Assessment of Endocan Serum Level in Patients with Behçet Disease: Relation to Disease Activity and Carotid Intima Media Thickness

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Behçet disease (BD) is a form of vasculopathy that can influence blood vessels of variable diameters. Endocan is a biomarker of endothelial activation and it is secreted from endothelial cells as a soluble proteoglycan. The aim of the work was to assess endocan serum level in patients with BD and to examine its relationship with disease activity parameters and carotid intima media thickness (IMT). The study encompassed 42 patients with BD (25 males and 17 females) diagnosed according to the International Study Group Criteria of BD and 42 age and sex matched apparently healthy volunteers as controls. Human Endothelial cell-specific Molecule–1 (Endocan) was assessed using ELISA. Carotid mean IMT was calculated by Color Doppler ultrasonography. Thickness measurement more than 1 mm was considered abnormal:BD patients had significantly increased endocan serum levels (median, 249.5; IQR, 174 – 445 ng/l) compared to healthy controls (median, 190.5; IQR, 128 – 235 ng/l, *P*=0.002), endocan serum level was increased in BD patients with active disease (median, 434; IQR, 246 - 617.5 ng/l) compared to those with inactive disease (median, 195.5; IQR, 145 – 235 ng/l, *P*<0.001) and healthy controls (*P*<0.001). Endocan serum levels showed significant positive correlations with erythrocyte sedimentation rate (*P*=0.04), C-reactive protein (*P*<0.001), BD Current Activity form (*P*<0.001) and carotid IMT (*P*=0.008). In conclusion, Endocan can be used to monitor disease activity and endothelial dysfunction in BD.

Behçet disease (BD) is considered a form of vasculitis that acts on blood vessels of varying diameters [1]. It can involve the arterial and venous circulatory systems with a reported higher predilection for the lower limb veins [2]. Its estimated prevalence is 3.6 per 100.000 population in a recent Egyptian study [3].

In addition to its characteristic recurrent oral and genital ulceration, BD has a remarkable ability to involve many other organs as, the central nervous system (CNS), eyes and the gastrointestinal tract [4]. The exact etiology of BD is still not obvious and its pathophysiology seems to be multifactorial including genetic predisposition and immune dysregulation with elevated inflammatory cytokines levels [5-6]. Yet, there is a strong evidence that endothelial dysfunction is among the main pathogenic mechanisms in BD [7].

Endothelial dysfunction can be assessed by many non-invasive methods as biochemical markers, flow mediated dilatation of the brachial artery using nitric oxide and measurement of carotid intima-media thickness (IMT). A strong correlation was linking between increased carotid IMT and endothelial dysfunction [8-9].

Endothelial cell specific molecule-1, known as endocan, is a marker of endothelial activation and it is secreted from endothelial cells as a soluble proteoglycan [10]. It is considered a key player in many endothelial related functions as neovascularization, cell adhesion and

migration [11]. Endocan secretion is increased in many diseases that are associated with endothelial dysfunction, such as malignancy [12], inflammatory conditions [13], infections [14], bronchial asthma [15] and chronic kidney disease (CKD) [16]. In addition, endocan levels are elevated in various rheumatic diseases including rheumatoid arthritis (RA) [17], juvenile idiopathic arthritis (JIA) [18], systemic lupus erythematosus (SLE) [19], sarcoidosis [20] and inflammatory bowel disease (IBD) [21].

Many studies found increased serum concentrations of endocan in BD and proposed that it has a basic role in vascular inflammation and angiogenesis as well as various organ involvement in BD patients [22-24]. Our aim was to assess endocan serum level in BD patients and to examine its relationship with different disease activity parameters as well as carotid IMT.

Patients and Methods

Participants

This study was conducted from February 2019 to December 2019 and the patients were enrolled from the inpatients and outpatients 'clinics of the Rheumatology, Rehabilitation & Physical Medicine department, Benha University Hospitals. The study has been accepted by the local ethics Committee.

This study was conducted on 42 patients with BD (25 males and 17 females) identified according to the International Study Group Criteria of BD [25] and their ages ranged between 21 to 54 years. In addition, 42–apparently healthy matched volunteers (Male 23 and Female 19) were included as controls.

Patients were excluded if they had malignancy, recent infection, diabetes mellitus, hypertension, coronary artery disease, hyperlipidemia, smoking, CKD, IBD, other autoimmune or rheumatic diseases. We also excluded obese patients who had body mass index (BMI) more than 30 kg/m^2.

Clinical Evaluation

Full clinical evaluation was done for all patients with emphasis on detailed medical history, ophthalmological examination, musculoskeletal and systemic examination. Disease activity was reported using BD Current Activity form (BDCAF) [26] and patients with BDCAF value of 2 or higher were considered to be active [27].

Laboratory Investigations

Eight ml of venous blood were taken by peripheral venipuncture after fasting for 10 hours from all patients and controls and were divided as follow:

-First: 1 ml was taken on di-potassium EDTA to make complete blood count (CBC) by Sysmex-XS-800i including: hemoglobin (HB), platelets, white blood cells (WBCs) and mean platelet volume (MPV).

-Second: blood were taken on sodium citrate to carry out erythrocyte sedimentation rate (ESR), and the remaining blood was reserved in plain tube, centrifuged for 10 min; then the serum was divided into 2 aliquots, one for C-reactive protein (CRP) and total lipid profile including triglycerides (TG), cholesterol, high density lipoproteins (HDL) and low density lipoproteins (LDL); the other for subsequent assay of serum endocan. Serum TG, cholesterol, HDL were measured using BioSystems reagent kits provided by BioSystems, Costa Brava (Barcelona, Spain). LDL cholesterol (LDL-C) was calculated according to "Friedwald's equation": LDL-C = Total cholesterol - (HDL-C + TG/5). Human Endothelial cell-specific Molecule–1 (Endocan/ECSM1): was calculated using human ELISA (sandwich technique) kits provided by SunRed Bio, Shanghai, China. (Cat. No. 201-12-1978), with assay range: 8→2000 ng/L and sensitivity: 7.506 ng/L. This assessment has high sensitivity and excellent specificity. Intra-assay CV < 10% and inter-assay CV < 12%. All samples were assayed at the same experiment, in logarithmic scale, mean absorbance of standard (X) was plotted versus known standard concentration (Y) to make standard curve.

Carotid Ultrasound (US)

Ultrasonography was performed by experienced radiologist for both common carotid arteries in a longitudinal orientation using (Logiq P7, GE, USA) scanner. The mean carotid intima-media thickness (IMT) was calculated as it is preferred than maximal value because of its more reproducibility. Abnormal

thickness was considered if the measurement was more than 1 mm.

Statistical Analysis

Data was presented as mean, standard deviation (SD) or median and interquartile range (IQR) for quantitative data and number and percentage for categorical data. Comparison between various groups was done using the Mann-Whitney U test or the Kruskall-Wallis test for non-parametric data and independent sample t-test or one way analysis of variance (ANOVA) for normally distributed data, as appropriate. Correlations between serum endocan levels and different variables of BD was executed using Spearman correlation coefficient. The diagnostic performance of serum endocan levels in predicting sBDCAF and carotid IMT was examined using receiver operating characteristics (ROC) curve and the sensitivity, specificity and best cutoff point were calculated. The used statistical packages was SPSS v23 (Chicago, ILL Company) and *P* value was considered significant if less than 0.05.

Results

Forty two BD patients were enrolled in our study with a mean age of 35.4± 7.8 years (range from 21 to 54 years). Also, 42 volunteers with comparable age (37.6± 8.5 years; *P* =0.21) and sex (*P*=0.66) were included as healthy controls. Characteristics of BD patients during the study were shown in table 1.

Thirty (71.4%) of our BD patients were receiving colchicine, 28 (66.7%) received variable doses of prednisone, 15 (35.7%) were receiving azathioprine, 6 (14.3%) were on anti-TNF, 4 (9.5 %) were receiving cyclosporine and 2 (4.8%) were receiving cyclophosphamide.

Carotid US scanning was done for all subjects and showed statistically significant increase in the mean carotid IMT in the BD patients (*P* < 0.001) in comparison to the controls (0.85± 0.3 and 0.64 ± 0.17mm respectively) (Figure 1).

Serum endocan levels showed significant elevation in BD patients (median, 249.5; IQR, 174 – 445 ng/l) compared to their serum levels in healthy controls (median, 190.5; IQR, 128 – 235 ng/l, *P* =0.002). Furthermore, BD patients with active disease had a significantly elevated endocan serum levels (median, 434; IQR, 246 - 617.5 ng/l) compared to those with inactive disease (median, 195.5; IQR, 145 – 235 ng/l, *P* <0.001) and healthy controls (*P* <0.001) (Figure 2).

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BD patients were divided into quartiles regarding to their serum endocan levels. Patients who had the highest (4^{th}) quartile of serum endocan concentrations showed significant higher values of CRP and BDCAF (Table 2).

Endocan serum levels had significant positive correlation with ESR (*P* =0.04), CRP (*P* <0.001), BDCAF (*P* <0.001) and carotid IMT (*P* =0.008) (Table 3).

Figure 3 showed the diagnostic performance of serum endocan, CRP and ESR in predicting BDCAF score (Figure 3.A) and carotid IMT (Figure 3.B) using ROC curve analysis. Regarding BDCAF, serum endocan had an area under the curve (AUC) of 0.81 at a cutoff point of 267 ng/l with a sensitivity of 66.7 % and specificity

of 94.4 %. For CRP, the AUC was 0.77 with a sensitivity of 50 % and specificity of 94.4% at a cutoff point of 32 mg/l. ESR had an AUC of 0.6 with a sensitivity of 50 % and specificity of 77.8 %.at a cutoff point of 47 mm/1st hour.

Regarding carotid IMT, serum endocan had an AUC of 0.74 at a cutoff point of 256 ng/l with a sensitivity of 78.6 % and specificity of 71.4 %. For CRP, the AUC was 0.69 with a sensitivity of 85.7% and specificity of 53.6 % at a cutoff point of 18 mg/l. ESR had an AUC of 0.46.

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Table 1. Baseline characteristics of BD patients and healthy controls.

Parameter	BD patients (n=42)	Controls (n=42)	*P value*
Age (years) [Mean ±SD]	35.4 ± 7.8	37.6 ± 8.5	NS
Sex (Male: Female)	(25:17)	(23:19)	NS
BMI (Kg/m^2) [Mean ±SD]	27.4 ± 1.5	27.7± 1	NS
Disease duration (years) [Median (IQR)]	4 (2-5)	----	----
Active uveitis [n (%)]	17 (40.5%)	----	----
Mouth ulcers [n (%)]	22 (52.4%)	----	----
Genital ulceration [n (%)]	13 (30.1%)	----	----
Erythema nodosum [n (%)]	6 (14.3%)	----	----
Arthralgia [n (%)]	18(42.9%)	----	----
Arthritis [n (%)]	8 (19%)	----	----
DVT [n (%)]	3 (7.1%)	----	----
Nervous system [n (%)]	3 (7.1%)	----	----
Gastrointestinal [n (%)]	7(16.7%)		
ESR (mm 1st hour) Median (IQR)	45 (35 – 56)	----	----
CRP (mg/l) Median (IQR)	24 (12 – 36)	----	----
Hemoglobin (gm/dL) (Mean ±SD)	10.74 ± 1.23	12.46 ± 0.93	< 0.001
WBC (10^3/μl) (Mean ±SD)	8.1 ± 2.47	7.63 ± 1.5	NS
Platelets (10^3/μl) (Mean ±SD)	276.2 ± 99.68	255.3 ± 64.9	NS
MPV (fl) (Mean ±SD)	9.71 ± 1.9	8.5 ± 1.3	< 0.001
Cholesterol (mg/dl) [Median (IQR)]	181 (177 - 189)	179 (170 - 186)	NS
Triglycerides (mg/dl) [Median (IQR)]	136 (129 – 142)	132 (125 – 138)	NS
HDL (mg/dl) (Mean ±SD)	49.5 ± 8.9	52.19± 7.92	NS
LDL (mg/dl) (Mean ±SD)	105.4 ± 11.5	99.12 ± 12.27	0.02
Endocan (ng/l) [Median (IQR)]	249.5 (174 - 445)	190.5 (128 - 235)	0.002
BDCAF [Median (IQR)]	3 (1-4)	----	----
Carotid IMT (mm) (Mean ±SD)	0.85± 0.3	0.64 ± 0.17	< 0.001

BD: Behçet disease; BMI: body mass index; DVT: deep venous thrombosis; WBC: white blood cells; MPV: mean platelet volume; HDL: High-density Lipoprotein; LDL: Low-density Lipoprotein; BDCAF: Behçet disease current activity form; IMT: intima media thickness. *P*>0.05 is not significant (NS).

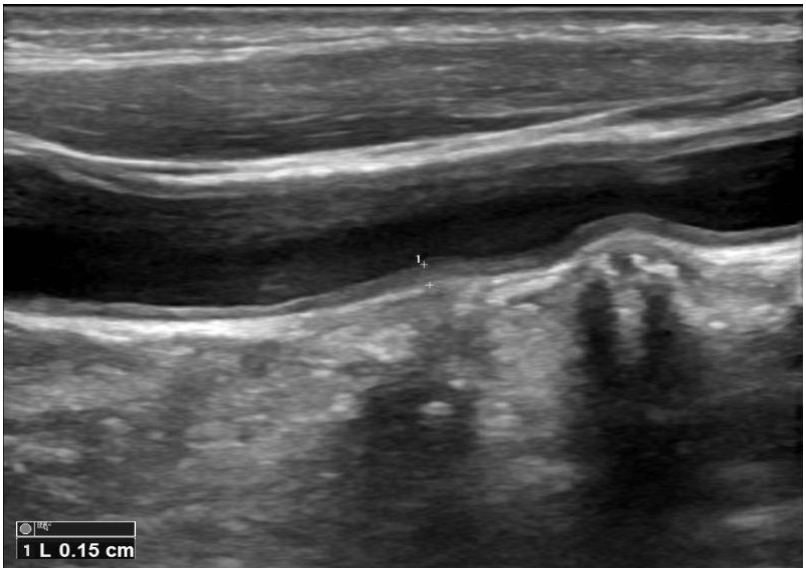

Figure 1. Ultrasonographic longitudial scanning of the right common carotid artery showed IMT is 1.5 mm (the distance between the marks + and 1+) in a 42 years old BD patient with increased serum endocan level (in 4th quartile). IMT: Intima media thickness; BD: Behçet disease.

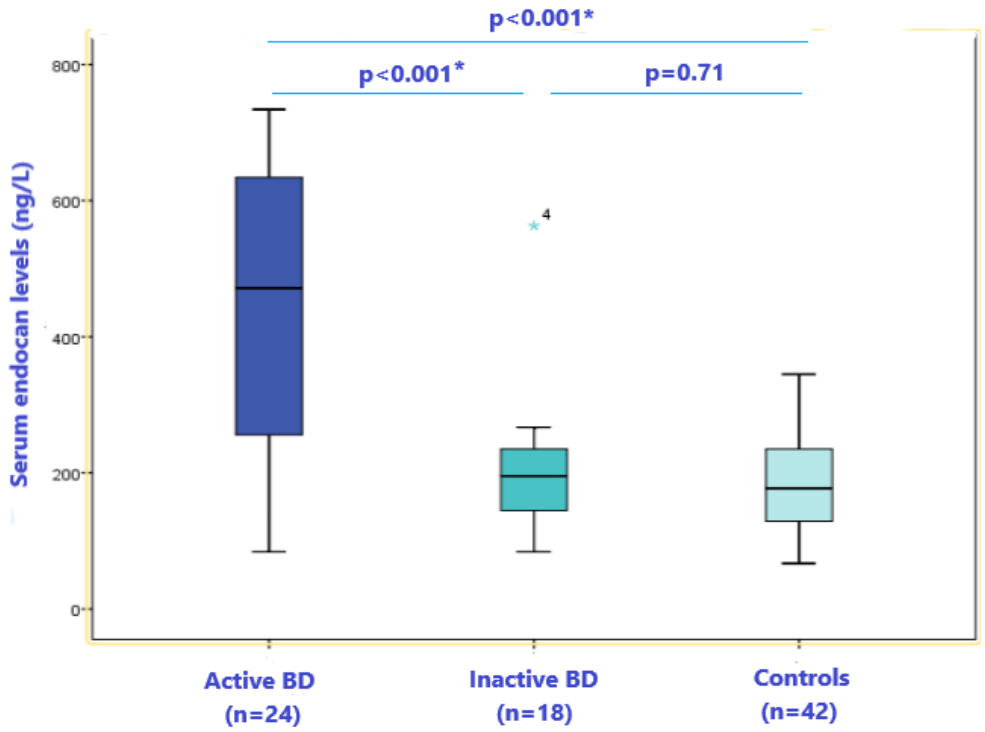

Figure 2. Comparison of serum endocan levels between active, inactive BD patients and controls showed significantly higher serum levels of endocan in active BD patient than those with inactive disease (*P*<0.001) and the controls (*P*<0.001). BD: Behçet disease.

Table 2. Characteristics of BD patients according to quartiles of their serum endocan levels.

Parameter	1st quartile (n=11)	2nd quartile (n=10)	3rd quartile (n=10)	4th quartile (n=11)	*P* value
Endocan (ng/l) [Median(IQR)]	143 (87.25-148)	219 (196-235)	376 (263-434)	623(568-642.75)	<0.001
Age (years) [Mean ±SD]	38 ± 7.4	32.9 ± 7.5	31.4 ± 5.7	38.6 ± 8.5	NS
Sex (Male: Female)	(6:5)	(7:3)	(5:5)	(7:4)	NS
BMI (Kg/m^2) [Mean ±SD]	27.3 ± 1.4	27± 1	27.6± 1.4	27.6± 2	NS
Disease duration (years) [Median(IQR)]	4 (2.25- 5)	2.5 (2- 4)	3.5 (2 - 4)	5 (3.25 – 7.5)	NS
Active uveitis [n (%)]	2 (18.2%)	3 (30%)	5 (50%)	7 (63.6%)	NS
Mouth ulcers [n (%)]	4 (36.4%)	4 (40%)	7 (70%)	7 (63.6%)	NS
Genital ulcerations [n (%)]	3 (27.3%)	1 (10%)	5 (50%)	4(36.4%)	NS
Arthritis [n (%)]	1(9.1%)	1 (10%)	1 (10%)	5 (45.5%)	NS
DVT [n (%)]	0	1 (10%)	0	2 (18.2%)	NS
ESR (mm 1ST hour) [Median(IQR)]	38 (29- 45)	44 (35 – 56)	53 (42 -56)	45 (34.75-77.25	NS
CRP (mg/l) [Median(IQR)]	8.2 (4.8-22.6)	13.5 (12-21.8)	28 (24-36)	46 (35.8 – 53.3)	<0.001 *P*1= NS, *P*2= 0.009, *P*3< 0.001, *P*4=0.009, *P*5=0.001, *P*6=0.02
Hemoglobin (gm/dL) [Mean ±SD]	11 ± 0.83	10.3 ±1.7	10.8 ± 1	10.8 ± 1.3	NS
WBC (10^3/μl) [Mean ±SD]	7.7 ± 2.5	7.2 ± 2.1	8.3 ± 2.8	9.1 ± 2.4	NS
Platelets (10^3/μl) [Mean ±SD]	266.7 ± 78.7	216.4 ± 94.5	318.2 ± 89.4	301.9 ± 114.7	NS
MPV (fl) [Mean ±SD]	9.3± 1.5	9.2 ± 1.8	10.1± 2.4	10.2± 1.9	NS
BDCAF [Median(IQR)]	1 (0-2.75)	1(0-1)	3.5 (2-5)	4 (3-5.75)	0.002 *P*1= NS, *P*2=0.03, *P*3= 0.003, *P*4=0.03, *P*5=0.003, *P*6=NS
Carotid IMT (mm) [Mean ±SD]	0.74 ± 0.24	0.73± 0.18	0.95± 0.33	0.96± 0.35	0.11

BMI: body mass index; DVT: deep venous thrombosis; MPV: mean platelet volume; BDCAF: Behçet disease current activity form; IMT: intima media thickness. ; p1: comparison between 1st and 2nd quartiles; p2: comparison between 1st and 3rd quartiles; P3, comparison between 1st ,4th quartiles; P4: comparison between 2nd and 3rd quartiles; P5: comparison between 2nd and 4th quartiles; p6: comparison between 3rd and 4th quartiles. *P*>0.05 is not significant (NS).

Table 3. Correlation between serum endocan level and different parameters in BD patients

Parameter	Serum endocan level	
	r	*P* value
Age	0.1	NS
Disease duration	0.28	NS
ESR	0.32	0.04
CRP	0.74	<0.001
HB	-0.09	NS
WBCs	0.29	NS
Platelet	0.27	NS
MPV	0.25	NS
Cholesterol	0.1	NS
Triglycerides	0.2	NS
BDCAF	0.65	<0.001
Carotid IMT	0.4	0.008

WBC: white blood cells; MPV: mean platelet volume; BDCAF: Behçet disease current activity form; IMT: intima media thickness. *P*>0.05 is not significant (NS).

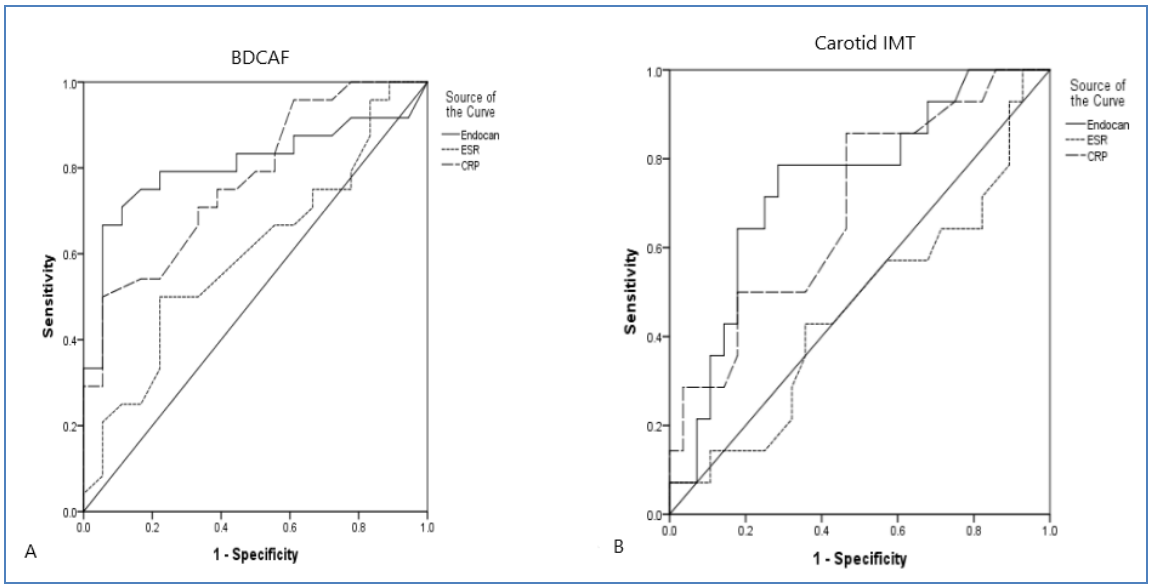

Figure 3. ROC curve analysis of the diagnostic performance of serum endocan levels to predict BDCAF showed that serum endocan had an AUC of 0.8 (figure 3A). Regarding carotid IMT, serum endocan had an AUC of 0.74 (figure 3B) in BD patients. ROC: Receiver operating characteristics; BDCAF: Behçet disease current activity form; IMT: Intima media thickness; BD: Behçet disease.

Discussion

The main function of normal endothelium is to maintain vascular stability that can be disturbed with chronic inflammation. Endothelial cells have the ability to release many cytokines that regulate immune cell recruitment toward inflammatory sites and regulate their function [28].

Endothelial dysfunction and activation was proposed to exert an interesting role in the pathophysiology of the inflammatory reaction associated with BD [28]. Many mechanisms can predispose to endothelial dysfunction in BD patients such as oxidative stress [29], hyper-homocysteinemia [30], anti-endothelial cell antibodies [31], many growth factors and cytokines [32-33]. Biomarkers related to endothelial dysfunction have been focused in researches and it was found that many of them were associated with disease activity and markers of systemic inflammation in various inflammatory conditions such as BD [22-24].

Our BD patients had increased endocan serum levels in comparison to healthy controls. Moreover, these levels were higher in BD patients with active disease compared to those with inactive disease. Also, serum endocan levels were correlated with CRP and BDCAF. These results are comparable to those reported with previous studies [22-24].

Inflammatory cytokines such as tumor necrosis factor-α or interleukin-1 beta can upregulate endocan expression [34-35].BD is characterized by intense inflammatory response and some authors classify BD as an auto-inflammatory disease in which vasculitis is the main pathological finding, however there is no consensus about its exact etiopathogenesis [36-37]. Many other mechanisms can explain increased endocan levels in BD patients, Shin et al. [38] suggested that increased vascular endothelial growth factor (VEGF) levels can increase the expression of endocan by endothelial cells. Also, increased endocan titers can be attributed to inhibition of lymphocyte function-associated antigen-1 /intracellular adhesion molecule-1 pathway [39]

In our study, we didn't find significant difference in the prevalence of different organ-specific involvement among BD patients regarding their serum endocan levels. On contrary, Hammad et al. [23] found that BD patients with papulopustular and genital ulcers to have elevated serum endocan levels, while Balta et al. [24] recorded that endocan levels were elevated in BD patients with active ocular involvement.

In this study, carotid IMT in our BD patients was significantly increased than in the healthy controls. This finding was similar to the results of previous studies [40-41]. Furthermore, serum endocan levels positively correlated with carotid IMT and it was reported that endothelial dysfunction can predispose to early atheroma formation that could be detected by measurement of arterial stiffness and/or carotid IMT [9, 42].

We did not find a significant difference in the frequency of DVT among BD patients with different endocan serum levels that can be attributed to the small number of cases with recent venous thrombosis who were recruited in our study and the exclusion of many conditions that can predispose to vascular thrombosis. There is a conflict about increased risk of thrombosis in BD; Koseoglu *et al.* suggested that vascular involvement in BD is multifactorial that includes increased thrombophilic factors and altered platelet activation in addition to the presence of endothelial dysfunction [28].On

contrary, Alkaabi et al. suggested that the increased thrombophilic factors didn't predispose to the thrombotic tendency in BD patients rather than being an acute phase response [43].

Although, previous studies reported the significant correlation between endocan and disease activity parameters in BD patients [22-24], none of them evaluated the association between endocan and any of the endothelial dysfunction tests in BD patients. A limitation to this work lies in the relatively small sized sample and the need of longitudinal follow-up of the patients before and after initiation of therapy to detect the precise effect of medications.

In conclusion, our BD patients have a remarkably elevated endocan serum levels that significantly correlated with disease activity and carotid IMT suggesting that it could be a useful marker to monitor both disease activity and endothelial dysfunction in BD patient.

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