Elevated Microparticle Tissue Factor Activity Differentiates Patients With Venous Thromboembolism in Anti-neutrophil Cytoplasmic Autoantibody Vasculitis

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Introduction: Venous thromboembolism (VTE) is a life-threatening complication of anti-neutrophil cytoplasmic autoantibody (ANCA) vasculitis whose mechanism remains incompletely elucidated. We tested the hypothesis that elevated microparticle tissue factor activity (MPTFa) or anti-plasminogen antibodies (anti-Plg) may identify patients at risk for VTE.

Methods: In this prospective study, patients were enrolled during active disease and followed longitudinally. Twelve patients who experienced a VTE (VTE[pos]) were compared with patients without VTE (VTE[neg], $n = 29$) and healthy controls (HC, $n = 70$). MPTFa, anti-Plg, interleukin-6, high-sensitivity C-reactive protein (hs-CRP), D-dimer, serum creatinine, and serum albumin were assessed. Fisher's exact tests and Wilcoxon tests compared categorical and continuous variables, respectively. Cox regression for time to VTE or last follow-up was performed.

Results: VTE[pos] patients had higher MPTFa (peak median = 14.0, interquartile range = 4.3–36.6) than HC (0, 0–3.5) and VTE[neg] patients (0, 0–1.4). In time-to-event analysis, MPTFa was associated with VTE when measured during both active disease (hazard ratio [HR]; 95% confidence interval [CI]: 1.04; 1.01–1.08) and remission (1.4; 1.11–1.77). Anti-Plg during remission was also associated with VTE (1.17; 1.03–1.33). Each g/dl decrease of serum albumin was associated with a 4-fold increase in VTE risk (4.4; 1.5–12.9). Adjusting for estimated glomerular filtration rate (eGFR), anti-Plg during remission remained significantly associated with VTE.

Conclusion: Elevated MPTFa and increased anti-Plg in remission are strong indicators of VTE independent of renal function. Association of anti-Plg during remission with VTE implies hypercoagulability even during disease quiescence. Hypoalbuminemia strongly portends VTE risk, which is a novel finding in ANCA vasculitis. A thrombotic signature would allow improved management of patients to minimize VTE risk and complications of anticoagulation.

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V TE is a complication of ANCA vasculitis that affects 8% to 16% of patients and confers significant morbidity and risk of mortality.[1–4] Mechanisms driving the high incidence of VTE in ANCA vasculitis are not fully understood. Moreover, current biomarkers of VTE lack specificity.

Anti-Plg antibodies potentially contribute to VTE by delaying conversion of plasminogen to plasmin, thus prolonging the dissolution time of fibrin clots and inhibiting fibrinolysis.[5] Bautz and colleagues[5] found that half of proteinase 3 (PR3)–ANCA patients who developed VTE were positive for anti-Plg. However, an association between anti-Plg and VTE in patients with

ANCA vasculitis has not been investigated prospectively.

Microparticles (MPs) and tissue factor (TF)–bearing microparticles have been implicated as risk factors for VTE in multiple clinical settings.[6–13] MPs are increased in patients with ANCA vasculitis during active disease.[14–17] Neutrophils treated with ANCA after priming with C5a or tumor necrosis factor-α release TF-bearing MPs that increase thrombin generation *in vitro*.[18,19] However, the contribution of monocyte-derived TF and association of MPTFa with VTE in patients with ANCA vasculitis has not been explored.

Because VTEs tend to occur during active disease,[1] we followed patients with active disease longitudinally, and prospectively measured anti-Plg, D-dimer, and C-reactive protein (CRP). Due to a putative role in thrombosis, MPTFa and interleukin (IL)-6[20] also were measured. We aimed to determine the role these markers of disease activity and inflammation play in development of VTE in ANCA vasculitis and to elucidate a thrombotic signature that may identify patients at increased risk for VTE.

METHODS

Cohort

A prospective longitudinal study was designed to determine if anti-Plg was associated with VTE in ANCA vasculitis. We aimed to enroll 60 patients at high risk for VTE based on historical estimates of willing participants with active disease, anti-Plg positivity, and elevated D-dimer. Fifty-eight patients with active disease (Birmingham Vasculitis Activity Score [BVAS] $\geq$2) were screened for D-dimer and anti-Plg from 2010 to 2015 at the University of North Carolina at Chapel Hill (UNC) (Figure 1a). All patients had ANCA vasculitis as defined by Chapel Hill Consensus Conference nomenclature[21] and a positive enzyme-linked immunosorbent assay (ELISA) for myeloperoxidase (MPO)- or PR3-ANCA at or before study screening. Patients with overlap disease, drug-induced ANCA vasculitis, eosinophilic granulomatosis with polyangiitis, or on dialysis were excluded ($n = 22$). All study patients ($n = 36$) were followed longitudinally by serial testing of anti-Plg, D-dimer, and hs-CRP with a primary outcome of VTE. Patients positive for anti-Plg or elevated D-dimer with a Well's score $\geq$2[22] underwent lower extremity compression ultrasonography. Five additional patients diagnosed with VTE during the study period were identified through our Clinical Core and included retrospectively because appropriate samples were available due to the longitudinal nature of our Clinical Core biorepository; samples were obtained when patients were seen in the clinic every 3 to 6 months

(Figure 1a). Patients who experienced VTE were defined as VTE^{pos}; patients who did not were designated VTE^{neg}. The sample date associated with the highest BVAS surrounding the time of initial screening (6 months before or after) was used as the active disease time-point for all analytes. The first BVAS = 0 without active disease within 3 months was used as the remission sample time-point. In addition, stored patient samples were tested for IL-6 and MPTFa as potentially relevant biomarkers of thrombosis. HCs were tested for anti-Plg, IL-6, and MPTFa; not all controls were tested for each analyte. The study was approved by the UNC Institutional Review Board. Informed consent was obtained from all patients and HCs.

Clinical Information and Research Tests

Clinical characteristics were assessed during active disease. Medications and organ involvement were obtained from electronic medical records. Standard-of-care clinical laboratory tests for serum albumin, serum creatinine, platelet number, hemoglobin, proteinuria, and ANCA were performed by UNC Hospitals McLendon Laboratories. eGFR was calculated using the 4-variable Modification of Diet in Renal Disease equation.[23,24] ANCA titers >20 U/ml (INOVA [San Diego, CA] QUANTA Lite MPO and PR3) were considered positive. D-dimer and hs-CRP were performed by UNC Hospital Laboratories. Positive D-dimer was defined as >229 ng/ml. The lower limit of detection for D-dimer is 150 ng/ml, and for continuous analyses we defined these values as 149 ng/ml. Hs-CRP values >10 mg/l indicate active inflammation. IL-6 levels were determined using stored serum and plasma samples following manufacturer's protocol in a commercial IL-6 ELISA (R&D Systems, Minneapolis, MN). For IL-6, values >1.57 pg/ml were considered positive based on the mean plus 2 SDs of 20 HCs.

Anti-Plg were detected by ELISA using the native glutamic acid isomer of plasminogen (Haematologic Technologies, Essex Junction, VT) as described previously[5] with some modifications. To decrease nonspecific background of the assay, glutamic acid isomer of plasminogen was depleted of contaminating IgG with Protein AG beads (Santa Cruz Biotechnology, Inc., Dallas, TX) before use as the coating protein of the ELISA. Ninety-six well white high-binding ELISA microplates (Greiner Bio-One, Monroe, NC) were coated with glutamic acid isomer of plasminogen at 5 μg/ml in 0.05M carbonate-bicarbonate coating buffer overnight at 4 °C. The next day, the plates were washed with Tris Buffered Saline with 0.1% Tween-20 and blocked with 1% goat serum in Tris Buffered Saline, 0.05% Tween-20, and 0.05% sodium azide (blocking buffer) for 1 hour. The plates were incubated with patient, HC, and
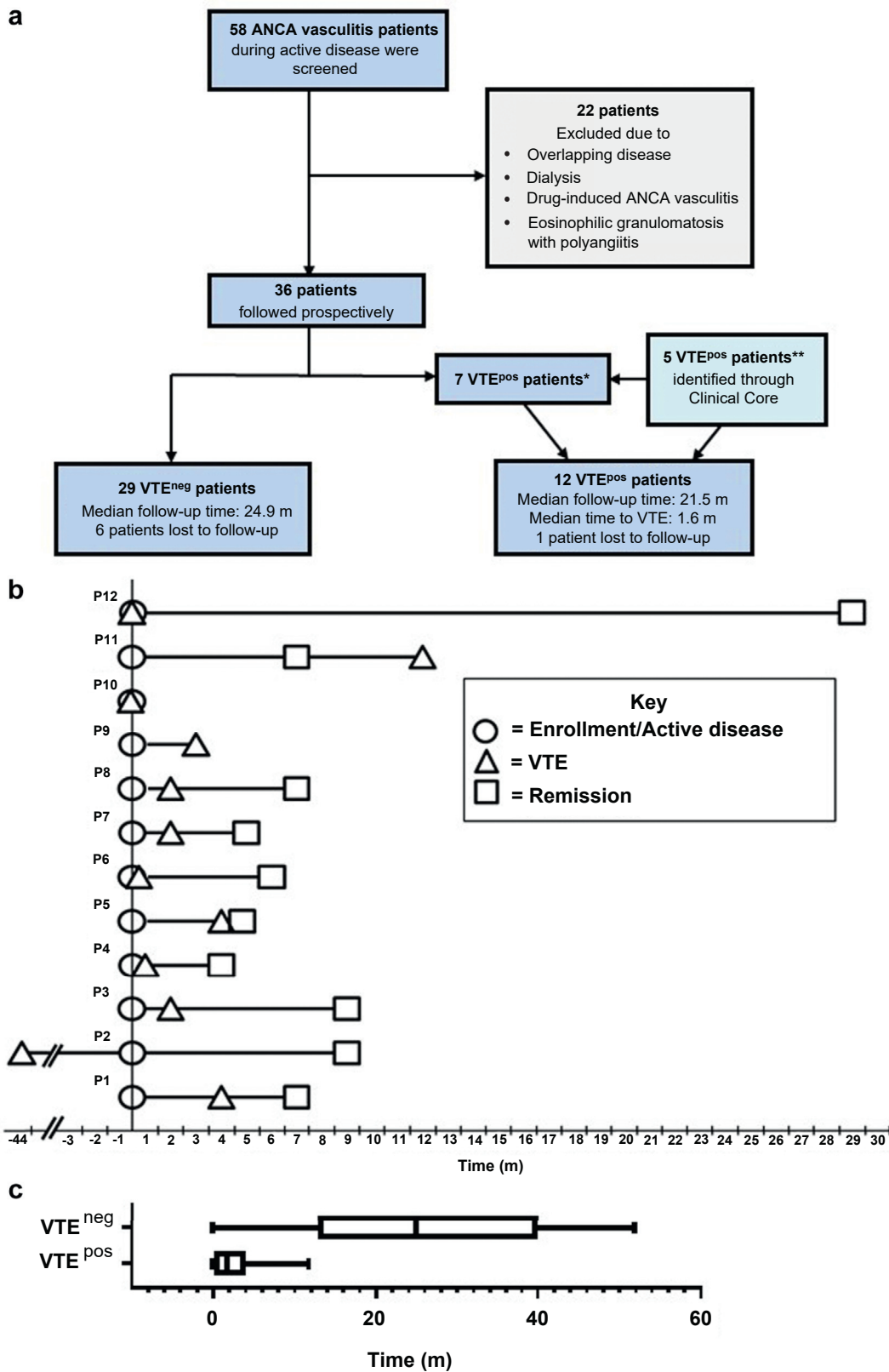
Figure 1. Patient population and timeline of disease activity and venous thromboembolism (VTE). (a) Flow diagram depicting patient screening, enrollment, and follow-up. VTE[neg] patients did not experience a VTE during the study, VTE[pos] patients did experience a VTE during the study. *One VTE[pos] patient had a remote VTE but was included in the VTE[pos] group except for time-to-event analyses. **These patients were not screened for enrollment but appropriate samples were available due to the longitudinal nature of our Clinical Core. (b) Diagram depicting timeline of active disease, VTE, and remission dates for VTE[pos] patients. Time 0 is the enrollment or active disease date. ○ is the enrollment or active disease sample, Δ is the date of the VTE, and □ is the remission sample. Patients 8 through 12 were added retrospectively. (c) Box and whiskers plot showing the follow-up time for VTE[neg] patients (median 24.9 month [m]) and time to VTE for the VTE[pos] patients (1.6 m). ANCA, anti-neutrophil cytoplasmic autoantibody; avg, average; neg, negative; pos, positive.

2 historical positive control sera diluted 1:800 in the blocking buffer in duplicate for 3 hours at room temperature or overnight at 4 °C. The plates were then incubated with an alkaline phosphatase conjugated goat anti-human IgG (Invitrogen, Carlsbad, CA) diluted 1:20,000 in blocking buffer. We used a Chemiluminescence detection (Sapphire-II Enhancer; Thermo Fisher Scientific, Waltham, MA; CDP-Star, Roche Applied Science, Indianapolis, IN) method to improve the signal-to-noise ratio and sensitivity of the ELISA. Based on the mean plus 2 SDs of 56 HC samples, the cutoff for positivity was established as 21.7% of the positive control (average of 2 historically positive samples included on each assay).

Microparticle TF Activity Assay

Citrated whole blood from patients and HCs was centrifuged within 1 hour after collection ($2500g$, 15 minutes, twice) to yield platelet-free plasma, then frozen at −80 °C until use (as recommended by the Scientific Collaborative Workshop of the International Society of Thrombosis and Haemostasis).[25] MPs were isolated from platelet-free plasma by centrifugation ($20,000g$, 30 minutes, 4 °C). MPTFa was determined as described previously.[26–28] In this kinetic assay, TF-dependent Factor Xa generation was determined by subtracting Factor Xa generation in the presence of anti-human TF from Factor Xa generation in the presence of control mouse IgG. TF concentration was determined by comparison with relipidated recombinant human TF (Innovin, Dade Behring; Siemens, Newark, DE). Unstimulated and LPS-stimulated platelet-free plasma from a healthy donor were included on each plate as negative and positive controls; values were expressed as a percentage of this positive control.

Flow Cytometry

To determine baseline TF on leukocytes, whole blood from 10 HCs, 6 VTE^{neg}, and 6 VTE^{pos} patients was stained with CD14-APC (clone 61D3), CD16-FITC (clone eBio6B16), and CD142-PE (clone HTF-1) antibodies (eBioscience, San Diego, CA). CD14 only and CD16 only samples were used to set proper positive/negative gates. Cells stained with all antibodies except CD142, a fluorescence minus 1 control, was used to set the negative gate and determine the threshold for TF positivity. Cells were examined by flow cytometry on a Becton Dickinson (Franklin Lakes, NJ) LSRII flow cytometer and data analyzed using FlowJo 10 (Tree Star, Ashland, OR). The percent TF-positive CD14^{+} monocytes, CD14^{+}CD16^{+} inflammatory monocytes, and CD16^{+} neutrophils were determined.

Statistical Analysis

Continuous variables were expressed as medians with interquartile range; categorical variables were expressed as counts (n) and percentage (%). Wilcoxon 2-sample tests or Kruskal-Wallis tests for continuous variables were compared across 2 or 3 groups, respectively. Fisher exact tests were used to compare groups for categorical measures. A signed-rank test was used for paired measures at active disease and remission within patients. Time-to-event analysis was performed by Cox regression to discern an HR of VTE associated with biological markers. Limited multivariable modeling of several statistically significant measures was done to evaluate independent associations of biomarkers, with only 2 variables in any given model at one time due to small sample size. Pearson correlation analysis measured the relationship of anti-Plg remission values from different time-points. Statistical analyses were conducted by SAS software (Version 9.4; SAS Institute, Cary, NC) and GraphPad Prism (Version 7.04; GraphPad Software, Inc., La Jolla, CA).

RESULTS

Baseline Characteristics

Forty-one patients with ANCA vasculitis (29 VTE^{neg} and 12 VTE^{pos}) and 70 HCs were included (Figure 1a). Eleven VTE^{pos} patients experienced a VTE during the study period. One patient in the study did not experience a VTE during the study period but is classified as VTE^{pos} due to a remote VTE (Figure 1b). Patients and controls did not differ in sex or race. HCs were younger (median = 36 years, interquartile range = 25–56) than patients (56, 45–70; $P = 0.0001$; Supplementary Table S1). However, the only analyte measured in this study that correlated with age was IL-6 (Supplementary Figure S1).

VTE^{pos} and VTE^{neg} patients were similar in age, sex, race, and distribution of MPO- and PR3-ANCA serotypes (Table 1). Comparable percentages of VTE^{pos} and VTE^{neg} patients were captured at new-onset disease and relapse. At presentation, skin involvement was more common among VTE^{pos} patients (9 of 12 = 75%) compared with VTE^{neg} patients (7 of 29 = 24%, $P = 0.004$). Patient groups did not differ in other organ involvement, eGFR, proteinuria, platelet count, or hemoglobin concentration. Serum albumin was lower in VTE^{pos} patients (2.9 g/dl, 2.6–3.5) than VTE^{neg} patients (3.8, 3.5–4; $P = 0.04$). Median time of longitudinal follow-up was similar between the 2 groups. Of the 7 patients lost to follow-up, 1 (8%) was a VTE^{pos} patient and 6 (21%) were VTE^{neg} patients (Figure 1a). More than 90% of the VTE^{neg} patients had longer

Table 1. Patient characteristics at active disease

Variable[a]	VTE^{neg} n = 29	VTE^{pos} n = 12	P value[b]
Age, yr, median (interquartile range)	56 (45, 69)	62 (51, 77)	0.28
Sex, male, n (%)	11 (38)	8 (67)	0.17
Race, W, n (%)	25 (86)	10 (83)	1.00
MPO-ANCA serotype, n (%)	11 (38)	4 (33)	1.00
New-onset active sample, n (%)	11 (38)	4 (33)	1.00
Organ involvement, n (%)	–	–	–
ENT	22 (76)	9 (75)	1.00
Eye	12 (41)	3 (25)	0.48
Pulmonary	20 (69)	9 (75)	1.00
Renal	26 (90)	12 (100)	0.54
Pulmonary and renal	17 (59)	9 (75)	0.48
Skin	7 (24)	9 (75)	0.004
Neurology	10 (35)	2 (17)	0.45
Musculoskeletal	17 (59)	7 (58)	1.00
Joints	17 (59)	7 (58)	0.73
Gastrointestinal	3 (10)	0 (0)	0.54
Serum albumin (g/dl)	3.8 (3.5, 4) [n = 16]	2.9 (2.6, 3.5) [n = 8]	0.04
UPCR (mg/mg)	0.5 (0.1, 1.1) [n = 19]	1 (0.5, 2.8) [n = 7]	0.27
Serum creatinine (mg/dl)	1.3 (0.9, 2.3) [n = 26]	1.8 (1.4, 2.7)	0.20
Estimated GFR (ml/min per 1.73 m^2)	52 (27, 61)	35 (24.5, 47)	0.21
Platelet count (10^9/l)	319 (239, 472) [n = 26]	291 (237, 379)	0.48
Hemoglobin (g/dl)	11.8 (10.5, 13.6) [n = 26]	11.3 (9.7, 12.6)	0.36

ANCA, anti-neutrophil cytoplasmic autoantibodies; ENT, ear, nose and throat; GFR, glomerular filtration rate calculated by Modification of Diet in Renal Disease equation; MPO, myeloperoxidase; UPCR, urine protein to creatinine ratio (mg); VTE, venous thromboembolism; W, white.
[a]Data are presented as median (interquartile range) for continuous variables and n (%) for categorical data. Variables with missing values are noted as [different n].
[b]P values were calculated using a Wilcoxon 2-sample test or Fisher exact test.

longitudinal follow-up time than the median time to VTE (1.6 months; Figure 1c).

The use of anticoagulants (aspirin, unfractionated heparin, warfarin, and low molecular weight heparin) in VTE^{pos} patients before the thrombotic event did not differ from VTE^{neg} patients (Supplementary Table S2). Immunosuppressive therapy was similar in VTE^{pos} and VTE^{neg} patients (Supplementary Table S3). Therapeutic plasma exchange was more prevalent in VTE^{pos} (50%) than VTE^{neg} patients (10%; Supplementary Table S3, $P = 0.01$); however, disease severity did not vary with VTE status based on BVAS, eGFR, or pulmonary and/or renal involvement (Table 1).

Putative Biomarkers of VTE Correlate With Disease Activity

Putative markers of VTE were first examined during active ANCA vasculitis when VTEs most often occur and compared with a time of disease quiescence. Patients had higher anti-Plg levels (% positive control) in active disease (17.8% positive control, 10.1–22.8) than during remission (10.0, 7.01–13.8; $P =$

0.0003; Table 2) and compared with HCs (12.4, 10.4–16.4; $P = 0.04$; Figure 2a). This difference was emphasized in paired active and remission samples ($P < 0.0001$; Figure 2b). The association of anti-Plg with disease activity was observed in MPO- ($P = 0.006$) and PR3-ANCA patients ($P = 0.0006$; Supplementary Figure S2).

Patients with active disease had elevated D-dimer levels (493 ng/ml, 183–1116) compared with those in remission (149, 149–170; $P = 0.0004$; Figure 2c); this was confirmed with paired active and remission samples ($P = 0.03$; Figure 2d). Comparing unpaired samples from patients experiencing active disease (8.6 mg/l, 3.5–16) and patients in remission, no difference in hs-CRP (6.4, 4–8.2; $P = 0.36$; Table 2; Figure 2e) was detected. However, considering paired samples, a decrease in hs-CRP ($P = 0.03$; Figure 2f) was observed.

In addition, IL-6 and MPTFa were tested on patient samples due to their contemporary relevance to thrombosis. IL-6 was elevated in both active disease and remission compared with HCs ($P < 0.0001$; Figure 2g). In paired samples, a marked decrease in IL-6 ($P = 0.004$; Figure 2h) from active disease to remission was detected. No difference in MPTFa values (Figure 2i) was observed between active patients and HCs or patients in remission. In available paired active and remission samples, no difference in MPTFa levels was discernable ($P = 0.46$; Figure 2j).

Biomarker Comparison in Patients With and Without VTE at Active Disease

We then investigated whether elevated levels of biomarkers during active disease differed in patients with and without VTE. We detected no differences in anti-Plg, D-dimer, IL-6, or MPTFa between VTE^{pos} and VTE^{neg} patients during active disease (Supplementary Figure S3A, B, D, and E, respectively). Interestingly, hs-CRP was increased in VTE^{pos} (16 mg/ml, 12–16;

Table 2. Comparison of biomarkers according to disease activity

Variable	Disease activity Active n = 41	Remission n = 30	P value
BVAS	9 (6, 13)	0 (0, 0)	<0.0001
Anti-Plg (%Pos Co)	17.8 (10.1, 22.8)	10 (7, 13.8)	0.0003
D-dimer (ng/ml)	493 (183, 1116) [n = 29]	149 (149, 170) [n = 16]	0.0004
hs-CRP (mg/l)	8.6 (3.5, 16) [n = 24]	6.4 (4, 8.2) [n = 13]	0.36
IL-6 (pg/ml)	2.5 (1.6, 6.5) [n = 28]	1.99 (1.33, 3.03) [n = 22]	0.27
MPTFa (%Pos Co)	0 (0, 1.8) [n = 23]	0 (0, 1.7) [n = 19]	0.82

%Pos Co, % positive control; anti-Plg, anti-plasminogen; BVAS, Birmingham Vasculitis Activity Score; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; MPTFa, microparticle tissue factor activity.
Data are presented as median (interquartile range). P values were calculated using a Wilcoxon 2-sample test. Variables with missing values are noted as [different n].
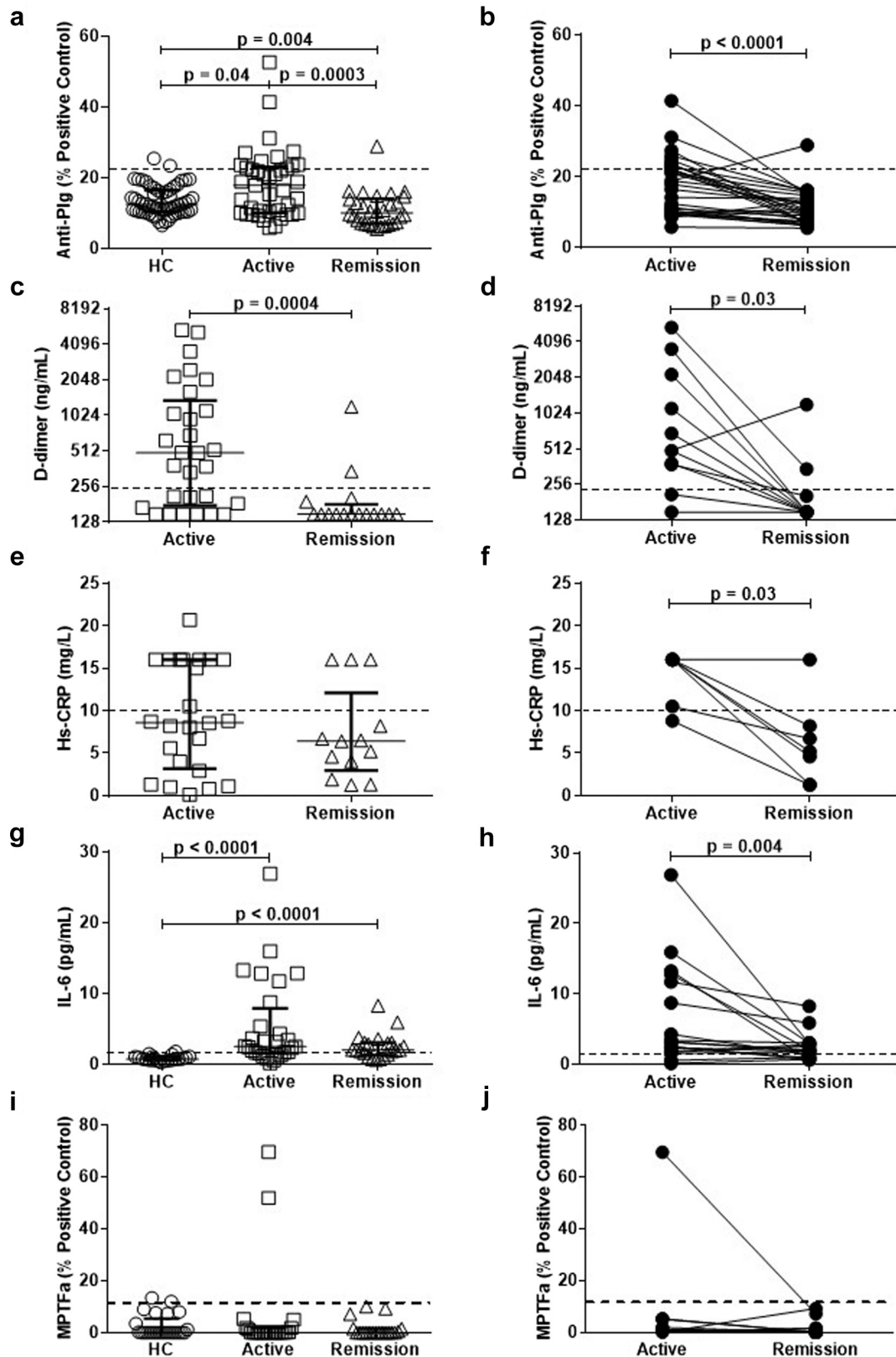
Figure 2. Putative biomarkers of venous thromboembolism correlate with disease activity. The dashed line represents the threshold for positivity in (a–h), as determined by the mean of healthy control values +2 SDs or values used for reporting clinical laboratory results. (a) Anti-plasminogen (Anti-Plg) were tested in 56 healthy controls (HCs), 41 patients were tested in active disease, and 30 patients were tested in remission. Patients in active disease had higher levels of anti-Plg than HCs and patients in remission. (b) Thirty paired active and remission samples emphasize the decrease in anti-Plg from active disease to remission. (c) Patients in active disease ($n = 29$) have significantly higher levels of D-dimer than patients in remission ($n = 16$). (d) Paired active and remission samples confirm that patients in remission have decreased levels of D-dimer ($n = 11$). (e) High-sensitivity C-reactive protein (hs-CRP) in a group of 24 patients did not differ from 13 patients in remission ($P = 0.36$). (f) In 6 of 7 patients (continued)
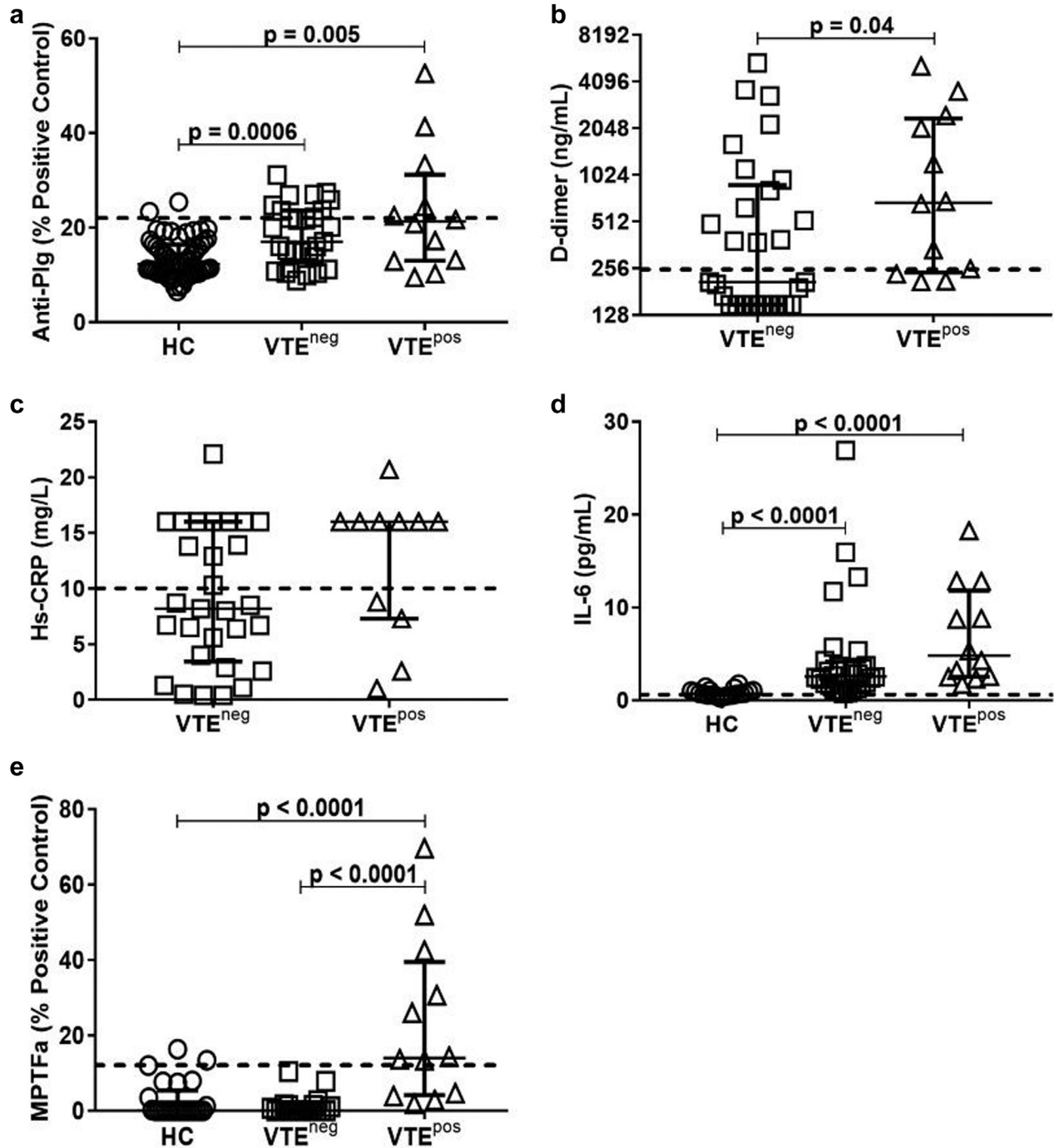
Figure 3. Comparison of candidate biomarkers in patients with and without venous thromboembolism (VTE). (a) Comparing peak values, VTE[pos] and VTE[neg] patients have significantly higher levels of anti-plasminogen (anti-Plg) than healthy controls (HCs). (b) VTE[pos] patients ($n = 12$) had significantly higher peak levels of D-dimer than VTE[neg] patients ($n = 29$). (c) There was no difference in peak high-sensitivity C-reactive protein (hs-CRP) in VTE[pos] ($n = 11$) and VTE[neg] patients ($n = 29$, $P = 0.1$). (d) VTE[pos] ($n = 12$) and VTE[neg] patients ($n = 28$) had significantly higher peak levels of interleukin 6 (IL-6) than HCs ($n = 20$). (e) Peak microparticle tissue factor activity (MPTFa) was significantly higher among VTE[pos] patients ($n = 12$) than VTE[neg] patients ($n = 21$) or HCs ($n = 25$), whereas peak MPTFa values were similar in VTE[neg] patients when compared with HCs ($P = 0.88$). The dashed line represents the threshold for positivity, as determined by the mean of HC values + 2 SDs or values used for reporting clinical laboratory results. neg, negative; pos, positive.

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Figure 2. (continued) with active and remission paired samples, patients in remission had lower levels of hs-CRP. (g) Patients in active disease ($n = 28$) and patients in remission ($n = 22$) have significantly higher levels of interleukin 6 (IL-6) than HCs ($n = 20$). (h) In 18 paired samples tested for IL-6, patients in active disease had significantly higher levels of IL-6 than during remission. (i) Microparticle tissue factor activity (MPTFa) tested in 25 HCs, 23 patients tested in active disease, and 19 patients tested in remission showed no difference between HCs and active patients ($P = 0.81$), active and remission patients ($P = 0.82$), or HCs and patients in remission ($P = 0.67$). (j) No significant difference in paired active and remission samples from 15 patients was observed ($P = 0.46$).

Table 3. Comparison of biomarkers according to VTE status

| | VTE status | | |
| | VTE^{neg} $n = 29$ | VTE^{pos} $n = 12$ | |
Variable			P value
BVAS	12 (8, 13)	13.5 (7.5, 19.5)	0.3
Anti-Plg (%Pos Co)	17 (13.8, 23.6)	21.3 (13.1, 28.9)	0.5
D-dimer (ng/ml)	208 (149, 811)	677 (245, 2263)	0.04
hs-CRP (mg/l)	8.2 (4, 16)	16 (7.3, 16) [$n = 11$]	0.1
IL-6 (pg/ml)	2.6 (1.6, 4) [$n = 28$]	4.8 (2.5, 11)	0.06
MPTFa (%Pos Co)	0 (0, 1.4) [$n = 21$]	14 (4.3, 36.6)	<0.0001

%Pos Co, % positive control; BVAS, Birmingham Vasculitis Activity Score; anti-Plg, anti-plasminogen; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; MPTFa, microparticle tissue factor activity; VTE, venous thromboembolism.
Data are presented as median (interquartile range) of highest values measured (peak values). P values were calculated using a Wilcoxon 2-sample test. Variables with missing values are noted as [different n].

Supplementary Figure 3C) compared with VTE^{neg} patients (7.4, 2.1–9.6; $P = 0.02$).

Anti-Plg Prevalence in VTE^{pos} Patients

To further elucidate the correlation between these markers and VTE, we evaluated the highest measured values for each biomarker (peak) to determine if they ever differed between VTE^{pos} and VTE^{neg} patients regardless of disease activity. Peak levels of anti-Plg in VTE^{pos} (21.3% positive control, 13.1–28.9; $P = 0.005$; Figure 3a) and VTE^{neg} patients (17%, 13.8–23.6; $P = 0.0006$) were higher than in HCs. VTE^{pos} and VTE^{neg} patients did not differ ($P = 0.5$, Table 3). Of 12 VTE^{pos} patients, 6 (50%) were positive for anti-Plg compared with 11 of 29 (38%) VTE^{neg} patients (Supplementary Figure S4A). Three VTE^{pos} patients were positive for anti-Plg before VTE (Figure 4a).

D-Dimer Higher in VTE^{pos} Patients

Peak values of D-dimer were higher in VTE^{pos} (677 ng/ml, 245–2263; Figure 3b) than VTE^{neg} patients (208, 149–811; $P = 0.04$; Table 3). Two patients who had elevated D-dimer with a Well's score ≥2 prompting compression ultrasonography had subclinical VTEs below the knee and one had pulmonary embolism. Peak values of hs-CRP were similar in VTE^{pos} and VTE^{neg} patients (Figure 3c). Although peak IL-6 values were higher in VTE^{pos} (4.8 pg/ml, 2.5–11.0; Figure 3d) and VTE^{neg} patients (2.6, 1.6–4.0) than HCs ($P < 0.0001$ for both), VTE^{pos} and VTE^{neg} patients did not differ ($P = 0.06$).

Elevated MPTFa Differentiates Patients With VTE

VTE^{pos} patients had higher levels of peak MPTFa (14.0, 4.3–36.6; Figure 3e) than VTE^{neg} patients (0, 0–1.4; $P < 0.0001$, Table 3) and HCs ($P < 0.0001$). Peak MPTFa was similar in VTE^{neg} patients and HC. Eight of 12 VTE^{pos} patients (67%) had at least one elevated MPTFa measurement, whereas 3 of 25 (12%) HCs and no
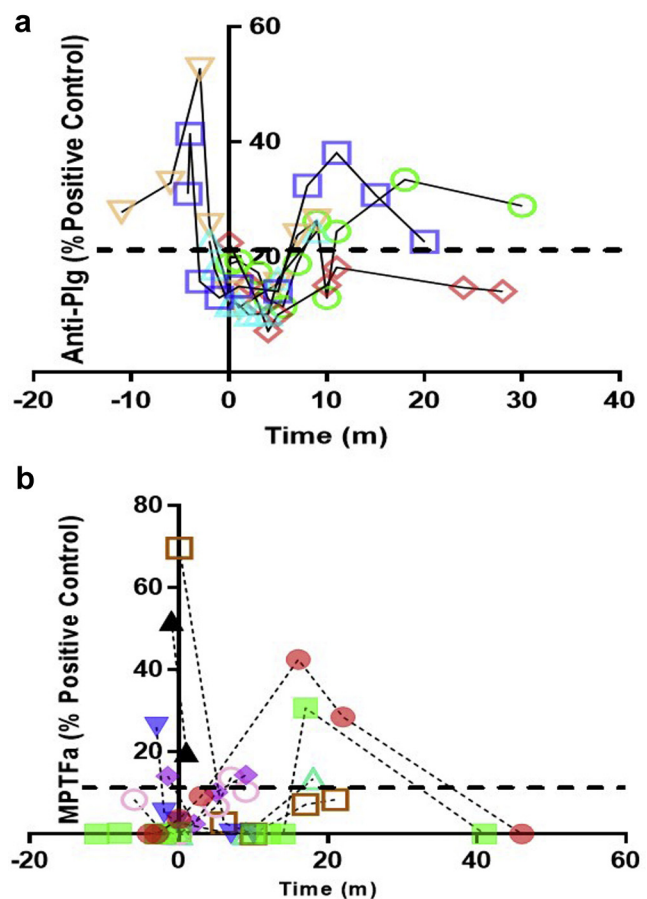
Figure 4. Longitudinal analysis of anti-Plg and MPTFa. (a) Shown are longitudinal samples from venous thromboembolism (VTE)^{pos} patients tested for anti-plasminogen (anti-Plg). Only VTE^{pos} patients who were ever positive (pos) for anti-Plg are shown ($n = 5$). Anti-Plg was elevated in all 3 patients with samples available before the VTE. One patient was elevated at the time of the VTE and one was elevated after the VTE. (b) Microparticle tissue factor activity (MPTFa) was tested on longitudinal samples from VTE^{pos} patients. Only the 8 of 12 VTE^{pos} patients who were ever pos for MPTFa are shown. In 3 of 6 patients with samples before the VTE, MPTFa was elevated before the diagnosis of the VTE. Five patients had elevated MPTFa after the VTE (3 days–18 months [m]). Elevated MPTFa was defined as greater than 2 SDs above the mean of healthy control (HC) values (11.1%). The dashed line represents the threshold for positivity, as determined by the mean of HC values + 2 SDs. Zero denotes the day of the VTE.

VTE^{neg} patients (0 of 20; $P < 0.0001$) had increased MPTFa in up to 46 months of follow-up (Supplementary Figure S4B).

To evaluate a temporal relationship, MPTFa was examined longitudinally relative to the time of VTE (Figure 4b). Considering the 8 patients who ever had elevated MPTFa, 3 of 6 patients (50%) with plasma available before VTE had increased MPTFa 1 to 3 months before the VTE. The other 3 patients had elevated MPTFa after VTE (7–17 months). Of 2 patients without plasma samples before VTE, 1 had the highest MPTFa in our cohort measured 3 days after VTE; the other had elevated MPTFa 18 months after VTE.
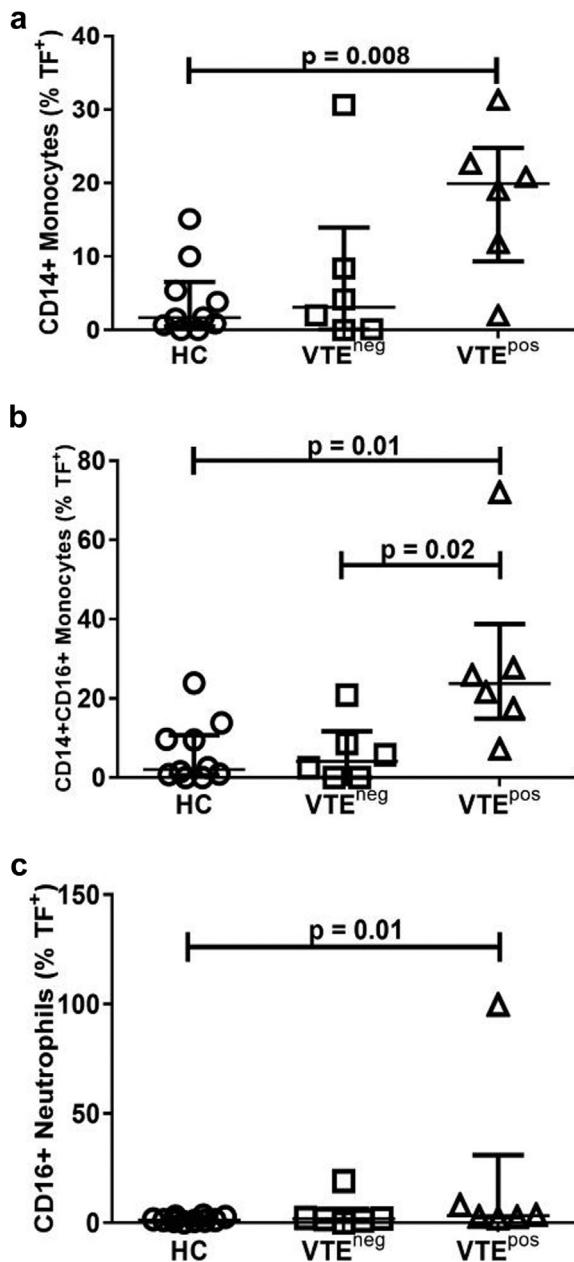
a

b

c

Figure 5. Tissue factor (TF) is increased on cells of venous thromboembolism (VTE)^{pos} patients. Whole blood from healthy controls (HCs), VTE^{pos}, and VTE^{neg} patients were stained with cluster of differentiation 14 (CD14), cluster of differentiation 16 (CD16), and CD142 (anti-TF) to determine the percentage of TF$^+$ leukocytes by flow cytometry. VTE^{pos} patients had a higher surface expression of TF on (a) CD14$^+$ monocytes, (b) CD14$^+$CD16$^+$ inflammatory monocytes, and (c) CD16$^+$ neutrophils than HC. (b) VTE^{pos} patients also had significantly higher surface expression of TF on CD14$^+$CD16$^+$ inflammatory monocytes than VTE^{neg} patients. neg, negative; pos, positive.

Increased TF-Bearing Leukocytes in VTE^{pos} Patients

Because MPTFa is elevated in VTE^{pos} patients, we examined whether cells that house ANCA antigens might have increased TF protein. Flow cytometry analysis demonstrated that the percentage of cells

Table 4. Biomarkers associated with VTE by univariate analysis

Variable	Univariate analysis		
	Hazard ratio	95% CI	P value
Anti-Plg (Act) (%Pos Co)	1.03	0.97–1.08	0.33
Anti-Plg (Rem) (%Pos Co)	1.17	1.03–1.33	0.02
MPTFa (Act) (%Pos Co)	1.04	1.01–1.08	0.01
MPTFa (Rem) (%Pos Co)	1.40	1.11–1.77	0.005
Serum creatinine (Act) (mg/dl)	1.29	1.00–1.66	0.05
Serum albumin^a (Act) (g/dl)	4.4	1.5–12.9	0.008
hs-CRP (Act) (mg/l)	1.17	1.01–1.35	0.04
Estimated GFR^a (Act) (ml/min per 1.73 m^2)	1.03	1.00–1.07	0.06

%Pos Co, % positive control; Act, active disease; Anti-Plg, anti-plasminogen; CI, confidence interval; GFR, glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MPTFa, microparticle tissue factor activity; Rem, remission; VTE, venous thromboembolism.
^{a}These variables, low serum albumin and low estimated GFR, are inverse associations.

positive for TF was increased in monocytes ($P = 0.008$), inflammatory monocytes ($P = 0.01$), and neutrophils ($P = 0.01$) from VTE^{pos} patients compared with HCs (Figure 5a–c). TF was also increased on inflammatory monocytes from VTE^{pos} patients compared with VTE^{neg} patients (Figure 5b, $P = 0.02$). To confirm that bioactive TF could be released by ANCA, we treated HC whole blood with PR3-ANCA IgG and found that MPs with TFa could be released (data not shown).

Association of Biomarkers With VTE Incidence

Time-to-event analysis was assessed to discern how biological markers relate to incidence of VTE. MPTFa was highly associated with VTE whether using MPTFa at active disease (HR: 1.04; 1.01–1.08; $P = 0.01$; Table 4) or MPTFa during remission (HR: 1.4; 1.11–1.77; $P = 0.005$). For every 10% increment of increase in MPTFa during active disease, the risk of VTE increased by 40%. This is a significant unit of measure, as the threshold for positivity was set at 11% of the positive control.

Increased anti-Plg during remission was associated with VTE (HR: 1.17; 1.03–1.33; $P = 0.02$). Due to the design of the study, some remission time-points were after VTE, but sensitivity analysis of VTE^{pos} patients with multiple remission samples indicated anti-Plg was comparable at all remission time-points ($n = 7$, Pearson correlation coefficient $= 0.93$, $P = 0.002$). Using minimum anti-Plg values during remission still resulted in an HR of 1.23 (1.06–1.43; $P = 0.008$). Increased hs-CRP (HR: 1.21; 1.02–1.45; $P = 0.03$) and serum creatinine (HR: 1.29; 1.00–1.66; $P = 0.05$) during active disease were also associated with VTE. Low baseline serum albumin was associated with an increased risk of VTE (HR: 4.4; 1.5–12.9; $P = 0.008$).

We performed limited multivariable modeling of statistically significant measures described previously to evaluate independence of associations. The association of anti-Plg at remission with VTE was independent of eGFR (HR: 1.17; 1.03–1.33; $P = 0.02$) or serum

Table 5. Biomarkers associated with VTE by multivariable analysis

Variable	Multivariable analysis		
	Hazard ratio	95% CI	P value
MPTFa (Act) (%Pos Co) Adjusted by serum creatinine	1.04	1.01–1.07	0.02
MPTFa (Act) (%Pos Co) Adjusted by eGFR	1.03	1.00–1.07	0.06
MPTFa (Rem) (%Pos Co) Adjusted by anti-Plg (Rem)	1.44	1.09–1.90	0.01
MPTFa (Rem) (%Pos Co) Adjusted by minimum anti-Plg (Rem)	1.48	1.08–2.04	0.01
Anti-Plg (Rem) (%Pos Co) Adjusted by serum creatinine	1.19	1.04–1.36	0.01
Anti-Plg (Rem) (%Pos Co) Adjusted by eGFR	1.17	1.03–1.33	0.02
Serum creatinine (Act) (mg/dl) Adjusted by anti-Plg (Rem)	1.40	1.02–1.92	0.04

%Pos Co, % positive control; Act, active disease; Anti-Plg, anti-plasminogen; CI, confidence interval; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MPTFa, microparticle tissue factor activity; Rem, remission; VTE, venous thromboembolism.

creatinine (HR: 1.19; 1.04–1.36; $P = 0.01$; Table 5). MPTFa during remission was consistently associated with VTE when adjusted for anti-Plg during remission (HR: 1.44; 1.09–1.9; $P = 0.01$) or minimum anti-Plg (HR: 1.48; 1.08–2.04; $P = 0.01$).

DISCUSSION

We explored several biological factors that we hypothesized might contribute to the increased incidence of VTE in patients with ANCA vasculitis. Our group and others identified anti-Plg in a subset of patients.[5,29,30] These antibodies inhibit fibrinolysis and could contribute to VTE. We corroborate detection of anti-Plg in patients with both MPO- and PR3-ANCA as seen in other cohorts.[29,30]

We confirmed that anti-Plg correlate with disease activity in MPO-ANCA as shown previously,[30] and extended this finding to patients with PR3-ANCA. Half of both MPO- and PR3-ANCA VTE^{pos} patients were positive for anti-Plg compared with one-third of VTE^{neg} patients. This proportion of VTE^{pos} patients with anti-Plg is similar to a prior observation in PR3-ANCA patients.[5] A subsequent study confirmed an anti-Plg–mediated decrease in fibrinolysis via reduced thrombin generation and noted an association of anti-Plg with decreased renal function.[29] Correlation of anti-Plg with both systemic and renal disease has been reported.[30] Although elevated during active disease, anti-Plg do not discriminate between VTE^{pos} and VTE^{neg} patients at this time-point. Importantly, our HR analysis demonstrated that increased anti-Plg during remission indicated patients more likely to have VTE independent of renal function. This concept of hypercoagulability during remission has been described[31] and suggests that some patients with ANCA vasculitis

and detectable anti-Plg, even within a normal reference range, may be at risk for VTE during remission.

Because anti-Plg are not universally detectable among VTE^{pos} patients and occur in VTE^{neg} patients, additional factors must contribute to mechanism(s) underlying the increased propensity for VTE in ANCA vasculitis. Multiple studies suggest a higher risk of VTE during active disease[1–4] when inflammation is high.[32] We confirmed that D-dimer[33] and CRP[34] are associated with disease activity in ANCA vasculitis. Unlike Tomasson et al.,[20] IL-6 was elevated during active disease in this study, but we did not observe a significant difference in IL-6 between VTE^{pos} and VTE^{neg} patients. These discrepancies may be attributable to fewer patients in our study and our strict definition of remission. While it is possible that increased IL-6 levels in patients compared with HCs may be less robust than shown because of the correlation of IL-6 with age, our analysis of active and remission samples within the same patient demonstrate a strong elevation of IL-6 during active disease. Although D-dimer differed in VTE^{pos} and VTE^{neg} patients, almost half of VTE^{neg} patients were positive for D-dimer, emphasizing its sensitive but nonspecific nature.[35] During active disease, hs-CRP distinguished VTE^{pos} from VTE^{neg} patients and was associated with VTE in our HR analysis similar to a prior study.[36] Interestingly, 75% of VTE^{pos} patients were positive for both D-dimer and hs-CRP at active disease. It is conceivable that hs-CRP measures may improve the prognostic value of D-dimer. It would be informative to examine this intriguing observation in a larger cohort. Of note, CRP triggers TF protein expression in human monocytes.[37]

We demonstrate that increased percentages of monocytes, inflammatory monocytes, and neutrophils from VTE^{pos} patients have surface TF than those of healthy individuals. VTE^{pos} patients also have more TF-positive inflammatory monocytes than VTE^{neg} patients. Leukocyte surface expression of TF can be driven by antibody-mediated cell activation.[12,18,19] ANCA bind both neutrophils and monocytes,[38] but ANCA-driven TF expression has not been examined previously in monocytes or inflammatory monocytes. As a proof of concept, we found PR3-ANCA IgG can stimulate release of MPs with TF activity. Because monocytes and inflammatory monocytes from VTE^{pos} patients exhibit elevated TF protein, these cells, in addition to neutrophils, may be sources of increased MPTFa observed in VTE^{pos} patients.

Microparticles and TF are implicated in development of VTE in autoimmune disorders,[39–41] including systemic vasculitis.[16,18,19,42] Because TF protein is not equivalent to TF activity,[43] a strength of our study is

direct measurement of TF activity of MPs. Our study is the first to examine MPTFa in ANCA vasculitis and ascertain an association with VTE. Patients with VTE have strikingly higher levels of MPTFa than patients without VTE. Two-thirds of VTE[pos] patients had elevated MPTFa during their disease course. In contrast, no VTE[neg] patients ever had elevated MPTFa, even during active disease when risk for VTE is expected to be highest. Another strength of our study is longitudinal data that allowed discernment of elevations in anti-Plg and MPTFa that might have gone undetected in cross-sectional samples.

A limitation of this small study is the seeming lack of temporal correlation of elevated MPTFa with VTE in some patients, possibly due to the timing of sample acquisition, as therapy around the time of VTE may affect measurement of MPTFa. Although MPTFa did not correlate with disease activity, we tested a limited number of paired samples in this assay. Nonetheless, HR analyses demonstrated a strong association of MPTFa with VTE in active disease and in remission. These data suggest that MPTFa may be a useful biomarker for identifying patients most prone to develop VTE. These findings are intriguing and should be replicated in a larger prospective study. Because a larger percentage of VTE[neg] patients were lost to follow-up than VTE[pos] patients, it is conceivable that some potential VTE events were missed in the 6 VTE[neg] patients lost to follow-up. However, only 2 of the 6 VTE[neg] patients had shorter follow-up time than the median time to VTE. An interesting finding and possible limitation of the study is that half of the VTE[pos] patients received therapeutic plasma exchange compared with 10% of VTE[neg] patients. Plasmapheresis could potentially contribute to the risk of VTE because of the use of a catheter in this procedure. Conversely, plasmapheresis removes clotting factors, anti-Plg, and microparticles that would theoretically decrease the risk of VTE. This is a complicated issue that our study was not designed to address and would be best explored in a large, randomized clinical trial. Additional biological factors not examined in our study may also contribute to thrombosis in ANCA vasculitis. For example, antibodies to anti-tissue plasminogen activator were frequent in patients with anti-Plg; IgG samples containing both antibodies were most effective in delaying fibrinolysis.[29]

In our cohort, VTE[pos] patients had 3-fold more skin involvement than VTE[neg] patients, similar to that reported recently.[36] Conversely, we found no over-representation of gastrointestinal or eye involvement in VTE[pos] patients.[36] Eye involvement was also similar between VTE[pos] and VTE[neg] patients in a UK cohort.[44] VTE[pos] and VTE[neg] patients had comparable renal involvement, eGFR, and serum creatinine. In time-to-

event analysis, serum creatinine was associated with VTE as reported recently[36]; however, eGFR did not reach significant association with VTE in our study. Increased MPTFa and anti-Plg at remission signify a propensity for VTE in ANCA vasculitis independent of renal function.

Low serum albumin was associated with a 4-fold risk of VTE. The inverse relationship between serum albumin and VTE is not surprising given that hypo-albuminemia is a predictor of VTE in nephrotic syndrome,[24] but has not been reported in ANCA vasculitis.[24,45,46] Although low plasma albumin (hypoalbuminemia) generally correlates with proteinuria, the relationship between these 2 measures may not only reflect the causal glomerular disease of the patient,[47] but also the risk of VTE.[24] In addition to proteinuria, hypoalbuminemia may be affected by nutritional status, inflammation, and vascular protein permeability.[48] Another mechanism that may predispose patients to thrombosis includes the hepatic over-production of fibrinogen and factors V and VIII as a compensatory response to hypoalbuminemia.[49,50] Decreased plasma levels of antithrombin III, α-1 anti-trypsin, and free protein S are known to correlate with serum albumin level.[49,51,52] In nephrotic patients, low plasma albumin contributes to a compact fibrin clot structure and impaired fibrinolysis; therapeutic albumin replacement improved fibrin clot structure and fibrinolysis.[53] Moreover, albumin is a cofactor for the binding of plasminogen to fibrin and their interaction with tissue plasminogen activator[54]; it is conceivable that anti-Plg may augment the reduced effectiveness of this interaction in the setting of low serum albumin, further impairing fibrinolysis. It will be important to confirm our finding that hypoalbuminemia is associated with an increased risk of VTE in a larger cohort.

Our study identified elevated MPTFa, increased anti-Plg during remission, and hypoalbuminemia as novel biomarkers associated with VTE in patients with ANCA vasculitis. These hypothesis-generating studies contribute to our understanding of the mechanisms of VTE and form a foundation for future studies. Discernment of a thrombotic signature would allow improved management of patients to minimize both risk of VTE and complications of anticoagulation.

AUTHOR CONTRIBUTIONS

DOB, PHN, VKD, RJF, and JCJ designed the study. AF consented patients and acquired data. EJB, CEM, and DOB performed experiments. DOB, CEM, EJB, VKD, MLM, YH, and SLH analyzed data. DOB and CEM prepared figures. DOB, CEM, EJB, and VKD drafted the paper. All authors revised and approved the final version of manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Table S1. Demographics of study population.

Table S2. Anticoagulation medications in study population.

Table S3. Immunosuppressive therapy in study population.

Figure S1. Correlation of candidate markers with age in healthy controls or patients.

Figure S2. Anti-plasminogen antibodies correlate with disease activity in MPO- and PR3-ANCA vasculitis.

Figure S3. Comparison of candidate biomarkers in patients during active disease.

Figure S4. Some VTEneg patients exceed threshold of positivity for anti-Plg but not MPTFa.

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