Endogenous thrombin potential in Behçet's disease: relationship with thrombosis and anticoagulant therapy

J.C. Mejía[1], G. Espinosa[1], D. Tàssies[2], J.C. Reverter[2], R. Cervera[1]

[1]*Department of Autoimmune Diseases;* [2]*Department of Haemotherapy and Haemostasis, Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Catalonia, Spain.*

Juan Carlos Mejía, MD
Gerard Espinosa, MD, PhD
Dolors Tàssies, MD, PhD
Joan-Carles Reverter, MD, PhD
Ricard Cervera, MD, PhD, FRCP

Please address correspondence to:
Dr Gerard Espinosa,
Department of Autoimmune Diseases,
Hospital Clinic,
Villarroel 170,
08036 Barcelona,
Catalonia, Spain.
E-mail: gespino@clinic.cat

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ABSTRACT

Objective. *To analyse the relationship between an automated thrombin generation test, the endogenous thrombin potential (ETP), and other hypercoagulability markers, with vascular involvement in patients with Behçet's disease (BD).*

Patients and methods. *We analysed 56 BD patients (30 men; mean age, 34.4 ± 14.3 years) without any known thrombophilic factor, of which 17 had previously suffered from thrombosis (deep venous thrombosis in 14 and ischaemic stroke in 3), and 56 controls matched for age and sex. Additionally, we also evaluated 20 plasma samples with an international normalised ratio (INR) between 1.5 and 5.0 obtained from patients with atrial fibrillation but without a history of embolic events that were under treatment with acenocumarol. Thrombin generation was measured as ETP with a chromogenic assay in an automated analyser. Factor VIII, von Willebrand factor antigen, prothrombin fragment 1.2, D-dimer and plasmin-antiplasmin complexes were also measured.*

Results. *BD patients showed higher ETP values than controls (471.3± 49.3 vs. 427.5± 31.3 mA; p<0.001). Additionally, BD patients with a history of thrombosis had higher ETP values than patients without thrombosis (496.6± 36.5 vs. 460.7± 50.5 mA; p<0.01). Factor VIII and von Willebrand factor antigen were also elevated in BD patients, but only von Willebrand factor antigen showed statistically significant differences between BD patients with and without thrombosis. Acenocumarol treatment reduced thrombin generation in BD patients in parallel to INR levels, reaching values similar to those of patients with atrial fibrillation and similar INR.*

Conclusions. *BD is associated with thrombosis, and increased thrombin generation (measured as ETP) is a promising marker of hypercoagulability.*

Introduction

Behçet's disease (BD), originally recognised by three-symptoms (uveitis, recurrent oral aphtae and genital ulcerations), is currently classified as a variable vessel vasculitis involving both venous and arterial vessels (1, 2). In fact, thrombosis in BD is common and venous involvement is much more frequent than arterial with a prevalence ranging from 14% to 39% (3).

Although the etiopathogenesis of vasculo-Behçet is poorly understood, there is evidence of activation of the haemostatic system with increase of procoagulant markers of thrombosis (4, 5) as well as defective fibrinolysis (6).

There is no consensus on the long-term treatment of thrombosis in BD. Recommendation from the European League Against Rheumatism (EULAR) advises the use of corticosteroids and immunosuppressive agents such as azathioprine, cyclophosphamide or ciclosporine A. Conversely, anticoagulants, antiplatelet or antifibrinolytic agents are not recommended (7). Furthermore, a recent study demonstrated that immunosuppressive agents significantly reduced venous thrombosis relapse in BD (8).

Therefore, it would be of interest to know if BD patients have a hypercoagulable state. Thrombin generation is considered to be a sensitive and reliable marker of hypercoagulability and of bleeding tendency (9). Endogenous thrombin potential (ETP) measures the overall tendency of a plasma sample to form thrombin after initiation of coagulation. High thrombin generation measured as ETP suggests the presence of a hypercoagulable state (10).

The objective of this study was to analyse the relationship between the ETP, an automated thrombin generation test, and other hypercoagulability markers, with vascular involvement in BD patients.

Patients and methods

Patients

We performed a prospective study that included 56 BD patients (30 men and 26 women; mean age, 34.4; standard deviation [SD], 14.3 years), without any thrombophilic factor including protein C, protein S or antithrombin deficiencies, factor V Leiden, prothrombin G20210A polymorphism or antiphospholipid antibodies (lupus anticoagulant, IgG and IgM anticardiolipin antibodies and IgG and IgM anti beta2 glycoprotein I antibodies). A total of 17 of these patients had previously presented episodes of thrombosis (deep venous thrombosis in 14 and ischaemic stroke in 3). Thrombotic episodes were assessed by objective methods. Diagnosis of deep venous thrombosis was confirmed by Doppler ultrasonographic scans or by venography. Pulmonary embolism was diagnosed by ventilation/perfusion scanning or by pulmonary angiography. Cerebrovascular ischaemic episodes were confirmed by computed tomography scanning or by magnetic resonance imaging techniques.

Patients were recruited in the outpatient clinic of the Department of Autoimmune Diseases of a tertiary university hospital. All patients fulfilled three or more of the International Study Group criteria for the diagnosis of BD (11). Of note, inclusion criteria were inactive BD in accordance to Behçet's Syndrome Activity Score (BSAS) (12). After informed consent, demographic and clinical data, with particular attention to prior development of thrombosis (arterial or venous), were collected. The main *cumulative* clinical characteristics of these patients are reflected in Table I. Regarding the treatment, glucocorticoids at variable doses were prescribed in the majority of patients (83%), followed by colchicine (74%), cyclosporine (54%), azathioprine (17%), intravenous pulses of cyclophosphamide (13%), and methotrexate (4%), respectively.

A sex- and age-matched control group of 56 volunteer healthy subjects (30 men and 26 women; mean age, 34.9 years; SD, 14.1 years), without previous history of thrombotic events, was also recruited. None of the control subjects reported taking oral contraceptives or other medication known to interfere with blood coagulation (13). Another control group was also included consisting of 20 plasma samples with an international normalised ratio (INR) between 1.5 and 5.0 obtained from patients with atrial fibrillation but without previous history of embolic events that were under treatment with acenocumarol.

Table I. Cumulative clinical manifestations of patients with Behçet's disease.

	n	(%)
Mouth ulcers	56	(100)
Genital ulcers	38	(68)
Cutaneous involvement	39	(70)
Erythema nodosum	16	(42)
Pseudofolliculitis	13	(34)
Eye involvement	32	(57)
Anterior uveitis	19	(34)
Retinal vasculitis	8	(14)
Posterior uveitis	9	(16)
Fever	19	(34)
Thrombotic events	17	(30)
Deep venous thrombosis – Pulmonary embolism	14	(25)
Ischaemic stroke	3	(5)
Neurologic manifestations	6	(11)
Central nervous system involvement	4	(9)
Peripheral nervous system involvement	2	(4)
Arterial involvement*	3	(5)
Artery aneurysm	1	(2)
Pseudoaneurysm	2	(4)
Gastro-intestinal involvement	3	(5)

*In BD, involvement of the vessel wall leads to formation of true aneurysms, or through disruption and haemorrhagic dissection of the layers results in the formation of false aneurysms or pseudoaneurysms.

Blood collection and plasma preparation

Venous blood samples were drawn from a clean antecubital venipuncture without venocclusion, in the morning, with the patient sitting and resting. Samples for coagulation and fibrinolysis studies were obtained in tubes containing 3.2% trisodium citrate (1:9, vol:vol) (Becton Dickinson, Rutherford, NJ, USA) and platelet-free plasma was immediately obtained by double centrifugation: first at 2,000 g for 10 min. at 22°C, and then at 5,000 g for 10 min. at 4°C. Plasma was aliquoted and stored at -40ºC. For genotype studies, samples were drawn in trisodium EDTA tubes (Becton Dickinson). Sera for biochemistry and for autoantibodies studies were drawn in tubes containing no anticoagulants (Becton Dickinson).

The 12 BD patients with previous thrombosis that received antivitamin K therapy were asked to stop antivitamin K therapy for two weeks to perform routine thrombophilia study. They received instead thromboprophylactic dose of low molecular weight heparin until 48h before blood sampling. In these patients, an additional sample was obtained during antivitamin K treatment.

Laboratory studies – General

Prothrombin and activated partial thromboplastin time were determined in an automated analyser (BCS-XP™; Siemens, Marburg, Germany) using standard reagents (Siemens). Fibrinogen was measured by the Clauss' technique.

Thrombophilia and hypercoagulability parameters

Protein C and antithrombin activities were quantified by chromogenic assays using standard commercially available reagents, free and total protein S by ELISA, and factor V Leiden and prothrombin G20210A by PCR. Factor VIII was quantified by a chromogenic assay, von Willebrand factor antigen, prothrombin fragment 1.2 and plasmin-antiplasmin complexes were measured by ELISA, and D-dimer was determined by a turbidimetric method. Lupus anticoagulant was detected fol-

Table II. Haemostatic parameters in patients with Behçet's disease and in controls.

	Behçet's disease (n=56)	Controls (n=56)
Protein C (%)	103 ± 23	101 ± 19
Antithrombin (%)	98 ± 17	101 ± 22
Free protein S (%)	97 ± 15	98 ± 17
Total protein S (%)	100 ± 21	99 ± 19
Factor V Leiden homocygous wild type (%)	56 (100%)	56 (100%)
Prothrombin G20210A homocygous wild type (%)	56 (100%)	56 (100%)
Factor VIII (U/dL)	116 ± 19*	97 ± 16
Von Willebrand factor antigen (U/dL)	114 ± 17*	98 ± 18
Prothrombin fragment 1.2 (nmol/L)	1.8 ± 1.2*	0.9 ± 0.3
Plasmin/alpha-2-antiplasmin complexes (µg/L)	347 ± 251*	224 ± 103
D-dimer (µg/mL)	0.2 ± 0.1	0.2 ± 0.1

*p<0.01 *vs*. controls.
Results expressed as mean ± SD. In genotypes results are in absolute numbers and proportions.

Table III. Haemostatic parameters in patients with Behçet's diseases with or without thrombosis.

	BD patients with thrombosis (n=17)	BD patients without thrombosis (n=39)
Protein C (%)	110 ± 16	100 ± 25
Antithrombin (%)	96 ± 15	99 ± 18
Free protein S (%)	95 ± 17	98 ± 14
Total protein S (%)	95 ± 29	102 ± 16
Factor V Leiden homocygous wild type (%)	17 (100%)	39 (100%)
Prothrombin 20210A homocygous wild type (%)	17 (100%)	39 (100%)
Factor VIII (U/dL)	120 ± 26	114 ± 15
Von Willebrand factor antigen (U/dL)	123 ± 13*	110 ± 17
Prothrombin fragment 1.2 (nmol/L)	2.5 ± 1.3*	1.5 ± 1.0
Plasmin/alpha-2-antiplasmin complexes (µg/L)	413 ± 278	318 ± 236
D-dimer (µg/mL)	0.2 ± 0.1	0.2 ± 0.1

*p<0.01 *vs*. patients with Behçet's disease without thrombosis.
Results expressed as mean ± SD. In genotypes results are in absolute numbers and proportions.

lowing the last update of the guidelines of the Subcommittee for the Standardisation of Lupus Anticoagulants of the International Society of Thrombosis and Haemostasis (14). IgG and IgM anticardiolipin antibodies and IgG and IgM anti-beta-2-glycoprotein I antibodies were measured using standardised ELISA.

Studies of thrombin generation

Thrombin generation was continuously monitored in plasma using an automated analyser (BCS-XP™) by a chromogenic method as described by the manufacturer (ETP test™; Siemens). In brief, 108 µl platelet poor plasma (PPP) is mixed with 40 µl of ETP reagent, containing a chromogenic substrate (H-β-Ala-Gly-Arg-pNA), and 89 µl buffer (Tris-HCl buffer, 50 mM, pH 7.4). Thrombin generation is activated in PPP by 15 µl diluted recombinant tissue factor (Innovin™, Siemens) and 8 µl of a 250 mM CaCl2 solution. Conversion of the chromogenic substrate was measured continuously over 20 min using a 405 nm filter. Substrate cleavage by the α2-macroglobulin-thrombin complex is automatically subtracted from the curve with the appropriate software (Siemens). In the assay, standard was lyophilised normal human plasma calibrated by the manufacturer. ETP was obtained in optical mA as the area under the thrombin formation curve. The coefficients of variation intra-and interassay for the ETP were less than 7%, (6.2% and 6.9%, respectively).

Statistical analysis

Results are shown as mean ± standard deviation (SD). Comparisons were performed by χ^2 test or analysis of variance (ANOVA). Data were analysed with the SPSS version 18.0.

Results

The haemostatic parameters detected in BD patients and controls are shown in Table II, while those observed in BD patients with and without thrombosis are presented in Table III. Factor VIII and von Willebrand factor antigen levels were both elevated in BD patients (p<0.01), but only von Willebrand factor antigen levels showed statistically significant differences between BD patients with and without thrombosis (Tables II and III).

Regarding prothrombin fragment 1.2 and plasmin-antiplasmin complexes levels, they were increased in BD patients in comparison with controls (Table II). Furthermore, prothrombin fragment 1.2 but not plasmin-antiplasmin complexes levels were higher in BD patients with previous thrombotic events (Table III). D-dimer levels did not show differences between BD patients and controls nor between BD patients with or without thrombosis (Tables II and III). The lupus anticoagulant was positive in 2 BD patients (3.6%), none of whom had thrombotic events. No one in the control group had antiphospholipid antibodies.

Patients with BD had higher ETP levels than controls (471.3±49.3 *vs*. 427.5±31.3 mA; p<0.001) (Fig. 1) in samples obtained after two weeks without antivitamin K therapy indicating an increased hypercoagulability. Furthermore, BD patients with a history of thrombosis had higher ETP levels than BD patients without thrombosis (496.6±36.5 *vs*. 460.7±50.5 mA; p<0.01) (Fig. 2).

We examined the effect of acenocumarol on the ETP levels in BD patients and in 20 samples from patients with atrial fibrillation but without previous embolism treated with this anticoagulant. Acenocumarol treatment reduced the ETP levels of BD patients in parallel to the INR (r=0.86, log-log regression), yielding values similar to those of patients with atrial fibrillation and similar INR (Fig. 3).

Discussion

In the present study, we have confirmed that patients with inactive BD have an increased thrombin generation meas-

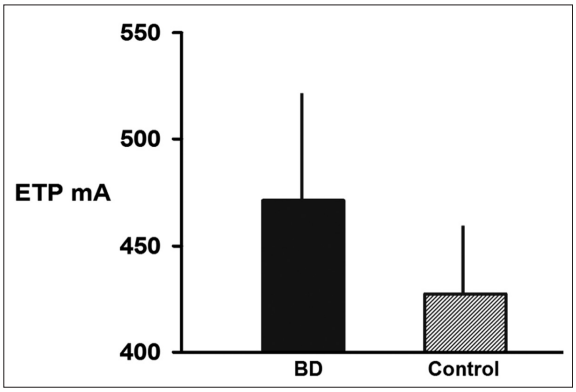

Fig. 1. Endogenous thrombin potential levels in patients with Behçet's disease and in controls.
ETP: Endogenous thrombin potential; BD: Behçet's disease

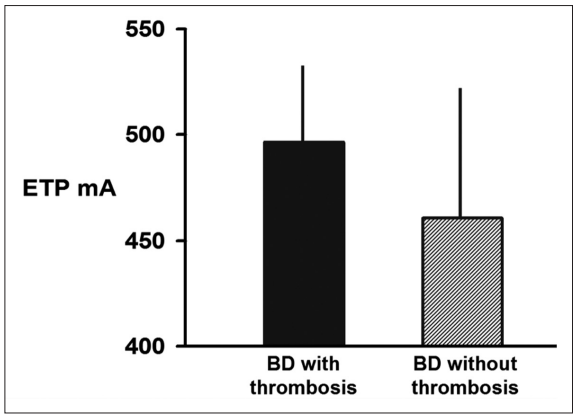

Fig. 2. Endogenous thrombin potential in patients with Behçet's disease with and without prior thrombosis.
ETP: Endogenous thrombin potential; BD: Behçet's disease

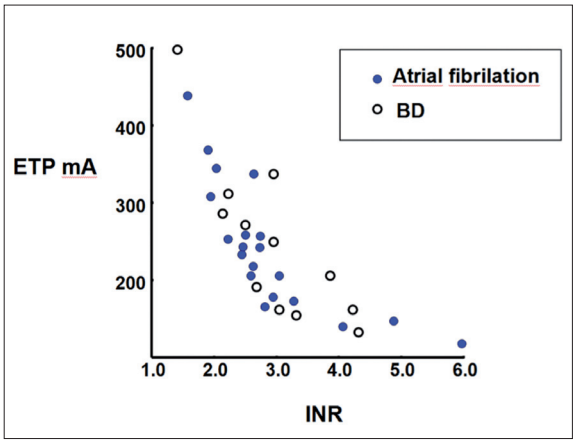

Fig. 3. ETP levels under acenocumarol treatment: patients with atrial fibrillation (solid circles, n=20) and patients with Behçet's disease (open circles, n=12).
ETP: Endogenous thrombin potential; BD: Behçet's disease; INR: international normalised ratio

ured by the ETP test. This increase is more important in patients with previous thrombosis. In addition, the use of acenocumarol reduced the ETP levels of BD patients in parallel to the INR levels, similarly to those of patients with atrial fibrillation and equivalent INR.

Thrombin generation is a global coagulation assay that indicates the final balance between different prothrombotic and antithrombotic factors present in plasma. It is globally accepted that increased thrombin generation indicates a hypercoagulable state. Although no prospective studies on Behçet disease and thrombin generation have been made, several prospective studies in other groups of thrombotic patients have demonstrated that high thrombin generation is associated with the risk of first and recurrent thrombosis (15-17). Based on the conclusions of these studies, we can hypothesise that BD patients with higher thrombin generation potential would have a hypercoagulable state, and probably a higher risk of thrombosis.

This is of paramount importance because the treatment of thrombotic complications in BD patients is controversial (7, 18). For some authors, since the pathogenic substrate would be endothelial inflammation and the thrombotic process would be a secondary event, the treatment should be based only on immunosuppressant agents (7). For other authors, this regimen should also include anticoagulant treatment (18). Moreover, the optimal duration of the latter is completely unknown (8). The current work has been performed in patients who suffered thrombosis several years ago, yet maintained an increased thrombin generation. Therefore, if confirmed in prospective studies, this technique can be a method to monitor hypercoagulability in these patients and help to decide whether or not to extend the anticoagulant treatment.

In the field of systemic vasculitis, ETP has been used to demonstrate a high thrombin generation in patients with antineutrophil cytoplasmic antibodies associated vasculitis (AAV) in remission (19). Similarly to BD patients, AAV patients have an increased incidence rate of venous thrombosis.

Another important point is the fact that factor VIII and von Willebrand factor antigen levels were both elevated in BD patients compared to healthy controls and that von Willebrand factor antigen levels were higher in BD patients than those without. This data may suggest persistent endothelial dysfunction in BD patients, even when they are inactive. Future studies should analyse the relationship between immune-inflammatory response and the coagulation system (20) in order to decide the best treatment (immunosuppressant agents, antithrombotic therapy or both) in selected BD patients.

The most prominent shortcomings of the present study are its sample size and the low number of BD patients with thrombotic manifestations. In addition, there was no control data of BD patients in active phase of the disease.

In spite of these limitations, the results of the present study showed that ETP test could be a useful a marker of hypercoagulability in BD patients and, therefore, provide important information when deciding the treatment.

References

1. JENNETTE JC, FALK RJ, BACON PA *et al*.: 2012 revised International Chapel Hill consensus conference nomenclature of vasculitides. *Arthritis Rheum* 2013; 65: 1-11.
2. HATEMI G, SEYAHI E, FRESKO I, HAMURYUDAN V: Behçet's syndrome: a critical digest of the 2012-2013 literature. *Clin Exp Rheumatol* 2013; 31(3 Suppl. 77): 108-17.
3. CALAMIA KT, SCHIRMER M, MELIKOGLU M: Major vessel involvement in Behçet's disease: an update. *Curr Opin Rheumatol* 2011; 23: 24-31.
4. ESPINOSA G, FONT J, TASSIES D *et al*.: Vascular involvement in Behçet's disease: relation with thrombophilic factors, coagulation activation, and thrombomodulin. *Am J Med* 2002; 112: 37-43.
5. NAVARRO S, RICART JM, MEDINA P *et al*.: Activated protein C levels in Behçet's disease and risk of venous thrombosis. *Br J Haematol* 2004; 126: 550-6.
6. YURDAKUL S, HEKIM N, SOYSAL T *et al*.: Fibrinolytic activity and d-dimer levels in Behçet's syndrome. *Clin Exp Rheumatol* 2005; 23: S53-8.
7. HATEMI G, SILMAN A, BANG D *et al*.: EULAR recommendations for the management of Behçet disease. *Ann Rheum Dis* 2008; 67: 1656-62.
8. DESBOIS AC, WECHSLER B, RESCHE-RIGON M *et al*.: Immunosuppressants reduce venous thrombosis relapse in Behçet's disease. *Arthrtis Rheum* 2012; 64: 2753-60.
9. TEN CATE H: Thrombin generation in clinical conditions. *Thromb Res* 2012; 129: 367-70.
10. CASTOLDI E, ROSING J: Thrombin generation tests. *Thromb Res* 2011; 127 (Suppl. 3): S21-5.
11. INTERNATIONAL STUDY GROUP FOR BEHÇET'S DISEASE: Criteria for diagnosis of Behçet's disease. *Lancet* 1990; 335: 1078-80.
12. FORBESS C, SWEARINGEN C, YAZICI Y: Behçet's Syndrome Activity Score (BSAS): a new disease activity assessment tool, composed of patient-derived measures only, is strongly correlated with the Behçet's Disease Current Activity Form (BDCAF). *Arthritis Rheum* 2008; 58: S54.
13. TANS G, CURVERS J, MIDDELDORP S *et al*.: A randomized cross-over study on the effects of levonorgestrel- and desogestrel-containing oral contraceptives on the anticoagulant pathways. *Thromb Haemost* 2000; 84: 15-21.
14. PENGO V, TRIPODI A, REBER G *et al*.: Update of the guidelines for lupus anticoagulant detection. *J Thromb Haemost* 2009; 7: 1737-40.
15. BESSER M, BAGLIN C, LUDDINGTON R, VAN HYCKAMA VLIEG A, BAGLIN T: High rate of unprovoked recurrent venous thrombosis is associated with high thrombin-generating potential in a prospective cohort study. *J Thromb Haemost* 2008; 6: 1720-5.
16. HRON G, KOLLARS M, BINDER BR, ELCHINGER S, KYRLE PA: Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. *JAMA* 2006; 296; 397-402.
17. TRIPODI A, LEGNANI C, CHANTARANGKUL V, COSMI B, PALARETI G, MANUCCI PM: High thrombin generation measured in the presence of thrombomodulin is associated with an increased risk of recurrent venous thromboembolism. *J Thromb Haemost* 2008; 6: 1327-33.
18. TAYER-SHIFMAN OE, SEYAHI E, NOWATZKY J, BEN-CHETRIT E: Major vessel thrombosis in Behçet's disease: the dilemma of anticoagulant therapy - the approach of rheumatologists from different countries. *Clin Exp Rheumatol* 2012; 30: 735-40.
19. HILHORST M, WINCKERS K, WILDE B, VAN OERLE R, TEN CATE H, COHEN TERVAERT JW: Patients with antineutrophil cytoplasmic antibodies associated vasculitis in remission are hypercoagulable. *J Rheumatol* 2013: 40: 2042-6.
20. AKSU K, DONMEZ A, KESER G: Inflammation-induced thrombosis: mechanisms, disease associations and management. *Curr Pharm Des* 2012; 18: 1478-93.