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Research Article

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Homocysteine Metabolism and Haemostatic Factor in Behcet's Disease

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Abstract Behçet disease (BD) is a rare multisystem, inflammatory disease of unknown origin that may lead to life-threatening events and is associated with a prothrombotic state. The aim of this study was to determine the role of homocysteine and haemostatic factors in the etiopathogenesis and the determination of the activity of BD. In line with this objective, the present study aimed to examine the levels of homocysteine, vitamin B6, vitamin B12 and folic acid which are play role of homocysteine metabolism, and haemostatic factors such as PAI-1, for the diagnosis and particularly the follow-up of BD.

The study group consisted of a total of 60 persons, 40 of whom were BD patients and 20 of whom were healthy individuals. We evaluated homocysteine levels and some vitamin levels which were associated with homocysteine metabolism and plasma levels of plasminogen activator inhibitor type 1 (PAI-1), C-reactive protein (CRP), and MDA, lipid peroxidation end product.

Plasma vitamin B12, folic acid, PAI-1 and homocysteine levels in the active patient group were statistically significantly higher than those in inactive and control group ($p<0.05$), there was no statistically significant difference in vitamin B_6 levels as such between active, inactive Behcet's patients and the control group ($p>0.05$).

An excess of PAI-1 and hyperhomocysteinaemia, which is a major and independent risk factor for vascular disease, and an accompanying evaluation of vitamin B6, vitamin B12, folic acid may be responsible for the endothelial damage in BD and assumed to be a risk factor and a marker for activation of BD.

Keywords Behcet's disease, homocysteine, PAI-1, vitamins

Introduction

Behcet's disease (BD) or Behçet syndrome, initially described by Turkish dermatologist Hulusi Behcet's in 1937, is a chronic multisystem disorder of unknown etiology. Many factors, including viral, bacterial, genetic, immunological, hematological and environmental factors and toxic response to organic chemicals are held responsible for the etiology of the disease [1-2]. The disease is clinically characterized by repeated oral aphtha, genital ulceration, uveitis and skin lesions. It involves a variety of organs including joints, the gastrointestinal tract, the central nervous system, and the vascular system. Vascular lesions in Behçet disease can involve both the arterial and venous vessels and are often complicated by thrombosis [3]. The main histopathology of BD is vasculitis, and neutrophilic or monocytic vascular inflammation can involve large, medium or small vessels [4]. Inflammation and haemostasis are interrelated pathophysiologic processes that considerably affect each other. During inflammatory response, inflammatory mediators, in particular proinflammatory cytokines, play a central role in the effects on haemostatic system such as endothelial cell dysfunction, coagulation cascade activation, impaired function of physiologic anticoagulants and suppression of fibrinolytic activity [5].

Endothelial cell injury due to the vasculitis of BD seems to be the key event in the prothrombotic state of this disease. Endothelial dysfunction relates to activation of platelets (for example, by vWF), inhibition of fibrinolysis (raised PAI-1), and inhibition of natural anticoagulants. It is speculated that a combination of abnormalities of procoagulants, anticoagulants, and fibrinolytic factors, together with the vasculitis and the endothelial injury, accounts for the clinical thrombosis in this subgroup of patients with BD [6].

In recent years, abnormalities in the methionine-homocysteine cycle and associated folic acid metabolism have attracted great attention due to the reported link between hyperhomocysteinemia and atherothrombotic diseases. Folic acid is a water-soluble B-vitamin and enzymatic cofactor that is necessary for the synthesis of purine and thymidine nucleotides and for the synthesis of methionine from homocysteine. [7]. Folic acid and vitamin B12 are involved in remethylation of homocysteine to methionine by methionine synthase, and vitamin B6 acts as a cofactor in the transsulfuration of homocysteine to cystathionine and cysteine. Genetic or nutritional (folate, vitamin $B_{12,}$ vitamin B_6 deficiency etc.) abnormalities in the methionine-homocysteine cycle can cause hyperhomocysteinemia by inhibiting this reaction. Several studies have shown an association of elevated plasma homocysteine levels with oxidant stress-induced pathology in the coronary arterial disease, cerebrovascular disease, deep atherothrombosis and vein thrombosis [8-10]. Increased oxidative stress has been suggested as causes of hyperhomocysteinemia in reactive oxygen and nitrogen species formation with auto-oxidation of homocysteine, can cause direct endothelial cytotoxicity, inhibition of pro-oxidant enzymes, suppression of antioxidant enzymes, interference with clotting factor, and LDL oxidation [11].

Diagnosis is usually based on its clinical presentations and there is, however, no any specific laboratory tests to make the diagnosis of BD. It is therefore of great importance to determine the factor(s) that may lead to disease and to find some rapid and useful laboratory test(s) for the diagnosis of this unique disorder. We investigated homocysteine, vitamin B6, vitamin B12, folat and PAI-1 levels in BD patients to evaluate their role in etiopathogenesis of BD.

Material and Methods

A total of 40 patients with BD who presented for the first time or were being monitored in the Dermatology Clinic of Firat University Hospital and were diagnosed according to International Study Group criteria for BD [12] were included in the study. According to the concerned criteria, presence of any two of the recurrent genital ulceration, uveitis or renal vasculitis, cutaneous pustules or erythema nodosum and positive pathergy test, in addition to recurrent oral ulcerations were diagnostic. All patients were interviewed in terms of symptoms at the onset of the Behcet's disease and the current symptoms. The 40 Behcet's disease patients' age range was between 20 and 69 (35.97±12.37 years), 29 (16 females and 13 males) were clinically active and 11 (6 females and 5 males) were inactive. Mean duration of BD was 4.88±4.11 months in the active patient group and 11.87±13.16 months in the inactive patient group. None of the patients had any evident disease except for Behcet's. The control group comprised 20 healthy volunteers (12 males and 8 females; mean age 28.30±5.75 years) from the hospital staff that had no disease and were not on any medication. To minimize the confounding effects of endothelial function, the study group did not have coronary risk factors and other systemic diseases. All selected patients were in the chronic state of the disease and were taking colchicines treatment. Patients in the acute phase of the disease or who were being treated with steroids and were on medications known to alter plasma lipid profiles and/or homocysteine levels were also excluded from the study. At the time of study, all medications had been discontinued for ~3 weeks.

All participants gave their informed consent before the study, and the local Ethics Committee of the Firat University, Faculty of Medicine approved the initial research proposal, and all subjects gave their informed consent to participation in the study.

Venous blood samples were drawn in the morning (8:00 A.M. to 10:00 A.M.) after an overnight fast using a 25-gauge needle from a peripheral vein, avoiding hemolysis into plain tubes. Because synthesis of homocysteine takes place in red cells after sampling, it is very important to centrifuge and separate plasma and serum from the blood cells as soon as possible. Therefore, samples were centrifuged within 30 minutes at 4,000 rpm for 5 minutes and the separated plasma was stored at -20°C until assay. Total homocysteine and vitamin B_6 levels were measured in the

plasma with EDTA according to high performance liquid chromatography (HPLC) method in Schimadzu 10A VP equipment using ClinRep (GmbH Munich, Germany) made commercial kits.

Vitamin B_{12} and folic acid measurements were made in Hitachi (Roche Diagnostics GmbH, Mannheim, Germany) immunoassay analyzer in accordance with electrochemiluminescence immunoassay (ECLIA) method. Vitamin B12 determination was based on competitive test principle using a specific intrinsic factor. Folic acid measurement was based on competitive test principle using a specific natural folat-bound protein.

Plasma PAI-1 levels were measured with ELISA technique with standardized commercial kits (bender Medsystems GmbH, Austria), with results expressed as pg per milliliter.

Serum C-reactive protein (CRP) level was determined by the nephelometric method (Beckman Array 360 Protein System, Minnesota, Brea, USA). Erythrocyte sedimentation rate (ESR) was determined according to the classic Westergren method using anticoagulant containing whole blood.

Statistical analysis was done using the software SPSS for Windows version 17.0. Statistical significance was evaluated by analysis of variance (ANOVA) and the least significant difference (LSD) post-hoc comparison was used where appropriate to analyze differences between the BD patients and control group. Discrete variables were evaluated with Chi-square test. $p<0.05$ was considered to indicate statistical significance. In addition, correlation coefficient was determined by linear regression analysis performed between measured parameters in study groups.

Results

General characteristics, disease and treatment duration results of the patients with BD and control subjects are shown in Table 1. There were no significant differences between the two groups with respect to gender and age.

Table 1: Demographic properties of the patients with Behcet's disease and controls

	Active Patients with BD (n:29)	**Inactive Patients with BD (n:11)**	**Control (n:20)**
Sex (M/F)	13/16	5/6	12/8
Age (year)	33.21±9.52	38.73±15.22	35.30±5.75
Disease duration(month)	4.88±4.11	11.87±13.167	-
Treatment duration (year)	1.34±0,48	1.55±0,52	-

Duration of the disease and the treatment were given in table 1, pathergy test positivity and findings that are Behcet's disease criteria (oral, genital aphtha, ocular symptoms, arthralgia, arthritis, acne) were given in table 2. Twenty-two patients with active BD had oral aphtous lesions (76%); genital ulcers were found in 9 patients (31%), eye lesions were found in 7 patients (24%), arthralgia was found in 11 patients (38%), arthritis were found in 5 patients(17%) and skin lesions were present in 9 patients (31%). As for the inactive Behcet's patient group, they had almost none of the Behcet's disease symptoms during the study. Of the active Behcet's patients, only 5 (17%) had positive pathergy test.

Table 2: Clinical properties of the patients with Behcet's disease

No	**Age/Sex (years)**	**Oral Ulcer**	**Genital Ulcers**	**Eye Lesion**	**Acneiform Nodules**	**Arthritis**	**Arthralgia**	**Pathergy Test**
1	38/ F	+	++	-	++	++	++	-
2	21/ M	++	++	-	-	-	+	++
3	38/ F	+	++	-	++	-	+	++
4	22/F	++	+	-	-	-	++	++
5	25/F	+	++	++	-	-	++	++
6	45/F	++	++	-	++	++	+	-
7	27/F	+	+	++	-	-	+	-
8	32/M	+	++	++	-	-	++	-
9	43/F	++	++	-	-	-	+	++

10	35/F	+	-	+	-	-	+	++
11	26/M	+	+	-	+	-	-	+
12	23/M	++	++	-	-	-	-	++
13	37/M	+	++	-	++	-	-	++
14	50/F	++	++	-	++	-	+	-
15	21/F	+	++	+	+	-	+	-
16	32/F	+	++	-	++	++	+	-
17	42/F	+	++	-	-	++	+	-
18	35/M	+	++	-	+	-	-	++
19	32/F	+	++	++	-	-	-	++
20	33/F	+	+	++	-	-	-	-
21	27/M	++	+	-	+	-	+	+
22	66/M	+	++	-	++	+	+	++
23	40/M	++	++	-	+	+	+	-
24	38/M	++	-	-	++	++	++	++
25	23/M	+++	++	+	++	-	++	++
26	34/M	+	+	++	+	++	-	++
27	46/F	+	+	+	-	+	+	+
28	30/M	++	++	+	-	-	+	+
29	38/M	+	++	-	++	++	++	++
30	24/F	++	-	++	++	-	++	++
31	47/F	++	-	+	++	-	++	-
32	21/F	++	++	-	-	-	-	++
33	43/F	++	++	++	-	++	++	-
34	20/F	+	++	-	+	+	+	-
35	36/M	+	+	-	++	+	+	-
36	25/F	+	++	++	-	-	++	++
37	69/M	++	++	-	++	++	++	++
38	49/F	++	++	++	++	++	+	++
39	27/F	+	+	-	+	++	++	+
40	29/M	++	-	+	+	-	+	-

-: Unripe, +: Past, **++:** Active, M: Male F: Female, All of the BD patients had oral

Table 3: Vitamin B6, B12, Folic acid, homocysteine and PAI-1 levels in patient and control groups

	Total Behçet's patients	**Active patients**	**Inactive patients**	**Control**
Vit B6 (μg/L)	20.64±14.67	21.83±15.53	17.49±12.19	23.39±31.76
Vit B12 (pg/mL)	318.99±92.34	347.95±85.62	242.64±62.32[a]	270.34±70.38[a,b]
Folic Acid (ng/mL)	8.06±4.52	8.73±5.02	6.29±2.05	6.34±1.86[c]
Homocysteine (μmol/L)	9.81±4.05	10.84±4.21	7.09±1.79[c]	7.87±2.53[c]
PAI-1 (pg/mL)	191.09±85.45	225.25±72.69	101.04±37.07[a]	108.20±35.84[a,b]
CRP (mg/L)	14.58±15.68	18.08±17.09	5.36±3.32[c]	3.25±0.76[a,b]
ESR (mm/h)	21.3±18.46	25.89±18.84	9.18±10.63[c]	9.2±10.61[a,b]

[a]$p<0.001$; when compared to the active patients

[b]$p<0,05$; when compared to total Behçet's patients

[c]$p<0.005$; when compared to the active patients

Vitamin B_{12}, folat, Hcy, PAI-1 and CRP levels were higher in active patients with BD when compared with inactive BD patients and controls. On the other hand, there was no statistically significant difference between groups in serum vitamin B_6 levels (Table 3).

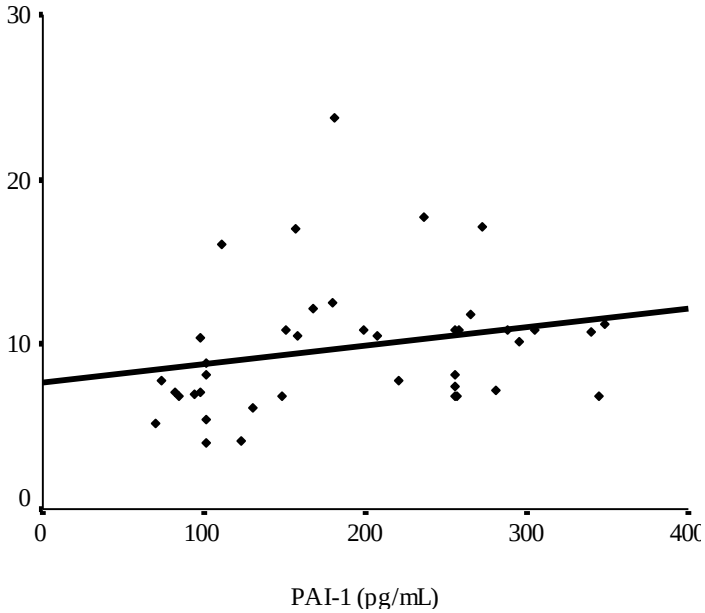

Figure 1: Correlation between plasma PAI-1 and total homocysteine levels in Behcet's disease. By nonparametric correlation analysis, PAI-1 and total homocysteine from all BD patients were found to be positively correlated (Spearman COEFFICIENT=0.608, p<0.05).

In addition, there was significant positive correlation between plasma PAI-1 and homocysteine levels ($p< 0,05$) (Figure 1).

The age range was between 20 and 66 years (33.21±9.52) in the clinically active Behcet's patients and between 21 and 69 years (38.73±15.23) in the inactive Behcet's patients. There was no statistically significant difference between the groups in terms of sex and mean age. The age range of the 18 females and 2 male that constituted the control group was between 21 and 42 years (28.30±5.75). Mean disease duration after diagnosis of the disease in the 40 Behcet's patients was 6.8±8.16 months (3 days-48 months), whereas the mean disease duration was 5.46±4.48 months (3 days-17 months) in the 20 patients (16 active and 4 inactive patients) who had recurrent oral aphtha. When the Behcet's disease group was examined in itself, it was seen that the mean disease duration was 0.1-17 months in the active group and 0.5-48 months in the inactive group.

Discussion

The etiology and pathogenesis of Behcet's disease are unknown. Non-specific vasculitis of the veins, arteries, and capillaries is common to all affected organs. Vascular disease and thrombosis is one of the most important features, and accounts for most of the mortality. Endothelial injury seconder to vasculitis of BD seems to be the key event in the thrombotic abnormalities of this disease. Endothelial dysfunction relates to inhibition of natural anticoagulants and inhibition of fibrinolysis (increased PAI-1) [13].

In this study, PAI-1 levels were higher in BD patients than in healthy controls. The fibrinolytic system is located at the crossroads of haemostasis and matrix formation. Therefore, pathological fibrinolysis may represent a link between thrombotic vasculopathy and the unique arthritis of BD. The active patient with the highest PAI-1 levels had been in an active episode of BD with orogenital ulcerations, arthritis, uveitis and erythema nodosum. However, vascular thrombosis was not present then or in the following course. Therefore, the interrelationship between circulating PAI-1 and vascular events does not seem to be absolute in all patients. There has been increased evidence that the coagulation system is activated in inflamed joints, which is followed by the formation of fibrin and consequently activation of the fibrinolytic system [14,15]. Apart from being the key enzyme of fibrinolysis and thrombolysis, the plasminogen activation system is also involved in the extracellular matrix and bone degradation [16,17]. There is also *in vitro* and *in vivo* evidence of the involvement of the plasminogen activation system in the

destructive phase of RA [18,19]. As PAI-1 can be synthesized from both platelets and the endothelial cells and both cell types are activated in BD (20), PAI-1 could be derived from both sources. Pathological alterations of the plasminogen activation system have been reported in various connective tissue disorders including systemic lupus erythematosus, systemic sclerosis and vasculitic syndromes [21-23]. We suggest that increased levels of PAI-1 may reflect the presence of endothelial injury and/or activation and fibrinolytic activation in patients with BD. The other way, Hcy generates free oxygen radicals such as superoxide and hydrogen peroxide, both of which have been linked to endothelial damage. It changes coagulation factor levels so as to encourage blood clot formation with aggregated platelets. Elevated Hcy level has been shown to be a risk factor for myocardial infarction, stroke, retinal vascular diseases such as retinal artery/vein thrombosis and occlusion. Hcy induced vascular problems may be multifactorial, including direct Hcy damage to the endothelium, enhanced LDL peroxidation, and increased platelet aggregation by the effects on the coagulation system [24-26]. The present study demonstrated an association between plasma total Hcy and BD over control subjects. The overall plasma Hcy levels in patients with BD (9.81±4.05μmol/L) were higher than healthy control subjects (7.86±2.25μmol/L), but the difference was not significant ($p = 0.055$). Plasma Hcy levels in active subjects (10.84±4.12μmol/L) were also significantly ($p = 0.007$) higher than in inactive patients (7.09±1.79 μmol/L) and control subjects ($p < 0.001$). We found that both PAI-1 and Hcy are higher in active disease. The main factor responsible for the increased frequency of thrombosis is thought to be endothelial dysfunction in BD. This increase in Hcy was associated and correlated with the increase in plasma PAI-1 levels in BD (Figure 1). Therefore, these findings suggest that PAI-1 and Hcy may be interrelated in endothelial cell activities in BD. Endothelial damage, venous stasis or occlusion, and thrombogenesis have been extensively documented in the course of BD. Therefore, our results support the suggestion that Hcy may be a risk factor for BD but also identify it as a risk factor for the development of complication.

Table 3 shows the plasma concentrations of vitamin B_{12}, vitamin B_6 and folic acid in Behçet patients with or without activity and control subjects. Plasma vitamin B_6 concentrations of Behçet patients were the same as those of controls. In our Behçet patients, in contrast to an increment in plasma Hcy values, plasma vitamin B_{12} concentrations and plasma folic acid concentrations were found to be within control ranges. Calıkoglu et. al. [25] did not observe a significant difference in vitamin B12 and folic acid levels of Behcet's disease patients and healthy controls. Similarly, Korkmaz et. al. [26] did not establish a significant difference between vitamin B12 and folic acid values of Behcet's patients and healthy controls. In the present study we could not find a statistically significant difference between Behcet's disease patients and healthy controls in terms of serum vitamin B_6, however B_{12} and folat values were higher in patients than in controls. Thus, high vitamin B_{12} and folic acid concentrations do not affect Hcy concentration, and endothelial dysfunction in Behçet disease may not be dependent on vitamin B_{12} and folat concentrations. The increased B_{12} and folat levels in patients with BD may depend on B-vitamin supplementation in active BD patients. On the other hand, in BD patients, vitamin B_6 concentrations were found to be no significantly difference, as observed by others [27]. Vitamin B_6 is an important regulator of protein and energy metabolism, since it is a cofactor in enzymatic reaction involving amino acids [28,29]. Bekpınar et. al. [27] examined vitamin B6, homocysteine and CRP levels in Behcet's disease patients and patients with rheumatoid arthritis. They interpreted this finding to the effect that mild inflammation would not influence vitamin B6 level and that endothelial dysfunction in Behcet's disease was independent of vitamin B6 level. Thus, no availability of vitamin B_6 could have a deleterious effect on the balance of protein synthesis and degradation, which may cause several dysfunctions of some tissues such as the vascular system. For this reason, vitamin B_6 treatment to BD patients might be advisable.

According to the data above, it was concluded that increased Hcy and PAI-1 levels may tend to promote endothelial damage in active patients than inactive patients and controls. In our study; levels of vitamins involved in homocysteine metabolism (Vitamin B_6, Vitamin B_{12} and folat), and PAI-1, a parameter relevant to endothelium and hemostasis, were evaluated together in Behcet's disease. Increased PAI-1 and Hcy levels in Behcet's disease, a multifactorial and multicomplicated disease, and the higher levels in active Behcet's patients suggest these parameters may be responsible for the complications and pathologic findings. However, we think that more extensive case studies would be helpful in defining the etiopathogenesis and complications. Further studies that

show the oxidative capacity of Hcy and PAI-1 in patients with Behcet's disease by considering disease activity and complications are required.

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