities and treatment condition.

Netrin-1 value, pg/mL, median (min-max)	**DAS-28 disease activity**					**p**
	Control (n=41)	Remission (n=35)	Low (n=11)	Moderate (n=11)	High (n=3)	
	65 (35.8-436.6)	67.7 (38.1-551.4)	61.2 (35.9-305.6)	60.8 (36.2-133.5)	70.6 (52.3-187.1)	0.809
	CDAI disease activity					**p**
	Control (n=41)	Remission (n=17)	Low (n=24)	Moderate (n=14)	High (n=5)	
	65 (35.8-436.6)	54.1 (38.1-501.9)	87.5 (35.9-551.4)	58.4 (41.6-133.5)	73.1 (41.8-187.1)	0.240
	Treatment status					**p**
	Naive treatment (n=5)	Alone cDMARD (n=43)	bDMARD with/without cDMARD (n=12)			
	70.6 (46.9-501.9)	57.3 (35.9-551.4)	71.3 (41.8-273.4)			0.735

bDMARD: Biologic disease/modifying rheumatic drug, CDAI: Clinical Disease Activity Index, cDMARD: Conventional disease/modifying rheumatic drug, DAS-28: Disease activity score-28, Min: Minimum, Max: Maximum, tsDMARD: Target specific disease/modifying rheumatic drug

Table 3. Netrin-1 levels according to clinical characteristics of RA patients

		n	Netrin-1, pg-mL, median (min-max)	p
Sex	Female	42	71.8 (35.9-501.9)	0.302
	Male	18	55.0 (36.3-551.4)	
Seropositivity	Seropositive	44	64.4 (35.9-551.4)	0.810
	Seronegative	16	65.5 (38.1-273.4)	
	Control group	41	65 (35.8-436.6)	
RF positivity	Absent	31	61.1 (35.8-551.4)	0.819
	Present	29	67.6 (32.3-501.9)	
Anti-CCP positivity	Absent	45	70.5 (35.8-551.4)	0.453
	Present	15	57.3 (36.3-305.6)	
Interstitial lung disease	Absent	56	61.0 (35.9-501.9)	0.083
	Present	4	284.4 (41.8-551.4)	
Pulmonary nodules	Absent	55	61.2 (35.9-551.4)	0.402
	Present	5	75.3 (41.8-295.4)	
Hand deformity	Absent	21	60.1 (35.8-305.6)	0.361
	Present	37	70.5 (38.5-551.3)	

Anti-CCP: Anti-citrullinated peptide, RA: Rheumatoid disease, RF: Rheumatoid factor, Min: Minimum, Max: Maximum

evaluated disease activity by DAS-28 CRP, DAS-28 ESR, and CDAI. When patients were subgrouped according to activity state, netrin-1 levels were similar. Furthermore, netrin-1 levels did not correlate with DAS-28 CRP, DAS-28 ESR, and CDAI scores.

The absence of RF or anti-CCP antibodies at a rate of 10-30% in RA patients[25] and the lack of markers that can be used in disease activation have revealed the necessity of investigating new autoantibodies that may be diagnostic or an indicator of activation in RA. In our population, 73.3% of the patients had seropositive RA, 26.7% had seronegative RA, and we observed no statistically significant difference between seropositive RA and seronegative RA in terms of netrin-1 level. To the best of our knowledge, there is no study in the literature regarding whether seropositivity affects netrin-1 levels in RA patients.

The effect of drugs on netrin-1 levels has not been evaluated before in the literature. RA patients included in our study were divided into three main groups according to the treatments they were receiving. The patients in the first group were treatment naïve; the second group was using conventional DMARDs but not biological DMARDs; the third group comprised RA patients using biological DMARDs with or without conventional DMARDs. There was no statistically significant difference between the treatments received by the patients and serum netrin-1 levels.

Acute phase reactants (CRP and ESR) are important markers in RA, correlating with disease severity and activity.[26] Similar to our study, Maraş et al.[11] did not detect a significant correlation between CRP and ESR and netrin-1 in SSc Kerget et al.[27] found a weak correlation between serum netrin-1 level and CRP levels of patients admitted during

Table 4. Correlations between clinical features, laboratory parameters, and netrin-1 levels in RA patients

	Netrin-1	
	r	p
Age, years	0.155	0.238
BMI, kg/m^2	0.117	0.371
Age at the onset of RA symptoms, years	0.009	0.943
Age at the diagnosis of RA, years	0.018	0.889
Disease duration, years	0.238	0.068
DAS-28 CRP	0.041	0.757
DAS-28 ESR	0.038	0.776
CDAI	0.039	0.769
RAID score	0.049	0.710
ESR	0.161	0.218
CRP	0.023	0.862
Creatinin	0.016	0.905
GFR	-0.183	0.162
RF	0.116	0.386
Anti-CCP	0.054	0.685

ALT: Alanine aminotransferase, Anti-CCP: Anti-citrullinated peptide, AST: Aspartate aminotransferase, BMI: Body mass index, CRP: C-reactive protein, DAS-28-CRP: Disease activity score-28/C-reactive protein, DAS-28-ESR: Disease activity score-28/erythrocyte sedimentation rate, ESR: Erythrocyte sedimentation rate, GFR: Glomerular filtration rate, HB: Hemoglobin, NEU: Neutrophil, RAID: Rheumatoid arthritis impact of disease, RF: Rheumatoid factor, WBC: White blood count

the active period of chronic obstructive pulmonary disease. It has not been previously evaluated in RA patients. Our results did not indicate such a relation between acute phase reactants and netrin-1 levels. Additionally, no correlations were observed between RF/anti-CCP and netrin-1 levels. However, a positive correlation was observed between netrin-1 and neutrophil levels.

RA primarily targets the synovium, yet various extra-articular manifestations may occur during the disease course. Accordingly, we subgrouped our patients regarding the presence of ILD, lung nodules, secondary Sjögren's syndrome, xerostomia, xerophthalmia, and Raynaud's phenomenon. The presence of extra-articular manifestations was not related to netrin-1 levels in our study except for the fact that, although no statistically significant results were found in terms of netrin-1 serum levels in patients with ILD (n=4) compared to patients without ILD (n=56), the median values of netrin-1 were found to be significantly higher in patients with ILD than in patients without ILD [respectively, 284.4 (41.8-551.4) pg/mL, 61.0 (35.9-501.9) pg/mL, p=0.083]. Although the small number of people in the ILD subgroup may affect the accuracy of the statistical results, some studies have provided evidence that netrin-1 may be associated with the development of lung fibrosis. These studies have shown that netrin-1 promotes the development of fibrosis in bleomycin-induced mouse lung and human SSc lung cell cultures.[28,29] It has also been reported that the expression of M2 macrophage, which increases extracellular matrix protein synthesis by stimulating profibrotic cytokine synthesis,[30,31] is induced by netrin-1.[32-34] These results can be speculated to reflect that netrin-1 may have a place in the process of lung fibrosis.

Study Limitations

Limitations of this study include the fact that it is a cross-sectional study, the small number of subjects in RA subgroups, selection bias of individuals in the control group, or generalizability to all RA populations. In our study, although the median values of netrin-1 were higher in the group with ILD, no statistically significant results were obtained. The reason for this may be that the statistical results are affected due to the small number of subjects in the subgroups. Although it was shown in this study that netrin-1 was not associated with the diagnosis of RA and disease activation, the close relationship of netrin-1 with fibrotic processes makes it necessary to investigate its relationship with ILDs due to RA with larger-scale prospective studies.

Conclusion

In conclusion, this study showed that netrin-1 was similar in the serum of RA patients compared to healthy controls and that there was no significant correlation between serum netrin-1 levels and RA disease activation scores. In addition, our results showed that the median values of netrin-1 were higher in patients with ILD, although there was no statistically significant difference.

Ethics

Ethics Committee Approval: The study was approved by the Ankara City Hospital Ethics Committee IRB no: E1/2087/2021, date: 03.11.2021.

Informed Consent: Informed consent form was obtained from all participants in the study.

Authorship Contributions

Concept: G.Ö., Ş.E., S.C.G., A.K., F.E., P.A.D., Y.M., İ.D., K.O., O.K., S.N., Ö.E., Design: G.Ö., Ş.E., S.C.G., A.K., F.E., P.A.D., Y.M., İ.D., K.O., O.K., S.N., Ö.E., Data Collection or Processing: G.Ö., Ş.E., S.C.G., A.K., F.E., P.A.D., Y.M., İ.D., K.O., O.K., S.N., Ö.E., Analysis or Interpretation: G.Ö., Ş.E., S.C.G., A.K., F.E., P.A.D., Y.M., İ.D., K.O., O.K., S.N., Ö.E., Literature Search: G.Ö., Ş.E., S.C.G., A.K., F.E., P.A.D., Y.M., İ.D., K.O., O.K., S.N., Ö.E., Writing: G.Ö., Ş.E., S.C.G., A.K., F.E., P.A.D., Y.M., İ.D., K.O., O.K., S.N., Ö.E.

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