

Impaired *in vivo* activated protein C response rates indicate a thrombophilic phenotype in inherited thrombophilia

Venous thromboembolism (VTE) is a multifactorial disease. Hereditary risk factors include the common mutations factor V Leiden (FVL) and prothrombin (FII) 20210G>A, with a prevalence of 3–15% among whites, as well as deficiencies of the coagulation inhibitors antithrombin (AT), protein C (PC), and protein S.¹ In the recent past, novel risk loci have been found by genome-wide association studies.^{2,3} However, their consideration in addition to the classical thrombophilic defects results in an estimated heritability of VTE of only 15%, in contrast to 40–60% heritability observed in family-based studies.⁴ In order to identify further unknown genetic thrombophilic defects, consideration of the laboratory phenotype of increased thrombin formation in addition to the clinical phenotype of VTE has been proposed, based on the observation of elevated *in vitro* thrombin generation parameters in families with unexplained thrombophilia and in carriers of genetic variations in hemostasis-related genes other than FVL and FII

20210G>A.⁵

It remains unclear, however, if increased *in vitro* thrombin formation rates indeed reflect increased *in vivo* thrombin formation. In order to investigate this, we comparatively analyzed *in vitro* and *in vivo* thrombin formation in a cohort of healthy individuals and in thrombophilic patients. *In vivo* coagulation activation was induced by low-dose recombinant activated factor VII (rFVIIa). Subsequent hemostasis biomarker-monitoring included measurement of activated PC (APC) as a measure of the endothelial-dependent anticoagulant response. Recently, using this stimulated hemostasis activity pattern evaluation (SHAPE) approach, we were able to show increased *in vivo* thrombin generation rates and a comparable APC response in FVL and FII 20210G>A carriers.^{6,7} Moreover, we found that APC response rates correlated with the thrombotic risk in FVL carriers.⁷

The study population consisted of 30 healthy individuals and 51 patients with a history of VTE, thereof 28 FVL or FII 20210G>A carriers (FVL/FII 20210G>A cohort), and 23 unrelated subjects with unexplained familial VTE (FH cohort). A diagram of patient recruitment and selection criteria is shown in Figure 1, along with a description of study procedures. Blood samples were drawn before

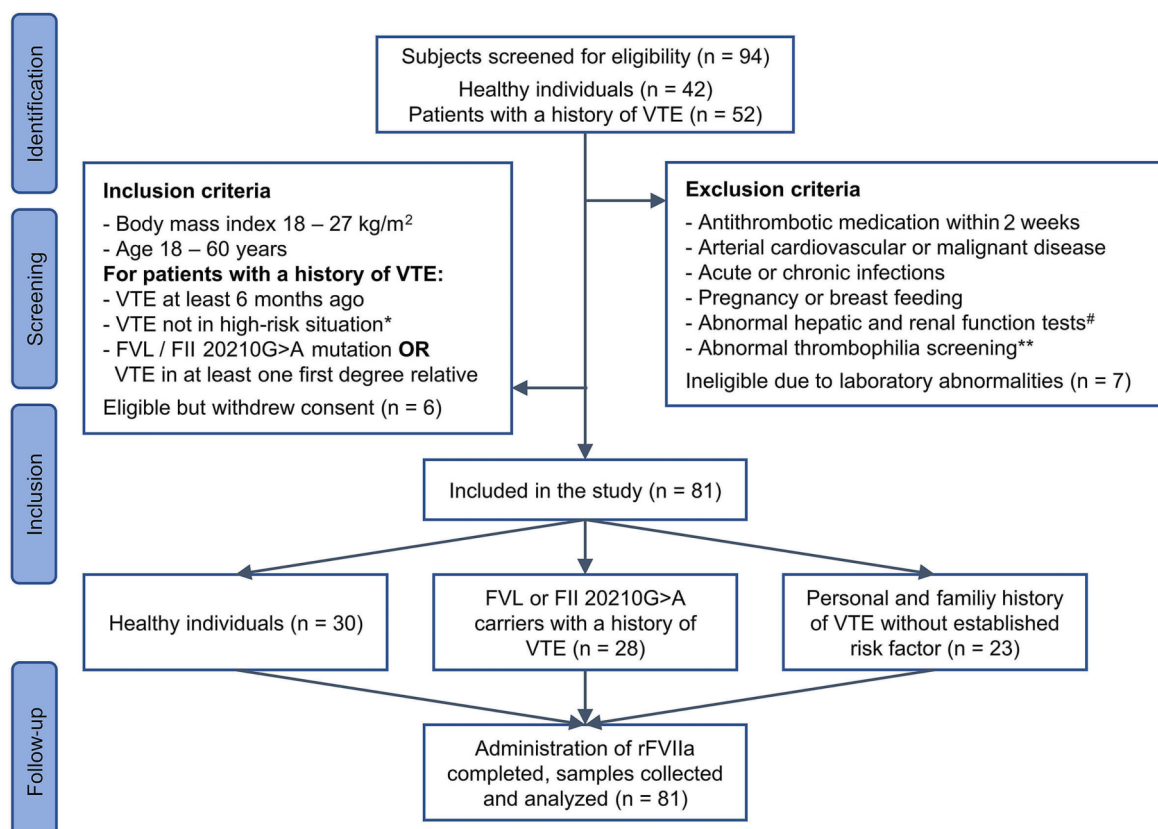


Figure 1. Eligibility criteria and study procedures. Healthy individuals were recruited from blood donors. Patients with a history of venous thromboembolism (VTE) were recruited from the thrombophilia outpatient clinic of our hospital. The study proposal was approved by the Ethics Committee of the Medical Faculty of the University Bonn (reference number O16/16). Written informed consent was received prior to participation. All finally included study participants (n=81) received morning administration of 15 µg/kg recombinant activated factor VII (rFVIIa) as single intravenous bolus injection after overnight fast. Blood samples were drawn immediately before and 10 minutes (min), 30 min, 1, 2, 3, 5, and 8 hours after administration, each from a new venipuncture. After discarding the first 2 mL, blood was drawn into citrate tubes (10.5 mmol/L, Sarstedt, Nümbrecht, DE). Citrate tubes were supplemented with aprotinin (10 µmol/L) and bivalirudin (250 µg/mL) for activated protein C (APC) measurement. Plasma samples were obtained by centrifugation (2,600 x g, 10 min) within 30 min and stored at less than -70°C until assayed. All finally included study participants completed rFVIIa administration and follow-up blood sampling. All collected samples were analyzed. *Surgery, trauma, immobilization, pregnancy, and puerperium. #Transaminases, γ-glutamyl transferase, urea, creatinine in serum. **Decreased plasma levels of antithrombin, protein C, protein S, anti-cardiolipin and anti-β2 glycoprotein I immunoglobulin G (IgG) and IgM, functional lupus anticoagulants (activated partial thromboplastin time, dilute Russell viper venom time), and factor V Leiden (FVL) and prothrombin (FII) 20210G>A mutation (except for inclusion into the FVL/FII 20210G>A cohort).

Table 1. Baseline characteristics and rFVIIa-induced biomarker changes.

	Healthy controls, N = 30	VTE, FVL or FII 20210G>A, N = 28*	P	VTE, family history of VTE, no RF, N = 23	P
Age, years (range)	35 (21-60)	41 (18-60)	-	38 (20-53)	-
Sex (male/female)	12 / 18	12 / 16	-	9 / 14	-
BMI, kg/m ² (range)	23 (18-27)	24 (18-27)	-	24 (19-27)	-
DVT / PE / both, n	-	15 / 3 / 10	-	10 / 6 / 7	-
Fibrinogen, g/L	252 (221-284)	262 (250-309)	-	267 (256-331)	-
FII, %	103 (98-116)	124 (115-135)	10⁻⁵	114 (103-120)	-
Factor XI, %	102 (90-115)	101 (95-107)	-	115 (100-127)	0.044
Antithrombin, %	107 (100-111)	98 (93-104)	-	100 (98-106)	-
sTM, ng/mL	1.62 (1.30-2.15)	1.61 (1.47-2.19)	-	1.62 (1.19-1.86)	-
sEPCR, ng/mL	45.6 (26.0-81.6)	57.0 (35.4-91.0)	-	72.5 (46.4-108.0)	-
PC, %	106 (97-118)	112 (103-122)	-	105 (97-116)	-
F1+2, nmol/L	0.16 (0.12-0.21)	0.25 (0.17-0.30)	0.002	0.15 (0.12-0.20)	-
AUC, nmol-h/L	0.29 (0.16-0.45)	0.34 (0.23-0.49)	-	0.42 (0.19-0.73)	-
TAT, ng/mL	<21.2 (<21.3-<21.3)	<21.3 (<21.3-29.7)	-	<21.3 (<21.3-24.7)	-
AUC, pmol-h/L	35.9 (0.81-109.5)	123.9 (45.1-188.2)	0.008	141.6 (12.8-332.3)	0.021
APC, pmol/L	0.68 (0.40-1.11)	1.13 (0.75-1.43)	0.022	0.79 (0.39-1.11)	-
AUC, pmol-h/L	6.55 (5.22-8.82)	15.1 (10.7-22.7)	<10⁻⁴	9.46 (5.50-14.55)	-

Age and body mass index (BMI) are shown as mean (range), all other variables as median (interquartile range). The area under the curve (AUC) quantifies changes of prothrombin activation fragment F1+2 (F1+2), thrombin-antithrombin complex (TAT), and activated protein C (APC) over 8 hours after intravenous injection of recombinant activated factor VII (rFVIIa). *P* describes significant (<0.05) differences to healthy controls. *P* was calculated using the unpaired Student *t*-test (prothrombin, FII; protein C, PC) or the Mann-Whitney test (all other parameters) and corrected for multiple testing using the Bonferroni method. DVT: deep vein thrombosis; FVL: factor V Leiden; PE: pulmonary embolism; sEPCR: soluble endothelial PC receptor; sTM: soluble thrombomodulin; VTE: venous thromboembolism. * 14 heterozygous FII 20210G>A carriers, 1 homozygous and 13 heterozygous FVL carriers, thereof 2 with HR2 haplotype.

and during 8 hours after administration of 15 µg/kg rFVIIa. No adverse events were observed. APC was measured using an oligonucleotide-based enzyme capture assay (OECA).⁸ The thrombin biomarkers prothrombin activation fragment 1+2 (F1+2), thrombin-antithrombin complex (TAT), and other hemostasis parameters were determined using commercially available assays. *In vitro* thrombin generation was assessed before rFVIIa administration, using the calibrated automated thrombogram (CAT) assay (Thromboscope, Maastricht, NL). Table 1 lists demographic features and measurement results of hemostasis parameters in the three cohorts at baseline, and rFVIIa-induced changes of F1+2, TAT, and APC over time, expressed as area under the curve (AUC). Hemostasis parameters at baseline were comparable in FVL and FII 20210G>A carriers (Online Supplementary Table S1).

In vitro thrombin formation kinetics were higher in the FH cohort than in FVL/FII 20210G>A carriers and healthy controls, indicated by an elevated endogenous thrombin potential (ETP) (Figure 2A). Additionally, peak thrombin concentration was increased compared with FVL/FII 20210G>A carriers, whereas lag time and time-to-peak did not differ significantly (Online Supplementary Figure S1A to C). The difference in the ETP was more pronounced at 1 pmol/L tissue factor (TF) concentration. This could be explained by higher FXI levels in the FH cohort, which have been shown to affect *in vitro* thrombin generation at a greater extent at lower TF concentrations.⁹ In the resting state, plasma levels of F1+2 were slightly increased in the FVL/FII 20210G>A cohort, giving additional evidence of increased thrombin formation.

After infusion of rFVIIa, plasma levels of F1+2 (Figure 2B) and TAT (Figure 2C) increased significantly in all three cohorts (peak vs. baseline values, Wilcoxon signed-rank test *P*<0.05 after Bonferroni correction). F1+2 increased in every participant, indicating that rFVIIa acti-

vates the clotting cascade, resulting in thrombin formation. Every FVL/FII 20210G>A carrier showed an increase of F1+2 and TAT, whereas four subjects in the FH group and seven healthy controls showed an isolated increase of F1+2. This absence of a TAT increase could indicate a comparably lower thrombin formation rate. The most probable explanation of this discrepancy is the longer F1+2 half-life of approximately 2 hours in comparison to the TAT half-life of 44 minutes,¹⁰ making F1+2 a more sensitive thrombin generation marker. The *in vivo* thrombin generation parameters F1+2 AUC and TAT AUC correlated with each other in healthy controls and patients with a history of VTE (Figure 2D). However, they did not correlate with *in vitro* thrombin generation (representatively shown for ETP and TAT AUC, Online Supplementary Figure S1D and E), suggesting that different factors determine and interfere with the outcome in both distinct and complex methodological approaches. In addition, compared with FVL and FII 20210G>A carriers, a more heterogeneous risk profile can be expected in the FH cohort.

If the endothelium is intact, the thrombin formation capacity is effectively controlled by APC formation. The extent to which thrombin formation induces an increase in APC might therefore indicate the functionality of the APC-generating pathway in an individual patient and, moreover, modulate the thrombotic potential of increased thrombin formation rates. In order to investigate the reactivity of the PC system to thrombin formation we measured plasma levels of APC. After infusion of rFVIIa, APC increased significantly in all cohorts (Wilcoxon signed-rank test, *P*<0.05 after Bonferroni correction). Changes in APC (and thrombin biomarkers) did not differ in FVL and FII 20210G>A carriers (Online Supplementary Figure S2). In contrast to thrombin formation rates the APC response was significantly lower in the FH cohort than in the FVL/FII 20210G>A cohort and

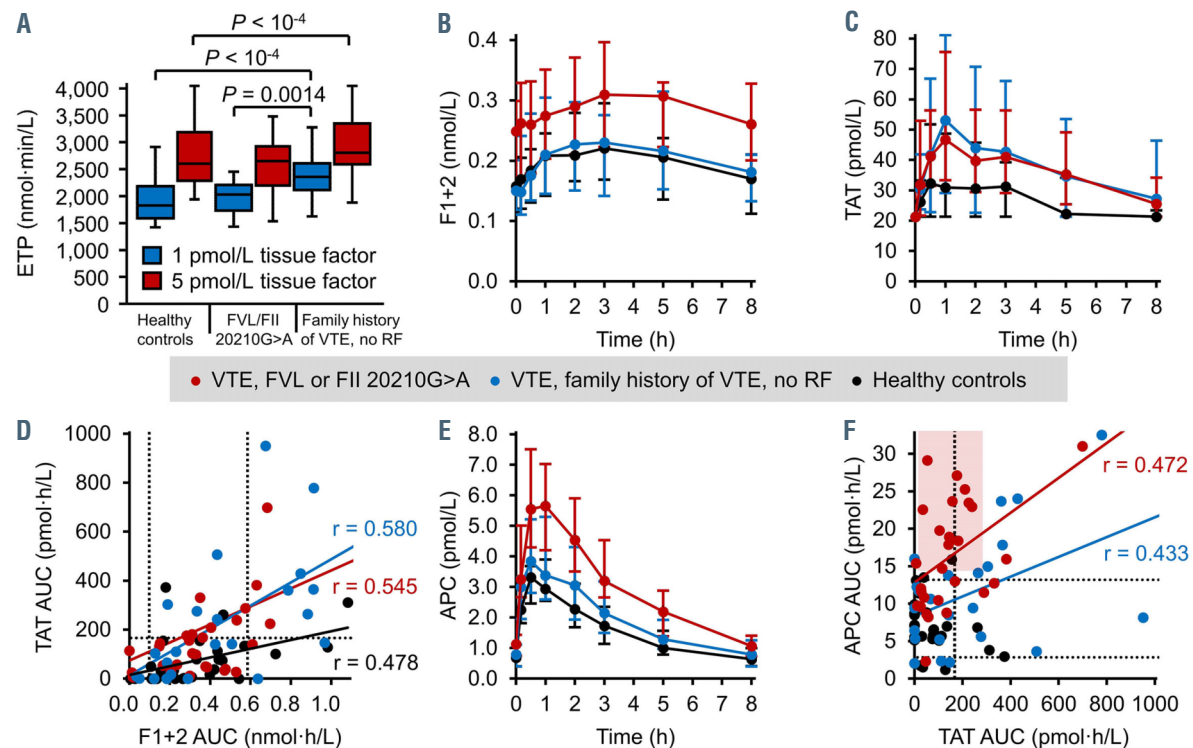


Figure 2. *In vitro* thrombin generation and *in vivo* thrombin-activated protein C response to rFVIIa. *In vitro* thrombin generation was measured by the calibrated automated thrombogram (CAT, Thromboscope, Maastricht, NL) in healthy controls (n=30) and in patients with venous thromboembolism (VTE) with factor V Leiden (FVL) or prothrombin (FII) 20210G>A mutation (n=28), or a family history of VTE without an established risk factor (RF, n=23). Plasma levels of prothrombin activation fragment 1+2 (F1+2), thrombin-antithrombin complex (TAT), and activated protein C (APC) were measured in the same population before (t=0) and after intravenous injection of 15 µg/kg recombinant activated factor VII (rFVIIa). (A) Endogenous thrombin potential (ETP) measured by CAT, presented as median and interquartile range (IQR, boxes), 1.5-fold IQR (whiskers), and outliers (circles). P-values <0.05 (Mann-Whitney test) are shown. (B) F1+2 and (C) TAT in plasma (median, IQR). (D) Area under the F1+2 generation curve (F1+2 AUC) in comparison to TAT AUC. Dotted lines indicate 90th percentiles of F1+2 AUC and TAT AUC, and 10th percentile of F1+2 AUC in healthy controls. (E) APC in plasma (median, IQR). (F) TAT AUC in comparison to APC AUC. Dotted lines indicate 90th percentiles of TAT AUC and APC AUC, and 10th percentile of APC AUC in healthy controls. The red area highlights the absence of a thrombin-related increase of APC in patients with unexplained familial thrombophilia (blue symbols). r: Pearson's correlation coefficient.

did not differ from healthy controls (Figure 2E). As the APC response is a direct marker of the APC formation capacity of the endothelium, the disproportionately low APC response in relation to the thrombin formation rate indicates an impaired endothelial APC-generating activity in the FH cohort. This relative APC deficiency after coagulation activation would consecutively result in increased thrombin formation. Several data support this conclusion: i) previously, reciprocal and opposite changes of indirect thrombin and PC activation markers were observed in patients with abnormalities of the PC pathway in a basal state;¹¹ ii) in a previous study, asymptomatic FVL carriers showed a higher APC response in the SHAPE approach than those with prior VTE;⁷ iii) in the present study, thrombin and APC formation rates (TAT AUC and APC AUC) correlated with each other in both FVL/FII 20210G>A carriers and patients with unexplained familial thrombosis, but not in healthy controls (Figure 2F). With seven subjects (25%) in the FVL/FII 20210G>A cohort and six subjects (26%) in the FH cohort, both TAT AUC and APC AUC lay above the 90th percentiles of the healthy controls in similar rates of patients. However, only two individuals (9%) in the FH cohort showed a disproportionately high APC formation rate, as evidenced by an APC AUC (slightly) above and TAT AUC within the 90th percentiles of the healthy controls. In the FVL/FII 20210G>A cohort such a pattern was observed more often (29%), and more distinctively

(Figure 2F). Thrombomodulin (TM) and endothelial PC receptor (EPCR) are two main factors that determine the APC formation capacity of the endothelium and variants in both genes have been suggested as thrombotic risk factors.^{12,13} In order to assess interindividual variations in TM and EPCR, we measured plasma levels of soluble EPCR and TM but did not find significant differences between cohorts.

Potential sources of bias or imprecision include the size of the study population, the precision of rFVIIa dosing and times of blood draw, and laboratory analysis. In order to account for these issues, sample size, rFVIIa dosage and blood sampling times were chosen in orientation to previous pharmacokinetic studies on rFVIIa, yielding expected pharmacokinetic results (*Online Supplementary Figure S1F*).¹⁴ The OECA for APC measurement has been extensively assessed.⁸ Except for sEPCR and sTM the other assays were covered by accreditation with the national accreditation body and were performed according to ISO standards. Moreover, the age and sex distribution, and the body mass index were similar in the different subgroups, ruling out a potential confounding effect of these variables. Finally, one might argue that instead of assessing a genetic hypercoagulable state in patients with unexplained familial thrombosis, an effect of the previous VTE may have been measured, as we did not include asymptomatic family members.

In conclusion, the data indicate that a dysbalanced

APC response characterized by increased thrombin formation rates and simultaneously decreased APC formation rates contributes to the increased thrombotic risk of patients with familial thrombosis. Further studies are now warranted to elucidate the pathophysiological and genetic basis of the described phenotype. Moreover, the data show that the SHAPE procedure is a useful tool to measure the functionality of the PC pathway, which is helpful to investigate prothrombotic mechanisms in patients with thrombophilia without an established risk factor.

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