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RESEARCH

The relationship between neuropathic pain and serum endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, VEGF-A levels in fibromyalgia patients and molecular docking results

Fibromiyalji hastalarında nöropatik ağrı ile serum endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, VEGF-A düzeyleri arasındaki ilişki ve moleküler docking sonuçları

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Abstract

Purpose: The most important clinical finding of fibromyalgia syndrome (FMS) is pain. Its etiology has not been fully elucidated. This study was planned to determine the relationship between endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-a, VEGF-A levels and pain/neuropathic pain in FMS patients.

Materials and Methods: Forty-four FMS patients who met the inclusion criteria and 44 age-matched premenopausal healthy controls were recruited. The fibromyalgia group was evaluated in terms of Visual Analog Scale, Beck Depression Scale, Beck Anxiety Scale, Fibromyalgia Impact Questionnaire and LANSS Pain Scale. Serum endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A values were determined by the ELISA method. Protein-protein interaction was evaluated by molecular docking analysis. Bioinformatics analysis was performed using the STRING v 11.5 protein interaction tool.

Results: Endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A were significantly higher in FMS patients than the control group. 24 of 44 patients had neuropathic pain. No correlation was found between pain/neuropathic pain and serum markers levels. High interaction and homology scores of the proteins were defined.

Conclusion: The pain/neuropathic pain relationship of these markers could not be determined, but the calculated binding energies and activities of the proteins provided important clues for future studies.

Keywords:. Cytokine, fibromyalgia, molecular docking, neuropathic pain

Öz

Amaç: Fibromiyalji sendromunun (FMS) en önemli klinik bulgusu ağrıdır. Etiyolojisi tam olarak aydınlatılamamıştır. Bu çalışma FMS hastalarında endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-a ve VEGF-A düzeylerinin ağrı/nöropatik ağrı ile ilişkisini belirlemek amacıyla planlandı.

Gereç ve Yöntem: Çalışmaya dâhil edilme kriterlerini karşılayan 44 FMS hastası ve yaşları eşleştirilmiş 44 premenopozal sağlıklı kontrol alındı. Fibromiyalji grubu Visual Analog Ölçeği, Beck Depresyon Ölçeği, Beck Anksiyete Ölçeği, Fibromiyalji Etki Anketi ve LANSS Ağrı Ölçeği açısından değerlendirildi. Serum endokan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α ve VEGF-A değerleri ELISA yöntemi ile belirlendi. Protein-protein etkileşimi, moleküler docking analizi ile değerlendirildi. Biyoinformatik analiz, STRING v 11.5 protein aracı kullanılarak yapıldı.

Bulgular: Endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α ve VEGF-A FMS hastalarında kontrol grubuna göre anlamlı olarak yüksekti. 44 hastanın 24'ünde nöropatik ağrı vardı. Ağrı/nöropatik ağrı ile serum belirteçleri arasında korelasyon saptanamadı. Proteinlerin yüksek etkileşim ve homoloji skorları tanımlandı.

Sonuç: Bu belirteçlerin ağrı/nöropatik ağrı ilişkisi belirlenememiştir ancak proteinlerin hesaplanan bağlanma enerjileri ve aktiviteleri ilerideki çalışmalar için önemli ipuçları sağlamıştır.

Anahtar kelimeler: Sitokin, fibromiyalji, moleküler yerleştirme, nöropatik ağrı

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INTRODUCTION

Fibromyalgia Syndrome (FMS) is a chronic musculoskeletal disease of unknown etiology, characterized by widespread body pain, leading to loss of work force and deterioration in quality of life. The most common symptoms in FMS are widespread pain, morning stiffness, morning tiredness and sleep disturbance. In most of the patients, complaints such as sleep disturbance, fatigue, stiffness, drowsiness, depression, dry mouth-dry eyes, irritable bowel syndrome, dysmenorrhea, urethral syndrome, palpitation, headache and cognitive disorders may accompany widespread pain[1,2].

Pain is one of the frequent complaints of patients in their daily lives. Acute pain is self-limited and occurs in response to a specific injury, while chronic pain is more commonly caused by direct neuronal tissue damage resulting in certain conditions such as Neuropathic Pain (NP) and FMS[3]. NP affects approximately 7-8% of the global population in recent years[4]. Untreated neuropathic pain causes mood and sleep disorders, obstacles in patients' work and social lives, and reduces quality of life. Therefore, it is important for public health[5]. Pro-inflammatory cytokines play a role in pain formation by both signaling in the central nervous system after they are released by glia in the brain and spinal cord[6]. Cytokines have been placed on the agenda of developments in pain pathophysiology with the understanding that they have a role in the formation of hyperalgesia and analgesia[7]. In recent years, the roles of molecules such as endocan, endothelin-1 (ET-1), monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor A (VEGF-A) have been mentioned in a limited number of important studies conducted in FMS patients with whole exome sequencing or other molecular analyzes[8-11]. The fact that the pain is unique to the individual and the occurrence of pain of different sizes in different people with the same lesion is attributed to the genetic difference in cytokine production[12].

Molecular docking and string analyzes provide a systematic approach to network analysis of biological systems using bioinformatics to reveal potentially complex relationships between multiple components. The aim of this study was to evaluate serum levels of endocan, ET-1, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), MCP-1, tumor necrosis factor-α (TNF-α), and VEGF-A molecules, which are associated with pain/neuropathic pain and have different results in the literature, and to investigate the relationship between FMS clinical effects and these parameters. Moreover, we wanted to reflect the protein-protein interactions and network topology of these molecules through molecular docking and string analyses.

MATERIALS AND METHODS

Study design and patients

A total of 44 premenopausal female patients with complaints of more than 3 months diagnosed with fibromyalgia for the first time according to American College of Rheumatology 2016 (ACR) criteria at Yozgat Bozok University Research Hospital, Physical Therapy and Rehabilitation Clinic between March 2019 and August 2019 were included in the study. American College of Rheumatology 2016 (ACR) diagnostic criteria of fibromyalgia are; i- Persistence of symptoms for about 3 months, ii- Widespread pain index ≥7, Symptom Severity Scale ≥5 points or Widespread pain index 4-6, Symptom Severity Scale ≥9 iii- Generalized pain in 4 of the 5 regions. Generalized pain in the jaw, chest and abdomen is excluded[13]. Demographic data of the patients were recorded. The FMS group were evaluated in terms of Body Mass Index (BMI), Visual Analog Scale (VAS), Beck Depression Scale, Beck Anxiety Scale, Fibromyalgia Impact Questionnaire (FIQ), Leeds Assessment of Neuropathic Symptoms, and Sings (LANSS) pain scale. Beck Depression Scale is a 21-item scale with a 0-3 scoring system for each question and is employed to reveal the characteristic features of depression and helps to understand the severity of depression in the person[14]. Beck Anxiety Scale is a 21-item scale with a scoring system between 0-3 for each question. It is a test to measure the level of anxiety[15]. The LANSS pain scale is a test used to differentiate neuropathic pain from nociceptive pain with pain and sensation assessment. The total score ranges between 0-24 (< 12 no neuropathic pain, ≥12 neuropathic pain)[16]. The control group consisted of 44 women of the same age group without complaints who agreed to participate in the study.

Those who had autoimmune and chronic inflammatory diseases (i.e. rheumatoid arthritis, patients with Ankylosing Spondylitis), systemic chronic diseases (i.e. diabetes, chronic heart disease, cardiovascular diseases, chronic lung disease, chronic kidney, and liver disease, etc.), diseases that caused

malabsorption (i.e. celiac, radiation enteritis, etc.), those with active infections, cancer, active osteoarthritis, thyroid and parathyroid disease, those with other serious somatic/psychiatric disorders, alcohol-consumers, smokers, those with glucocorticoid, hormone replacement therapy, long-term analgesic, those who used hypnotic-derivatives, anti-lipidemics, antioxidant or anti-coagulant drugs, those with traumas, those who were pregnant and in lactation, those with lumbar or cervical disc disease, and those with neck injuries were excluded from the study.

This study was approved by the Ethics Review Board of Yozgat Bozok University, Faculty of Medicine (2017-KAEK-189_2019.02.28_08). The study was carried out in accordance with the principles of medical research provided by Helsinki and International Charter Guidelines and the International Conference on Harmonization Guideline for Good Clinical Practice. Written informed consent was obtained from each participant.

Blood sample collection

Venous blood samples were taken from patients and the control group in the morning following 12-hours of fasting. Baseline blood samples were collected from the subjects and blood samples were centrifuged for 10 min at 3000 rpm, after which the supernatant was quickly removed and kept frozen at -80°C until the assays were performed by a specialist who was blinded to patient status.

ELISA

Serum endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A levels were measured with commercially available Enzyme-Linked Immune Sorbent Assay (ELISA) kits (Sunlong Biotech Co., Ltd., China and Elabscience, Wuhan, China) with detectable concentration range 3.3-200 pg/ml, 3-120 pg/ml, 3-200 pg/ml, 7.81-500 pg/mL, 6-200 pg/ml, 10-500 pg/ml, 7.81-500 pg/mL and 3-200 pg/ml, respectively according to the manufacturer's instructions. Optical density values for samples and standard samples were detected on Thermo Scientific (USA) Multiscan Go Microplate Reader ELISA reader at 450 nm. The results are presented as pg/mL.

Molecular docking analysis

The Protein Data Bank (PDB) (https://www.rcsb.org/) was used to obtain the crystal structures of eight proteins. The human proteins (endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF- α, and VEGF-A) were chosen and preferred for their high-resolution structures with no mutations. In PyMOL v.2.5.2 heteroatoms such as solvent molecules and ligands were eliminated during protein production[17]. PyMOL was then used to add missing hydrogens in protein structures. Table 1 displays the PDB code, sequence length, and crystallographic resolution of proteins.

ClusPro 2.0 was utilized to conduct rigid protein docking experiments on human proteins (endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A)[18]. This server includes three computational steps; rigid body docking using the FFT (Fast Fourier Transform) correlation approach, RMSD (Root Mean Square Deviation)-based clustering of the generated structures to find the largest cluster that will represent the complex's likely models, and refinement of selected structures[19]. By default, ClusPro 2.0 generates four types of models based on the scoring algorithms balanced, electrostatic-favored, hydrophobic-favored, and van der Waals + electrostatic.

To execute molecular docking, two proteins were uploaded to the server as receptor and ligand depending on sequence length. Initially, each protein was docked with itself to form homodimeric complexes, which were subsequently docked with each other to form heterodimeric complexes. As a result, 8 homodimers and 28 heterodimers were collected for further investigation. ClusPro returns the optimal 30 complexes for each job following CHARMM energy minimization based on clustering probability and energy-based (balanced, electrostatic-favored, hydrophobic-favored, and VDW+electrostatic-favored) parameters. In this study, the best complexes were obtained by taking into account the balanced energy parameter and the higher clustering probability of complexes. Table 1 shows the number of cluster members as well as the model cluster scores (cluster center and lowest energy). The cluster center-weighted score represents the structure in the cluster with the greatest number of neighbor structures, whereas the lowest energy score designates the structure in the cluster with the least energy. This model score can be calculated using following equation.

$$E = 0.40Erep + (-0.40Eattr) + 600Eelec + 1.00EDARS \quad (1)$$

Where Erep and Eattr indicate the repulsive and attractive terms of the van der Waals energy respectively whereas Eelec denotes electrostatic energy term. The desolvation energy contribution is represented by EDARS, a paired structure-based potential derived by the decoys as the reference state approach[20]. The PRODIGY web tool, which can be found at https://wenmr.science.uu.nl/prodigy/ was used to examine the protein binding affinity of these docked complexes[21]. The binding energy evaluated at the PRODIGY server was calculated using following equation[22].

$$\Delta Gcalc = 0.09459ICscharged/charged + 0.10007ICscharged/apolar - 0.19577ICspolar/polar + 0.22671ICspolar/apolar - 0.18681\%NISapolar - 0.13810\%NIScharged + 15.9433 \quad (2)$$

Where, ICs (Inter-residue contacts) and NIS (Non-interacting surface) terms indicate the contribution of various types of residues to the overall binding affinity. The first ten docking structures with the relative low energies were selected. PyMol software was used for visual representation and assessing of the complex interaction, measuring of the distances between the interacting amino acid residues.

Table 1. Structural information of proteins

Proteins	Sequence length	PDB Code	Resolution (Å)
Endocan	193	1LQV	1.63
ET-1	21	6DK5	1.85
IL-1	158	4DEP	3.10
IL-6	220	4CNI	2.20
IL-8	72	1QE6	2.35
MCP-1	76	2RA4	1.70
TNF-α	160	7JRA	2.1
VEGF	102	6D3O	3.10

ET-1: Endothelin-1, IL-1: interleukin-1, IL-6: interleukin-6, IL-8: interleukin-8, MCP-1 monocyte chemotactic protein-1, TNF-α: tumor necrosis factor alpha, VEGF-A: vascular endothelial growth factor.

STRING v11.5 analysis of proteins

STRING, which is available online at (https://string-db.org/) has been used to interpret the interaction of endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A proteins with the proteins of Homo sapiens as organism involved in the biological activity.

Statistical analysis

Statistical analysis was performed using SPSS software (version 20, SPSS, Chicago, IL). The data were expressed as mean ± SD, median and interquartile range. Continuous variables were investigated using visual (histograms, probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk's Test) to determine whether or not they are normally distributed. The Student's t-Test was used to compare continuous variables with normal distributions and the Mann Whitney U-Test was used to compare variables with non-normal distributions. Relationships between categorical variables were analyzed by the Chi-Square Test. The level of statistical significance was taken as 0.05 in all tests.

RESULTS

The mean age of females with FMS and healthy controls was 38.49 ± 5.04, 36.35 ± 5.38 years, respectively. There were no differences in terms of age and Body Mass Index between the groups ($p > 0.05$). The demographic and clinical characteristics of all subjects enrolled in the study are presented in Table 2. When serum concentrations of endocan (3.9 ±1.2), ET-1 (16.8 ±5.3), IL-1 (70.3 ±19.3), IL-6 (4.6 ±1.5), IL-8 (68.0 ±20.6), MCP-1 (397.4 ±124.5), TNF-α (56.4 ±17.7), and VEGF-A (18.9 ±5.9) were compared between patients with FMS and healthy individuals, all values were found to be significantly higher in the FMS group ($p < 0.001$) (Table 2, Figure

1, Figure 2). Also, endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A levels were positively correlated with each other (Table 3). According to the LANSS pain score, 24 of 44 patients had neuropathic pain. There was no statistically significant difference in terms of endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A levels between the patient group with and the group without neuropathic pain complaint ($p > 0.05$) (Table 4). When evaluated in terms of correlations, no relations were detected between pain/neuropathic pain, FIQ and proteins (endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, VEGF-A) levels ($p > 0.05$).

Protein-protein docking is used in a variety of biomolecular applications, including enzyme conformation exploration[23], interactome prediction[24], molecular recognition[25], protein dimerization[26], design of specific probes for protein targets[27], amyloid aggregation[28], protein binding mechanism prediction[29], peptide design against diseases[30], and vaccine design[31]. Various computational tools were used in this study to examine the binding energies and types of interactions of docked vascular endothelial dysfunction protein complexes (8 homodimers and 28 heterodimers). The complex binding energies estimated by the PRODIGY online server are provided in Table 5, and the complex structures are displayed in Figure 3. Protein-protein binding affinity ranges from -5.1 kcal/mol to -21.4 kcal/mol among 28 complexes. Furthermore, of the eight proteins tested, VEGF, IL-6, and IL-1 have the highest affinity to connect with their protein partners (Table 6).

We performed STRING network analysis to determine the functional interactions of these proteins in cellular processes. In the study, Multiple Proteins STRING network analysis was performed with endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A proteins. Protein-protein interaction scores of these proteins (excluding endocan) were in the range of 0.999-0.900 homology score (Table 7) (Figure 4).

Table 2. Demographic data, clinical parameters and ELISA levels of all subjects.

	FMS Group (n= 44)		Control Group (n= 44)		*p*
	Mean ± SD	**Median (IQR)**	**Mean ± SD**	**Median (IQR)**	
Age (year)	38.49 ± 5.04	39 (8)	36.35 ± 5.38	36 (9)	0,054**
BMI (kg/m²)	23.2 ± 2.1	23.5 (25.3-19.5)	22.6±1.7	22.8 (25.6-18.8)	0.083**
VAS	6.3 ±1.6	6 (3)	-	-	-
LPS	11.7 ±4.3	13 (9)	-	-	-
Beck dep.	18.2 ±7.4	19 (10)	-	-	-
Beck anx.	14.1 ±6.6	12 (8)	-	-	-
FIQ	66.9 ±10.5	66.9 (14.35)	-	-	-
WBC (10^3/mm)	8.2 ±1.8	7.9 (2.44)	7.8 ±2.6	7.6 (3.04)	0.158*
Hbg (g/dL)	11.65 ±0.8	11.7 (1.2)	12.8 ±1.5	12.8 (2.2)	**0.000***
CRP (mg/L)	5.3 ±2.8	5.5 (3.45)	4.8 ±2.9	4.1 (5.6)	0.442*
ESR (mm/h)	13.6 ±5.8	13 (6.54)	13.3 ±6.9	12 (7.9)	0.462**
Endocan (ng/mL)	3.9 ±1.2	4.2 (1.89)	1.2 ±0.3	1.2 (0.37)	**0.000****
ET-1 (pg/mL)	16.8 ±5.3	17.9 (7.89)	3.9 ±1.1	3.9 (1.22)	**0.000****
IL-1 (pg/mL)	70.3 ±19.3	69.8 (28.5)	37.7 ±14.1	37.7 (14.43)	**0.000****
IL-6 (pg/mL)	4.6 ±1.5	4.7 (2.45)	2.0 ±1.0	1.7 (1.77)	**0.000****
IL-8 (pg/mL)	68.0 ±20.6	71.3 (31.1)	27.8 ±8.5	28.0 (9.72)	**0.000****
MCP-1 (pg/mL)	397.4 ±124.5	424.2 (191.6)	123.1 ±35.1	123.8 (37.06)	**0.000****
TNF-α (pg/mL)	56.4 ±17.7	59.7 (28.56)	23.0 ±6.9	22.9 (7.58)	**0.000****
VEGF-A (pg/mL)	18.9 ±5.9	20.2 (9.0)	6.5 ±1.7	6.6 (1.83)	**0.000****

Beck Ank: beck anxiety, Beck Dep: beck depression, BMI: body mass index, CRP: C-reactive protein, ESR: Erythrocyte sedimentation rate; ET-1: Endothelin-1; FIQ: Fibromyalgia Impact Questionnaire; Hgb: Hemoglobin; IL-1: Interleukin-1; IL-6: interleukin-6; IL-8: Interleukin-8; IQR: Interquartile range; LPS: LANSS pain scale; MCP-1 monocyte chemotactic protein-1; TNF-α: Tumor necrosis factor alpha; WBC: White blood cell; VAS: Visual analog scale; VEGF-A: Vascular endothelial growth factor; SD: standard deviation. Values presented as mean ± standard deviation; $p < 0,05$; *normal distributions; **non-normal distributions.

Table 3. Correlation between Endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α and VEGF-A values in FMS group

			Endocan	ET-1	MCP-1	IL-1	IL-6	IL-8	TNF-α	VEGF-A
Spearman's rho	Endocan	r		0.999**	1.000**	0.915**	0.965**	0.998**	0.998**	1.000**
		p		0.000	0.000	0.000	0.000	0.000	0.000	0.000
	ET-1	r	0.999**		0.999**	0.923**	0.962**	0.999**	0.997**	0.999**
		p	0.000		0.000	0.000	0.000	0.000	0.000	0.000
	MCP-1	r	1.000**	0.999**		0.915**	0.965**	0.998**	0.998**	1.000**
		p	0.000	0.000		0.000	0.000	0.000	0.000	0.000
	IL-1	r	0.915**	0.923**	0.915**		0.840**	0.933**	0.902**	0.915**
		p	0.000	0.000	0.000		0.000	0.000	0.000	0.000
	IL-6	r	0.965**	0.962**	0.965**	0.840**		0.961**	0.967**	0.965**
		p	0.000	0.000	0.000	0.000		0.000	0.000	0.000
	IL-8	r	0.998**	0.999**	0.998**	0.933**	0.961**		0.994**	0.998**
		p	0.000	0.000	0.000	0.000	0.000		0.000	0.000
	TNF-α	r	0.998**	0.997**	0.998**	0.902**	0.967**	0.994**		0.998**
		p	0.000	0.000	0.000	0.000	0.000	0.000		0.000
	VEGF-A	r	1.000**	0.999**	1.000**	0.915**	0.965**	0.998**	0.998**	
		p	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

ET-1: Endothelin-1, IL-1: interleukin-1, IL-6: interleukin-6, IL-8: interleukin-8, MCP-1 monocyte chemotactic protein-1, TNF-α: tumor necrosis factor alpha, VEGF-A: vascular endothelial growth factor. *: Correlation is significant at the 0.05 level. **: Correlation is significant at the 0.01 level.

Table 4. Distribution of Endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α and VEGF-A values of patient groups with and without neuropathic pain according to LANSS pain score.

	LANSS pain score		
	NP (-) (n=20)	NP (+) (n=24)	p
Endocan (ng/mL)	3.8 ± 1.2	4 ± 1.3	0.551
ET-1 (pg/mL)	16.2 ± 5.1	17.2 ± 5.5	0.521
IL-1 (pg/mL)	67.5 ± 17.5	72.5 ± 20.6	0.395
IL-6 (pg/mL)	4.4 ± 1.5	4.7 ± 1.6	0.494
IL-8 (pg/mL)	66 ± 19	69.7 ± 21.9	0.548
MCP-1 (pg/mL)	384.8 ± 118.3	407.4 ± 130.7	0.551
TNF-α (pg/mL)	54.8 ± 16.5	57.6 ± 18.8	0.602
VEGF-A (pg/mL)	18.3 ± 5.5	19.4 ± 6.2	0.556

ET-1: Endothelin-1, IL-1: interleukin-1, IL-6: interleukin-6, IL-8: interleukin-8, LPS: LANSS pain scale, MCP-1 monocyte chemotactic protein-1, NP**:** Neuropathic pain, TNF-α: tumor necrosis factor alpha, VEGF-A: vascular endothelial growth factor. Values presented as mean ± standard deviation. Statistical significance: $p < 0,05$.

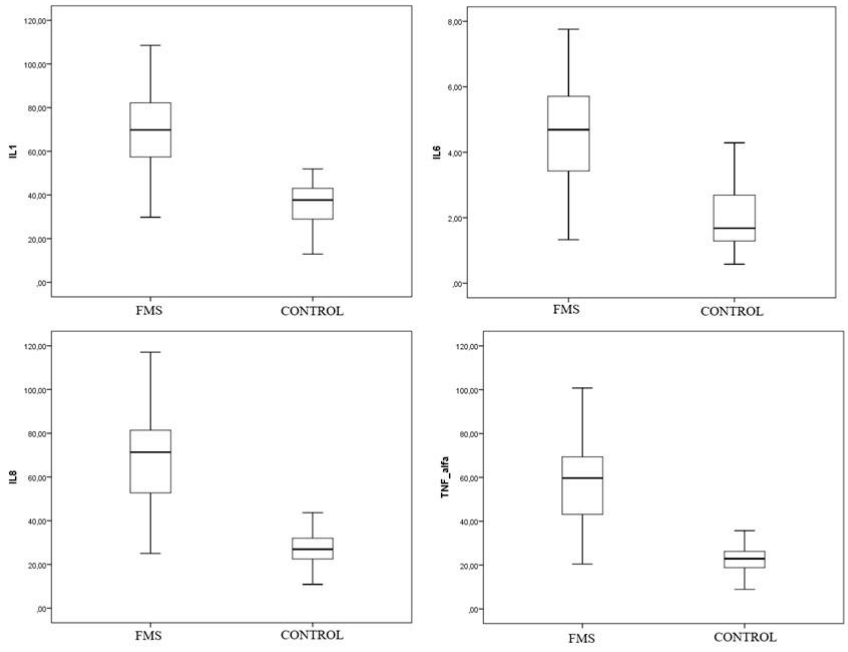

Figure 1. Distribution of serum IL-1, IL-6, IL-8 and TNF-α result between FMS patients and control groups.

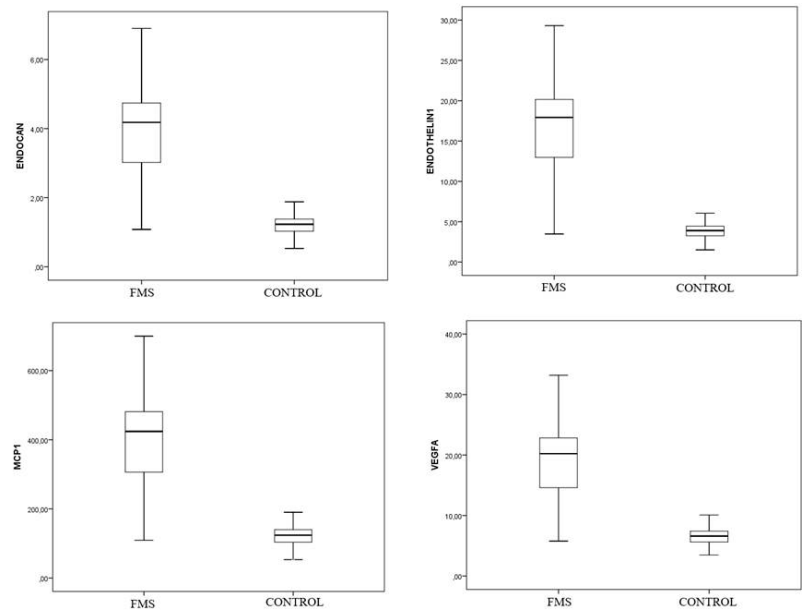

Figure 2. Distribution of serum Endocan, ET-1, MCP-1 and VEGF-A results between FMS patients and control groups.

Table 5. Binding affinity (kcal/mol) of proteins docked complexes calculated using PRODIGY web server.

Complex Number	Complex name	Binding Energy(Kcal/mol)	K_P Prediction (K_d (M) at 25.0˚C
Endocan			
	Endocan-endocan	-5.6	8.0E-05
	Endocan-ET-1	-9.4	1.2E-05
	Endocan-IL-1	-7.1	6.1E-06
	Endocan-IL-6	-6.0	4.0E-05
	Endocan-IL-8	-5.7	6.3E-05
	Endocan-TNF-α	-5.1	1.9E-04
	Endocan-MCP-1	-5.6	7.5E-05
	Endocan-VEGF-A	-5.7	6.4E-05
Endothelial-1 (ET-1)			
	ET-1-ET-1	-7.3	4.5E-06
	ET-1-IL-1	-14.5	2.5E-11
	ET-1-IL-6	-21.4	2.0E-16
	ET-1-IL-8	-11.8	2.4E-02
	ET-1-TNF-α	-14.6	1.9E-11
	ET-1-MCP-1	-7.4	3.5E-06
	ET-1-VEGF-A	-13.5	1.3E-10
Interleukin-1 (IL-1)			
	IL-1-IL-1	-17.2	2.5E-13
	IL-1-IL-6	-17.9	7.5E-14
	IL-1-IL-8	-17.7	1.0E-13
	IL-1-TNF-α	-17.5	1.5E-13
	IL-1-MCP-1	-17.6	1.2E-13
	IL-1-VEGF-A	-17.7	1.1E-13
Interleukin-6 (IL-6)			
	IL-6-IL-6	-17.1	2.8E-13
	IL-6-IL-8	-18.3	3.9E-14
	IL-6-TNF-α	-17.9	7.8E-14
	IL-6-MCP-1	-18.3	4.1E-14
	IL-6-VEGF-A	-18.3	4.1E-14
Interleukin-8 (IL-8)			
	IL-8-IL-8	-9.9	5.5E-08
	IL-8-TNF-α	-19.8	3.0E-15
	IL-8-MCP-1	-9.7	7.6E-08
	IL-8-VEGF-A	-9.7	7.1E-08
Tumor Necrosis Factor-α (TNF-α)			
	TNF-α- TNF-α	-10.9	1.0E-08
	TNF-α-MCP-1	-21.4	1.9E-16
	TNF-α-VEGF-A	-21.2	2.9E-16
Monocyte Chemoattractant Protein-1 (MCP-1)			
	MCP-1-MCP-1	-13.1	2.6E-10
	MCP-1-VEGF-A	-15.8	2.7e-12
Vascular Endothelial Growth Factor (VEGF-A)			
	VEGF-A-VEGF-A	-14.4	2.9E-11

ET-1: Endothelin-1, IL-1: interleukin-1, IL-6: interleukin-6, IL-8: interleukin-8, MCP-1 monocyte chemotactic protein-1, TNF-α: tumor necrosis factor alpha, VEGF-A: vascular endothelial growth factor.

Table 6. Protein-protein binding energies and interactions of top-three complexes, TNF-α/MCP-1, TNF-α/VEGF-A, and ET-1/IL-6.

Energies Component (Kcal/mol)	Complexes		
	TNF-α-MCP-1	TNF-α-VEGF-A	ET-1-IL-6
Binding Affinity	-21.4	-21.2	-21.4
Balanced coefficient	-592.9	-918.4	-854.2
Electrostatic favored	-718.8	-976.5	-936.6
Hydrophobic energy	-585.6	-1089.6	-1366.9
vWd energy	-223.9	-205.0	-216.1

ET-1: Endothelin-1, IL-6: interleukin-6, MCP-1 monocyte chemotactic protein-1, TNF-α: tumor necrosis factor alpha, VEGF-A: vascular endothelial growth factor.

Table 7. Predicted functional proteins associated with endocan, ET-1, IL-1, IL-6, IL-8, MCP-1, TNF-α, and VEGF-A.

Proteins	Proteins Associated	Homology score
Endocan (ESM-1)	VEGF-A	0.535
ET-1 (EDN-1)	VEGF-A	0.923
IL-1	IL-6	0.997
IL-6	IL-8 (CXCL8)	0.997
IL-8 (CXCL8)	IL-1	0.998
MCP-1 (CCL-2)	IL-8 (CXCL8)	0.995
TNF-α	IL-6	0.994
VEGF-A	IL-6	0.987

ET-1: Endothelin-1, IL-1: interleukin-1, IL-6: interleukin-6, IL-8: interleukin-8, MCP-1 monocyte chemotactic protein-1, TNF-α: tumor necrosis factor alpha, VEGF-A: vascular endothelial growth factor.

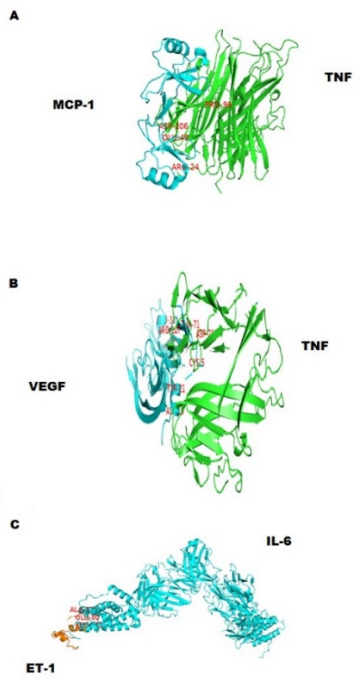

Figure 3. Ribbon Structures of the complexes, (A) TNF[green]-MCP-1[Blue], (B) TNF [green]-VEGF [Blue], (C) ET-1 [Orange]-IL-6 [Blue].

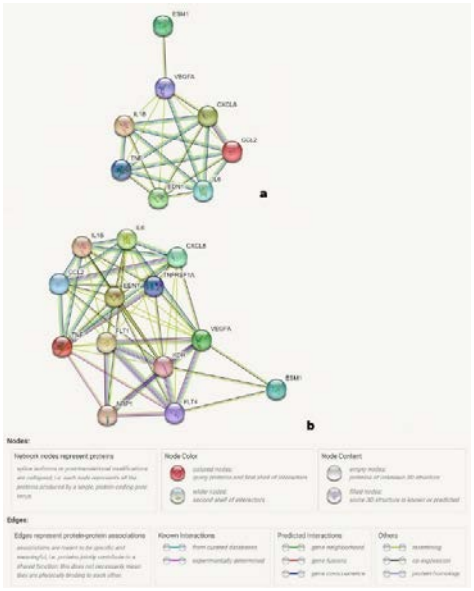

Figure 4. Interactions of endocan (ESM-1), ET-1 (EDN-1), IL-1, IL-6, IL-8 (CXCL8), MCP-1 (CCL-2), TNF-α, and VEGF-A proteins are analyzed in the STRING database. Each line has features. [Red line: gene fusions evidence; Green line: gene neighborhood; Blue line: gene co-occurrence; Yellow line: text mining; Black line: co-expression; Purple line: experimentally determined; Light blue line: from curated databases; Lilac line: protein homology].

DISCUSSION

Pro-inflammatory cytokines, endocan, ET-1, VEGF-A levels and pain/neuropathic pain parameters of patients with FMS were investigated in this study. Statistically significant differences were found between FMS and the control group in all parameters examined. However, no relations were detected between these parameters and pain/neuropathic pain scores and FIQ.

Many studies have been done on the etiopathogenesis of FMS for many years. Genetic causes, immunological mechanisms and central and peripheral theories are collected under sub-titles. The relationship between NP and FMS has been shown in few studies in the literature. Some of these investigations reported that IL1, IL-6, IL8, MCP-1 and TNF-α are associated with pain severity in FMS[6,12,32,33]. Wang et al. evaluated the serum IL-6, IL-8 and TNF-α levels and their relationship with pain intensity in 20 FMS patients at admission and 10, 21, and 180 days after starting treatment. As a result of the analysis, IL-8 and TNF-α were found to be high in patients with FMS at admission. After 6 months of pain treatment, IL-8 serum level was found to be correlated with pain intensity in patients[34]. In a study conducted at the University of Indiana, when the Brief Pain Inventory (BPI) pain severity and plasma concentrations of IL-8 and MCP-1 from week 1 to week 12 were evaluated in 16 FMS patients, BPI pain severity changes were significantly associated with changes in IL-8 and MCP-1 plasma concentrations[35]. ET-1 is a strong physiological vasoconstrictor released after the activation and/or impairment of endothelial cells playing important roles in connective tissue diseases, inflammatory processes, and rheumatic diseases[36,37]. There are studies reporting significantly higher plasma ET-1 and/or expression levels in patients with FMS than in healthy control group[38,39]. ET-1 activates macrophages causing excessive secretion of inflammatory mediators like IL-6, IL-8, TNF, PGE2, and superoxide anions[40]. As for the relationship between pain and ET-1, ET-1 has been shown to stimulate both nociceptors and sensitize them to painful stimuli. Selective stimulation of ET receptors has been implicated as the cause of inflammatory, neuropathic and tumoral pain[41]. Although we found the serum concentration of ET-1, IL-1, IL-6, IL-8, MCP-1, and TNF- α to be higher in the FMS group compared to the healthy controls, we could not detect any relationship with NP.

Endocan is a proteoglycan that consists of a dermatan sulfate chain. The prognostic value of endocan is indicated in diseases such as inflammatory disorders, tumor progression, sepsis, hypertension, diabetes, and heart diseases. In previous research, high endocan levels were determined in patients with FMS[42]. Few studies were detected conducted on endocan levels and pain in the literature. In this study, serum endocan levels were significantly higher in FMS patients than in the control group. Furthermore, there was no significant difference in endocan levels between patients with and without NP. Endocan expression has been reported to be intensely up-regulated via proangiogenic molecule VEGF-A[43]. Endocan and VEGF values were found to be higher in patients with recurrent aphthous ulcers compared to controls. In the same study, it was emphasized that an increase in endocan was associated with an increase in pain score and vice versa[44]. It was reported that VEGF-A directly affects neurons, microglia, astrocytes, and Schwann cells, and has neurotrophic and neuroprotective activity in peripheral and central nervous systems[45]. Recent studies have shown that VEGF-A is effective in peripheral neuropathy that stems from nerve damage[46]. It has been reported that it helps to improve functional healing reducing pain in the nerve injury model[47]. In a study conducted with rats, it was shown that the central VEGF-A pathway plays key roles in trigeminal neuropathic pain development[48]. There are limited studies in the literature evaluating the relations between FMS and VEGF. The FMS patients with inherited alpha1-antitrypsin deficiency had lower serum VEGF levels compared to normal population[9]. In another clinical study, serum VEGF levels were not different in patients with FMS compared to healthy controls[49]. This study is the first to report elevated VEGF-A serum levels in FMS patients. It showed a positive correlation with all other markers.

As the result of the STRING analysis, we determined that proteins except endocan have protein-protein interactions in the range of 0.999-0.800 homology score. The homology scores of these proteins were found in the range of 0.998-0.923, which was an important finding for us. Since there are very limited numbers of articles on this subject in the literature, the evaluation of our data was also limited. In addition, being one of these limited studies increased the importance of our research data. Taş et al. evaluated STRING analysis findings in their study in FMS patients and the score of VEGFR-1 with

potassium ion channel protein-2 (KCNH) was found to be 0.949[50].

Consequently, when we evaluated all the analysis data, although molecular docking and string analyzes gave us significant results in the protein-protein relationship, we could not find a statistical result regarding the relationship with NP. The significant serum levels of these molecules may indicate that they are still effective in inflammation and/or endothelial dysfunction. This study has a few limitations. Firstly, the study group was small. Secondly, only female patients were included in the study. In addition, only serum samples were analyzed. Different results can be obtained with different analyzes in plasma, cerebrospinal fluid or blood cells.

These findings indicate the important role of these proteins and their protein-protein interaction in fibromyalgia pathophysiology. All parameters were found to be high in FMS patients; however, there were no statistically significant differences in the parameters examined in FMS patients with NP complaints and those who did not have these complaints. However, it is still not clear at what level and how these high serum levels affect the etiology and pain complaints. More studies are needed to evaluate the pain and other clinical symptoms in FMS patients, and examine the relations between the relevant parameters and other markers that cause pain. In addition, the results of molecular docking and string analyzes will be a source for in silico analyzes to be studied in fibromyalgia.

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REFERENCES

1. Bazzichi L, Giacomelli C, Consensi A, Giorgi V, Batticciotto A, Di Franco M et al. One year in review 2020: fibromyalgia. Clin Exp Rheumatol. 2020;38:3-8.
2. Küçükşen S, Genç E, Yılmaz H, Sallı A, Gezer İA, Karahan AY et al. The prevalence of fibromyalgia and its relation with headache characteristics in episodic migraine. Clin Rheumatol. 2013;32:983-90.
3. Alles SRA, Smith PA. Etiology and pharmacology of neuropathic pain. Pharmacol Rev. 2018;70:315-47.
4. Jensen TS, Baron R, Haanpää M, Kalso E, Loeser JD, Rice ASC et al. A new definition of neuropathic pain. Pain. 2011;152:2204-05.
5. Galer BS, Gianas A, Jensen MP. Painful diabetic polyneuropathy: epidemiology, pain description, and quality of life. Diabetes Res Clin Pract. 2000;47:123-28.
6. Kelley KW, Bluthé RM, Dantzer R, Zhou JH, Shen WH, Johnson RW, et al. Cytokine-induced sickness behavior. Brain Behav Immun. 2003;17:112-118.
7. Watkins LR, Maier SF. The pain of being sick: implications of immune-to-brain communication for understanding pain. Annu Rev Psychol. 2000;51:29-57.
8. Feng J, Zhang Z, Wu X, Mao A, Chang F, Deng X et al. Discovery of potential new gene variants and inflammatory cytokine associations with fibromyalgia syndrome by whole exome sequencing. PLoS One. 2013;8:e65033.
9. Blanco I, Janciauskiene S, Nita I, Fernández-Bustillo E, Cárcaba V, Gallo C et al. Low plasma levels of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNFalpha), and vascular endothelial growth factor (VEGF) in patients with alpha1-antitrypsin deficiency-related fibromyalgia. Clin Rheumatol. 2010;29:189-97.
10. Mertoglu C, Gunay M, Yerligok O. Could endocan, a marker of inflammation and endothelial dysfunction, be a new diagnostic marker for fibromyalgia?. Clin Lab. 2018;64:405-10.
11. Nah SS, Lee H, Hong Y, Im J, Won H, Chang SH et al. Association between endothelin‐1 and fibromyalgia syndrome. Mol Med Rep. 2017;16:6234-39.
12. Sommer C. Zytokine bei neuropathischen Schmerzen [cytokines in neuropathic pain]. Anaesthesist. 2001;50:416-26.
13. Wolfe F, Clauw DJ, Fitzcharles MA, Goldenberg DL, Häuser W, Katz RL, Mease PJ, Russell AS, Russell IJ, Walitt B. 2016 Revisions to the 2010/2011 fibromyalgia diagnostic criteria. Semin Arthritis Rheum. 2016;46:319-29.
14. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. Arch Gen Psychiatry. 1961;4:561-71.
15. Beck AT, Epstein N, Brown G, Steer RA. An inventory for measuring clinical anxiety: psychometric properties. J Consult Clin Psychol. 1988;56:893-7.
16. Bennett M. The LANSS Pain Scale: the Leeds assessment of neuropathic symptoms and signs. Pain. 2001;92:147-57.
17. Schrödinger LLC. The PyMOL molecular graphics system. Version 2.5.2. 2010.
18. Rakhmetov A, Lee SP, Grebinyk D, Ostapchenko L, Chae HZ. Simulation of peroxiredoxin II and brain-type creatine Kinase protein-protein interaction using

the on-line docking server ClusPro 2.0. J App Pharm Sci. 2015;5:011-016.
19. Kozakov D, Beglov D, Bohnuud T, Mottarella SE, Xia B, Hall DR et al. How good is automated protein docking? Proteins. 2013;81:2159-66.
20. Chuang GY, Kozakov D, Brenke R, Comeau SR, Vajda S. DARS (Decoys As the Reference State) potentials for protein-protein docking. Biophys J. 2008;95:4217-27.
21. Xue LC, Rodrigues JP, Kastritis PL, Bonvin AM, Vangone A. PRODIGY: a web server for predicting the binding affinity of protein-protein complexes. Bioinformatics. 2016;32:3676-78.
22. Vangone A, Bonvin AM. Contacts-based prediction of binding affinity in protein-protein complexes. Elife. 2015;4:e07454.
23. Valdés H, Díaz N, Suárez D, Fernández-Recio J. Interdomain Conformations in the full-length MMP-2 enzyme explored by protein-protein docking calculations using pyDock. J Chem Theory Comput. 2010;6:2204-13.
24. Kastritis PL, Bonvin AM. Are scoring functions in protein-protein docking ready to predict interactomes? Clues from a novel binding affinity benchmark [published correction appears in J Proteome Res 2011 Feb 4;10:921-2]. J Proteome Res. 2010;9:2216-25.
25. Mottin M, Souza PC, Skaf MS. Molecular recognition of PPARγ by kinase Cdk5/p25: insights from a combination of protein-protein docking and adaptive biasing force simulations. J Phys Chem B. 2015;119:8330-39.
26. Qiao B, Lopez L, Olvera de la Cruz M. "Mirror"-like protein dimers stabilized by local heterogeneity at protein surfaces. J Phys Chem B. 2019;123:3907-15.
27. Soulier JL, Russo O, Giner M, Rivail L, Berthouze M, Ongeri S et al. Design and synthesis of specific probes for human 5-HT4 receptor dimerization studies. J Med Chem. 2005;48:6220-28.
28. Koldsø H, Andersen OJ, Nikolajsen CL, Scavenius C, Sørensen CS, Underhaug J et al. Early events in the amyloid formation of the A546T mutant of transforming growth factor β-induced protein in corneal dystrophies compared to the nonfibrillating R555W and R555Q mutants. Biochemistry. 2015;54:5546-5556.
29. Kahler U, Kamenik AS, Waibl F, Kraml J, Liedl KR. Protein-protein binding as a two-step mechanism: preselection of encounter poses during the binding of BPTI and trypsin. Biophys J. 2020;119:652-66.
30. Cui W, Wei Z, Chen Q, Cheng Y, Geng L, Zhang J et al. Structure-based design of peptides against G3BP with cytotoxicity on tumor cells. J Chem Inf Model. 2010;50:380-87.
31. Yang Z, Bogdan P, Nazarian S. An in silico deep learning approach to multi-epitope vaccine design: a SARS-CoV-2 case study. Sci Rep. 2021;11:3238.
32. Littlejohn G, Guymer E. Neurogenic inflammation in fibromyalgia. Semin Immunopathol. 2018;40:291-300.
33. Xu X, Wang B, Ren C, Hu J, Greenberg DA, Chen T, et al. Age-related impairment of vascular structure and functions. Aging Dis. 2017;8:590-610.
34. Wang H, Moser M, Schiltenwolf M, Buchner M. Circulating cytokine levels compared to pain in patients with fibromyalgia - a prospective longitudinal study over 6 months. J Rheumatol. 2008;35:1366-70.
35. Ang DC, Moore MN, Hilligoss J, Tabbey R. MCP-1 and IL-8 as pain biomarkers in fibromyalgia: a pilot study. Pain Med. 2011;12:1154-61.
36. Yoshio T, Masuyama J, Mimori A, Takeda A, Minota S, Kano S. Endothelin-1 release from cultured endothelial cells induced by sera from patients with systemic lupus erythematosus. Ann Rheum Dis. 1995;54:361-65.
37. Pache M, Schwarz HA, Kaiser HJ, Wüest P, Klöti M, Dubler B, et al. Elevated plasma endothelin-1 levels and vascular dysregulation in patients with rheumatoid arthritis. Med Sci Monit. 2002;8:CR616-CR619.
38. Pache M, Ochs J, Genth E, Mierau R, Kube T, Flammer J. Increased plasma endothelin-1 levels in fibromyalgia syndrome. Rheumatology (Oxford). 2003;42:493-94.
39. Bauer V, Sotníková R. Nitric oxide--the endothelium-derived relaxing factor and its role in endothelial functions. Gen Physiol Biophys. 2010;29:319-40.
40. Cunningham ME, Huribal M, Bala RJ, McMillen MA. Endothelin-1 and endothelin-4 stimulate monocyte production of cytokines. Crit Care Med. 1997;25:958-64.
41. Hans G, Deseure K, Adriaensen H. Endothelin-1-induced pain and hyperalgesia: a review of pathophysiology, clinical manifestations and future therapeutic options. Neuropeptides. 2008;42:119-32.
42. Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. Pharmacol Rev. 2004;56:549-80.
43. Delehedde M, Devenyns L, Maurage CA, Vivès RR. Endocan in cancers: a lesson from a circulating dermatan sulfate proteoglycan. Int J Cell Biol. 2013;2013:705027.
44. Mahmoud EA, Moneim WA, Shaker OG, Ghalwash DM. Expression of endocan and vascular endothelial growth factor in recurrent minor aphthous ulcers. J Clin Exp Dent. 2019;11:e534-e541.
45. Ruiz de Almodovar C, Lambrechts D, Mazzone M, Carmeliet P. Role and therapeutic potential of VEGF in the nervous system. Physiol Rev. 2009;89:607-48.
46. Lin J, Li G, Den X, Xu C, Liu S, Gao Y et al. VEGF and its receptor-2 involved in neuropathic pain transmission mediated by $P2X_2(/)_3$ receptor of primary sensory neurons. Brain Res Bull. 2010;83:284-91.

47. Lee HL, Oh J, Yun Y, Lee HY, You Y, Che L, et al. Vascular endothelial growth factor-expressing neural stem cell for the treatment of neuropathic pain. Neuroreport. 2015;26:399-404.
48. Lee GW, Son JY, Lee AR, Ju JS, Bae YC, Ahn DK. Central VEGF-A pathway plays a key role in the development of trigeminal neuropathic pain in rats. Mol Pain. 2019;15:1744806919872602.
49. Kim SK, Kim KS, Lee YS, Park SH, Choe JY. Arterial stiffness and proinflammatory cytokines in fibromyalgia syndrome. Clin Exp Rheumatol. 2010;28:S71-S77.
50. Tas A, Hayta E, Karadag A, Zontul C, Ozmen E, Aydin S, et al. Potassium ion channel protein (KCNH) levels in patients with fibromyalgia syndrome. Cell Mol Biol. 2022;67:451-57.