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# 6

## *Decreased plasma levels of activated factor VII in patients with deep vein thrombosis*

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## Abstract

**Background:** The initiating trigger in the development of deep vein thrombosis (DVT) remains unidentified. It has been suggested that tissue factor-bearing microparticles play a key-role, which indicate a role for the tissue factor (TF)-pathway in the initiation of DVT. **Objective:** To assess the role of the TF-pathway in the initiation of venous thrombosis, we measured plasma levels of factor VII and VIIa in patients with acute DVT and in controls. **Methods:** we included 148 patients diagnosed with acute DVT and 179 controls in this study. Antigen levels of FVII and FVIIa were measured using assays recently developed in our laboratory. **Results:** Median FVII levels in patients were 109.8% (interquartile range [IQR] 86.0-153.2) compared with 102.2% (IQR 76.1-141.7) in controls. Individuals with FVII levels in the upper quartile had a 1.6-fold increased risk for the presence of a DVT (odds ratio 1.6, 95% confidence interval 0.8-3.1). Median FVIIa levels in patients were 50.2 ng/ml (IQR 25.2-86.1) compared with 96.6 ng/ml (IQR 69.9-168.9) in controls. Individuals with FVIIa levels in the lowest quartile had a more than 5-fold increased risk for the presence of a DVT (odds ratio 5.5, 95% confidence interval 2.8-10.6). Both risks did not change substantially after adjustment for potential confounders. **Conclusion:** Decreased plasma levels of FVIIa in patients with deep vein thrombosis may indicate ongoing consumption of FVIIa and suggest a contributory role for TF in venous thrombus formation.

## Introduction

Venous thrombosis is a multi-causal disease for which many genetic and acquired risk factors have been firmly established (reviewed in [1]). These risk factors generally increase hemostatic potential by either enhancing activation of coagulation or by decreasing inhibition of coagulation [2]. In addition, a hypofibrinolytic status has been repeatedly shown to increase the risk for venous thrombosis [3-5]. Although it is clear that alterations in the hemostatic balance may contribute to the development of venous thrombosis, the initiating trigger remains unidentified. In contrast to arterial thrombosis, in which exposure of thrombogenic material present in atherosclerotic plaques initiates thrombus formation, such lesions are absent in veins. Remarkably, the endothelial lining of a thrombosed vein appears to be intact, suggesting that initiation of thrombus formation proceeds via components present within the bloodstream [6,7]. It has been suggested that tissue factor (TF)-bearing microparticles present in the circulation play a key-role in the initiating of deep vein thrombosis (DVT) [8-12]. It has been proposed that activation of the venous endothelial lining, for example by venous stasis, leads to the recruitment of TF-bearing microparticles via P-selectin expressed on the activated endothelium and P-selectin glycoprotein ligand-1 (PSGL-1) on the TF-bearing microparticles. This process results in accumulation of TF, which, possibly after fusion with platelets or endothelial cells, initiates venous thrombus formation [13]. Because high plasma levels of FVII and low levels of TFPI are risk factors of venous thrombosis [14,15], a role of the TF pathway in the initiation of coagulation during venous thrombosis is plausible. Recently, however, a clinical study suggested a pivotal role for the intrinsic pathway in venous thrombosis. A novel anticoagulant strategy using antisense nucleotides toward FXI was effective in venous thrombosis prevention after knee replacement surgery [16]. It has, however, not yet been established whether this central role of FXI in venous thrombosis relates to FXIIa- or thrombin-mediated activation of FXI. It is tempting to speculate that thrombin-mediated activation of FXI is the dominant mechanism because elevated FXI levels are a risk factor for venous thrombosis [17], whereas FXII levels are not associated with venous thrombosis risk [18]. The latter scenario, again, is compatible with the TF pathway as the initiator of venous thrombus formation. To assess the contribution of TF in the initiation of venous thrombosis, we measured plasma levels of factor VII and VIIa in patients with acute DVT and in controls. Because TF-mediated activation of coagulation starts with the conversion of zymogen to activated FVII, we hypothesized that there would be increased plasma levels of FVIIa in patients with DVT, which would be indicative of a major role of the TF-pathway in the initiation of thrombus formation.

## Methods

### *Study design*

The study design was described earlier [19]. In short, patients ( $\geq 18$  years old) with suspicion of acute symptomatic DVT who were between September 1999 and May 2006 referred to the Academic Medical Center in Amsterdam, the Netherlands, were eligible for the study. We included 148 patients diagnosed with acute proximal DVT and 179 patients in whom DVT was objectively excluded (controls) in the present study. DVT was diagnosed with compression ultrasound. DVT was ruled out in patients with a low pretest probability as assessed by the Wells score in combination with a negative D-dimer test and in patients with a negative serial compression ultrasound. In addition, controls did not have a history of previous venous thromboembolism, and controls with alternative diagnoses that may be associated with activation of coagulation (thrombophlebitis, calf vein thrombosis, calf muscle vein thrombosis, venous insufficiency, erysipelas, baker's cyst, and muscle bleeding) were excluded from the study. Patients with DVT were selected regardless of the presence of thrombophilia or provoking comorbidities. All patients gave written informed consent, and collection of blood was approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam, the Netherlands.

### *Blood collection and plasma preparation*

Blood was drawn into 3.2% sodium citrate (9:1, v/v) prior to the initiation of anticoagulant treatment. Platelet-poor plasma was obtained by double centrifugation at 1500 g for 15 min at room temperature. Plasma was aliquoted and stored at  $-80^{\circ}\text{C}$  until use.

### *Plasma FVII and FVIIa levels*

The plasma FVII and FVIIa levels were measured using in-house developed assays as described earlier [20]. Briefly, plasma levels of FVII and FVIIa were determined using a semi-automated ELISA on a Tecan Freedom EVO robot (Tecan, Männedorf, Switzerland). A sheep anti-FVII antibody ( $1\ \mu\text{g}/\text{ml}$ ; Stago, Leiden, the Netherlands) or a llama-derived antibody fragment directed against FVIIa ( $2\ \mu\text{g}/\text{ml}$ ) was immobilised on a 384-well plate (Thermo Fisher Scientific Inc., Waltham MA, USA) at  $4^{\circ}\text{C}$  overnight. After blocking, diluted plasma samples were added to the plate and incubated on a shaker set at 180 rpm for 2 h at room temperature. Bound FVII was detected using horseradish peroxidase (HRP)-labeled sheep anti-FVII antibody ( $0.5\ \mu\text{g}/\text{ml}$ ; Affinity Biologicals, Ancaster, Canada). FVIIa was detected using a chicken anti-FVII ( $0.16\ \mu\text{g}/\text{ml}$ ; Abcam, Cambridge, UK) antibody followed by a HRP-labeled donkey anti-chicken antibody

(2.2 µg/ml; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Subsequently, a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific Inc., Waltham MA, USA) was added, and after 60 min the luminescence was measured using a Spectramax reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). The luminescence values of the plasma samples were translated into FVII or FVIIa levels by using an 8 point calibration curve of pooled normal plasma or recombinant FVIIa (NovoSeven, Novo Nordisk, Bagsvaerd, Denmark). The intra-assay and interassay coefficients of variation of the ELISA were: <10% (n = 40) for FVIIa and <8% for FVII (n = 40). The FVII and FVIIa levels were determined in 144 (FVII) and 148 (FVIIa) patients diagnosed with acute DVT and in 174 (FVII) and 177 (FVIIa) controls as insufficient plasma was available for some patients.

### *Plasma FVIIa-antithrombin complex levels*

The plasma FVIIa-antithrombin (AT) complex levels were measured using an in-house developed assay, adapted from the previously described assay to measure FVIIa levels. In short, after diluted samples were added to plates coated with a llama-derived antibody fragment directed against FVIIa (2 µg/ml), FVIIa-AT complexes were detected with 0.5 µg/ml HRP-labeled sheep anti-human antithrombin (Affinity Biologicals, Ancaster, Canada). The assay was calibrated using a standard, which was generated by incubating recombinant FVIIa and human AT (Enzyme Research laboratories, South Bend, IN, USA) in the presence of TF (Innovin, Siemens Healthcare Diagnostics, Marburg, Germany) and fondaparinux (Arixtra, GlaxoSmithKline, Zeist, the Netherlands) for 1 h at 37°C. The standard was diluted in FVII-deficient plasma (Precision Biologic Inc., Dartmouth, NS, USA) to obtain a 15-point calibration curve. The plasma FVIIa-AT complex levels were determined in 145 patients diagnosed with acute DVT, and in 176 controls as insufficient plasma was available for some patients.

### *Statistical analysis*

The FVII, FVIIa and FVIIa-AT levels in patients diagnosed with acute DVT and in the control group are shown as median values. We present differences between median values with corresponding 95% bootstrap confidence interval (CI). The associations between plasma levels of FVII or FVIIa and the presence of DVT were analysed by means of logistic regression analysis and are expressed as odds ratios (ORs) with corresponding 95% CIs. Levels were grouped into quartiles based on the distribution among controls. The lowest quartile of FVII and the highest quartile of FVIIa were taken as the reference

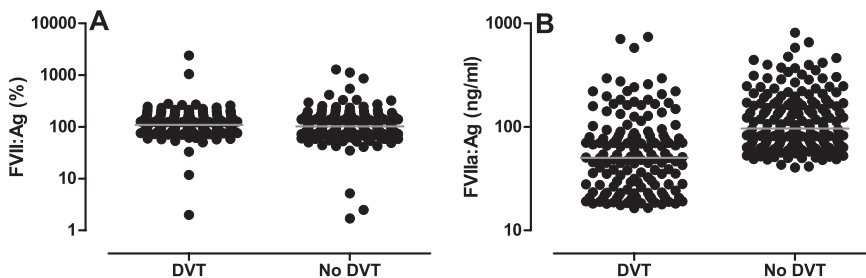
group. The influence of potential confounders (age, sex, smoking status, malignancy, paralysis, paresis or recent plaster immobilization of the lower extremities, recently bedridden >3 days and/or major surgery within 4 weeks and hospitalization <6 months) on the association between plasma levels of FVII or FVIIa and DVT were analysed by means of multivariable logistic regression models. For the analyses, we used the software package R (version 3.1.1, R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS Statistics, PC (release 20, IBM, New York, USA).

## Results and discussion

Table 1 shows the characteristics of the patients diagnosed with acute DVT (n = 148) and patients in whom this diagnosis was excluded (n = 179, controls). Established risk factors for DVT were present more frequently in patients compared with controls.

Antigen levels of FVII and FVIIa in patients and controls are shown in Figure 1. FVII levels were comparable between patients and controls (Figure 1A). Median FVII levels were 109.8% (interquartile range [IQR] 86.0-153.2) in patients compared to 102.2% (IQR 76.1-141.7) in controls (a difference of 7.6%, 95% CI -4.6-20.2).

FVIIa levels were substantially lower in patients compared to controls (Figure 1B). Median FVIIa levels were 50.2 ng/ml (IQR 25.2-86.1) in patients compared to 96.6 ng/ml (IQR 69.9-168.9) in controls (a difference of 46.4 ng/ml, 95% CI 29.6-69.7).



**Figure 1** Plasma levels of FVII antigen (A) or FVIIa antigen (B) in patients diagnosed with DVT or in the control group. Horizontal lines indicate median values.

High levels of FVII were associated with a slightly increased risk for the presence of a DVT as shown in table 2. Individuals with FVII levels in the upper quartile had a 1.6-fold increased risk (OR 1.6, 95% CI 0.8-3.1) for the presence of a DVT compared with individuals with FVII levels in the lowest quartile, and this risk did not change after adjustment for potential confounders.

**Table 1** Characteristics of Study Population.\*

	<b>n (acute DVT/ controls)<sup>†</sup></b>	<b>Acute DVT</b>	<b>Controls (no DVT)</b>
Mean age, y (range)	148/175	58 (19-90)	59 (19-100)
Male sex, n (%)	148/179	69 (47)	81 (45)
Body mass index, kg/m <sup>2</sup> (SD)	146/172	26.6 (4.5)	28.6 (5.8)
Malignancy (on treatment/ recently treated), n (%)	141/172	24 (16)	22 (12)
Smoking, n (%)	148/177	39 (26)	39 (22)
Paralysis, paresis, or recent plaster immobilization lower extremities, n (%)	147/173	15 (10)	14 (8)
Recent immobilization (bedridden >3 days, major surgery <4 wk), n (%)	147/171	26 (18)	13 (7)
History of recent trauma (<60 d), n (%)	146/172	18 (12)	20 (11)
Hospitalization (<6 mo), n (%)	147/173	35 (24)	24 (13)
Oral contraceptives use, n females (%)	148/179	21 (14)	11 (6)

\* Parameters are at time of blood sample collection. † Some variables were not available for all patients. Indicated are the numbers of patients for whom values were available.

Low levels of FVIIa were associated with an increased risk for the presence of a DVT. Individuals with FVIIa levels in the lowest quartile had a more than 5-fold increased risk for the presence of a DVT (OR 5.5, 95% CI 2.8-10.6) compared with individuals with FVIIa levels in the highest quartile, and this risk did not substantially change after adjustment for potential confounders.

We found substantially decreased levels of FVIIa in patients in the acute phase of a DVT, which at first glance is paradoxical since it has been well established that there is active coagulation activation in such patients, as indicated by, for example, elevated levels of prothrombin fragment 1+2 and thrombin-antithrombin (TAT) complexes [21-23].



We thus hypothesized to find elevated levels of FVIIa as a result of TF-mediated FVII to FVIIa conversion similar to previous findings on elevated FVIIa levels in sepsis and acute coronary syndromes [20,24]. The decreased FVIIa levels in patients, however, may indicate ongoing consumption of FVIIa.

We detected FVIIa-antithrombin complexes only in a minority of patients (n = 16, 11%) and controls (n = 58, 33%). In those individuals in whom detectable levels of the FVIIa-AT complex were detected, levels were substantially higher in patients compared to controls. Median levels were 25.8 pM (IQR 9.1-54.9) in patients compared to 7.2 (IQR 5.3-12.5) in controls (a difference of 18.6 pM, 95% CI 1.9-38.5).

Our results, thus, do not exclude that active, TF-mediated FVII to FVIIa conversion takes place in patients during an acute venous thrombosis, as it may be that the FVIIa does drive coagulation activation but is rapidly inhibited and/or cleared by the AT-dependent mechanisms as increased FVIIa-AT complex levels were measured in these patients. In line with our results, Spiezia et al. also showed reduced levels of FVIIa in patients with acute venous thrombosis [25]. In contradiction to our results, this study showed that FVIIa-AT complex levels were decreased in these patients, which led these authors to conclude that in acute thrombosis the formation of TF-FVIIa-FXa-TFPI complexes may be the preferred inhibitory route, which results in decreased levels of FVIIa-AT complexes. Although we find elevated levels of FVIIa-AT complexes in patients compared to controls, the proportion of controls in whom detectable levels were measured was more than 3-fold higher compared to patients with detectable levels. This latter finding may be in line with the findings of Spiezia et al. Notably, another study with a similar design found no difference in FVIIa levels between patients and controls [26].

The combined results of our study suggest the TF pathway to be activated during venous thrombus formation. Since levels of FVII, TFPI and FXI are, and levels of FXII are not associated with the risk of venous thrombosis, a role of FXIIa-mediated activation of coagulation in the development of venous thrombosis appears minor. We therefore hypothesize that initiation of venous thrombus formation proceeds via the TF pathway. Subsequent thrombin-mediated activation of FXI appears vital in propagation of venous thrombus formation given the profound antithrombotic effect of a pharmacological decrease in FXI levels.

**Table 2** Association between plasma levels of FVII or FVIIa and the presence of a DVT.

	<b>Acute DVT</b>	<b>Controls (no DVT)</b>	<b>Odds Ratio</b>	<b>Adjusted Odds Ratio*</b>
	<b>(n = 144)</b>	<b>(n = 174)</b>	<b>(95% CI)</b>	<b>(95% CI)</b>
<b>Levels of FVII</b>				
Quartile 1; n ( $\leq 76.1\%$ )	25	43	1.0 (reference)	
Quartile 2; n ( $> 76.1, \leq 102.2\%$ )	37	44	1.4 (0.7 to 2.8)	1.3 (0.6 to 2.6)
Quartile 3; n ( $> 102.2, \leq 141.7\%$ )	42	44	1.6 (0.9 to 3.1)	1.7 (0.9 to 3.4)
Quartile 4; n ( $> 141.7\%$ )	40	43	1.6 (0.8 to 3.1)	1.6 (0.8 to 3.2)
<b>Levels of FVIIa</b>				
Quartile 4; n ( $\geq 168.9$ ng/ml)	17	44	1.0 (reference)	
Quartile 3; n ( $\geq 96.6, < 168.9$ ng/ml)	15	45	0.9 (0.4 to 1.9)	1.3 (0.5 to 3.3)
Quartile 2; n ( $\geq 69.9, < 96.6$ ng/ml)	23	44	1.4 (0.6 to 2.9)	1.4 (0.6 to 3.6)
Quartile 1; n ( $< 69.9$ ng/ml)	93	44	5.5 (2.8 to 10.6)	6.4 (3.0 to 13.4)

\* Adjusted for age, sex, smoking status (non-smoker, currently smoking, quit smoking), malignancy, paralysis, paresis or recent plaster immobilization of the lower extremities, recently bedridden  $> 3$  days and/or major surgery within 4 weeks, hospitalization  $< 6$  months.

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