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Original Article

Serum netrin-1 and netrin receptor levels in fibromyalgia and osteoarthritis

Alparslan Köhle[1], Arif Gülkesen[2], Tuba Kaya Karataş[3], Gürkan Akgöl[2], Ahmet Karataş[4], Necip İlhan[3], Süleyman Serdar Koca[4]

[1]Department of Internal Medicine, Maçka Ömer Burhanoğlu Physical Medicine and Rehabilitation Hospital, Trabzon, Türkiye
[2]Department of Physical Medicine and Rehabilitation, Fırat University Faculty of Medicine, Elazığ, Türkiye
[3]Department of Biochemistry, Fırat University Faculty of Medicine, Elazığ, Türkiye
[4]Department of Internal Medicine, Division of Rheumatology, Fırat University Faculty of Medicine, Elazığ, Türkiye

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ABSTRACT

Objectives: This study aims to define serum levels of netrin-1 and netrin receptors in patients with fibromyalgia (FM) and osteoarthritis (OA).

Patients and methods: This cross-sectional study was conducted with a total of 150 female participants (mean age: 47.2±16.1 years; range, 18 to 89 years) at Fırat University between June 2016 and December 2016. The participants were evaluated in three groups: the FM group with 50 patients, the OA group with 50 patients, and the control group, which included 50 healthy volunteers. Netrin-1, netrin receptors (DCC, UNC5B, and UNC5D), interleukin (IL)-6, IL-10, and IL-17 levels were analyzed by the enzyme-linked immunosorbent assay from the serum samples of the participants.

Results: The level of serum netrin-1 was significantly lower in the FM group than in the control and OA groups ($p<0.01$ and $p<0.001$, respectively). However, the difference between patients with OA and healthy controls in terms of netrin-1 was not statistically significant ($p>0.05$). In addition, serum levels of netrin receptors and cytokines in the FM group were similar to the control group ($p>0.05$). However, serum DCC, UNC5D, IL-6, and IL-10 levels were higher in the OA group compared to the control group ($p<0.001$, $p<0.05$, $p<0.01$, and $p<0.001$, respectively).

Conclusion: Serum netrin-1 level is suppressed in FM, which suggests that netrin-1 is influential in FM pathogenesis.

Keywords: Cytokine, fibromyalgia, netrin-1, osteoarthritis.

Fibromyalgia (FM) is a recurrent pain syndrome affecting the musculoskeletal system characterized by widespread pain and joint stiffness in sensitive areas, accompanied by systemic symptoms such as anxiety, fatigue, sleep disorders, depression, dysmenorrhea, headache, paresthesia, and cognitive disorders, without a defined organic disease and inflammatory cause.[1-3] Fibromyalgia has been identified in all ethnic groups, ages, and both sexes, but it is more common in females.[4-6] It has been suggested that the etiology and pathogenesis of FM are multifactorial. Additionally, in the light of recent studies, central nervous system sensitization is thought to be a significant pathophysiological mechanism of FM.[4-6]

Osteoarthritis (OA), the most common joint disease, is a progressive, noninflammatory disease that concerns the entire joint, including the joint cartilage, subchondral bone, ligaments, meniscus, and synovium.[7-9] It is characterized by the progressive destruction of articular cartilage due to the

Corresponding author: Süleyman Serdar Koca, MD. Fırat Üniversitesi Tıp Fakültesi İç Hastalıkları Anabilim Dalı, Romatoloji Bilim Dalı, 23119 Elazığ, Türkiye.
e-mail: sskoca@firat.edu.tr

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disintegration of the balance between construction and destruction in cartilage homeostasis for mechanical, biological, chemical, molecular, and enzymatic reasons. Enzymes (metalloproteinases), cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-1β, and IL-6), and growth factors (transforming growth factor [TGF]-β and insulin-like growth factor play a crucial role in the dynamic balance between anabolic and catabolic processes in the joint cartilage.[7-9]

Netrins are proteins related to the extracellularly located laminin and manage cell and axon migration during embryogenesis.[10] Netrins have a role in embryonic development, including functions in cell migration, axon guidance, angiogenesis, and morphogenesis, in animals and humans. Secreted netrin-1 is attractive for some cells and repulsive for some other cells.[11] In addition, netrin-1 is an autocrine and paracrine organizer of osteoclast differentiation. Moreover, it promotes inflammation by affecting macrophages at inflamed areas and inhibits migration of neutrophils, lymphocytes, and monocytes.

Netrin receptors belong to the family of immunoglobulins encoded by a large group of proteins that direct cell adhesion and signal transduction. Netrin receptors belong to three main families of receptors called Down Syndrome Cell Adhesion Molecule (DSCAM), Deleted in Colorectal Cancer (DCC), and UNC5 (UNC5A, B, C, and D).[12-14] The DCC gene has been found at high rates in the central nervous system. It was also observed in the prostate, basal lamina of the skin, lung, gastrointestinal tract, and bladder epithelium, and it participates in the axon and transmembrane protein guidance. Vertebrae have four homologous netrin-1 receptors, UNC5A through UNC5D. The UNC5 proteins are transmembrane proteins. The DCC receptors act attractive and also participate in the repulsive function, while UNC5 acts repulsive.[10-13] Netrin-1 and DCC receptors have been shown to have a possible role in inducing sensory innervation and OA pain.[15] It has also been demonstrated that UNC5B and UNC5C increased in patients with OA.[16] In addition, some cytokines, such as IL-8 and IL-6, have been reported to be responsible for hyperalgesia in patients with FM.[17] Considering the effects of netrin and its receptors on cytokines and their relationship with pain, we aimed to evaluate their levels in FM and OA patients in our study.

PATIENTS AND METHODS

The cross-sectional study was conducted with a total of 150 female participants (mean age: 47.2±16.1 years; range, 18 to 89 years), who were evaluated in three groups (the FM group, OA group, and control group), at Fırat University Faculty of Medicine between June 2016 and December 2016. The FM group included 50 patients diagnosed with FM according to the 2010 ACR diagnostic criteria[14] who applied to the Rheumatology and Physical Medicine and Rehabilitation outpatient clinics. The OA group was contained 50 patients diagnosed with OA according to the ACR criteria[18] who applied to the Rheumatology and Physical Medicine and Rehabilitation outpatient clinics. The control group included 50 healthy volunteers in the same age range as the other two groups. Patients under the age of 18, patients with systemic inflammatory rheumatic disease, patients who could not be contacted, and those with acute or known clinically interfering disease, such as malignancy, thyroid or endocrine disease, and osteoporotic fracture, and pregnant women were excluded from the study. A locomotor system examination with medical history-taking was conducted in all participants. The patients were questioned for their treatments and complications. The body weight and height of all participants were measured.

Blood samples were obtained from all participants after 12 h of fasting for laboratory tests. Complete blood count, urea, creatinine, alanine aminotransferase, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) values of the participants were recorded from the files. Antinuclear antibody, rheumatoid factor, CRP, uric acid, and thyroid hormone levels were determined to exclude possible autoimmune and rheumatological diseases. In addition, 5 mL of blood was obtained from the patients and centrifuged for 5 min at 5000 rpm, and the serum sample was stored at -20°C until laboratory analysis. In these serum samples, netrin-1, UNC5 homologous family, DCC family, IL-17, IL-10, and IL-6 levels were analyzed by the enzyme-linked immunosorbent assay (ELISA).

Serum netrin-1 (Elabscience, Wuhan, China; catalog no: E-EL-H2328), Human UNC5B (Elabscience, Wuhan, China; catalog no: E-EL-H2631), Human UNC5D (Elabscience, Wuhan, China; catalog no: E-EL-H2628), Human DCC (Elabscience, Wuhan, China; catalog no: E-EL-H2639), IL-6 (DIAsource ImmunoAssays S.A, Ottignies-Louvain-la-Neuve, Belgium; catalog no: KAP1261), IL-10 (DIAsource ImmunoAssays S.A, Ottignies-Louvain-la-Neuve, Belgium; catalog no: KAP1321), and IL-17 (Elabscience, Wuhan, China;

catalog no: E-EL-H0105) levels were studied in accordance with the ELISA method using the appropriate commercial kit.

Statistical analysis

Statistical analyses were conducted using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). The continuous data were presented as mean ± standard deviation. Normal distributions were tested with the Kolmogorov-Smirnov test. The difference of continuous variables among the groups was examined by one-way analysis of variance (ANOVA) and Kruskal-Wallis variance analysis for parametric data with normal distributions and nonparametric data, respectively. In dual comparisons, Mann-Whitney U test was used for nonparametric data, and Student's t-test was utilized for parametric data. Categorical data were evaluated by the chi-square test. Pearson correlation analysis was used for the correlation analysis. ANCOVA test were used for the comparison of the netrin-1 levels by means of age, ESR, CRP, creatinine, and fasting blood glucose parameters. The diagnostic efficacies of netrin-1 and netrin receptors for FM were examined by the receiver operating characteristic analysis. A p value of <0.05 was considered significant.

RESULTS

The OA patients were older due to the nature of the disease, and the mean age of the control group was lower (p<0.001). Similarly, the mean age of the FM patients were higher than the healthy controls (p<0.001; Table 1). Fasting blood glucose and creatinine levels were higher in the OA group compared to the control group and FM group (p<0.001 and p<0.05, respectively; Table 1). In terms of these parameters, the differences between the FM group and the control group were not significant.

There was no difference between groups in hematological tests. Compared to the FM group and the control group, CRP (p>0.05 and p<0.05, respectively) and ESR (p<0.05 and p<0.01, respectively) levels were higher in the OA group (Table 1). There was no significant difference between the FM group and the control group in these parameters. Similarly, there was no significant difference in serum IL-17, IL-10, and IL-6 levels between the FM group and the control

TABLE 1
Laboratory results of the study groups

	Healthy controls (n=50)	Osteoarthritis (n=50)	Fibromyalgia (n=50)	
Variables	Mean±SD	Mean±SD	Mean±SD	p^a
Age (year)	34.2±13.1	62.7±9.4***	44.9±10.7***/×××	<0.001[b]
FBG (mg/dL)	91.3±11.8	113.3±40.3***	92.2±14.3×××	<0.001[b]
Creatinine (mg/dL)	0.6±0.1	0.7±0.1*	0.62±0.2×	0.005[b]
Hemoglobin (g/dL)	13.1±0.9	13.1±1.1	12.8±1.2	0.323[b]
Leukocyte (10^3/μL)	6.4±1.6	7.1±2.2	6.3±2.1	0.066[b]
Platelet (10^3/μL)	281±89.6	283.7±76.9	295.4±81.8	0.672[b]
ESR (mm/h)	13.2±11.2	24.4±14.6*	16.2±10.6××	0.001[c]
CRP (mg/L)	4.7±3.6	6.6±6.3	4.3±2.5×	0.047[c]
IL-6 (pg/mL)	39.0±21.6	102.7±145.6**	57.9±64.7×	0.003[c]
IL-17 (pg/mL)	157.0±124.4	158.5±81.6	180.5±109.6	0.468[c]
IL-10 (pg/mL)	11.6±1.9	15.8±7.4***	12.8±2.2××	<0.001[c]
Netrin-1 (pg/mL)	134.7±23.5	138±14.1	121.7±26.2**/×××	0.001[b]
DCC (pg/mL)	475.6±159.8	906.0±422.2***	631.4±349.4×××	<0.001[c]
UNC 5B (ng/mL)	1.9±1.4	2.2±1.1	1.7±1.1	0.105[c]
UNC 5D (ng/mL)	5.3±1.0	5.7±0.6*	5.5±0.7	0.023[b]

SD: Standard deviation; FBG: Fasting blood glucose; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; IL: Interleukin; DCC: Deleted in colorectal cancer; UNC: Netrin receptor.
[a] Parametric data with normal distribution were analyzed by ANOVA and Kruskal Wallis variance analysis was performed for analysis of nonparametric data. Moreover, Mann-Whitney U test for nonparametric data[c] and student's t test for parametric data[b] were selected for dual comparisons.
Compared with the control group: * p<0.05; ** p<0.01; *** p<0.001.
Compared with the osteoarthritis group; × p<0.05, ×× p<0.01, ××× p<0.001.

group. However, IL-10 ($p<0.001$, $p<0.01$, respectively) and IL-6 ($p<0.01$ and $p<0.05$, respectively) levels in the OA group were higher than in the control group and the FM group.

The serum netrin-1 level in the FM group was significantly lower than in the control and OA groups ($p<0.01$ and $p<0.001$, respectively; Figure 1). There was no significant difference in serum netrin-1 levels between the control and OA groups (Table 1). The statistical analyzes were reperformed by the ANCOVA test as there were differences between the groups in terms of mean age, ESR, CRP, creatinine, and fasting blood glucose levels. Even though the mean age, ESR, CRP, creatinine, and fasting blood glucose levels were corrected, serum netrin-1 level was lower in the FM group than in both the control and OA groups ($p<0.01$ for both groups).

No significant differences were found between netrin receptors (DCC, UNC5B and UNC5D) levels between the FM and control groups (Figure 2a-c). However, the DCC in the OA group was higher compared to the control and FM groups ($p<0.001$ for both groups), whereas UNC5D level was higher compared to the control group only ($p<0.05$; Table 1). Even after correction for age and fasting blood glucose with the ANOVA test, the DCC level was statistically significantly higher in the OA group compared to the control and FM groups ($p=0.001$ and $p=0.005$, respectively).

Serum netrin-1 level and DCC level correlated in the control and OA groups ($r=0.285$, $p=0.045$ and $r=0.388$, $p=0.005$, respectively). In the FM group, netrin-1 level and UNC5B level ($r=0.332$, $p=0.018$) correlated positively. In addition, in the control group, serum netrin-1 level and IL-6 level correlated positively ($r=0.326$, $p=0.012$). Serum UNC5B level correlated negatively with fasting blood glucose level in the control group ($r=-0.347$, $p=0.015$). In the OA group, serum UNC5D level correlated negatively with age ($r=-0.282$, $p=0.049$).

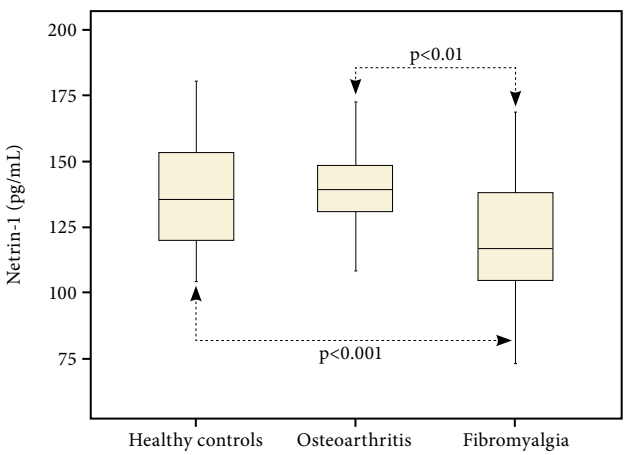


Figure 1. Netrin-1 levels of the groups.

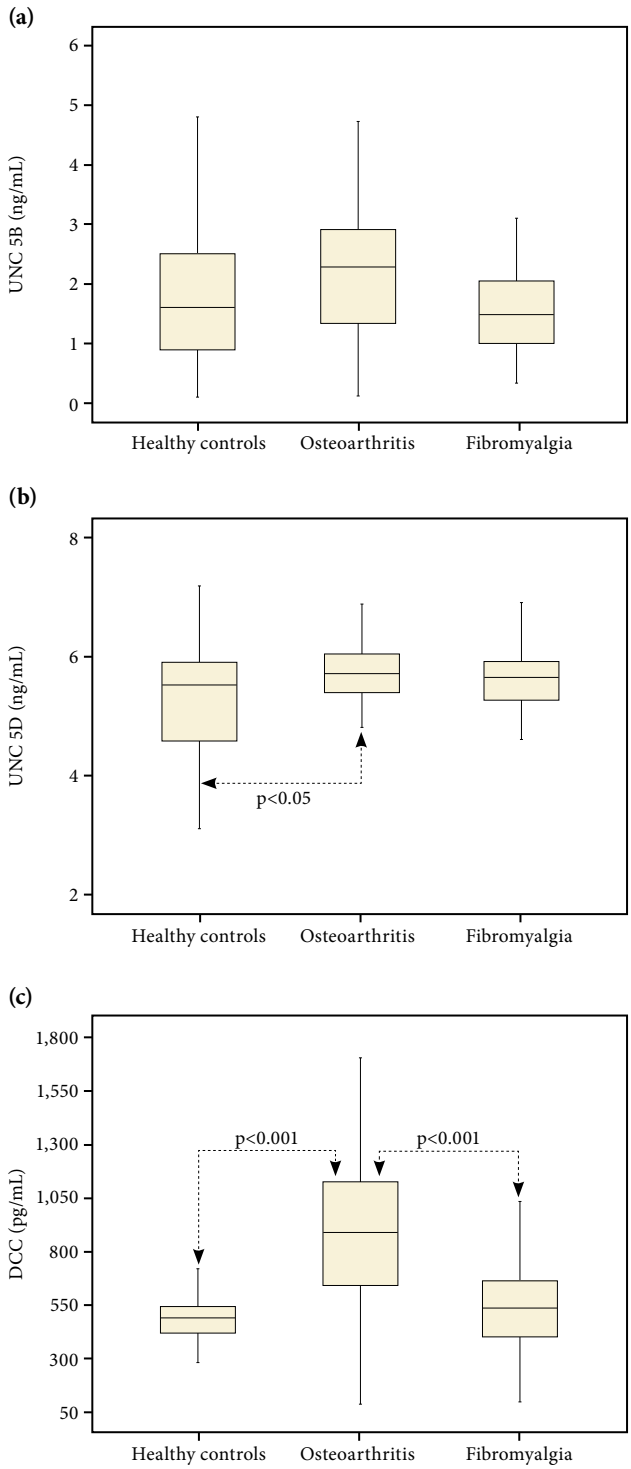
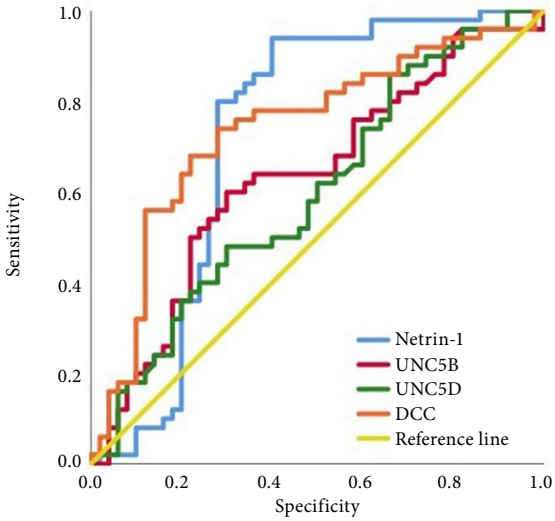


Figure 2. The levels of netrin receptors **(a)** UNC5B, **(b)** UNC5D, and **(c)** DCC.

UNC: Netrin receptor; DCC: Deleted in colorectal cancer.



	Area under curve	p values	95% CI
Netrin-1	0.726	<0.001	0.617-0.835
UNC5B	0.633	0.022	0.523-0.743
UNC5D	0.598	0.090	0.487-0.710
DCC	0.739	<0.001	0.638-0.839

Figure 3. Receiver operating characteristic curves of netrin-1 and DCC levels differentiate FM from OA.

CI: Confidence interval; DCC: Deleted in colorectal cancer; UNC: Netrin receptor; FM: Fibromyalgia; OA: Osteoarthritis.

Based on the receiver operating characteristic analysis, the netrin-1 level (using a cut-off of 128.8 pg/mL) differentiated FM from OA, with a sensitivity and specificity of 80% and 72%, respectively. Moreover, the DCC level (using a cut-off of 700.9 pg/mL) differentiated FM from OA, with a sensitivity and specificity of 72%. These results indicate that netrin-1 (area under the curve [AUC]: 0.726; 95% CI: 0.617-0.835; p<0.001) and DCC (AUC: 0.739; 95% CI: 0.638-0.839; p<0.001) were sensitive and specific markers for FM (Figure 3).

DISCUSSION

Netrin-1 is a critical molecule in the development of cortical neurons and spinal commissural neurons. During embryonic development, the attractive function of netrin-1 requires the DCC receptor. The UNC5H2 receptor is required for the repulsive task of netrin-1. The expression of the DCC receptor in adult spinal cord neurons is less than UNC5H2 compared to embryonic spinal cord neurons. Netrin-1 release increases after spinal cord injuries. Inflammatory cells in the wound play an important role in increasing the expression of netrin-1. Suppression of netrin-1 release from spinal dorsal horn neurons (sensory nerves) has been shown to increase induced mechanical allodynia.[19] Widespread pain is frequently reported in FM.[3] In our study, serum netrin-1 level was found to be low in FM patients.

Bennett et al.[20] discovered that circulating somatomedin C levels are low in FM patients. Somatomedin C is the mediator of the anabolic effects of the growth hormone and is required for normal muscle hemostasis. Chronic low level of somatomedin reduces growth hormone release and causes muscle pain. In addition, the effects of netrin-1 on growth have been established.[12,13] It is possible that the effects of low netrin-1 levels are more prominent compared to the growth hormone in the pathogenesis and clinical severity of FM.

Bengtsson et al.[21] reported a decrease in the levels of adonesintriphosphateand phosphocreatine in FM patients. This can cause postexercise pain and other painful conditions in patients. Netrin-1 binds directly to DCC; however, in some systems, adenosine A2b receptor (A2bR) acts as a coreceptor.[22] Activation of A2bR increases the level of cyclic adenosine monophosphate (cAMP) and increases the attractive chemical response to netrin-1.[12,13,22] In another study, it was found that netrin-1 limits neutrophil accumulation in the intestinal mucosa and increases this effect by binding to A2bR and increasing the level of cAMP.[23] Furthermore, Galeotti et al.[24] demonstrated an increase in the level of cAMP in FM patients. The low netrin-1 detected in the FM patients may have altered the cAMP level in our study.

Netrin-1 manages the inflammatory response of macrophages and neutrophils. Ischemia in the kidney increases prostaglandin E2 (PGE2) production from neutrophils, and PGE2 increases IL-17 production. Interleukin-17 production also increases interferon-gamma production. Netrin-1 suppresses this path by blocking the production of PGE2. It protects the kidney by suppressing inflammation and neutrophil infiltration and preventing apoptosis from ischemia-reperfusion injury.[25] Interleukin-17A receptor activation has been found to induce the release of chemokines and matrix metalloproteinases (MMPs).[26-28] Moreover, IL-17 has been shown to induce pain and neutrophil migration.[29]

Netrin-1 is broken down by MMPs located in the region close to the plasma membrane. Therefore, MMP inhibitors increase the effectiveness of netrin-1.[12,22]

Matrix metalloproteinases can be held responsible for decreasing the netrin-1 level in FM patients. Netrin-1 plays a crucial role not only in neural development but also in tissue organization, differentiation, cell survival, mobility, proliferation, cell adhesion, and cancer.[30] Netrin-1 only acts as axon attraction when connected to DCC. When the UNC5 receptor and DCC receptor bind, the UNC5 receptor closes and acts as a repellant.[31] In a study, UNC5B and UNC5C levels were determined to be high in synovial fluids of patients with OA and rheumatoid arthritis.[16] In our study, serum netrin-1 levels did not increase in OA, whereas DCC and UNC5D levels, which were among the netrin receptors, increased.

The etiopathogenesis of FM is unknown. Viral infections, trauma, psychopathological causes, immunological mechanisms, and genetic predisposition are thought to be effective in its pathogenesis.[32,33] It has been reported that FM symptoms are induced by the interaction between the sympathetic nervous system, the immune system, and the hypothalamic-pituitary-adrenal axis.[34] Although various cytokines are suspected of playing a role in pathogenesis, the possible roles of cytokines have not been adequately clarified.[33,35] Cytokines, such as TNF-β, IL-8, IL-6, and IL-1α, have been shown to directly cause peripheral and central neuropathic pain.[32,33] Pain is one of the most crucial symptoms of inflammation and also the most important symptom in FM. Wallace et al.[17] reported that IL-6 and IL-8, which play a role in inflammation, are responsible for hyperalgesia in FM patients. In patients with FM, serum IL-8 level of proinflammatory cytokines was higher than in healthy controls. No significant difference was found between the control group and FM patients in IL-10 and IL-2 levels. Gür et al.[33] did not find a difference in serum IL-1 and IL-6 values. However, they found a significant difference in IL-8 and IL-2 levels. They reported that the role of IL-8 is major in the development of pain in FM.

Chronic inflammation of cells is not evident in OA. The number of leukocytes is usually not high in the joint fluid. Cytokines play an important role in the inflammatory component of OA.[8] The role of inflammatory cytokines, such as TNF-α, IL-18, IL-15, IL-1β, and IL-6, and anti-inflammatory cytokines, such as IL-13, IL-10, and IL-4 in OA pathogenesis is still being investigated.[36]

Six cytokines (IL-17A-F) and five receptors (IL-17RA-E) belonging to the IL-17 family have been identified. Interleukin-17 was found to be high in synovial fluid and serum in OA patients. Interleukin-17 inhibits the production of proteoglycans synthesized by chondrocytes and supports the production of MMPs. It causes the secretion of other cytokines that negatively affect the cartilage. Interleukin-6 was determined to be high in serum and synovial fluids of OA patients. It reduces the synthesis of type 2 collagen and increases enzyme production from MMPs. One of the major cytokines causing changes in subchondral bone is IL-6. Interleukin-10 acts as a chondrocyte protector in OA, activates type 2 collagen synthesis, and increases proteoglycan synthesis. It also blocks the synthesis of MMPs and prevents the apoptosis of chondrocytes.[36] In a study, IL-6 and IL-10 levels were revealed to be higher in OA.[37]

Netrin-1 may demonstrate different effects through receptors DCC and UNC5B.[38,39] In our study, a correlation was found between netrin and its receptors in patients with FM and OA but not in the control group. This result may be a sign of the complex relationship between netrin and its receptors.

The inflammatory cells and various cytokines may be involved in the pathogenesis of FM and OA. Thus, serum IL-17, IL-10, and IL-6 levels were investigated in FM and OA patients to evaluate their possible role in the pathophysiology of FM and OA. In our study, serum cytokine levels did not increase in the FM group. However, serum IL-6 and IL-10 levels were higher in the OA group. Conversely, IL-10 was decreased in OA patients in studies.[40] However, it has been shown that the IL-10 level of OA patients increased with exercise.[41] Our result may be related to the exercise habits of the OA patients included in the study.

The main limitations of this study are its cross-sectional design and that the power analysis was not performed before enrolment in the study. However, the sample size is accepted as satisfactory since the power is above 0.8.

In conclusion, it was found that netrin-1 and its receptors were low in FM patients, which suggests that netrin-1 is involved in the pathogenesis of pain in FM patients. More comprehensive studies with a large sample size are needed to explore the role of netrin-1 and its receptors in FM.

Ethics Committee Approval: The study protocol was approved by the Fırat University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee (2016-17/08). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient Consent for Publication: A written informed consent was obtained from all participants.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Conception and design: A.K., S.S.K.; Designed and performed experiments: A.K., A.G., T.K.K., G.A., A.K., N.İ.; Wrote and revised of the manuscript: A.K.., S.S.K.; Conducted the laboratory procedures: T.K.K., N.İ.; Data acquisition and analysis: A.K., A.G., T.K.K., G.A., A.K., S.S.K.; Writing and revising of the manuscript critically: S.S.K.; All authors read and approved the final version of the manuscript.

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