https://doi.org/10.48047/AFJBS.6.12.2024.6222-6236

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Role of Neutrophil Extracellular Traps and Femoral Vein Wall Thickness in Diagnosis of Behcet Syndrome and Their Relation to Disease Activity

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Article History

Volume 6, Issue 12, 2024

Received:22 June 2024

Accepted: 31July 2024

doi:

10.48047/AFJBS.6.12.2024.6222-6236

Abstract:

Behcet's disease is a type of systemic vasculitis characterized by auto-inflammatory processes. The exact etiology is yet unknown and the condition is distinguished by mucocutaneous symptoms. Additionally, it involves systemic vasculitis affecting both large and small arteries and veins.

We aimed to assess the levels of NETs and femoral vein wall thickness in patients with BD, and compare them to patients with vasculitis, venous insufficiency, and healthy controls.

Ninety patients (n=90) were divided into four groups; group (1) included thirty patients with Behçet's disease, group (2) included twenty-five patients with vasculitis, group (3) included twenty-five patients with venous insufficiency and group (4) included ten healthy controls. CBC, creatinine, urea, ALT, AST, ESR and CRP were measured. In addition, NETs were measured in all patients. Veins of both lower limbs were examined using doppler ultrasonography.

There was statistically significant difference between the studied participants regarding the ESR and CRP levels. NETS level had a non-significant role in diagnosis of the Behçet syndrome and disease activity. Moreover, thickness of the right and left femoral vein wall were significantly higher in Behçet patients than vasculitis patients, venous insufficiency patients and healthy controls at p values (0.001).

It could be concluded that femoral vein wall thickness measurement is useful for Behçet's disease diagnosis but does not show disease activity. While NETs are probably not relevant, further studies are required to explore its role in Behcet's disease..

Keywords:Behçet's syndrome, Femoral vein, NETs, Ultrasonography, Vasculitis.

1. Introduction

Behçet disease (BD) is an inflammatory autoimmune disease that affects multiple systems in the body. It can either go into remission or recur spontaneously, similar to other autoimmune disorders. BD causes damage to the eyes, skin, joints, and other organs, leading to a range of clinical symptoms **(Mendoza et al., 2010)**.The incidence of different clinical manifestations varies among individuals with Behçet's syndrome. The most prevalent indicators in the population affected by Behçet's syndrome are mucocutaneous and ocular symptoms, which are consequently regarded as the characteristic features of the illness **(Rotondo et al., 2015**).The latest nationwide survey in Egypt revealed a geographical spread of male predominance with a male-to-female ratio of 2.6:1 **(Gheita et al., 2019)**.Veins, including the smallest venules and the superior and inferior vena cava, are affected by BD in 30–40% of patients. This leads to thrombosis in the veins and thrombophlebitis on the surface **(Desbois et al., 2014)**.

The development of Human leukocyte antigen (HLA)-B51 and HLA-B27 and the occurrence of BD may be interconnected, potentially leading to the activation of a Th1 immune response, either provoked or spontaneous (**Ombrello et al., 2014)**.

In addition, infections caused by viruses and bacteria are believed to potentially activate Behçet's disease by inducing the creation of heat shock proteins (HSP) **(Zierhut et al., 2003)**.

Neutrophils, as innate immunological phagocytes, play a crucial role in immune defense. In recent years, there has been a notable progress in our understanding of the involvement of neutrophils in immunological regulation, pathogen elimination, and disease pathogenesis **(Brinkmann et al., 2004).** Neutrophil extracellular traps (NETs) are chromatin structures that resemble webs. They have gained significant attention in the study of neutrophil biology. The discovery of chemicals that govern the release of NETs has enhanced understanding of the role of NETs in immunological defense, inflammatory and autoimmune illnesses, and cancer **(Le Joncour et al., 2019).**

The elevated levels of NETs in the bloodstream can be attributed, in part, to the heightened propensity of neutrophils from individuals with BD to generate NETs.1. The scientists discovered that NETs were present around damaged blood arteries. They also observed that BD patients with vascular involvement had significantly higher levels of NETs. This suggests a potential connection between these structures and the vascular symptoms of the disease **(Yazici et al.,2018)**.

Diagnosing Behçet's disease (BD) can be challenging, especially in countries where the disorder is rare. Recent research has shown that individuals with bipolar disorder (BD) exhibit increased thickness of the venous walls (VWT) in their lower limbs **(Alibaz-Oner et al., 2021)**. Moreover, **Alibaz-Oner et al., (2021)**,added that CFV thickness assessment using ultrasonography a non-invasive radiological approach could be used to diagnose BD. It has a high sensitivity and specificity rate of over 80% when using cut-off values below 0.5 mm.

This study aims to assess the levels of NETs and femoral vein wall thickness in patients with BD, and compare them to patients with vasculitis, venous insufficiency, and healthy controls. The study also aims to determine the potential use of these measurements for diagnosing BD and evaluating their correlation with disease activity.

Materials and Methods:

This was a case control study that aimed to investigate the potential roles of NET and femoral vein wall thickness in Behçet's Syndrome affected patients. The study was approved by Ethical Committee as well as informed written consents were signed by all patients to be included in the study.

Study Population:

The patients were selected from the clinics of internal medicine department, especially rheumatology and immunology clinic, Beni-Suef university hospital for six months from March 2023 to August 2023.

The patients were included in the study if they were; Adult BD patients with a confirmed diagnosis, adult patients diagnosed with vasculitis and adult Patients diagnosed with vascular insufficiency. While patients were excluded from the study if they were suffering from end stage renal disease, chronic liver cell failure patients as well as Malignancy patients.

The selected patients (n=90) were divided into four groups; group (1) included thirty patients with Behçet's disease (n=30), group (2) included twenty-five patients with vasculitis (n=25), group (3) included twenty-five patients with venous insufficiency (n=25) and group (4) included ten healthy controls of matched age and sex (n=10) (healthy controls).

Diagnosis of Behçet's Syndrome was done according to the international Study Group for BD criteria updated in 2013 and patients diagnosed with vasculitis classified according to chapel hill consensus of vasculitis.

Methods:

Patients in all groups as well as controls were subjected to; history taking, including family history of autoimmune disease, vascular diseases and drug history. Clinical Examination was performed including comprehensive physical examination, appropriate venous and arterial vascular examination, ophthalmic assessment Anterior segment with Slit-lamp Biomicroscopy and posterior segment with indirect opthalmoscope and fundus examination, evaluation of height, body mass index, and weight. In addition, laboratory investigations of complete blood count (CBC), creatinine, urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were measured.

Neutrophil extracellular traps were quantitatively evaluated using ELISA test kits **(Matta et al., 2023)**.Briefly, the kit designed for the quantitative level of NET in the sample, adopt purified Human NET to coat microliter plate, make solid-phase antibody, then added NET to wells, Combined NET antibody with labeled HRP to form antibody-antigen-enzyme-antibody complex, after washing completely, TMB substrate solution was added, TMB substrate becomes blue color at HRP enzyme-catalyzed. The reaction was terminated by the addition of a stop solution and the color change was measured at a wavelength of 450 nm. The concentration of NET in the samples was then determined by comparing the O.D. of the samples to the standard curve.

The veins of both the lower limbs were examined using doppler ultrasonography. Several veins in the lower extremities were examined with a high-resolution ultrasound Doppler system. These included the CFV, popliteal vein, deep and superficial femoral veins, great and small saphenous veins, and a GE logic P9 device with a probe frequency of 8-12 MHz.

Venous insufficiency was evaluated after Valsalva manoeuver both at the saphenofemoral junction and at the popliteal veins in supine and prone positions. B-mode ultrasound in the supine position was performed to measure CFV wall thickness. The wall thickness was measured at 2 cm distal from the saphenofemoral junction.

Statistical analysis:

Statistical analyses were performed by SPSS. T-test was used to compare continuous numerical variables that are given as mean and standard deviation. The Mann-Whitney U test used to compare the two groups, and skewed numerical data was displayed as the median and interquartile range. Fisher's exact test was used to compare categorical data that are given as numbers and percentages. A receiver-operating characteristic (ROC) curve was used to evaluate the tests' diagnostic efficacy. $p < 0.05$ were deemed noteworthy.

Results:

Table (1): Baseline characteristics of the studied participants.

		Behçet (n=30)		Vasculitis (n=25)		Venous insufficiency (n=25)		Healthy Controls (n=10)		P-value
		Count	%	Count	%	Count	%	Count	%	
Age in years	Mean±SD	36.7±9.1		38.6±13.6		33.36±7.7		41.3±15.1		0.207
	Range	22-62		14-71		22-45		18-64		
Sex	Female	11	36.7	11	44.0	19	76.0	4	40.0	0.022*
	Male	19	63.3	14	56.0	6	24.0	6	60.0	
Marital status	Single	8	26.7	4	16.0	6	24.0	1	10.0	0.612
	Married	22	73.3	21	84.0	19	76.0	9	90.0	
Disease duration	Mean±SD	3.83±4.1		2.28±2.7		2.96±1.1		--	--	0.004*
	Range	1-20		1-13		1-5		--	--	
Diabetes Mellitus		2	6.7	2	8.0	0	0.0	1	10.0	0.531
Hypertension		1	3.3	3	12.0	0	0.0	1	10.0	0.250
Smoking		6	20.0	6	24.0	2	8.3	2	20.0	0.524

The results of baseline characteristics of all participants were recorded in table (1). There was no statistically significant difference between the studied participants regarding age, marital status, diabetes, hypertension, and smoking history. Regarding disease duration, the mean duration in behçetpatients were longer than vasculitis (3.83±4.1) and venous insufficiency patients (2.28±2.7) and (2.96±1.1), respectively.

Table (2). Diagnosis of Behçet patients according to ISCG (International Society group guidelines) and Pathergy Skin Test and BSAS (Behçet Syndrome Activity Scale) for the studied Behçet patients.

		Frequency	Percentage
Pathergy	**Positive**	28	93.3
	Negative	2	6.7
Total		30	100.0

	Minimum	Maximum	Mean	SD
BSAS	6.0	50.0	22.0	11.3

Table (2) demonstrated that 30 out of 30 participants showed positive ISCG criteria (100.0%) and 28 out of 30 showed positive pathergy skin test (93.3%). In addition, the mean BSAS for the studied Behçet patients was 22.0±11.3 and ranging from 6-50.

Table (3): Distribution of the studied vasculitis patients regarding the nature of the disease

	Count (n=25)	Percentage (%)
Small vessel affection	12	48.0
Medium vessel affection	5	20.0
Large vessel affection	4	16.0
Secondary vasculitis	4	16.0

Distribution of the studied vasculitis patients regarding the nature of the disease was recorded in table (3). There were 26 patients diagnosed with primary vasculitis (84%) and 4 patients were diagnosed with secondary vasculitis (16%). According to chapel hill consensus the patients were classified into; large vessel vasculitis included 3 patient with Takayasu Arteritis and 1 patient with giant cell arteritis. Medium vessel vasculitis included 5 patients with Polyarteritis nodosa. Small vessel vasculitis included 5 patients with buergers disease, 5 patients with Leucocytoklastic vasculitis and 2 patients with Granulomatosis with Polyangitis. Secondary vasculitis included 2 patients secondary to SLE and 2 patients secondary to systemic sclerosis.

Table (4). Hematological parameters of the studied Behçet patients and healthy controls.

	Behçet (n=30)		Healthy controls (n=10)		
	Mean	**SD**	**Mean**	**SD**	***P value***
Hemoglobin (gm/dl)	12.32	1.76	12.90	0.88	0.183
MCV (fL/cell)	78.27	6.93	79.40	6.72	0.645
MCH (pg/cell)	26.27	3.63	23.80	2.66	0.056
TLC ($x10^3/mm^3$)	8.76	3.39	5.50	0.71	0.001*
Platelet ($x10^3/mm^3$)	276.47	92.46	344.30	73.26	0.042*
MPV (fL)	8.90	1.34	7.50	2.12	0.189

Table (4) demonstrated that there was statistically significant difference between the studied Behçet patients and healthy controlsregarding the total leucocytic count and platelet count (P value=0.001 and 0.042), respectively.

Table (5). Hematological parameters of the studied vasculitis patients vs healthy controls.

	Vasculitis (n=25)		Healthy controls (n=10)		
	Mean	**SD**	**Mean**	**SD**	***P value***
Hemoglobin (gm/dl)	59.36	228.38	12.90	0.88	0.528
MCV (fL/cell)	79.14	15.24	79.40	6.72	0.959
MCH (pg/cell)	28.76	10.62	23.80	2.66	0.157
TLC ($x10^3/mm^3$)	7.42	3.73	5.50	0.71	0.118
Platelet ($x10^3/mm^3$)	280.84	99.00	344.30	73.26	0.076
MPV (fL)	8.33	.82	7.50	2.12	0.677

Table (6). Hematological parameters of patients with venous insufficiency vs healthy controls.

	Venous insufficiency (n=25)		Healthy controls (n=10)		
	Mean	**SD**	**Mean**	**SD**	***P value***
Hemoglobin (gm/dl)	12.11	0.84	12.90	0.88	0.587
MCV (fL/cell)	80.12	5.60	79.40	6.72	0.747
MCH (pg/cell)	25.28	3.88	23.80	2.66	0.278
TLC ($x10^3/mm^3$)	5.64	1.11	5.50	0.71	0.661
Platelet ($x10^3/mm^3$)	278.88	89.68	344.30	73.26	0.049*
MPV (fL)	7.80	.84	7.50	2.12	0.779

In tables 5 and 6, there was no statistically significant difference between the studied vasculitis and Venous insufficiency patients when compared with healthy controls as regards the hematological parameters.

Table (7): Inflammatory markers, liver and renal functions of patients with Behçet's disease vs controls.

	Behçet (n=30)		Healthy controls (n=10)		
	Mean	**SD**	**Mean**	**SD**	***P value***
ESR (mm/hr)	32.73	12.58	20.60	4.65	0.005*
CRP (mg/l)	7.45	3.11	5.50	0.71	0.066
AST (U/L)	30.38	12.72	30.30	8.37	0.062
ALT (U/L)	26.46	7.43	21.00	8.42	0.985
Creatinine (mg/dL)	0.86	0.19	0.94	0.22	0.285

Table (7) demonstrated that the ESR level for Behçet patients was significantly greater than healthy controls. While there was no statistically significant difference between the studied participants as regards liver and kidney functions.

Table (8): Inflammatory markers, liver and renal function of patients with vasculitis vs controls.

	Vasculitis (n=25)		Healthy controls (n=10)		
	Mean	**SD**	**Mean**	**SD**	***P value***
ESR (mm/hr)	49.68	27.49	20.60	4.65	0.002*
CRP (mg/l)	26.67	16.98	5.50	0.71	0.001*
AST (U/L)	31.68	20.97	30.30	8.37	0.153
ALT (U/L)	29.88	18.30	21.00	8.42	0.843
Creatinine (mg/dL)	1.15	1.44	0.94	0.22	0.654

ESR and CRP level for vasculitis patients was significantly greater than healthy controls. While liver and kidney functions showed no statistically significant difference between the studied participants.

Table (9): Inflammatory markers, liver, and renal function of patients with venous insufficiency vs controls.

	Venous insufficiency (n=25)		Healthy controls (n=10)		
	Mean	**SD**	**Mean**	**SD**	***P value***
ESR (mm/hr)	24.12	7.64	20.60	4.65	0.185
CRP (mg/l)	5.50	0.71	5.50	0.71	1.000
AST (U/L)	30.32	7.93	30.30	8.37	0.061
ALT (U/L)	27.80	9.70	21.00	8.42	0.995
Creatinine (mg/dL)	0.85	0.11	0.94	0.22	0.249

In venous insufficiencypatients, there was no statistically significant difference between the studied participants as regards inflammatory markers, liver and kidney functions.

Table (10): NETS level among the studied participants.

NETS level	Groups				*P value*
	Behçet (n=30)	**Vasculitis (n=25)**	**Venous insufficiency (n=25)**	**Healthy controls (n=10)**	
Mean±SD	253.26± 318.23	431.90± 1008.10	288.24± 150.51	287.95± 131.80	P=0.689 P1=0.362 P2=0.658 P3=0.966
Range (MIN-MAX)	67.3 - 1682.0	110.8 - 5242.0	103.9 - 636.1	145.0- 561.0	

NETS: Neutrophil Extracellular Traps/ Statistical analysis was done using Two-tailed independent samples T test and Analysis of Variance (ANOVA test). P values represent the following; P= difference across all studied groups, P1= difference between Behçet group and healthy controls, P2= difference between Vasculitis group and healthy controls and P3= difference between Venous insufficiency group and healthy controls.

The levels of NETs were illustrated in table (10). There was no statistically significant difference between the studied participants regarding the NETS level.

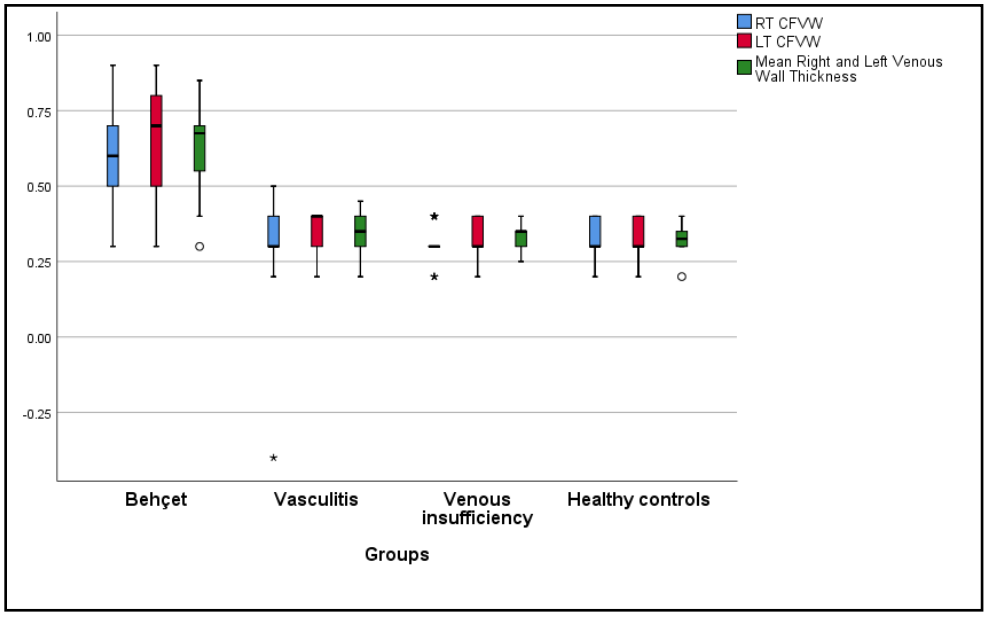

Figure (1). Common femoral venous wall thickness among the studied participants.

RT CFVW: Right common femoral vein wall thickness/ LT CFVW: Left common femoral vein wall thickness/ Statistical analysis was done using Two-tailed independent samples T test / *P value ≤0.05 is considered statistically significant.

Figure (1) demonstrated that the mean venous wall thickness of the right and left common femoral vein were significantly higher in behçetpatients (0.6±0.15 and 0.66±0.16) than vasculitis patients (0.31±0.17 and 0.34±0.08), venous insufficiency patients (0.31±0.06 and 0.34±0.06) and healthy controls (0.32±0.06 and 0.32±0.06) at p values (0.001). In addition, there was no statistically significant difference between vasculitis patients and controls regarding the venous wall thickness.

Table (11). Sensitivity and specificity of Neutrophil Extracellular Traps (NETS) and Femoral Vein Wall Thickness in Diagnosis of Behçet Syndrome.

Test	Area under the curve	Sensitivity	Specificity	Cut off	P value	95% Confidence Interval	
NETS	0.324	6.7%	98.3%	≥ 818.5	0.007*	0.203	0.446

Statistical analysis was done using Receiver Operating Characteristics (ROC) curve / *P value ≤0.05 is considered statistically significant.

Table (11) and Fig. (2) showed that the sensitivity and specificity of NETS level for diagnosis of Behçetsyndrome was 6.7% and 98.3% at a cutoff point 818.5.

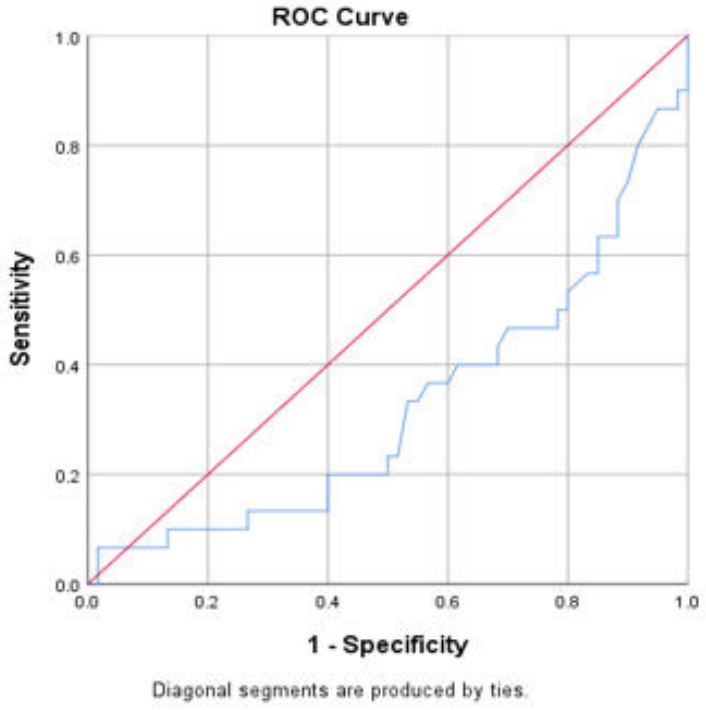

Fig. (2). ROC curve of NETS for the diagnosis of Behçet syndrome

Table (12): Sensitivity and specificity of Femoral Vein Wall Thickness in Detection of Behçet Syndrome activity

Test	Area under the curve	Cut off	P value	Sensitivity	Specificity
RT CFVW	0.309	0.65	0.154	16.7%	58.3%
LT CFVW	0.521	0.75	0.876	50.0%	66.7%
Mean right and left	0.420	0.63	0.551	66.7%	45.8%

NETS level, RT CFVW and LT CFVW thickness had non-significant role in detection of the Behçetsyndrome activity as shown in table (12) and figure (3).

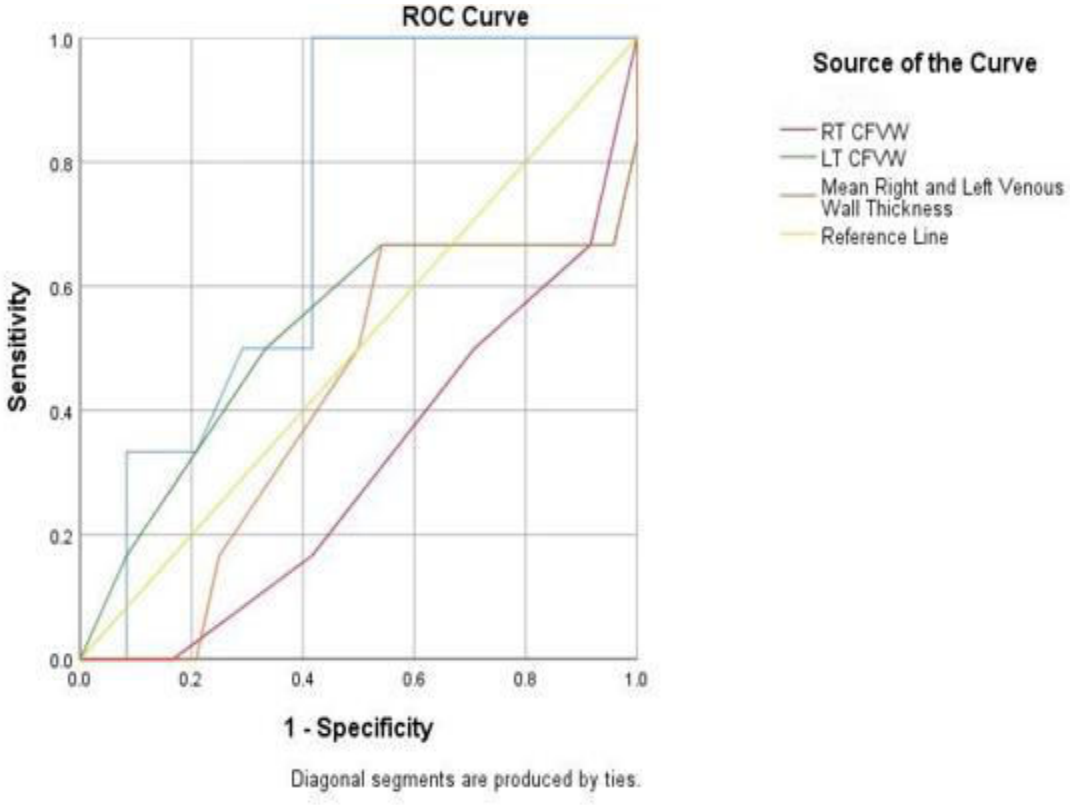

Fig. (3). ROC curve of femoral venous wall thickness for the detection of Behçet activity

Table (13): Correlation between BSAS and venous wall thickness for the studied Behçet patients

		RT CFVW	LT CFVW	Mean Right and left Venous Wall Thickness
BSAS	**Pearson Correlation (r)**	0.140	0.104	0.104
	P value	0.459	0.584	0.584
	Count	30	30	30

Statistical analysis was done Pearson Correlation analysis test.

Table (13) demonstrated that there was no significant correlation between BSAS for the studied Behçetpatients and their venous wall thickness.

Discussion:

Behçet's disease (BD) is categorized as a "variable-vessel vasculitis" according to the Chapel Hill classification. This means that it is a systemic, inflammatory disorder that impacts blood vessels of different sizes, including both veins and arteries. It should be emphasized that vascular involvement is seen in up to 40% of individuals with Behçet's disease. Both superficial thrombophlebitis and venous thrombosis indicate inflammation of the veins' walls in BD. Lower limb vein thrombosis is the most prevalent kind of vein involvement, accounting for up to 80% of cases. Nevertheless, it is possible that several other sites, including the pulmonary arteries, supra-hepatic vessels, dural/sagittal sinuses, inferior and superior vena cava, and cardiac cavities, could also be impacted **(Kaymaz et al., 2021; Giannessi et al., 2022)**.

Diagnosing Behçet's disease (BD) can pose challenges, especially in countries where the condition is rare. Recent evidence has shown that individuals with BD exhibit elevated venous wall thickness (VWT) in their lower extremities. The objective of our study was to determine the potential diagnostic use and performance of measuring the thickness of the common femoral vein (CFV) in BD **(Alibaz-Oner et al., 2022)**.

Our study determined that the NETS level did not have a major role in diagnosing behçet syndrome or disease activity. We identified no statistically significant variation in the NETS level among the participants we tested (The p-value was 0.326).

In contrast to our findings, a study conducted by **Le Joncour et al. (2023)**. Based on the presence of venous thrombosis, neutrophil activation, and ROS creation as characteristic features of BD, it may be inferred that one possible role of NETs in this disease is to promote thrombosis and vascular damage. This is supported by the fact that ROS generation, which is one of the primary molecular pathways leading to NETosis, also contributes to thrombosis and vascular damage. Neutrophils have the ability to generate NETs in situations of inflammation or infection by means of a distinct process of cell death called NETosis (**Le Joncour et al.,2023)**.

Another study conducted by **Li et al. (2020)** found that an excessive presence of BD NETs facilitated the development of Th1 cells, excessive production of proinflammatory cytokines, and hyperactivation of macrophages. The increased generation of IL-8 by macrophages was partially

caused by the higher levels of Histone H4 in BD NETs. Additionally, the presence of enriched oxidized DNA in BD NETs may have also contributed to the excessive activation of macrophages.

Additionally, research has demonstrated that neutrophil extracellular traps (NETs) have the ability to stimulate the formation of antibodies and influence the activities of other molecules, including pro-proliferative ligands. Hence, studying and explaining the process of NETosis and the regulatory mechanisms that impact the innate immunity of vasculitis patients can offer innovative treatments for managing the disease **(Li et al., 2019)**.

Our study demonstrated that the thickness of the right and left femoral vein wall were significantly higher in behçet patients (0.6±0.15 and 0.66±0.16) than vasculitis patients (0.31±0.17 and 0.34±0.08), venous insufficiency patients (0.31±0.06 and 0.34±0.06) and healthy controls (0.32±0.06 and 0.32±0.06) at p values (0.001). Moreover, the right and left common femoral vein wall thickness had a significant role in prediction of the presence of behçet syndrome at a cut off 0.45 with sensitivity (86.7%) for right vein and (90.0%) for left vein and specificity (98.3%) for right vein and (100.0%) for left vein.

These findings align with the research conducted by **Alibaz-Oner et al. (2021)**. When comparing BD to the control groups, there was a significant increase in the thickness of bilateral CFV ($P < 0.001$). In all comparison groups, the area under the receiver operating characteristic curve for bilateral CFV thicknesses exceeded 0.95, using a cut-off value of 0.5 mm. With a sensitivity rate of 90%, this cut-off value also performed favorably compared to all control groups.

The study conducted by **Erturk et al. (2023)** suggests that measuring the entire wall thickness of the common femoral vein can be used as a diagnostic method to differentiate between Behçet's disease and recurrent apthous stomatitis, particularly when presenting with oral ulcers. The study proposes that ultrasound can be a useful technique for determining the thickness of the common femoral vein's wall, as it can be challenging to distinguish between these two conditions in a clinical setting. The study found that ultrasound had a sensitivity of 79.8% and specificity of 64.7% in distinguishing between the two conditions. The wall thickness of the common femoral vein (>0.58 mm) may be helpful in differentiating Behçet's disease from recurrent apthous stomatitis, but further investigations are needed to confirm these findings.

Another meta-analysis study by **Merashli et al., (2023)** comprised 9 case-control studies and 1 cohort study. The VWT (vascular wall thickness) was found to be significantly higher in individuals with BD ($n = 650$) compared to controls ($n = 396$) ($p < 0.0001$), and there was a high level of variation among the studies ($I2 = 94.4\%$). The VWT (vessel wall thickness) is higher in individuals with BD compared to individuals without the disorder (controls).

The findings we obtained align with the research conducted by **Alibaz-Oner et al. (2021)**. CFV thickness measurements were repeated after a follow-up period ranging from 10 to 36 months. There was no observable change in the thickness of the CFV during the follow-up period. The thickness of the right CFV was 0.79 (0.2) mm at the beginning and 0.78 (0.1) mm at the end, with a p-value of 0.65. Similarly, the thickness of the left CFV was 0.79 (0.3) mm at the beginning and 0.78 (0.1) mm at the end, with a p-value of 0.78.

Conclusion:

The measurement of femoral vein wall thickness can be utilized as a valuable diagnostic tool for Behçet's illness, while it is not indicative of disease activity. Neutrophil extracellular traps likely do not play a role in diagnosing Behçet's disease vasculitis, chronic venous insufficiency, or disease activity. However, additional research is needed to investigate their potential role.

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