Patients with Antineutrophil Cytoplasmic Antibodies Associated Vasculitis in Remission Are Hypercoagulable

Marc Hilhorst, Kristien Winckers, Benjamin Wilde, René van Oerle, Hugo ten Cate, and Jan Willem Cohen Tervaert

ABSTRACT. ***Objectives.*** The risk of venous thromboembolism (VTE) is increased in patients with antineutrophil cytoplasmic antibodies (ANCA) associated vasculitides (AAV) as compared to healthy subjects. The mechanisms underlying this increased occurrence of VTE are not completely understood. We hypothesize that AAV patients in remission are more procoagulant than healthy controls.

Methods. Patients with AAV in remission and no VTE for the last 6 months were included. Patients with severe renal impairment (serum creatinine > 250 μmol/l) were excluded. Age and sex matched healthy controls were included. The endogenous thrombin potential (ETP) was determined together with hemostatic variables: fibrinogen, D-dimers, factor VIII (FVIII), tissue factor pathway inhibitor (TFPI), protein C, and free protein S.

Results. Thirty-one patients were included. In 27 patients not taking anticoagulants, ETP was measured and found to be elevated: 137.1% as compared to a median of 90.0% for healthy controls ($p < 0.01$). Fibrinogen and D-dimer levels were not elevated in patients (median 3.5 g/l and 279 μg/l, respectively). FVIII and TFPI levels were also significantly increased in patients as compared to healthy controls (159% vs 137%; 122.5% vs 101%, respectively), whereas protein C and free protein S levels were not elevated (126.5% vs 118.6% and 124.6% vs 118.3%, respectively).

Conclusion. Patients with AAV in remission are more procoagulant than healthy controls, as indicated by an increased ETP. The increased FVIII level measured in these patients suggests persistence of endothelial activation and/or dysfunction. This endothelial dysfunction may cause a continuous low-grade procoagulant state. (First Release Oct 15 2013; J Rheumatol 2013;40:2042–6; doi:10.3899/jrheum.130200)

Key Indexing Terms:
ANCA ASSOCIATED VASCULITIS THROMBOSIS ENDOGENOUS THROMBIN POTENTIAL

Antineutrophil cytoplasmic antibodies (ANCA) associated vasculitides (AAV) belong to the small vessel vasculitides. ANCA in these vasculitides are directed to proteinase-3 (PR3) or myeloperoxidase (MPO), both myeloid enzymes primarily located in the primary granules. Treatment of AAV has improved over past decades, but comorbidities remain significant[1]. Previous studies demonstrated an increased incidence rate for venous thromboembolism (VTE) in patients with AAV compared to the general population[2,3,4,5,6] and not only during active AAV, but also when patients are in remission[4].

The mechanisms responsible for the increased incidence of VTE among patients with AAV are not completely understood. Several risk factors, such as genetic susceptibility and/or endothelial cell activation and/or damage, have been suggested[7]. Further, inflammation induces a procoagulant state[8]. Since AAV patients also have a higher incidence of VTE during periods when they are in remission, we hypothesize that these patients are hypercoagulable during remission.

MATERIALS AND METHODS

Patients. Consecutive patients who visited the outpatient vasculitis clinic between December 2005 and December 2007 and had a diagnosis of AAV were included in this study[9]. All patients had either proteinase-3 (PR3) ANCA or myeloperoxidase (MPO) ANCA at the time of diagnosis[10]. Inclusion criteria were quiescent disease in accordance to European Vasculitis Study Group/European League Against Rheumatism guidelines for definitions of disease activity[11]. Exclusion criteria were the occurrence of a VTE within 6 months prior to the study and/or severe renal impairment (defined as serum creatinine > 250 μmol/l). We included age and sex

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matched healthy controls as a control group. Healthy controls were volunteers from the hospital staff without a history of thrombotic events. The local ethics committee approved the study.

Methods. Fasting venous blood samples from patients and controls were drawn at inclusion. By 2-step centrifugation (2000 ×g for 15 min followed by 11,000 ×g for 10 min), platelet poor plasma (PPP) was prepared.

Baseline measurements. At the time of inclusion, additional data were obtained: high sensitivity C-reactive protein (hs-CRP), serum creatinine, leukocyte, and platelet count. Proteinuria was determined in urine samples. In addition, ANCA[10] and anticardiolipin antibodies[12] were measured. Further, patients were checked for cytomegalovirus (CMV) positivity by microparticle enzyme immunoassay (Abbott).

Endogenous thrombin generation potential. Thrombin generation in PPP was determined using the calibrated automated thrombogram method. Thrombin generation was triggered with 1 pM recombinant tissue factor and 4 μM phospholipid, as described[13]. In addition, ETP was measured in a separate assay with the presence of 1 nM thrombomodulin[14]. Three variables were derived from the corrected thrombin generation curves: lag time (minutes) defined as time between coagulation initiation and thrombin formation (1/6 of peak height level); endogenous thrombin potential (ETP, nM*min), represented by the area under the curve; and peak height (nM), the maximal height of the curve. All ETP values and peak values were normalized using normal pooled plasma and expressed in percentage of normal to minimize interassay variation.

Factor VIII (FVIII) activity. FVIII activity was measured using a 1-stage clotting assay according to the manufacturer's instructions (Dade Behring).

Tissue factor pathway inhibitor (TFPI), protein C, and protein S activities. Full length TFPI and total protein S were measured by ELISA, as described[15]. Protein C levels were measured in assays with chromogenic substrate performed on a Sysmex CA-7000 automated coagulation analyzer with reagents (Dade Behring). Free protein S was measured using an ELISA following the manufacturer's instructions (Asserachrom).

Statistics. Continuous variables were checked for normality using the Pearson test and presented as mean ± SD or by median and interquartile range (25th to 75th percentile, IQR), where appropriate, and categorical variables by percentage. Differences in continuous and categorical variables were checked using the independent samples t test or Mann-Whitney U-test and the chi-square or Fisher's exact test, respectively. Associations between 2 continuous variables were tested using linear regression. $p < 0.05$ was considered significant.

Table 1. Baseline patient characteristics (n = 31) of antineutrophil cytoplasmic antibodies (ANCA) associated vasculitis patients in remission at the time of endogenous thrombin generation (ETP) potential measurement.

Characteristic	n
Age, yrs, ± SD	51.4 ± 15.4
Male/female	16/15
Diagnosis	
GPA (%)	29 (93.5)
MPA (%)	1 (3.2)
EGPA (%)	1 (3.2)
ANCA subtype	
MPO-ANCA (%)	7 (22.6)
PR3-ANCA (%)	24 (77.4)
Disease duration, yrs (range)	6.6 (3.1–9.0)
Immunosuppressive medication†	
None (%)	8 (25.8)
Prednisone with azathioprine (%)	8 (25.8)
Azathioprine alone (%)	2 (6.4)
MMF (%)	2 (6.4)
Prednisone with MMF (%)	3 (9.7)
Prednisone with methotrexate (%)	5 (16.1)
Prednisone alone (%)	3 (9.7)
VTE before study (%)	5 (16.1)
Anticoagulant use (%)	4 (12.9)
C-reactive protein, mg/l, (range)	3.9 (2.7–7.6)
D-dimers, ng/ml (range)	291 (180–463)
BVAS	0
Serum creatinine, μmol/l (range)	84 (71–97)
Leukocyte counts, cells × 10^9/l (range)	6.3 (5.4–7.9)
Platelet counts, cells × 10^9/l (range)	274 (206–309)
IgG anti-CMV antibodies present (%)	18 (58.1)

† Median amount of prednisone use daily was 5 mg (range 2 mg to 17.5 mg). GPA: granulomatous polyangiitis; MPA: microscopic polyangiitis; EGPA: eosinophilic granulomatosis with polyangiitis; MPO: myeloperoxidase; PR3: proteinase-3; MMF: mycophenolate mofetil; VTE: venous thromboembolism; BVAS: Birmingham Vasculitis Activity Score; CMV: cytomegalovirus; HDL: high-density lipoprotein; LDL: low-density lipoprotein; Ig: immunoglobulin.

RESULTS

Thirty-one patients with AAV (mean age 51.4 ± 15.4 yrs, 51.6% male) and 36 age and sex matched healthy controls (mean age 54.8 ± 15.0 years, 50% male) were included in the study. Five patients (16.1%) had had a VTE in the past, of whom 3 were still taking vitamin K antagonists at study inclusion. One patient received vitamin K antagonists for an aortic valve replacement. Eight of the 27 (29.6%) patients did not receive any immunosuppressive medication at the time of inclusion in the study. The other 19 patients received different immunosuppressive medications (Table 1). Two patients included had a relapse 4 and 5 months, respectively, before ETP measurement. All other patients had not had active disease > 1 year before ETP measurement (Appendix 1).

Median D-dimer level was 291 (range 180–463) at inclusion. This was comparable to healthy controls (median 259, range 157–447; p = 0.28). All patients were negative for immunoglobulin (Ig)G and IgM anticardiolipin antibodies. Further, all patients tested negative for anti-β_2-GPI antibodies. Eighteen patients (58.1%) were positive for IgG anti-CMV antibodies.

Endogenous thrombin generation potential. ETP was measured in all 27 patients who did not use vitamin K antagonists. The overall ETP in these patients was significantly higher than in healthy controls (median 137.1%, IQR 119.0–160.3 vs 90.0%, IQR 69.1–102.4; $p < 0.0001$; Table 2; Figure 1). Median normalized peak values in these patients were found to be 193.5% (IQR 150.2–244.9) compared to 81.6% (IQR 63.8–103.5) in healthy controls ($p < 0.0001$; Table 2). Median lag time of thrombin generation was found to be 7.3 (IQR 6.3–7.7) minutes in patients compared to 7.7 (IQR 6.7–9.3) minutes in healthy controls (p = 0.035).

No difference in ETP between ANCA subtypes could be detected (p = 0.978 for both ETP and peak values, p = 0.06 for lag times; Table 2). ETP in the presence of thrombomodulin was increased in patients compared to healthy controls ($p < 0.0001$; Table 2).

CMV status did not influence ETP values (mean ETP

Table 2. Hematological characteristics of antineutrophil cytoplasmic antibodies (ANCA) associated vasculitis patients (n = 31) and healthy controls (n = 36). Subgroups of PR3-ANCA and MPO-ANCA were analyzed.

	PR3 (n = 24)	MPO (n = 7)	All (n = 31)	HC (n = 36)
ETP, % (range)*	138.1 (120.0–158.8)	129.8 (109.7–174.7)	137.1 (119.0–160.3)	90.1 (69.1–102.4)
Peak ETP, % (range)*	200.8 (148.7–248.5)	186.1 (152.9–244.9)	193.5 (150.2–244.9)	81.6 (63.8–103.5)
Lag time, min (range)*	7.5 (6.4–7.7)	6.7 (5.3–7.3)	7.3 (6.3–7.7)	7.7 (6.7–9.3)
FVIII, % (range)*	156.5 (148.5–222.5)	173.0 (141.0–181.0)	159.0 (148.0–210.0)	137.0 (110.3–158.3)
TFPI, % (range)*◇	130.5 (114.3–154.0)	107.5 (94.0–118.5)	122.5 (108.8–145.0)	101.0 (95.0–139.0)
Protein C, % (range)	131.0 (123.1–148.4)	122.0 (101.6–124.2)	126.5 (119.7–145.8)	118.6 (112.1–136.4)
Free protein S, % (range)	130.8 (118.3–151.1)	121.5 (105.2–121.5)	124.6 (118.3–140.0)	118.3 (108.5–130.8)
Total protein S, % (range)*	112.6 (104.1–132.3)	105.6 (82.7–127.4)	111.3 (102.7–130.3)	99.8 (88.2–117.0)
Protein S ratio	1.1 (0.9–1.2)	1.1 (1.0–1.3)	1.1 (0.9–1.2)	1.2 (1.0–1.2)
ETP reduction with TM, % (range)*	25.5 (13.0–36.4)	18.6 (14.6–32.4)	25.2 (13.9–31.9)	51.1 (44.9–60.3)

* $p < 0.05$ between all patients and healthy controls (HC). ◇ $p < 0.05$ between PR3-ANCA and MPO-ANCA patients. ETP: endogenous thrombin potential; FVIII: factor VIII; TFPI: tissue factor pathway inhibitor; PR3: proteinase-3 ANCA; MPO: myeloperoxidase-ANCA; TM: thrombomodulin. Data are presented as medians (IQR, 25th to 75th quartile range). In the case of ETP, peak ETP, lag time, and ETP in the presence of TM, only 27 patients could be analyzed statistically due to anticoagulant use in the other 4 patients.

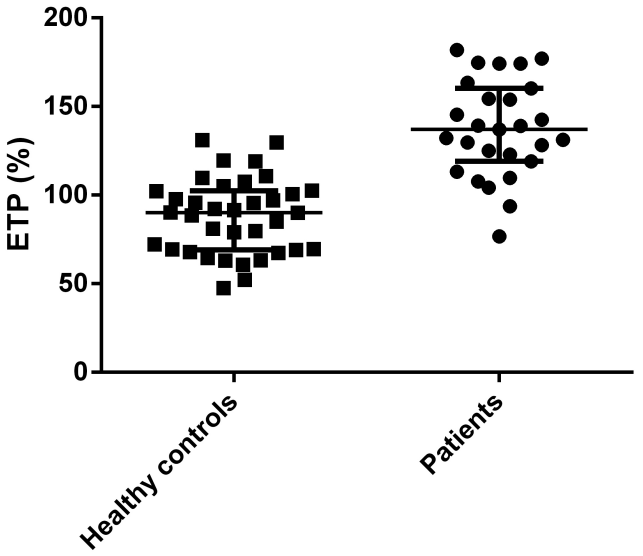

Figure 1. Endogenous thrombin potential (ETP) characteristics of ANCA associated vasculitis patients in remission (n = 27) and age and sex matched healthy controls (n = 36). ETP could not be measured in 4 patients due to anticoagulant use; median ETP with interquartile ranges (IQR) from patients and healthy controls. ETP was higher in patients ($p < 0.0001$).

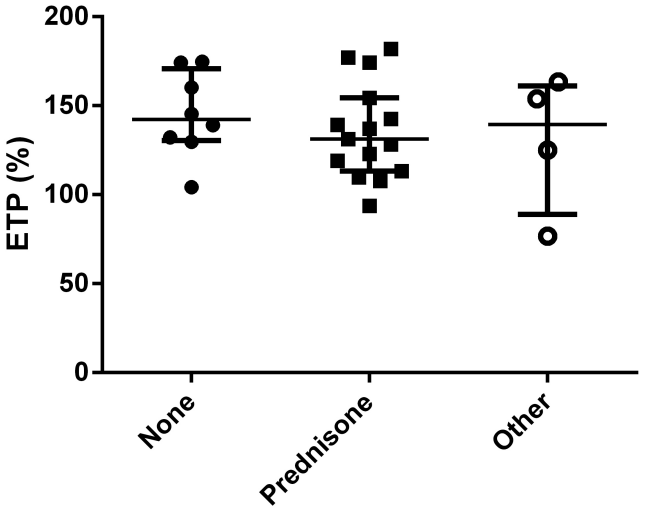

Figure 2. Endogenous thrombin potential (ETP) characteristics of ANCA associated vasculitis patients (n = 27) divided by the medication used. There was no difference in ETP between different medication groups and/or no immunosuppressive use ($p = 0.344$). Patients in the 'none' group (n = 8) used no immunosuppressive medication. Patients in the 'prednisone' group used prednisone alone (n = 3), in combination with azathioprine (n = 7), with mycophenolate mofetil (n = 2) or with methotrexate (n = 3). Patients in the 'other' group used mycophenolate mofetil (n = 2) or azathioprine (n = 2).

138.7% in patients who were CMV negative compared to a mean ETP of 137.3% in patients who were CMV positive; $p = 0.314$). There was no difference in ETP between groups of patients who received corticosteroids versus immunosuppressive medication versus none, (median ETP 142.5%, IQR 139.3–174.2 vs 126.7%, IQR 110.6–154.3, vs 142.2%, IQR 130.4–170.7, respectively; $p = 0.344$; Figure 2).

FVIII activity. Median FVIII activity in patients was 159.0% (IQR 148–210) and was significantly higher than in healthy controls (median 136.9%, IQR 110–158; $p < 0.0001$; Table 2). FVIII was found to positively correlate with ETP upon linear regression analysis in patients ($R^2 = 0.15$; $p = 0.044$) and in healthy controls ($R^2 = 0.33$; $p < 0.0001$).

TFPI, protein C, and protein S activities. TFPI (median 122.5% with IQR 108.8–145.0) was significantly higher in patients compared to healthy controls (median 101.0% with IQR 95.0–139.0; $p = 0.025$; Table 2).

Median protein C activity was 126.5% (IQR 119.7–145.8), similar to healthy controls (median 118.6%, IQR 112.1–136.4; $p = 0.189$; Table 2).

Total protein S activity was found to be higher in patients

(median 111.3%, IQR 102.7–130.3) than in healthy controls (median 99.8%, IQR 88.2–117.0; p = 0.037). In contrast, free protein S was not higher in patients (median 124.6%, IQR 118.3–140.0) compared to healthy controls (median 118.3%, IQR 108.5–130.8; p = 0.116). Protein S ratios, as calculated by dividing free protein S by total protein S, were 1.1 (IQR 0.9–1.2) in patients and 1.18 (IQR 0.9–1.2) in healthy controls (p = 0.170; Table 2).

Followup of patients. Patients were followed for a median of 5 years after ETP measurement. Two patients had a relapse 4 months after ETP measurement whereas all other patients remained in remission ≥ 1 year afterwards. During a total of 157 patient-years, 4 patients developed a venous thromboembolic event (VTE rate 2.5/100 person-years). One patient developed a VTE during active vasculitis, 1 patient after a total hip replacement, and 2 patients had an idiopathic VTE.

DISCUSSION

The main finding in our study is that patients with AAV in remission have a high thrombin generation potential compared to age and sex matched healthy controls. Thrombin generation is an elegant *in vitro* method to assess potential thrombogenicity in PPP, and a high ETP *in vitro* strongly suggests a hypercoagulable state *in vivo*[16]. We observed high ETP values both in patients with PR3-AAV and in patients with MPO-AAV. The reduction of ETP in the presence of thrombomodulin was significantly less in patients than in healthy controls. This supports the notion of hypercoagulability in patients where the activated protein C pathway is insufficiently able to suppress thrombin generation[17]. In addition, higher levels of FVIII were detected in patients when compared to healthy controls, suggesting persistent endothelial dysfunction in AAV patients with quiescent disease.

In line with our findings, Hergesell, *et al* found elevated von Willebrand factor (vWF) activity in patients with inactive AAV[18]. Moreover, we found that FVIII activity correlated positively with ETP values. The increased FVIII and vWF activities most probably point towards endothelial cell activation, dysfunction, and/or damage[19].

Recently, we found that free protein S, one of the natural coagulation inhibitors, was decreased in SLE patients compared to healthy controls[20], possibly contributing to a higher thrombogenicity in SLE. However, our study showed that free protein S levels were not decreased. Other anticoagulant proteins analyzed in our study, such as protein C and TFPI levels, were comparable, and respectively higher in patients compared to healthy controls. This suggests that the anticoagulant effects of TFPI and protein C and protein S in patients with AAV in remission are intact.

During active disease states of AAV, inflammation and other additional factors may contribute to hypercoagulability. Antiplasminogen antibodies have been found in active AAV and may contribute to the higher incidence of VTE in AAV patients during active disease[21,22].

We found that 4 patients developed a VTE after ETP measurement. One of these patients developed a VTE during active disease. The VTE rate during followup was 2.5/100 person-years. This rate is much higher than in the general population[6].

Most patients in our study (93.5%) had a diagnosis of granulomatous polyangiitis (GPA). We included only 1 patient with MPA and 1 patient with eosinophilic GPA. Therefore, not much can be concluded regarding these latter 2 diseases.

Our study shows that in patients with AAV there is evidence of hypercoagulability events even when patients are in remission, which may explain the protracted risk of VTE during apparently quiescent periods of disease. It may be that different factors are at play during active and inactive disease states. Future studies should further elucidate the complex interactions between the immune system and the coagulation system in AAV, which may facilitate better prophylactic antithrombotic treatment in those at highest risk of VTE.

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APPENDIX 1. Additional clinical characteristics of the patients with antineutrophil cytoplasmic antibodies (ANCA) associated vasculitis included (n = 31). Data of diagnosis, as well as disease relapses before and after endogenous thrombin potential (ETP), are reported.

Date of Diagnosis	Sample Collection	Prednisone, mg/day	Other Immunosuppression, mg/day	Last Period of Active Disease before ETP	Relapse after ETP	ETP, %	Thrombosis after ETP	Comments
1-5-2003	10-7-2007	No	AZA 125	25-10-2005		163.4		
1-8-2004	23-5-2006	No	AZA 150	1-6-2005		153.9		
1-8-1999	28-11-2007	No	No	5-2-2005	1-2-2012	129.8		DVT before ETP**
1-4-2001	19-11-2007	No	No	1-4-2001		104.2		
1-5-2005	7-8-2007	No	No	1-5-2005	16-6-2009	160.3		
1-5-2005	27-11-2007	No	No	1-5-2005		132.3		MI 1-8-2012
1-6-1996	4-9-2007	No	No	9-8-2004	9-9-2011	139.0		
1-6-1994	7-3-2006	No	No	1-6-1994		174.7	1-6-2011 DVT	DVT before ETP**
1-7-2004	26-6-2007	No	No	1-7-2004		174.2		
1-1-2000	10-7-2007	No	No	1-1-2000		145.4		
1-6-2001	30-10-2007	No	MMF 1000	9-8-2006		76.8		
1-8-2006	31-7-2007	No	MMF 1000	1-8-2006		125.0		
1-1-2004	22-1-2008	10	MMF 2000	1-8-2006	1-1-2011	137.1		
1-6-1991	4-9-2007	10	MTX 15/week	1-4-2007		131.3		
1-11-2000	26-6-2007	12.5	MMF 1000	7-2-2006	26-4-2011	119.0		
1-8-2002	26-6-2007	17.5	No	1-2-2007	1-1-2010	139.3		
1-1-2006	3-8-2006	2.5	AZA 100	1-1-2006	1-12-2006	122.9	20-12-2006 DVT	
1-5-2000	19-6-2007	2.5	AZA 25	1-5-2000	26-9-2009	113.2		
1-12-2004	19-6-2007	2.5	AZA 75	25-8-2005		107.8		
1-2-2000	24-10-2006	2	MTX 10/week	1-2-2000		154.4		
1-7-2002	19-6-2007	3	No	11-1-2005		174.2		
1-1-1999	19-6-2007	5	No	3-3-2003	27-8-2010	142.5		
1-6-2002	24-7-2007	5	AZA 100	1-3-2006	1-8-2012	128.3		
1-5-2006	13-11-2007	5	AZA 100	1-5-2006		177.1	14-4-2009 DVT	
1-6-1997	11-12-2007	5	AZA 150	1-10-2001		181.9		DVT before ETP**
1-6-1995	10-7-2007	5	AZA 75	30-8-2005	6-4-2012	93.7		
1-6-1999	6-11-2007	5	MTX 15/week	1-12-2003		109.7	1-2-2010 PE	
1-9-2005	19-6-2007	2.5	MMF 500	1-9-2005	30-10-2007	*		Aortic valve
1-12-2003	16-4-2007	3	AZA 75	1-12-2003		*		TIA
1-1-1998	19-6-2007	5	MTX 15/week	1-1-1998		*		DVT before ETP**
1-6-1997	23-10-2007	7	MTX 15/week	1-1-1999		*		DVT before ETP**

* These patients used vitamin K antagonists during ETP measurement, therefore ETP measurement was not reliable. ** Patients included who had had a DVT in the past, had always had this DVT > 6 months before ETP measurement. ETP: endogenous thrombin potential; DVT: deep venous thrombosis; PE: pulmonary embolism; MI: myocardial infarction; TIA: transient ischemic attack.

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