MJMR, Vol. 35, No. 3, 2024, pages (82-95)

Open Access
ISSN: 2682-4558

Value of flowcytometric determination of circulating endothelial progenitor cells in Behcet's disease patients.

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DOI: 10.21608/MJMR.2023.224689.1477

Research Article

Abstract

Background and aim of the study: Behcet's disease (BD) defined as a systemic vasculitis condition with multifactorial immunopathogenesis. Endothelial progenitor cells (EPCs) are essential for vascular repair and hemostasis. Our current study aimed to evaluate role of circulating EPCs in BD and correlate it with lab findings & clinical manifestations /disease activity. **Methodology:** The study included 30 patients (group I) suffering from BD and 25 apparently healthy subjects group(II) matched with group(I). For BD patients, clinical features and activity assessed by current disease activity form (**BDCAF)**. For All subjects, clinical and laboratory investigations including routine tests and the determination of circulating EPCs using flowcytometry (Identified as live nucleated CD45 negative/CD34 bright/CD309positive events) were recorded. **Results:** There was statistically significant increase in group I regarding EPCs,TLC,AST CRP and ESR compared to group II.Circulating EPCs showed positive significant correlation with hemoglobin,AST and ALT However there were negative significant correlations with TLC, Monocytes ,Neutrophils, ESR and NLR and negative insignificant correlation with CRP and ANCA and ANCA C& P, There was negative non-significant correlation between EPCs and eye , skin and joint symptoms and BDCAF score .**Conclusion:**Our study demonstrated increased level of circulating EPCs in BD than controls with negative correlation with inflammatory markers and negative(nonsignificant) correlation with clinical manifestations and BDCAF score. High levels of circulating EPCs could be a good sign of endothelial healing suggesting its potential, after further studies on large cohort of patients, to serve as a maker of repair and recovery of damaged blood vessels in BD patients.

Key words: Circulating endothelial progenitor cells, ANCA, Behcet's disease, BDCAF.

Introduction

Vasculitis could be defined as a large number of diseases in which inflammation of vessels commonly took place. They are similar in LAB findings, clinical manifestations, and pathophysiologic characters. The clinical manifestations and pathological characters are widely variable and closely related to the size and site of the damaged blood vessel. Vasculitis could be primary or secondary prowess to another systemic disorder. When vasculitis presented as a primary process in a disease it called primary systemic vasculitis one of the commonest disease of primary vasculitis is Behcet's disease [1].

Behçet's disease is known as chronic multiple systemic vasculitis, affecting from the smallest to the largest vessel[1]. It is characterized by recurrent aphthous ulcers attack oral mucosa , genitalia , skin and eyes[2]. It can occur in young ages especially those under 30 years old, men are more prone than women to have major issue damage. Behçet's disease is also often discovered in the third and fourth life decades [3].

The prevalence of BD all over the world is 10.3 per 100,000 population [4]While in Egypt the prevalence is 3.6/100,000 population with no huge difference between north and south [5].

Behcet's disease is multifactorial autoimmune disorder as there are both environmental factors including infections, microbiome, additional triggering factors and genetic factors could be involved in it[6].

HLA-B51 allele is a genetic factor with strong correlation with BD. Pro-inflammatory cytokines ,for example: tumor necrotic factor-alpha (TNFα), interleukin-6 (IL6) are massively increased in serum of in active BD patients, But unfortunately most of these tests are less specific and hardly available in most laboratories[7]. There are many lines of treatments of BD.

EPCs are basically take place in bone marrow and migrate to the circulation[8]. EPCs are very essential in vascular repair and homeostasis, where it vary in their numbers and functions. It is widely recognized that during endothelial activation or stress, EPCs can be migrate, in large numbers to the peripheral circulation. That could suggest that EPCs have strong link between endogenous repair or a defective homeostatic [9].

Aim of the work:

The present study aimed to assess the concentration of circulating EPCs using a standardized flowcytometry protocol in patients with BD and correlate the results with the laboratory findings and clinical manifestations/disease activity.

Subjects and Methods

This prospective cross-sectional study was done basically at the Clinical Pathology Department, Rheumatology department, Faculty of Medicine, Minia University, Minia, Egypt through the period from December 2021 to January 2023. It was conducted on 65 individuals. Our subjects were divided to Group I (Patient group) which included thirty (30) patients (seventeen males & thirteen Females) suffering from Behcet's disease. Group II (control group) which included twenty-five (25) apparently healthy subjects (gender, age and smoking habits) matched with group I. The hospital ethical committee kindly approved our study and a written consent was taken from each subject (Approval number: 205:12/2021, Date of approval: 2٧ December 2021).

All subjects included in the study were subjected to the following:

- Careful history taking emphasis international criteria for Behcet's disease (ICBD).
- Clinical examination with fulfillment of (BDCAF) score of disease activity
- Laboratory Investigations: - Collection protocol of Blood samples: Peripheral blood was drawn from the antecubital vein with 21 G needles in EDTA vacutainer tubes. The first 3 ml of drowned blood was excluded from the direct analysis EPCs and used for the other investigations, to avoid enumeration of cells released from vascular damage caused by venipuncture. -routine investigations was done: complete blood count (CBC), Renal functions serum urea and serum creatinine (Cr)), Liver enzymes (Alanine transaminase (ALT), Aspartate transaminase (AST)), Erythrocyte sedimentation ratio (ESR), C-reactive protein (CRP) and ANCA (C and P).

- Calculation of EPCs: calculation was done within 4 hours from the specimen collection by the standardized flow cytometry protocol, One hundred microliter of EDTA blood was used for each control tube and test tube for evaluation of EPCs. Ten microliter of markers (Human CD45 PE conjugated antibody/Human CD34APC-conjugated antibody) were added and mixed with vortex, covered with plastic cap and incubated for 30 minutes in dark area at room temperature. One ml. of diluted lyse (ammonium chloride) 1:10 by distilled water was added to 100 of sample and control tube, tubes were mixed well by vortex, then incubate 20 mins. in dark area at room temperature. Tubes were centrifuged at 1200 Rpm for 5 mins. then supernatant was removed. Two mL of Phosphate -Buffered Saline (PBS) was added to each tube. One hundred microliter of reagent A (Intrasure kits) for intracellular introduction of marker was added, then incubated for 5 mins at room temperature. Fifty microliter of reagent B was added to each tube. Then incubated again for 5 mins. at room temperature. Ten microliter from intracellular marker (Human VEGFR2/KDR/CD309/FIK-1 PreCP-conjugated antibody) was added then incubated for 5 mins in dark place at room temperature.

All tubes were centrifuged again at 1200 RPM for 5 mins and supernatant was removed. Five hundred of PBS was added to each tube, then mixed by vortex. Control tube was interpreted first by flowcytometry **(BD Bioscience FacsCanto II Flow Cytometer, USA**) then test tube was interpreted as EPCs were defined as alive nucleated CD45 negative / CD34 bright / CD 309 positive cells.

Statistical analysis: Statistical Package of Social Science (SPSS), version 20, was used to conduct the statistical analysis. Frequency (%) was used to describe qualitative data, and mean + SD (SD) was used to summarise quantitative data. When necessary, the Chi square test (X) and Fisher exact tests were used to analyse qualitative data, and the Kruskal-Wallis test or Analysis of Variance (ANOVA) with Bonferroni correction was used to compare quantitative results. Spearman correlations were employed in correlation research. A Z-score comparison tool was used to compare two proportions. For all analyses, a p-value of less than 0.05 was considered statistically significant, while one of less than 0.001 was considered highly significant. Using SPSS, scatter plot diagrams were created. Data was compiled using Excel 2013.

Results

The present study included two groups , group I (BD patients group) and group II (healthy control group) , The age ranged between (23-53 years) in Patients group with Mean ±SD of 35.8±7.9 and (20-48 years) in control group with Mean ±SD of 32.4±6.7, there was no significant difference between studied groups (p =<0.096).(Table I)

Group I included 16 males and 14 females while group II included 13 males and 12 females, there was no significant difference between studied groups (p=<0.921)(Table I).

Table (I): Demographic information about patients and control group

		Patients group I	**Control group II**	**P value**
		N.: 30	N.: 25	
Age	**Range** **Mean ± SD**	(23-53) 35.8±7.9	(20-48) 32.4±6.7	0.096
Sex	**Male** **Female**	16(53.3%) 14(46.7%)	13(52%) 12(48%)	0.921

There was significant statistically increase in TLC count in group I compared to group II with Mean ± SD of group II (7.2±1.9 $x10^3$/uL) and (9.4±4.4 $x10^3$/uL) of group I with (P=< 0.024*).(Table II) (Figures 2,3)

While other CBC parameters showed no significant statistically difference between Patient group (I) and control group (II) regarding HB, Platelet, lymphocytes count, monocytes count and neutrophilic count as following:

- HB level in group I ranged from 7.1 to 16.8 gm /dl while in group II ranged from 9.8 to 15.7 gm/ dl with (Mean ± SD 12.6±1.7 g/dl) with (Mean ± SD 12.7±0.5 g/dl) (P=< 0.558).
- Platelets count in group I ranged from 219.8 to 304 $x10^3$/uL, while in group II ranged from 192 to 310.5 $x10^3$/uL(P=< 0.946).
- lymphocytes count in group I ranged from 1150 to 2606.3 cell\mm3, while in group II ranged from 1500 to 2250 cell\mm3 (P=< 0.826).
- Monocytes count in group I ranged from 339 to 650 cell\mm3, compared with group II was from 255 to 500 cell\mm3 (P =< 0.186).
- Neutrophils count in group I ranged from 3456 to 10125 cell\mm3, and in group II ranged from 3450 to 6000 cell\mm3 (P =< 0.120).
- Neutrophil/lymphocytic ratio in group I was 3.3, compared to group II 2.8 (P=< 0.339).

Table (II): Comparison between BD patient and control group regarding CBC:

		Patients	Control	P value
		N=30	N=25	
Hb	**Range** **Mean ± SD**	(7.1-16.8) 12.3±2.4	(9.8-15.7) 12.6±1.7	0.558
TLC	**Range** **Mean ± SD**	(2.5-21.8) 9.4±4.4	(4-10) 7.2±1.9	**0.024***
PLT	**Median** **IQR**	250.5 (219.8-304)	260 (192-310.5)	0.946
Lymph	**Median** **IQR**	1977.5 (1150-2606.3)	1975 (1500-2250)	0.826
Mono	**Median** **IQR**	500 (339-650)	500 (255-500)	0.186
Neutrophils	**Median** **IQR**	5992 (3456-10125)	5000 (3450-6000)	0.120
Neut. / lymph. Ratio	**Median** **IQR**	3.3 (1.6-6.2)	2.8 (2.2-3.3)	0.339

There was significant statistically increase in AST level in group I (IQR. 14.8-20 IU/L) compared to group II (IQR 12-20 IU/L) (Table III)

There was no statistically significant differences between the two groups regarding ALT level (IQR 16-23.5 IU/L) versus (IQR. 15-21.5IU/L (P=0.218)(Table III).

Table (III): Comparison between BD patient and control group regarding Liver Enzymes:

		Group(I) Cases	Group (II) Control	P value
		N=30	N=25	
AST (IU /L)	**Median** **IQR**	19.5 (14.8-20)	15 (12-20)	0.030*
ALT (IU /L)	**Median** **IQR**	20 (16-23.5)	18 (15-21.5)	0.218

There was no statistically significant difference in group I regarding serum creatinine level (IQR 0.5-0.7 mg/dl) versus group II (IQR 0.5-0.7 mg/dl) (P= 0.062) (Table IV).
There was no statistically significant difference between group I regarding blood urea level (IQR 22.5-31.3mg/dl) versus group II (IQR 22.5-27.5 mg/dl) (P= 0.450) (Table IV).

Table (IV): Comparison between BD patient and control group regarding renal functions:

		Group (I) Patients	Group (II) Control	P Value
		N=30	N=25	
Creatinine (mg /dl)	**Median IQR**	0.6 (0.5-0.7)	0.6 (0.5-0.6)	0.062
Urea (mg /dl)	**Median IQR**	26 (22.5-31.3)	25 (22.5-27.5)	0.450

There was statistically significant increase in CRP level in group I (IQR 5-36 mg/dl) compared to group II (IQR 3-5 mg/dl) (P=<0.001*) (Table V) (Figure 4).
There was statistically significant increase in group I regarding ANCA C and ANCA P level (IQR 10-30 Units) versus group II (P=<0.001*****) (TableV).

Table (V): Comparison of Inflammatory markers between the BD patients and HC groups.

		Group (I) patients	Group (II) Control	P value
		N=30	**N=25**	
CRP (mg\dl)	**Median IQR**	10.3 (5-36)	3 (3-5)	**<0.001***
ESR 1st (mm\hr)	**Median IQR**	15 (10-30)	5 (5-10)	**<0.001***
ANCA c (Unit)	**-Ve +Ve**	0(0%) 30 (100%)	25(100%) 0(0%)	**<0.001***
	Median IQR	20 (10-30)	----	**----**
ANCA p (Unit)	**-Ve +Ve**	0(0%) 30 (100%)	25(100%) 0(0%)	**<0.001***
	Median IQR	20 (10-30)	----	**----**

There was statistically significant increase in group I regarding EPCs (CD45 negative/CD34 bright /CD309 positive) (Mean ± 2.7±1.1%**)** versus group II (Mean ± 7.3±1.5%) **(**P=<0.001*****) (TableVI).

Table (VI): Comparison of EPCs between the BD patients and HC groups.

		Group (I) patients	Group (II) Control	P value
		N=30	N=25	
EPCs	Range (%) Mean ± SD	(5.1-10.7) 7.3±1.5	(1-5.7) 2.7±1.1	**<0.001***

There was statistically significant positive correlation between circulating EPCs percentages in group I and Hemoglobin (r=**0.367**, P=**0.046***),AST (r=**0.402**, **0.028***) and ALT (r=**0.373**, P=**0.042***) However there were negative significant correlations between Circulating EPCs percentage and TLC (r=**-0.542**, P=**0.002***), Monocytes (r=**-0.433**, **0.017***),Neutrophils (r=**-0.521** , p=**0.003***), ESR (r=**-0.373** , P=**0.042****) and NLR (r=**-0.472 , P=0.008*)** (Table VII)(Figures 5,6,7,8).

Table (VII): Correlation of Circulating EPCs with other lab. variables in patients with BD

Patients with BD	Circulating EPCs	
	r	P value
Circulating EPCs		
Age	0.060	0.752
Hb	**0.367**	**0.046***
TLC	**-0.542**	**0.002***
PLT	-0.148	0.434
Lymph	-0.023	0.905
Mono	**-0.433**	**0.017***
Neutrophils	**-0.521**	**0.003***
Neut. / lymph. ratio	**-0.472**	**0.008***
CRP	-0.234	0.214
ESR 1st	**-0.373**	**0.042***
AST	**0.402**	**0.028***
ALT	**0.373**	**0.042***
Creatinine	-0.309	0.097
Urea	-0.298	0.110
ANCA c	-0.276	0.139
ANCA p	-0.300	0.107

There was negative non-significant correlation between circulating EPCs and ocular manifestations, skin manifestations , joint manifestations and BDCAF score **(r= -0.15, p=0.423) (r= -0.240, p=0.202) (r=-0.043, p=0.820) (r=-0.152 , p= 0.820)** respectively (Table VIII).

Table (VIII): Correlation of Circulating EPCs with manifestations of BD in patients 'group.

	Circulating EPCs	
	r	**P value**
Ocular manifestations	-0.152	0.423
Skin manifestations	-0.240	0.202
Joints manifestations	-0.043	0.820
BDCAF Score	-0.152	0.820

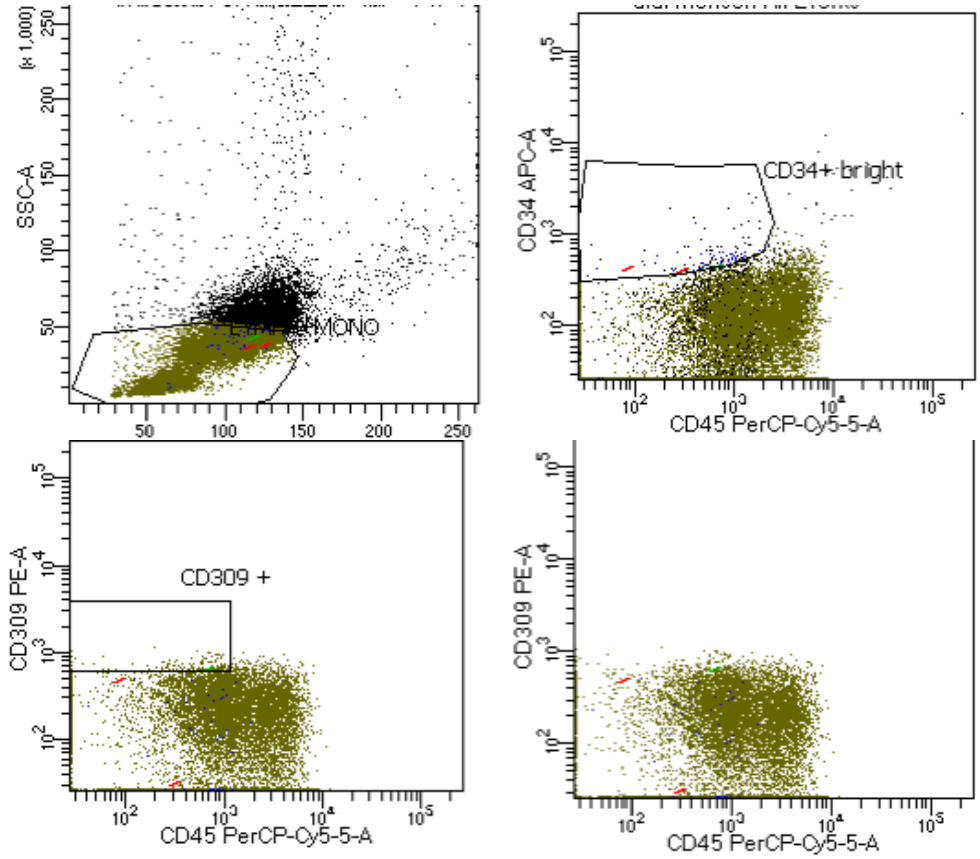


Fig 1 : Enumeration of circulating EPCs (identified as a live nucleated CD45 negative/CD34 bright and CD309 positive) in healthy control group .

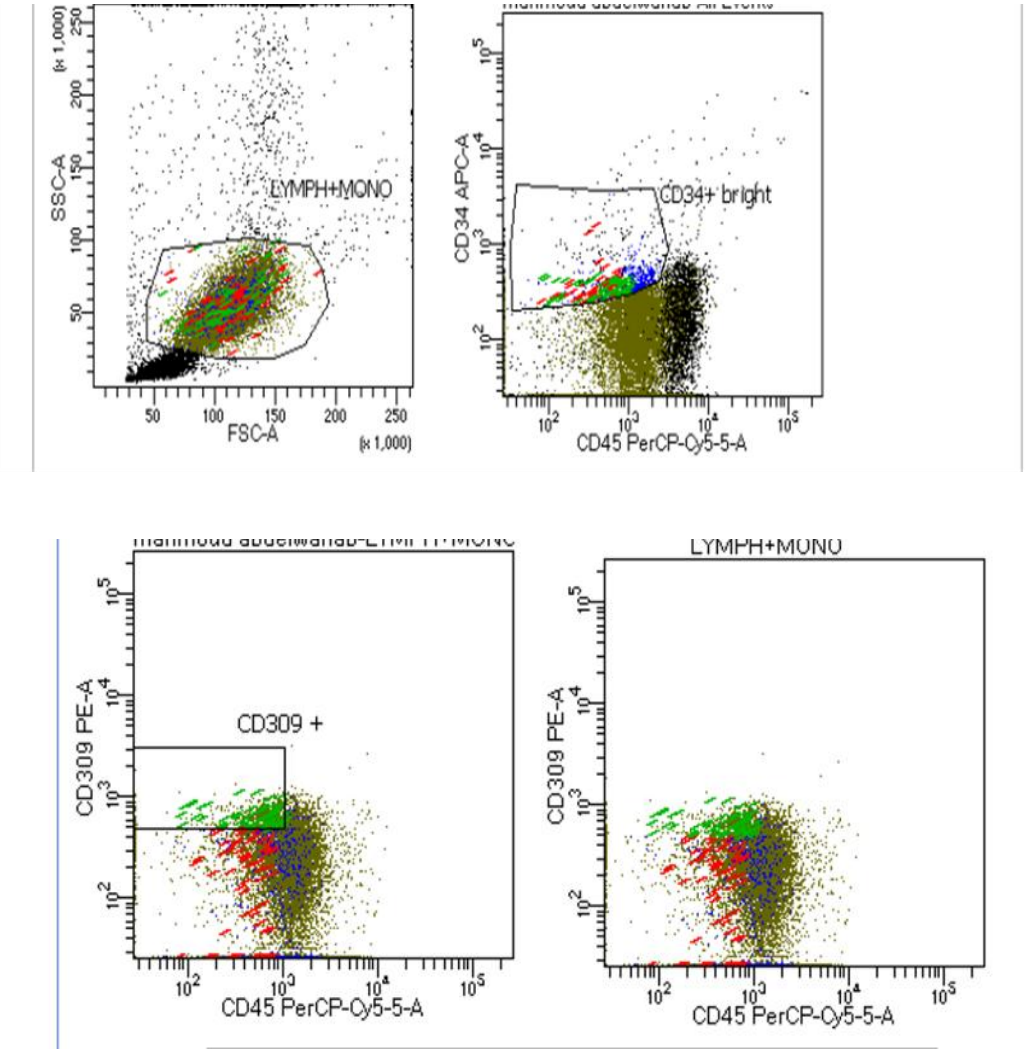
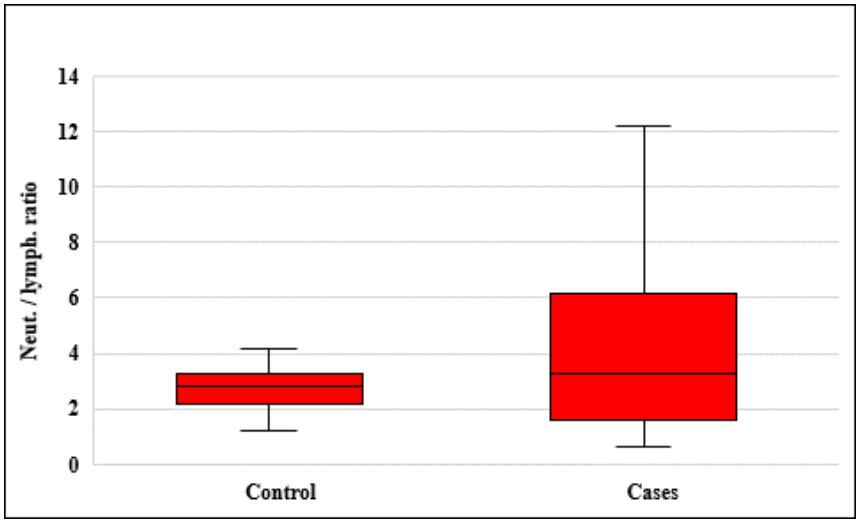


Fig 2 : Enumeration of circulating EPCs (identified as a live nucleated CD45 negative/CD34 bright and CD309 positive) in patients with BD .



- **Fig. 3: Difference between BD patients and HC groups regarding N/L ratio**

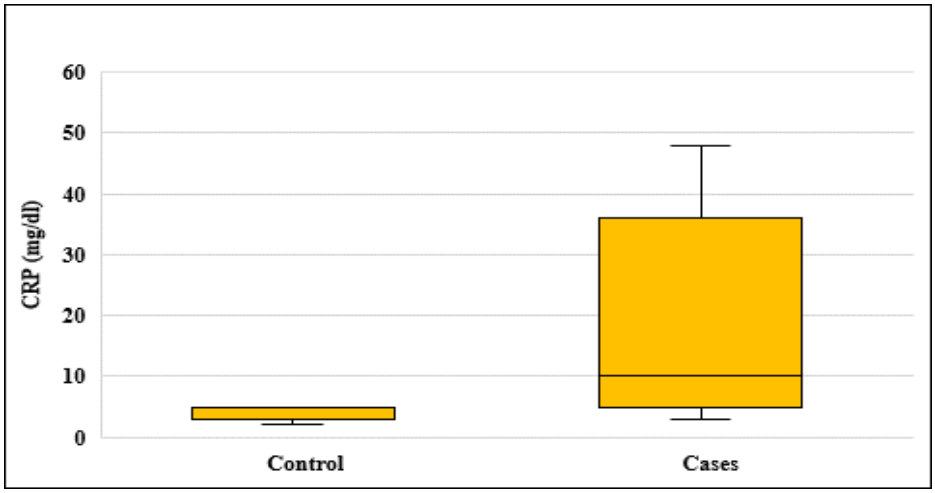


Fig. 4: Difference between BD patients and HC groups regarding CRP

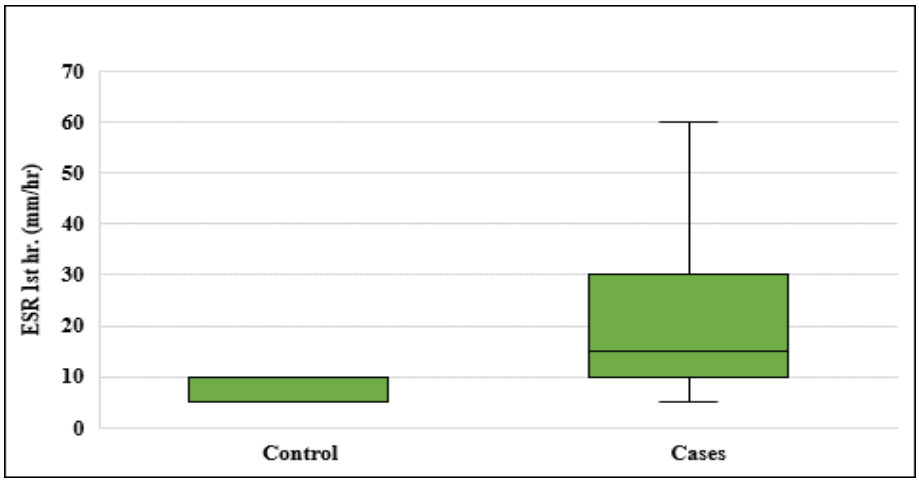


Fig. 5: Difference between BD patients and HC groups regarding ESR

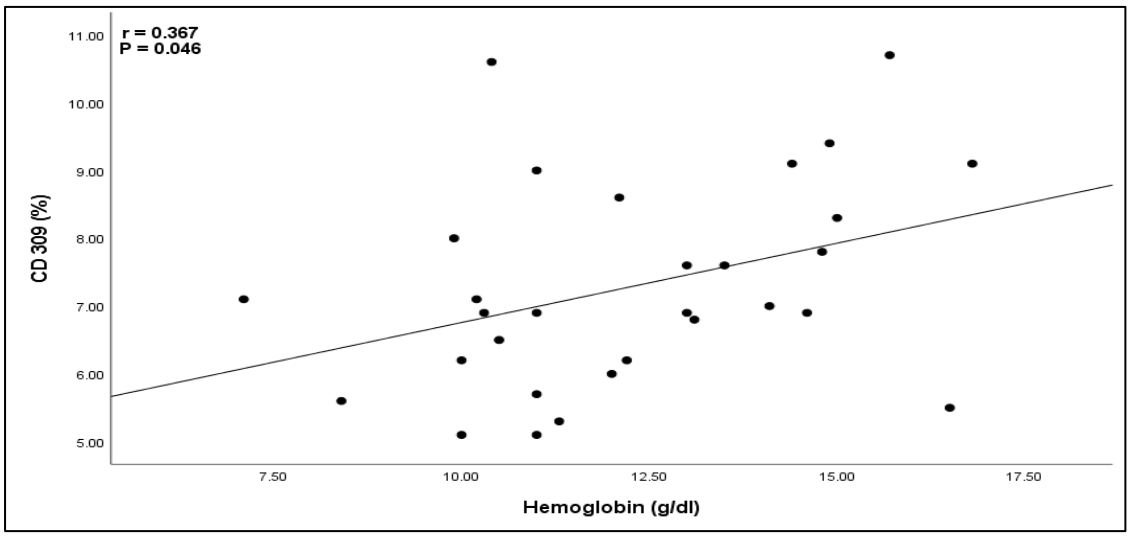


Fig. (6): Positive significant correlation between Hemoglobin(g\dl) and Circulating EPCs % in patients with BD.

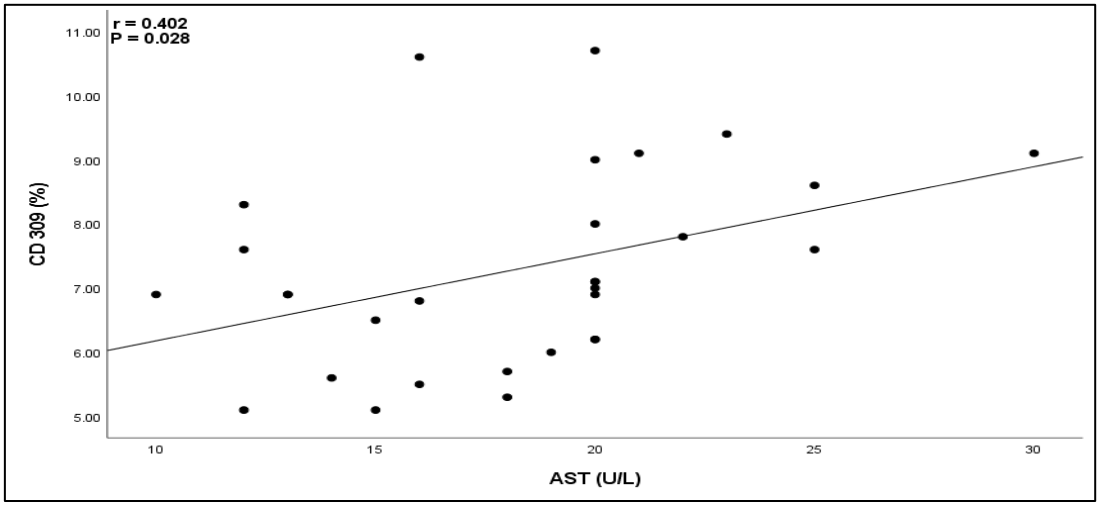


Fig. (7): Positive significant correlation between AST and Circulating EPCs % in patients with BD.

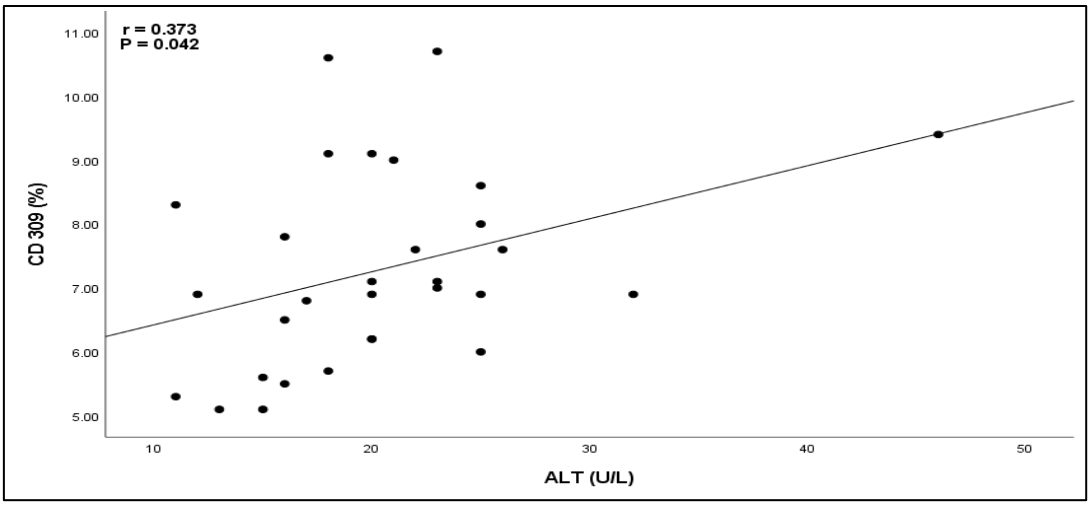


Fig. (8): Positive significant correlation between ALT and circulating EPCs % in patients with BD .

Discussion

Patients with Behçet's disease vary widely from one another in terms of their prognosis, organ presentations, numbers and severity of attacks, course of their illness, and response to treatment[10].

The ICBD can be used to identify patients with BD because there is currently no test for the condition [11].

It is possible to detect circulating EPCs in diseases that cause vascular inflammation, such as systemic vasculitis and BD. EPCs may therefore be useful in determining the extent of vascular involvement in BD [12].

In the present study, thirty patients were included suffering from BD sixteen males and fourteen females (Group I), and twenty-five were included as apparently healthy subjects as control they were thirteen males and twelve females (Group II).

Statistically there was no significance difference in age and sex between BD patients group and controls in this study (P-Value for age is 0.096 and sex is 0.921). This was

contrary to Ryu and other colleagues findings, which stated that patients with late-onset age had a lower chances of ocular involvement, pan uveitis, and folliculitis and a higher oncets of intestinal involvement, particularly intestinal ulcers, compared to adult patients with BD involvement before the age of 40[13].

Unlike Zou and other coauthors, who found that ocular disease, papulopustular lesion-type dermal lesions, cardiovascular disease, and neurological involvement are characteristics of male BD patients, whereas a high prevalence of vaginal ulcers is a characteristic of female BD patients, we disagree that these conditions are related to sex differences in the phenotypes of BD as there was no significant difference regarding sex in patients group and type of affected organ [14].

And Chen and Yao talked about how sex has an impact on early-onset (30 years) BD and how it manifests more frequently and severely in the eyes[15].
In the current study the male to female ratio was 8:7 with male predominance, this was similar to Attia and Adel Noor, study the disease activity score was obtained. The male to female ratio in the study was 6.7:1.0, indicating a clear male predominance[16].

In our study, TLC was significantly higher in patients when compared with healthy individuals (with p value 0.024), this was similar to Zhang and others who demonstrated that The TLC was reported to be high in patient with Behcet's disease [17]. This study was concomitant with Tanacan and his colleagues who found that TLC was significantly higher in patients with BD than in control group especially in the active phase of the disease due to inflammatory response and neutrophils activation [18].

This was in disagreement with Kim and his coauthors, who found that there was no significant difference in white blood cells count in patients and control groups even during uveitis[19].

The study also was in disagreement with Tezcan and others ,Who proved that there were no significant vary in all parameters of CBC between BD patients especially those on treatment and HC group[20].

In the present study, AST was significantly high in patients' group when compared with control group (with p value 0.030), This was in agreement with Bettiol who explained that the increased of liver enzymes in BD patients is mostly due to liver failure which occurs in budd-chaiari syndrome which is associated with BD especially in young age, this is may be due to multi organ damage and affection of disease on heart, lungs and brain in late complicated cases [21].

Alkhurassi, however, examined blood samples from BD patients and found no difference in the liver functions of patients and healthy controls[22].

In the other hand the present study there were no significant statistical difference between patients with BD and HC group regarding hemoglobin, platelets, neutrophils, lympho-cytic, monocytic count and Neutrophil/ Lymphocyte ratio with P value (0.558 , 0.946, 0.120, 0.826 , 0.186 and 0.339) respectively, this was in disparagement with Alan and others who found that patients with BD had greater levels of platelets, neutrophils, and leukocytes than healthy controls. By producing prion-flammatory substances, neutrophils actively contribute to both local and systemic inflam-matory reactions. Leukocytes, and neutrophils, play important roles in atherogenesis and atherothrombosis. Neutrophils are the primary tissue-damaging agents and exhibit intrinsic hyperactivation in BD patients. Platelets play an essential role for hemostasis and the development of venous and arterial thrombosis, respectively. Also platelet count is an indicator of systemic host inflammation[23].

Regarding the present study ESR level is higher in patients during disease activity than patients in remission and healthy controls. This was similar to Tanacan et al. who noted that in ophthalmic inactive BD patients and controls participants compared to ophthalmic active BD patients' ESR, CRP, and NLR (neutrophil-lymphocyte ratio) levels in BD active patients were significantly higher. This is could be explained by inflammation and vascular

affection that is usually present in active BS patients [18].

The current study also revealed that CRP level was higher in active patients with Bechet's disease, it was similar to Miyazaki et al. who reported that higher CRP level. As CRP is an acute phase reactant protein and it is highly correlated with disease activity and treatment response in BD patients[24].

In our study, EPCs is present in higher levels in patients of Behcet's disease than in healthy control group. This was similar to Arica et al., who proved that in cases of Behçet's disease there were increased mean plasma levels of endothelial progenitor cells . this process is a response of pathogenic stimulation as EPCs leave to the circulating blood and mature into endothelial cells which is highly important in healing endothelial damage in BD[25].

The present study shows Positive significant correlation between Hemoglobin and Circulating EPCs in patients with BD. This is mostly due to anemia of the chronic illness, On the reverse of Solomon who proved that poorer chance of vascular healing may be indicated by the decreased number of peripheral endothelial progenitor cells in patients with vascular disease with anemia[26].

Also in the present study there was positive significant correlation between liver enzymes and circulating EPCs in patients group. This could be explained by vascular affection of hepatic blood supply. This was in agreement with Zahran et al. who proved that circulating EPCs level was significantly higher in patients of HCC (hepatocellular carcinoma) with elevated liver enzymes due to major affection of hepatic cells and the process of repair and tumor control [27].

In our study there was negative significant correlation between TLC, Neutrophils count, monocyte count NLR, ESR and circulating EPCs in patients group as the high levels of circulating EPCs could be a good sign of healing and good patient survival rate as the mobilization of EPCs from the bone marrow in BD patients potentially help in the repair and recovery of damaged blood vessels.

In our study Circulating EPCs level had nonsignificant negative correlation with the disease activity (according to BDCAF score). This is in agreement with Kul et al., who found that active BD patients had significantly lower levels of circulating EPCs, Low circulating EPCs percentage , could reflect diminished vascular repair, which could be also associated with increased morbidity and mortality rate as reported in many conditions of cardiovascular diseases[28]. The present study was in disagreement with Manetti et al., who found that the circulating EPCs levels increase in patients of active vasculitis diseases such as systemic sclerosis due to active inflammation, but the reduction of EPCs numbers in active BD patients may be induced by insufficient immune response to the chronic inflammation[29].

Conclusion

Our study demonstrated increased level of circulating EPCs in BD patients than control subjects with positive correlation with Hb,ALT and AST but negative correlation with inflammatory markers(ESR, TLC,NLR, neutrophils and monocytes)and negative (nonsignificant) correlation with clinical manifestations and BDCAF score. High levels of circulating EPCs could be a good sign of healing endothelial damage. We suggest further extension of our study on wider numbers of participants to validate the role of EPC as a potential marker of repair and recovery of damaged blood vessels in BD patients.

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