Circulating endothelial cells in Behçet's disease: is there a relationship with vascular involvement?

N.Ş. Yaşar Bilge[1], O.M. Akay[2], E. Gunduz[3], M. Bilgin[4], T. Kasifoglu[1]

[1]Division of Rheumatology, Department of Internal Medicine, Eskisehir Osmangazi University, Eskisehir;
[2]Division of Haematology, Department of Internal Medicine, Koc University, Istanbul;
[3]Division of Haematology, Department of Internal Medicine, Eskisehir Osmangazi University, Eskisehir;
[4]Department of Biostatistics, Eskisehir Osmangazi University, Eskisehir, Turkey.

Nazife Şule Yaşar Bilge, MD
Olga Meltem Akay, MD
Eren Gunduz, MD
Muzaffer Bilgin, PhD
Timucin Kasifoglu, MD

Please address correspondence to:
Dr N. Şule Yaşar Bilge,
Sümer Mahallesi, Atalı Sokak 4/2,
26400 Eskisehir, Turkey.
E-mail: suleyasar@yahoo.com

Received on June 20, 2019; accepted in revised form on October 4, 2019.

Clin Exp Rheumatol 2019; 37 (Suppl. 121): S105-S110.

Key words: Behçet's disease, circulating endothelial cells, endothelial progenitor cells, vasculitis, thrombosis

Competing interests: none declared.

ABSTRACT

Objective. *Circulating endothelial cells (CEC) are identified in conditions with vascular damage such as systemic vasculitis. Our aim was to investigate if EPC, CEC, and/or its subgroups activated CEC (aCEC) or resting CEC (rCEC) related with vascular involvement in Behçet's disease (BD).*

Methods. *In total 60 patients were included in this study, divided into 4 groups: 1) Behçet patients with a history of vascular involvement: vascular BD; 2) Behçet patients with mucocutaneus involvement: mucocutaneus BD; 3) patients with history of thrombosis due to other causes: thrombosis; 4) 20 healthy controls were also included: control group. Percentages of CEC, aCEC, rCEC and EPCs in peripheral blood mononuclear cells were measured by flow cytometry.*

Results. *CEC (3.75 (1.80-7.20), 1.80 (0.70-3.53), 3.50 (1.83-7.23), 2.45 (1.28-4.60)) and aCEC (2.40 (1.28-4.28), 1.10 (0.77-2.20), 3.15 (1.48-7.20), 3.20 (1.15-9.80) levels were did not show a statistically significant difference between groups (p:0.077 and p:0.054, respectively). EPC and rCEC levels were higher in vascular BD and thrombosis groups than mucocutaneus BD and control groups (EPC:10.5 (7.20-18.3), 11.6 (7.30-20.9) vs. 7.15 (5.55-8.25), 10.2 (5.93-18.6), rCEC: 5.35 (3.13-7.90), 6.45 (4.60-10.8) vs. 4.95 (3.05-7.55), 3.40 (1.88-4.30), p:0.042 and p:0.007, respectively).*

Conclusion. *CEC, EPC, aCEC and rCEC may have role in the assesment of vascular involvement in BD. Longitudinal studies would be needed to identify the utility of these cells for the follow up and risk stratification of BD patients with vascular involvement for recurrences or identify BD patients at risk of vascular involvement.*

Introduction

Behçet's disease (BD) is a systemic vasculitis mostly known with recurrent oral and genital ulcerations, uveitis and mucocutaneus lesions. On the other hand vascular involvement (deep vein thrombosis, cerebral sinus thrombosis and pulmonary artery aneurysm, etc.) is an important clinical finding of disease which may cause mortality and morbidity (1). The mechanisms leading to vascular lesions are incompletely understood (2).

The endothelial layer has the major role in maintaining vascular homeostasis. The endothelial damage may trigger an immun reaction causing vasculitis (2). Microvascular endothelial cell damage is the hallmark of small-vessel vasculitis and BD is known to effect all sizes of vessels (1, 3). Endothelial progenitor cells (EPCs) are mobilised from bone marrow to peripheral circulation in response to situations causing vascular damage (2). EPCs are involved in both physiologic and pathologic vascular processes (4). Low levels of EPC is supposed to be associated with vascular injury (2).

Vascular damage may cause endothelial cells to detach from the site of injury and release into the circulation (5). Circulating endothelial cells (CEC) are defined in conditions which vascular damage is seen in course of diseases such as systemic vasculitis, coronary artery disease and chronic renal failure. The relation between ANCA-associated vasculitis (AAV) and CEC has been presented in a study by Woyvodt *et al*. (6). CEC was found to be correlated with active disease and thought to be an indicator of vascular damage in ANCA-associated vasculitis, Kawasaki disease and large-vessel vasculitis (6-8). Depending on the previous studies CEC would reflect disease extent and activity in vasculitis (9). For this reason we have investigated CEC as a possible marker of vascular involvement in BD.

Endothelial cells may be resting or active and the difference may be pre-

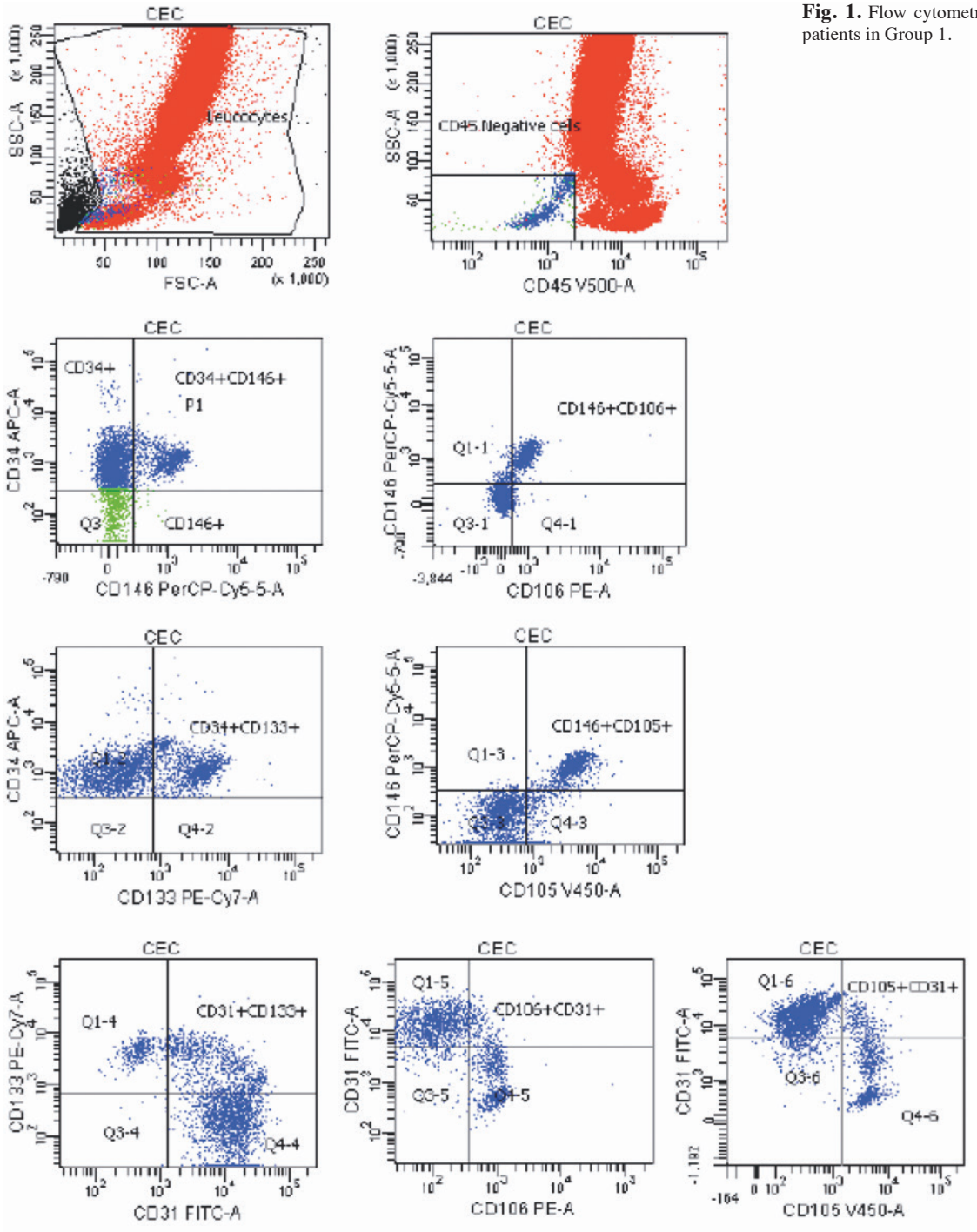

Fig. 1. Flow cytometry analysis of one of the patients in Group 1.

sented with secretion of chemokines and cytokines or express increased adhesion molecules (10). In a previous study, resting and activated CECs were increased in patients with lymphoma and breast cancer compared with healthy controls and decreased after therapy (11). The clinical significance of these subsets has not been determined yet (10).

Our aim in the current study was to investigate if EPC, CEC, or its subgroups aCEC or rCEC has a relationship with vascular involvement in BD. To answer these questions we have analysed CEC levels in patients with BD, compared them between patients with vascular and mucocutaneous involvement. Also we have compared the results of Behçet patients with patients with thrombosis due to other causes and healthy controls.

Materials and methods

Patients

Current study included 20 Behçet patients with a history of vascular involvement (vascular BD group), 20 Behcet patients with mucocutaneus involvement (mucocutaneus BD group), 20 patients with history of thrombosis due to other causes (thrombosis group) and 20 healthy controls (control group). Behcet patients were diagnosed accord-

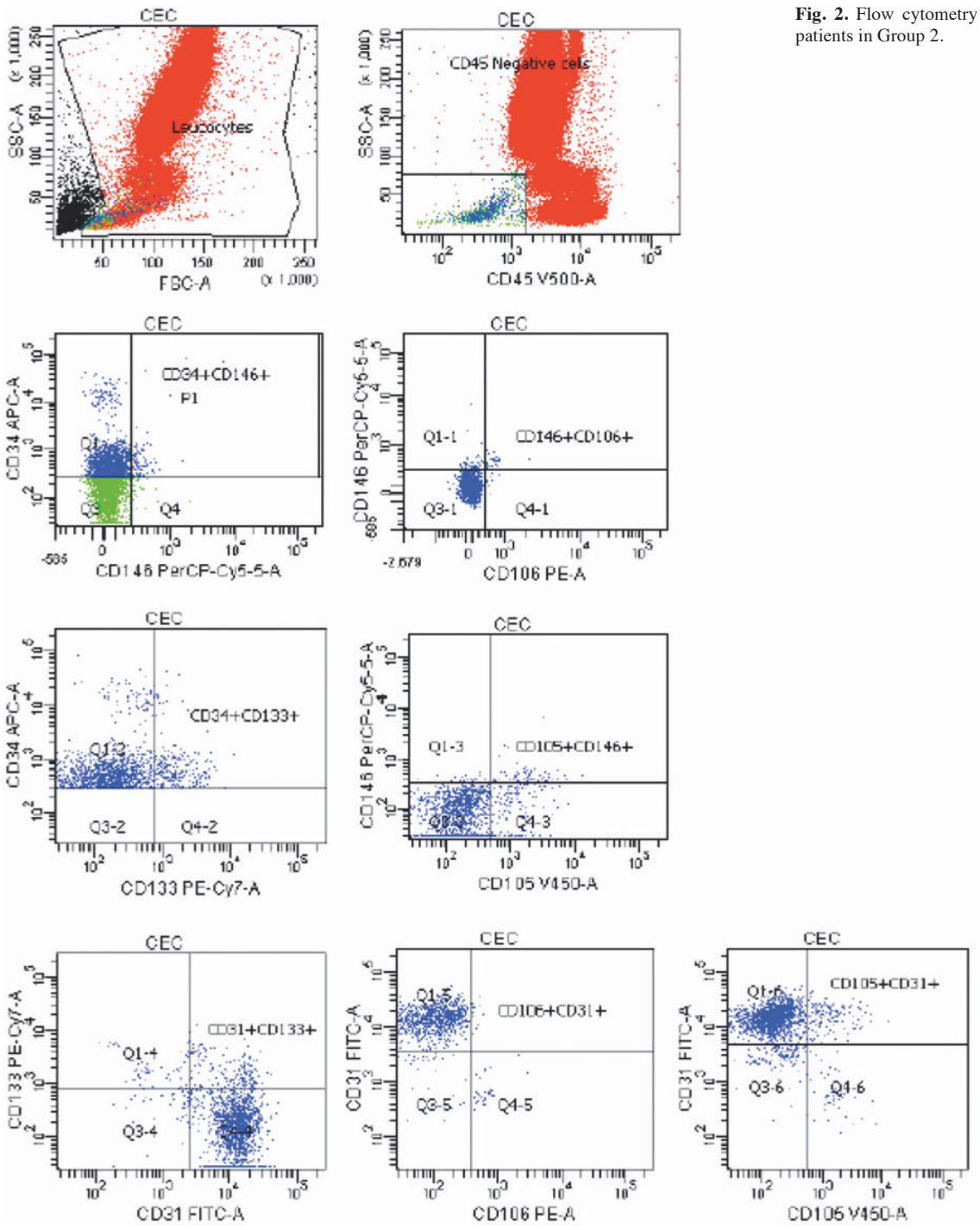

Fig. 2. Flow cytometry analysis of one of the patients in Group 2.

ing to the International Study Group criteria (12). Patients with established chronic renal failure, coronary artery disease, diabetes mellitus, hypertension, hyperlipidemia, malignancy and smokers were excluded. Diagnosis of thrombosis was based on doppler ultrasound or computed tomography which were evaluated by experienced radiology specialist. Clinical activity was assessed for activity signs and symptoms according to the BD Current Activity Form (13). The study was approved by local ethics committee (approval number: 25.08.2016/80558721/G).

CEC analysis

Blood samples of the patients and healthy controls were drawn into tubes containing etylene-diamine-tetra-acetic acid (EDTA). A panel of monoclonal antibodies, including anti-CD45 to exclude hematopoietic cells, anti-CD31, -CD34, -CD36, -CD105, -CD106, -CD133,and –CD 146 and appropriate analysis gates were used to enumerate resting and activated CECs and endothelial progenitor cells (EPC) (BD Pharmingen).

A hundred microlitre complete blood was added and incubated for 20 minutes at room temperature in the dark. After incubation for 10 minutes with

Table I. Demographic and laboratory features of the study population.

		Group				*p*
		Vascular BD	Mucocutaneus BD	Thrombosis	Control	
		n (%)				
Sex	Female	9 (% 45.0)	12 (% 60.0)	11 (% 55.0)	14 (% 70.0)	0.447***
	Male	11 (% 55.0)	8 (% 40.0)	9 (% 45.0)	6 (% 30.0)	
		Mean ± Standard deviation Median (Q1 – Q3)				
Age (yrs)		43.55 ± 8.31 43.5 (38.0 - 50.0)	46.85 ± 9.43 49.0 (42.0 - 53.5)	42.50 ± 15.22 44.0 (33.3 - 50.0)	41.65 ± 6.54 40.5 (38.8 - 45.0)	0.323**
Duration of disease (yrs)		14.90 ± 8.69 13.0 (10.0 - 18.5)	11.30 ± 8.80 10.0 (5.75 - 18.0)	4.25 ± 3.71 3.0 (1.25 - 5.0)	-	0.109*
Haemoglobin (mg/dl)		13.0 ± 1.30 12.8 (12.0 - 14.2)	14.1 ± 1.60 13.7 (13.0 - 14.9)	12.0 ± 2.54 11.6 (10.5 - 14.0)	13.8 ± 1.35 13.5 (13.0 - 14.5)	0.018**
Leucocytes (10^3/ul)		8.79 ± 2.47 8.45 (6.94 - 10.5)	8.42 ± 2.39 7.69 (6.89 - 9.55)	8.25 ± 3.10 8.00 (6.09 - 9.05)	7.04 ± 1.66 6.87 (6.40 - 7.85)	0.168**
Platelet (10^3/ul)		261 ± 68.1 248 (220 - 294)	250 ± 70.1 244 (207 - 279)	294 ± 150 265 (207 - 332)	260 ± 62.5 248 (219 - 281)	0.893**
ESR (mm/h)		28.4 ± 25.3 22.0 (8.00 - 43.8)	17.8 ± 15.7 13.0 (4.75 - 28.3)	32.2 ± 23.7 31.5 (19.8 - 37.0)	20.78 ± 12.8 14.0 (13.2 - 22.1)	0.089**
CRP (mg/dl)		3.53 ± 6.35 0.845 (0.33 - 3.72)	1.08 ± 0.970 0.695 (0.33 - 1.43)	2.96 ± 4.15 1.75 (0.95 - 3.12)	2.32 ± 3.22 1.25 (0.90 – 3.05)	0.135**

BD: Behçet's disease; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.
*Mann Whitney U-test. **Kruskal Wallis H-test. ***Pearson Chi Square Test.

erythrocyte lysing solution at room temperature, centrifugation at 1,800 rpm for 5 minutes was performed. Supernatant was removed and washed with phosphate buffer saline (PBS) for two times. Pellet was resuspended with PBS and 1000000 cells were counted with BD FACSCantoII flow cytometry device.

CD146 positive and CD 45 negative cells were defined as CEC, CD146, CD 105 or CD 106 positive cells were defined as activated CECs (aCEC), CD146 positive, CD 105 or CD 106 negative cells were defined as resting CECs (rCEC), CD146 and CD 133 positive cells were defined as EPC (Fig. 1, 2).

Percentages of CEC, aCEC, r CEC and EPCs in peripheral blood mononuclear cells were measured by flow cytometry. Flow cytometric analysis of CEC has been described in detail previously (10).

Statistical analysis

Continuous data are given as mean ± standard deviation, median (Q1-Q3). Categorical data are given as percentage (%). Shapiro Wilk's test was used to investigate the appropriateness of the data to normal distribution. The Mann-Whitney U-test was used for the two groups, and the Kruskal-Wallis H test was used for the cases with a group number of three groups to non normal distrution. Pearson's chi-square analysis was used in the analysis of the cross tables. Box plot was used to see the distribution of the data points per study group. IBM SPSS Statistics 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, v. 21.0. Armonk, NY: IBM Corp.) was used in the implementation of the analyses. A *p*-value of <0.05 was considered as a criterion for statistical significance.

Results

Age, distribution of sex and duration of disease did not show any difference among groups. Laboratory parameters (haemoglobin, leucocyte and platelet counts, erythrocyte sedimentation rate and C-reactive protein levels were also compared between groups. Haemoglobin levels were lower in Group 3 than all other groups. All remaining parameters did not show any difference between groups (Table I).

Of the 20 patients in vascular BD, 7 patients had deep vein thrombosis (DVT), 4 patients had cerebral sinus thrombosis, 3 patients had pulmonary vasculitis, 1 patient had inferior vena cava thrombosis, 1 patient had thrombosis in coronary arteries, 1 patient had both DVT and pulmonary vasculitis, 1 patient had thrombosis in iliac vein and common femoral vein, 1 patient had cerebral sinus thrombosis, inferior and superior vena cava thrombosis and 1 patient had DVT, hepatic vein thrombosis, inferior vena cava thrombosis and pulmonary artery aneurysm. Eight patients were receiving immunosupresive treatment at the time of the blood sample collection; 2 mycophenolate mofetil and 6 azathiopurin. The remainig 12 patients' immunosuppresive therapy was stopped at least 6 months prior to the study.

The thrombosis group consisted of 20 patients with thrombosis related to other causes; 6 patients had systemic lupus erythematosus (SLE) and anti-

Table II. Comparision of CEC, EPC, aCEC and rCECs levels between groups (%).

	Group				*p*	Multiple comparison
	Vascular BD	Mucocutaneus BD	Thrombosis	Control		
	mean ± standart deviation Median (Q1 – Q3)					
CEC	5.09 ± 4.7 3.75 (1.80 - 7.20)	2.52 ± 2.55 1.80 (0.70 - 3.53)	4.89 ± 3.83 3.50 (1.83 - 7.23)	4.09 ± 4.9 2.45 (1.28 - 4.60)	0.077**	-
EPC	13.49 ± 9.38 10.5 (7.20 - 18.3)	7.62 ± 4.02 7.15 (5.55 - 8.25)	15.6 ± 11.21 11.6 (7.30 - 20.9)	13.71 ± 10.1 10.2 (5.93 - 18.6)	0.042**	1-2: 0.015 2-3: 0.009
aCEC	4.39 ± 5.78 2.40 (1.28 - 4.28)	2.24 ± 2.14 1.10 (0.77 - 2.20)	8.17 ± 13.26 3.15 (1.48 - 7.20)	8.78 ± 13.21 3.20 (1.15 - 9.80)	0.054**	-
rCEC	6.44 ± 5.4 5.35 (3.13 - 7.90)	5.43 ± 3.49 4.95 (3.05 - 7.55)	9.03 ± 7.79 6.45 (4.60 - 10.8)	3.52 ± 2.34 3.40 (1.88 - 4.30)	0.007**	1-4: 0.034 3-4: <0.001

CEC: circulating endothelial cell; EPC: endothelial progenitor cell; aCEC: activated circulating endothelial cell; rCEC: resting circulating endothelial cell.

phospholipid antibody syndrome, 4 patients had polyctyhemia vera, 2 patients had rheumatoid arthritis, 1 patient had psoriatic arthritis, 1 had SLE, 1 patient had essential thrombocytemia and 5 patients had thrombosis with unknown causes. Eight patients had DVT, 3 had patients pulmonary thromboembolism, 2 patients had DVT and pulmonary thromboembolism, 1 patient had thrombosis in popliteal, femoral and iliac veins, 1 patient had DVT and splenic vein thrombosis, 1 patient had pulmonary thromboembolism and portal vein thrombosis, 1 had haepatic vein thrombosis, 1 had femoral vein thrombosis, 1 patient had splenic vein thrombosis and 1 had temporal vein thrombosis.

CEC levels did not show a statistically significant difference between groups. EPCs, aCECs and rCECs were also compared between groups. EPCs were higher in vascular BD patients and in thrombosis group than mucocutaneus BD patients and control group (p=0.042). Activated CECs levels did not show a difference between groups (p>0.05). Resting CECs were higher in vascular BD and thrombosis groups than mucocutaneus BD patients and control groups. The detailed analysis of CEC, EPC, activated and resting CECs of groups is given in Table II and Figure 3. Correlation of activity of BD with CEC, EPC, aCEC and rCEC was evaluated in vascular BD and mucocutaneus BD groups and there was a positive correlation with EPC in mucocutaneus BD group (r:0.635, p=0.003).

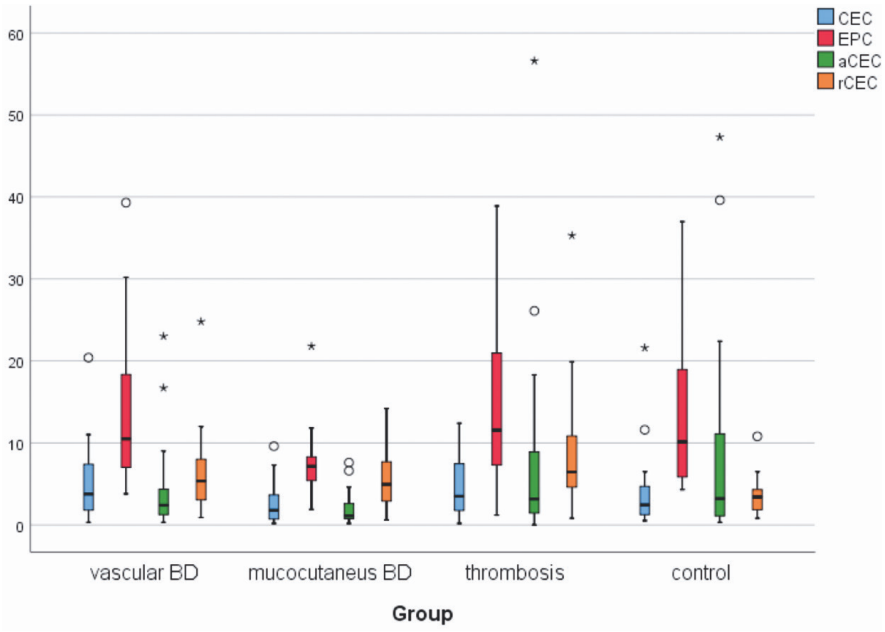

Fig. 3. The distribution of CEC, EPC, aCEC and rCEC per study group.

Discussion

The difference in total CEC numbers was not statistically significant in Behçet patients with mucocutaneus or vascular involvement, patients with thrombosis related with other factors and control group. But EPC and rCEC levels were higher in both patient groups with thrombosis.

CEC elevation in the blood of patients is supposed to be a useful marker for vascular dysfunction (14). Elevated CEC levels were demonstated in active phase of vasculitis such in ANCA-associated vasculitis and Kawasaki disease (6, 7). In the current study CEC levels tended to be higher in BD patients with history of vascular involvement but the difference did not reach a significant level. Lack of significance might have been caused by; 1. none of the patients had acute thrombosis, 2. all patients recieved immunosupresive therapy which may decline CEC levels, 3. the small number of patients in each groups. According to the results of the previous studies, increased levels of aCECs may be an indicator of active vascular involvement in BD. But in the current study aCEC levels did not show a difference between groups, even though aCEC levels were higher in vascular BD than mucocutaneus BD. None of the patients had recent vascular events

which may be the cause of the failure to find a significant difference.

Resting EPC and resting CEC levels were elevated in both groups of patients with thrombosis. In peripheral blood, while CECs are regarded as a marker of endothelial damage, EPCs are a marker of repair. Due to their important role in endothelial maintenance and vascular healing, bone marrow-derived EPCs are supposed to decrease in vascular damage. In the current study, however, the levels of EPCs were higher in both thrombosis group than Behçet patients with mucocutaneus involvement. Similarly, in a study by Del Papa *et al*. EPC levels were found to be increased in patients with systemic sclerosis (15). Zavada *et al*., however, (14) found decreased number of CEC and EPC in AAV and Fadini *et al*. (2) reported decreased levels of EPC in BD and both concluded that low number of EPCs could reflect an impaired mechanism of vascular repair and EPCs do not correlate with markers of inflammation. High number of EPCs in group 1 and 3 may suggest; 1. healing and recovery process of inflammation in the vessel wall, 2. inactive period of vascular injury (none of the patients were in active phase). There was a positive correlation with diseae activity and EPC in mucocutaneus BD group, which also supports the increase is caused by healing process.

In a study by Holmen *et al*. (16) inflammatory CEC levels were increased in active AAV patients whereas EPC levels were decreased and the authors have concluded as increased CEC levels has an inhibitory effect on EPC levels. This may be another explanation in increased ECP levels for the current study, as CEC levels were not increased in our patient group.

Sometimes it may be difficult to establish disease activity in vasculitis. Today assesment of disease activity in BD is mainly based on clinical findings and there is no hint to predict which patient group will develop vascular involvement. Even though we could not show an exact relation, based on the literature, measuring CEC, EPC and rCEC levels may provide to anticipate vascular disease in patients with BD. Also, aCEC may be a marker for active vascular inflammation.

Our study has some limitations; one of them is the design of the study as we do not have a follow-up period to show changes in the number of CEC with disease duration. In addition, we did not have a chance to include any patient with acute thrombosis, which is a major limitation of our study. It would be valuable if we could present the results of BD patients with active thrombosis and compare them with patients who had a history of thrombosis. The relative small number of patients in each group is another limitation.

In conclusion, CEC may be used as screening test. Increased levels of EPC and rCEC may help us to identify a high-risk patient group for vascular involvement. Resting CECs may be a vascular dysfunction marker in patients with BD.

Asfar as we know, this is the first study analysing all CEC, EPC, aCEC and rCEC in BD. More research is essential to clearly elucidate the biology of CEC, EPC, aCEC and rCEC in BD.

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