Is there an association of plasma homocysteine levels with vascular involvement in patients with Behçet's syndrome?

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ABSTRACT

Objective. *To assess whether or not plasma homocysteine levels play a part in vascular involvement in Behçet's syndrome (BS).*

Methods. *74 consecutive BS patients fulfilling the criteria of the International Study Group for BS, 35 healthy control (HC) and 14 rheumatoid arthritis (RA) patients on methotrexate (MTX) were studied. BS patients were then classified as those with and without vascular involvement. Fasting plasma homocysteine, folate, and vitamin B_{12} concentrations were measured by enzyme immunoassay and chemiluminescent immunoassay methods respectively.*

Results. *Plasma homocysteine levels were found to be higher in the BS patients than in the healthy control (16.08 ± 7.5 vs. 12.9 ± 6.3 mol/L, $p < 0.03$). The homocysteine levels in the RA group on MTX were higher compared with both the BS and HC groups (28.7 ± 9.9; $p < 0.0001$). No remarkable difference pertaining to homocysteine levels was found between BS patients with or without thrombosis ($p < 0.86$). Hyperhomocysteinemia was also detected in 11 out of 22 (50%) of the patients with vascular involvement, which proved to be of no significant difference in comparison with those without vascular involvement (20/52, 38%; $^2 = 0.26$, $p > 0.05$). Active BS smokers exhibited a higher concentration of homocysteine in contrast to non-smoker BS sufferers (20 ± 8.4 vs 14.1 ± 6.1 mol/l; $p < 0.004$). Smoking was determined to have a positive correlation with vascular involvement ($r = 0.26$, $p < 0.046$), as well as with homocysteine levels ($r = 0.31$, $p < 0.012$) in BS. Upon logistic regression analysis, smoking was found to have a significant relationship with vascular involvement (odds ratio 3.12 [95% CI 2.02 - 4.22] $p = 0.04$). There was no significant difference between the study groups with respect to their B_{12} vitamin and folate levels. We were unable to make any correlation between homocysteine and vitamin B_{12} or folate in any of the groups ($p > 0.05$).*

Conclusions. *No association was found between homocysteine levels and vascular involvement in our BS patients. We determined that smoking seems to pose a risk for vascular involvement in BS patients.*

Introduction

BS is a multisystem relapsing vasculitis of unknown aetiology (1). Mucocutaneous, ocular, articular, genital, central nervous system and gastrointestinal involvement may occur during the course of BS. Vascular complications have been reported in approximately 12-40% of the patients with BS (2). The most frequent involvement types occur as superficial thrombophlebitis and deep venous thrombosis (2).

What triggers off the development of thrombosis in this disease remains to be ascertained, but endothelial injury/dysfunction is believed to be an important factor (3, 4). None the less, protein C and S deficiencies, as well as factor V Leiden mutation (5-7), have been reported to be contributing factors for vascular involvement in BS.

Homocysteine is a sulphur-containing aminoacid. It may lead to early atherothrombotic disease by exerting a direct toxic influence upon endothelial cells, as well as contributing to an increase in the proliferation of vascular smooth muscle cells (8, 9). Homocysteine has to date been determined to reduce prostacyclin synthesis (10), enhance the efficiency of the factor V (8), inhibit thrombomodulin synthesis (11), and render protein C to be less efficient in arterial and venous endothelial cells (12). A number of factors influence homocysteine levels, including end-stage renal failure, inflammatory bowel disease and diabetes mellitus (13-15). It may also occur due to vitamin B_{12} or folate deficiency (16) and can be easily

corrected by treatment with these vitamins.

In this study, we aimed to investigate whether or not homocysteine poses a risk for vascular involvement in patients with BS.

Patients and methods

Subjects

This study, conducted between January 1999 and May 2001, involved 90 consecutive cases already being followed, along with newly diagnosed patients. These patients were included in the study due to their fulfilling the criteria issued by the International Study Group for BS (17). Four patients using cyclosporin, four patients taking vitamin pills, and a further 8 patients diagnosed as having hyperlipidaemia were excluded. The remaining 74 cases with BS (49 males, 25 females, mean age 35 ± 8.4, range: 18-58 yrs; disease history: 81 ± 58.9 months), 14 cases of RA, each of whom was on MTX in doses of 7.5-17.5 mg/week but who had not previously been administered folic acid (4 males, 10 females; mean age 42 ± 13, range: 19-62 yrs) (MTX group), and 35 healthy people (18 males, 17 females; mean age 31 ± 3, range: 24-39 yrs) were studied. Patients with renal failure, diabetes mellitus, hyperlipidaemia, psoriasis, hepatitis and patients suffering from alcoholism, as well as those on vitamin pills, were not considered for this study. Clinical activity at the time of venipuncture was assessed on the basis of the active signs and symptoms of oral ulcers, genital ulcers, eye lesions, and vascular involvement. Patients with activity of at least 2 signs or symptoms were considered to have active BS.

Vascular involvement had already been verified prior to our study, but two of the cases developed vascular involvement while the study was being carried out. Deep venous thrombosis (DVT) was diagnosed in two patients by venography and in 11 patients by doppler ultrasonography. Pulmonary and popliteal artery aneurysms were diagnosed by computed tomography in four patients and angiography in one patient respectively. Thrombosis of intracerebral vessels was diagnosed in two patients by magnetic resonance imaging

Table I. Clinical features of patients with Behçet's syndrome.

	In the past (%)	At the time of clinical evaluation (%)
Oral aphtous	74 (100)	17 (23)
Genital ulceration	66 (89)	3 (4)
Uveitis	10 (14)	-
Arthritis/Arthralgia	65 (88)	1 (1)
Skin manifestations	70 (95)	10 (7)
Cerebral involvement	1 (1)	-
Intestinal involvement	1 (1)	-
Vascular involvement	20 (27)	2 (3)
PAA	3 (4)	1# (1)
FAA	1 (1)	-
DVT*	13 (18)	1 (1)
DST	2 (3)	-
VCSS	1 (1)	-

PAA: Pulmonary artery aneurysm; FAA: Femoral artery aneurysm; DVT: Deep venous thrombosis; DST: Dural sinus thrombosis; VCSS:Superior vena cava syndrome. *8 patients with DVT had a positive history of thrombophlebitis. # This patient had also DVT at the same time.

scan. Smoker was defined according to the criteria established by World Health Organisation (WHO) (Smoker: someone who smokes at least once a day) (18).

Analytical procedure

Blood samples, having been taken for the purpose of measuring homocysteine levels after 12 h of fasting, were centrifuged at 2000 g for 15 min at +4° C, and the obtained plasma was then stored at -70°C until the commencement of the study. Aside from this, blood samples for B_{12}, folate, glucose and total cholesterol levels were also obtained. Vitamin levels were assessed by means of the chemiluminescent immunoassay method (Beckman, Access immunoassay system; Sanofi Diagnostic Pasteur, Marnes La Coquette, France). A normal interval for Vitamin B_{12} is 180-914 pg/ml, while it is > 3 ng/ml for folate. Homocysteine was assessed by the Enzyme Immunoassay Method (Axis Homocysteine enzyme immunassay, Germany). This method has a correspondence of 94% with high pressure liquid chromatography (HPLC) (19).

Statistical analysis

Calculations were performed with the statistical package SPSS for windows 9.0 (SPSS Inc; Chicago, Illinois, USA). The values of homocysteine were not distributed normally. Kruskal-Wallis analysis, the Mann-Whitney U test, and

Table II. Demographic and laboratory findings in all groups.

	BS (n = 74)	BS (VI) (n = 22)	BS (NVI) (n = 52)	MTX group* (n = 14)	HC (n = 35)
Age (mean ± SD)	35 ± 8.4	37.13 ± 8.5	33.7 ± 8.6	42 ± 13.5	31.2 ± 3.8
M/F	50/24	21/1	29/23	4/10	17/18
Homocysteine (N=5-15 mol/l)	16.08 ± 7.5[a]	16.3 ± 7.6	15.9 ± 7.9	28.7 ± 9.9[b]	12.9 ± 6.3
Hyperhomocysteinemia (n,%)	31 (42)	11 (50)	20 (38)	12 (86)	11 (31)
Vit B_{12} (N=180-914 pg/ml)	213 ± 80	205 ± 64	221 ± 93	140 ± 38	232 ± 120
Folate (N > 3 ng/ml)	7.8 ± 7.5	6.2 ± 2.4	9.2 ± 9.4	8.8 ± 3.6	7 ± 5.9

[a] $p < 0.03$ BS vs. HC; [b] $p < 0.0001$ MTX group vs. HC, $p < 0.0001$ MTX group vs BS.
VI: Vascular involvement; NVI: Non-vascular involvement. *No matching for age and sex distribution in the MTX group was achieved with the other groups.

Table III. Homocysteine levels associated with vascular involvement in smoker and non-smoker BS patients.

	VI+Smoking (n = 11) (Group I)	VI+Non-smoking (n = 11) (Group II)	NVI+Smoking (n = 14) (Group III)	NVI+Non-smoking (n = 38) (Group IV)
Homocysteine levels (N=5-15 mmol/L)	20.1 ± 6.8*	14.4 ± 6.7	18.2 ± 8.5	13.9 ± 5.6

* $p < 0.01$ group I vs. group IV. Abbreviations: VI: Vascular involvement; NVI: Non-vascular involvement.

the Wilcoxon signed rank test were used for the comparisons. Homocysteine levels were first transformed logarithmically, and then simple correlation were performed using Spearman's correlation coefficient. For vascular involvement, logistic regression analysis was used to calculate odds ratios with 95% CI for homocysteine and smoking. The ^{2} test was used to scrutinise the relationship between the two independent variables. Statistical significance was defined as $p < 0.05$.

Results

In the BS group, 11 (14.8%) patients were accepted as clinically active. There was no correlation between the plasma homocysteine levels and disease activity ($p > 0.05$) The clinical features of BS patients are shown in Table I. The mean homocysteine levels in all groups are shown in Table II. No matching for age and sex distribution in the MTX group was achieved with the other groups.

Comparison between all the groups in accordance with their homocysteine levels

Homocysteine levels were higher in the whole BS group than in HC ($P < 0.03$), whilst it was lower than in patients on MTX ($p < 0.0001$). No statistical difference pertaining to homocysteine levels was determined between BS patients with/out thrombosis ($p < 0.86$). No relationship was found between the clinical manifestations of BS and mean homocysteine levels ($p > 0.05$). Neither of the two separate comparisons of cases with and without vascular involvement with in HC revealed a statistical difference ($p > 0.05$). The homocysteine levels were determined to be higher in male patients than in female ones in both BS and HC ($p < 0.01$).

Assessment of the number of patients with hyperhomocysteinemia in all the groups

Hyperhomocysteinemia was detected in 31 out of 74 in BS (42%), 11 out of 35 in HC (31%), and 12 out of 14 in the MTX group (86%) ($p > 0.05$ BS vs. HC; $p < 0.05$ MTX vs. BS and HC). Hyperhomocysteinemia was also detected in 11 out of 22 (50%) patients with vascular involvement in accordance with kit's reference values (5-15 mol/l), which was of no significant difference in comparison with those without vascular involvement (20/52, 38%; ^{2} = 0.26, $p > 0.05$).

The 95th percentile of homocysteine level was 25.48 mol/l in HC. When we take this as the cut-off level, only 4 BS patients with vascular involvement had a level above this threshold. All of the four patients were smokers, with two of them having dural sinus thrombosis and the other two DVT. No significant difference was determined between BS patients with and without vascular involvement (^{2} = 0.41; $p > 0.05$). We were unable to determine a significant difference in respect to homocysteine level between patients with DVT and those with only arterial involvement (16.3 ± 8.3; 16.9 ± 1.7, $p = 0.49$ respectively).

The impact of cigarette smoking upon homocysteine levels

The number of active smokers was 11/22 (50%) in BS with vascular involvement, 14/52 (27%) in BS patients free from vascular involvement, 19/35 (54%) in HC, and 3/14 (21%) in the MTX group. Incidentally, there was no statistical difference between the involved groups with respect to the number of active smokers. Active BS smokers had a higher concentration of homocysteine when compared to non-smoker BS sufferers (20 ± 8.4 mol/l in smokers vs. 14.1 ± 6.1 mol/l in non-smokers; $p < 0.004$). A positive correlation was determined between not only smoking and vascular involvement ($r = 0.26$, $p < 0.046$), but also smoking and homocysteine levels ($r = 0.31$, $p < 0.012$) in BS. Smoking was significantly associated with vascular involvement (OR: 3.122 [95% CI 2.02 - 4.22], $p < 0.04$)

Homocysteine levels in BS patients both smoking and developing vascular involvement at the same time (group I) were determined to be higher in comparison with those who neither smoked nor developed vascular involvement (group IV). On the other hand, despite the fact that the same levels were found to be higher in BS patients who smoked but had not developed vascular involvement (group III) than those in group IV, this difference did not reach a level of statistical significance (Table III). No significant difference was determined for either the MTX group or HC in terms of smoking and plasma homocysteine level.

Comparison between all the groups in accordance with their vitamin levels

There was no significant difference between the study groups in respect to B_{12} vitamin and folate levels. No correlation between homocysteine and vitamin B_{12}, and folate was able to be determined in any of the groups ($p > 0.05$).

Discussion

BS is well known to involve the vascular system, the exact reason for which remains obscure. Apart from suggesting an endothelial dysfunction to account for this enigma (3, 4), protein C and S deficiencies, as well as factor V Leiden mutation, have all been reported to be contributing factors for vascular involvement in BS (5-7).

Hyperhomocysteinemia may lead to arterial and venous thrombosis (20, 21). In this study, we aimed to investigate whether or not homocysteine posed a risk for vascular involvement in patients with BS.

Aksu *et al*. reported plasma homocysteine levels to be higher in BS than in HC and hyperhomocysteine to be an

independent risk factor for thrombosis in BS (22). Despite the fact that our study results were consistent with their result in regard to high homocysteine levels in BS, we could find no evidence that hyperhomocysteine is a risk factor for vascular involvement in patients with BS. Although the mean plasma homocysteine level in BS were found to be higher than in HC, it was notably lower than that of patients' on MTX. Still, hyperhomocysteine was not determined to pose a risk for vascular involvement, inasmuch as no significant difference was observed with respect to homocysteine levels in those with and without vascular involvement among BS patients.

Clinical features of patients in both studies comply with each other. However, homocysteine determination was undertaken by Enzyme Immunoassay (EIA), whereas the other study determined homocysteine levels by HPLC. Judging from the notable consistency between these two methods in terms of homocysteine determination (19), we can speculate that these different results may not be due to the application of different methods.

This was a cross-sectional study and the median time between the moment of the thrombotic event and tested time was 39 ± 53.9 months (range 0.5-204 months). Since homocysteine levels can vary with time and it is well-documented that plasma homocysteine levels may be affected by such factors as diet, smoking, gender and so forth (23). The homocysteine levels have also been reported to decrease just after an acute thrombotic event (24). In addition to, the longer the interval between the time of analysis and of cardiovascular incident is, the less significant the association between homocysteine and atherosclerosis becomes (25). In this respect, the change in plasma homocysteine levels may well depend upon whether the determination process is undertaken before or after the development of thrombosis. Considering that our study was a cross-sectional one, it is possible that the median time elapsing between the occurrence date of the thrombotic incidence and of inclusion in our study was 39 ± 53.9 months might indicate the absence of a relationship between hyperhomocysteinemia and vascular involvement.

We can propose that a serial monitoring process of plasma homocysteine levels may be of help in revealing a possible relationship between hyperhomocysteinemia and thrombosis. For instance, Petri *et al.* (26) concluded that there was a link between hyperhomocysteinemia and arterial thrombosis, and that hyperhomocysteinemia had no link with venous thrombosis in SLE, which is in common with BS by virtue of their inflammatory natures, by means of a serial measurement of plasma homocysteine levels. Another study of patients with SLE made by Fijnheer *et al.* (27) arrived at conclusions in agreement with those of Petri *et al.* Additionally, cardiovascular cases which increase the mortality rate in RA patients in comparison with the general population (28) have been attributed to hyperhomocysteinemia (29, 30).

Although hyperhomocysteine has a tendency to cause thrombotic events when it coincides with another thrombophilic event in patients with RA (31), no solid evidence exists as to whether it independently gives rise to DVT. Therefore, these studies lead us to conclude that the likelihood of the relationship between hyperhomocysteinemia and isolated DVT, which is commonly seen in BS patients, is remote in diseases with an inflammatory nature.

There also exist some controversial results obtained from studies into the curious relationship between arterial and venous thrombosis attributable to hyperhomocysteinemia. For instance, den Heijer *et al*. have reported a positive relationship between hyperhomocysteinemia and DVT (32), while two other studies arrived at an opposite result (33, 34). These discrepancies have been mentioned to account for the fact that hyperhomocysteinemia is a weak factor for DVT unless accompanied by other thrombophilic factors (35). With all this taken into account, despite the existence of hyperhomocysteinemia in the ongoing of inflammatory diseases, no solid evidence that hyperhomocysteinemia leads to DVT has been provided in the literature as yet.

We therefore speculate that, hyperhomocysteinemia might not have a part in the development of venous thrombosis when independent of any other thrombophilic factors in BS. However, the fact that hyperhomocysteinemia is likely to act as an additive factor in thrombosis when it coincides with a thrombophilic condition should not be disregarded. Unfortunately, we were unable to assess other thrombophilic conditions such as protein C resistance and anticardiolipin antibodies. Altinbas *et al.* (36) reported activated protein C resistance and hyperhomocysteinemia in 11 and 12 out of 43 BS patients respectively. They reported that while thrombosis history was present in 5 patients with protein C resistance, hyperhomocysteinemia was present in only 2 of the patients, who also had activated protein C resistance. The authors concluded that hyperhomocysteinemia concomitant with activated protein C resistance might also contribute to vascular involvement. Nevertheless, Misgav *et al.* (37) also reported a case of BS with arterial and venous thrombotic events, which showed the co-existence of hyperhomocysteinemia with factor V Leiden mutation in this patient.

In our study, in contrast to the results obtained by Aksu *et al.*, we found plasma homocysteine levels to be higher in smokers than in non-smokers. Additionally, there was a positive correlation between smoking and vascular involvement, apart from a positive correlation between smoking and homocysteine levels. However, the fact that vascular involvement is seen in non-smoker BS patients suggests the probability that some other factors do also contribute to vascular involvement in BS patients. Apart from causing changes in platelet functions and blood viscosity, smoking is a cardiovascular risk factor due to its destructive effect on endothelial cells (38). Smoking, along with abdominal obesity, has been reported to be an independent risk factor for venous thromboembolism among middle-aged men (39). Although a negative relationship between smoking and mucocutaneous manifestations was reported for BS patients (40), our results seems to suggest that smoking may act as an

additional factor for vascular involvement in BS.

We herewith suggest that hyperhomocysteinemia may not act as an independent risk factor for vascular involvement. On the other hand, considering the cut-off value for the high homocysteine levels in the healthy controls, the 4 actively-smoking BS patients had vascular diseases and had high values of homocysteine outside 95% percentile might indicate that there might be some association between vascular disease and high homocysteine levels. However, we believe further studies with a greater number of patients may help bring this to light. Judging from our study results, we assume that smoking has some effect upon vascular involvement in patients with BS.

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