Development of a Factor VII Activating Protease (FSAP) generation assay and its application in studying FSAP in venous thrombosis

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Main text: **3879 words** without legends and references

Highlights

- Development of a robust and highly specific FSAP generation assay (FGA)
- Carriers of the Marburg I-SNP can be easily identified by strongly delayed FSAP generation, low peak FSAP and low FSAP potential
- No significant differences in any FGA parameter exist between individuals with a history of venous thrombosis and a healthy control group
- Excluding putative Marburg I carriers from both groups, delayed FSAP generation significantly correlated with venous thrombosis in post-menopausal women

Abstract

Human genetic studies based on the Marburg I polymorphism in the factor VII activating protease (FSAP) encoding gene, analysis of FSAP activity in plasma and biochemical characterization of FSAP substrates indicate a possible causal link between FSAP activity and venous thrombosis. We hypothesized that a direct standardized assay to measure FSAP activity in plasma could provide convincing arguments for or against such a potential link. Using Ac-*D*Tyr-Lys-Arg-AMC as a highly specific and sensitive substrate, histones as a trigger to activate pro-FSAP and plasma-purified active FSAP as a calibrator, we have developed a fluorogenic kinetic assay that reveals the FSAP generating potential in human plasma in real time. This assay is similar to the thrombin generation assay and allows analysis of lag phase, time to peak and velocity, as well as peak FSAP and the endogenous FSAP potential (EFP) of plasma samples. Carriers of the Marburg I polymorphism showed clearly delayed FSAP generation and lower peak FSAP and EFP level. There were no significant differences in all FSAP activity parameters between plasma from patients with a history of venous thrombosis and controls. When excluding Marburg I carriers, which were evenly distributed between groups, delayed FSAP generation significantly correlated with venous thrombosis in postmenopausal women. The novel FSAP activity assay is robust and easy to perform and will be a useful tool for analyzing plasma FSAP activity also in other pathophysiological conditions.

Keywords: FSAP, HABP2, FSAP generation assay, Marburg I single nucleotide polymorphism, venous thrombosis

Abbreviations:

AMC	aminomethyl coumarin	MI-SNP	Marburg I single nucleotide polymorphism
AUC	area under the curve		
CTI	corn trypsin inhibitor	RFU	relative fluorescence unit
EFP	endogenous FSAP potential	TGA	thrombin generation assay
FG(A)	FSAP generation (assay)	TtPk	time to peak
FSAP	Factor VII Activating Protease	VT	venous thrombosis

Introduction

The factor VII activating protease (FSAP, also known as hyaluronic acid-binding protease or plasma hyaluronan-binding protein PHBP) [1–3] is produced mainly in the liver and is found in circulation in its single chain zymogen form (pro-FSAP). The mean protein concentration in normal human pooled plasma is 12 µg/ml or 200 nmol/l [4]. Activation of pro-FSAP in plasma is triggered by tissue damage and/or inflammation, presumably through the release of histones [5].

Many potential physiologic targets of FSAP in plasma have been described, in particular factors related to hemostasis and inflammation. These include FVII [3], pro-urokinase [6], tissue factor pathway inhibitor [7], fibrinogen [8] and complement proteins [9]. A rare single nucleotide polymorphism (SNP) in the FSAP gene (G534E, Marburg I) with strongly reduced proteolytic activity against small synthetic peptides and physiologic substrates [10,11] is present in 6-9 % of the Caucasian population. This SNP has been associated with carotid stenosis [12], stroke and mortality [13], cardiovascular disease [14] and liver fibrosis [15]. A role of this SNP in venous thrombosis (VT) is still uncertain, because there have been reports on a positive association [16,17], while others failed to do so [18–22]. However, plasma FSAP activity shows large variations [23], and there are also other genetic factors influencing FSAP activity in plasma [24]. Thus, any conclusions about the involvement of FSAP in VT based solely on the analysis of MI-SNP may be misleading. Moreover, there are situations with substantial changes in FSAP antigen and activity levels e.g., trauma [9], stroke [25,26], pregnancy [27] and sepsis [28].

To date, FSAP activity in the plasma has been measured by many groups, including us, using an immune-capture activity assay [4,29,30]. This involves immune-capture of pro-FSAP from plasma, its activation by heparin, and conversion of added pro-uPA to uPA as a readout. As the activity of FSAP in plasma is a function of its concentration, activation state

and ambient inhibitor concentration, this assay has some limitations. Heparin may lead to enhanced inhibition in plasma due to its co-factor effect and the washing steps remove any influence of plasma inhibitors. Pro-uPA activation as readout may not accurately reflect original FSAP activity and there may be immobilization-related artefacts. Moreover, discrepancies in FSAP activity have been observed between a direct immune-capture/chromogenic substrate activity assay and the indirect pro-uPA based activity assay [30]. Measuring FSAP-serpin complexes by ELISA has also been described to assess FSAP activation in plasma [28]. This assay is pre-selective as it covers only FSAP that formed a complex with the respective serpin, thus depending on the levels of serpins present [31]. Moreover, clearance kinetics are altered after complex formation between proteases and inhibitors, which will influence the readout.

We have recently developed a second generation fluorogenic substrate that shows high selectivity, specificity and sensitivity for FSAP [32]. Here, we describe for the first time a kinetic assay that reveals the histone-triggered FSAP generating potential in human plasma in real time, based on the principle of a thrombin generation assay (TGA) [33,34]. Alterations in the scu-PA activating properties of FSAP are not markedly involved in the development of acute deep vein thrombosis [23] . We reinvestigated this point by measuring endogenous FSAP activity in plasma samples from patients with or without a history of venous thrombosis.

Material and Methods

Materials

The fluorogenic substrate Ac-Pro-*D*Tyr-Lys-Arg-AMC (7-amino-4-methylcoumarin) was synthesized at GenScript (Leiden, The Netherlands), free 7-amino-4-methyl coumarin (AMC) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Citrated pooled control plasma ("standard human plasma" SHP), Actin FS and Thromborel were from Siemems Healtheneers (Marburg, Germany). Normal reference plasma (NRP), a normal donor set, and α2 antiplasmin depleted plasma were obtained from Precision Biologic/CoaChrom (Maria Enzersdorf, Austria). Unfractionated calf thymus histones (Type II-A) (Cat. # H9250), unfractionated heparin, polybrene, human thrombin, α2 antiplasmin and PKSI-527 were from Merck (Darmstadt, Germany), tissue plasminogen activator (tPA) was from Technoclone (Vienna, Austria). Corn trypsin inhibitor (CTI) was a gift from G. Praefke, Langen. Aprotinin was from CSL Behring, Marburg, Germany. Two-chain FSAP (tc-FSAP) was prepared as described earlier [2].

Patient population and control subjects

The patient material comprised 50 postmenopausal women with at least one prior venous thromboembolic event (deep vein thrombosis and/or pulmonary embolism), who were included in a randomised clinical trial on the effect of postmenopausal hormonal therapy on the risk of subsequent VT [35]. The controls comprised 50 healthy postmenopausal women without a history of VT, who were included in a randomised clinical study on the effects of different postmenopausal hormonal therapies on biomarkers of activated coagulation [36]. In the present study we used blood samples collected at baseline before the women were given any study drug. Details on blood collection, centrifugation, storage, and clinical data of the cases and the control subjects have been reported earlier [35,36]. Both studies were approved by a Norwegian Regional Committee on Medical and Health Research Ethics and the

Norwegian Medicines Agency. All participants gave their written informed consent to participate. For establishing reference ranges for the assay, standard plasma (n=50) was obtained from Cryocheck/Precision Biologic.

Determination of kinetic parameters K_m, k_{cat} and $k_{cat/Km}$

An in house preparation of tc-FSAP [2] was active site titrated with aprotinin of defined activity, using the chromogenic substrate S-2288 (H-D-lle-Pro-Arg-pNA, Chromogenix, Mölndal, Sweden) under tight-binding inhibition conditions [37] using the GraFit Software package [38]. All fluorescence readings were performed in black, flat-bottom microtiter plates (ThermoFisher Scientific, Carlsbad, CA, USA) in an Infinite 200 fluorescence reader (Tecan, Crailsheim, Germany) at 360 nm excitation and 450 nm emission wavelength, using the Magellan Software package. Kinetic parameters were determined in 100 µl reaction volume in TNB buffer (20 mM Tris, 150 mM NaCl, 1% BSA, pH 7.5) by incubating active-site-titrated tc-FSAP at 5-20 nM with 0.5 to 500 µM of substrate Ac-Pro-*D*Tyr-Lys-Arg-aminomethyl coumarin (AMC). Alternatively, FSAP was activated in 25 % plasma with 25 µg/ml histones in the presence of 0.5 to 500 µM fluorogenic substrate. The K*m* value was calculated using the GraFit Software package. The substrate turnover rate k_{cat} was calculated from the fluorescence signal intensity of the released AMC fluorophore, measured against an AMC calibration curve prepared in 100 µl TNB buffer or in 25 % plasma/25µg/ml histones at identical fluorimeter settings as used in the FGA.

FSAP generation assay (FGA)

FSAP generation (FG) was measured in duplicate wells in a final volume of 100 µl. In a single well, 25 µl of control plasma or test plasma was mixed with 55 µl of TNB buffer and 10 µl of 2 mM fluorogenic substrate Ac-Pro-*D*Tyr-Lys-Arg-AMC, unless indicated otherwise. Prior to adding starting reagent, the endogenous fluorescence of each plasma sample was

recorded for 15 min as a baseline. At t=15 min 10 µl of 0.25 mg/ml histones were added and fluorescence formation was recorded every 30 s for further 75 min. In some experiments, histones were replaced by activators of coagulation, 1:20 diluted Actin FS or 1:20 diluted Thromborel, and 10 mM $CaCl_2$ (end concentrations) or were replaced by 1.5 IU/ml thrombin/10 mM $CaCl_2$/0.68 nM tPA to study fibrinolysis. If other reagents were present, the buffer volume was reduced accordingly.

From the raw data (RFU) the maximum dF/min was calculated within the Magellan software or within a Microsoft Excel spreadsheet. For determining the FGA parameters, the raw data were transferred into an Excel spreadsheet of the Technothrombin® TGA software package (Technoclone, Vienna, Austria) using a 4-point calibration curve of active-site-titrated tc-FSAP (0.78 nM-50 nM), which was included on each plate in duplicate. The following parameters of the FGA were analysed automatically by the software: lag time, time to peak (TtPk), peak FSAP, velocity (peak FSAP/[TtPk-lag time]) and endogenous FSAP potential (EFP, "area under the curve" AUC). Data for lag time and TtPk represent the net time after histone addition. The standard immuno-capture-based FSAP activity assay followed the procedure as previously described [39].

Statistics

Intra-and interassay coefficient of variation was calculated using the SAS®/STAT Software package, Version 9.4. Comparison of FSAP generation in plasma from VT patients and healthy controls was performed by means of non-parametric Hodges-Lehmann estimates for median differences (with 95% Confidence Intervals) and Wilcoxon-Rank-Sum test.

Results

Development of an FSAP generation assay (FGA)

We first optimized the assay conditions with respect to the concentrations of substrate, plasma, activator and cofactor calcium to limit substrate consumption, fluorescence quenching, as well as preventing activation of potentially interfering proteases.

Substrate concentration: FSAP generation (FG) was monitored using a highly selective fluorogenic substrate, Ac-Pro-*D*Tyr-Lys-Arg-AMC [32]. A high affinity of FSAP for this substrate was confirmed by low Km values (purified FSAP: ~10 µM; histone-activated FSAP in 25 % plasma: ~37 µM) and k_{cat} values of ~45 s^{-1} in buffer and ~4 s^{-1} in 25 % plasma (Supplementary data 1). In our studies, in 25 % plasma, a substrate concentration well above K_m was selected (200 µM), showing only minor substrate consumption at the time when peak FSAP was reached (after 7-8 min). The AMC fluorescence curve remained fairly linear up to 50 µM and was similar to the AMC fluororescence in calibrator buffer, limiting the discrepancy between plasma sample and calibrator (see Supplementary data 2 A).

Plasma concentration: The plasma concentration in commercial fluorogenic TGA varies between 67 % and 40 % [33], but also 25 % plasma has been successfully used [40]. Here 25 % plasma was selected, because optimum FSAP activation was seen at this dilution (Figure 1 A), similar to reports that in TGA the highest AUC values were seen at 20 % to 30 % plasma [41]. Except for total height (maximum activity), almost identical FSAP activation and inactivation kinetics (peak form) was seen in 25 % and 50 % plasma (Figure 1 A, right). Thus, the lower plasma concentration was selected, as this also required a smaller sample volume.

Histones: Histones were selected as a trigger in the FGA as they are potent natural activators of pro-FSAP in plasma [5]. Activation followed an optimum curve, reaching the highest level

at 25-50 µg/ml histones (Figure 1 B), a range as reported by others [5,32] In a sub-sampling experiment, where 200 µM fluorogenic substrate was replenished at selected time points, maximum FSAP activation occurred within a few minutes after adding 25 µg/ml histones, followed by rapid inactivation (Figure 1 C). This demonstrated that the rapid loss in the FSAP fluorescence signal was not due to substrate consumption or fluorescence quenching, but the result of enzyme inactivation/ inhibition processes in plasma.

Calcium ions: Calcium ions stabilize FSAP zymogen and enhance the proteolytic activity of active FSAP [42,43], but are also co-factors for coagulation. Here we speculated that low mM level of calcium may be sufficient for FSAP generation, but insufficient to activate coagulation factors. Unexpectedly, histones triggered FSAP generation most effectively already at 0-1 mM of exogenous calcium (Figure 1 D) and calcium could be completely omitted, preventing potential interference by activation of coagulation.

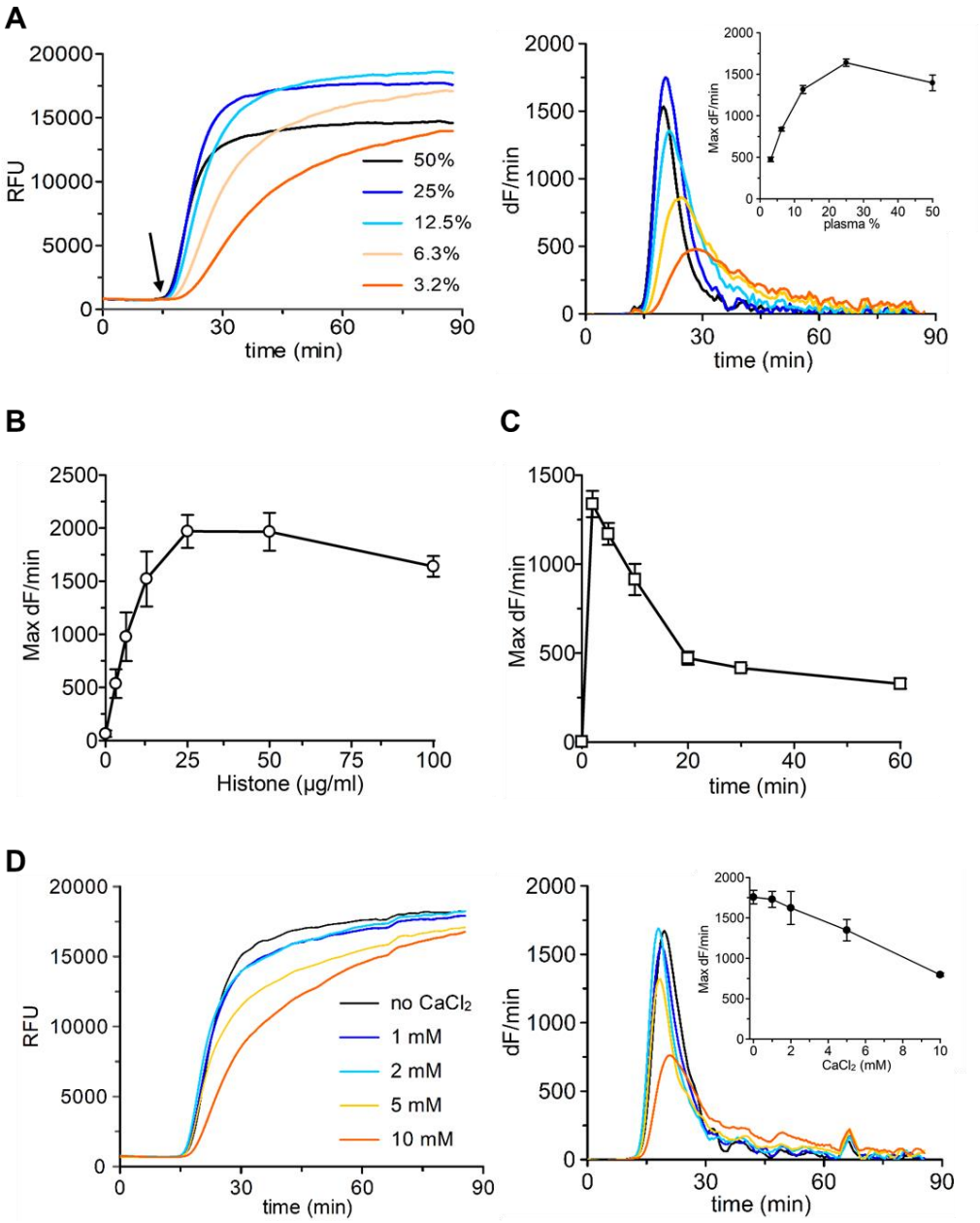


Figure 1: Optimization of FSAP generation assay parameters: **A)** Plasma dilution: FSAP generation was initiated with 25 µg/ml histones at t=15 min (indicated by arrow) in pooled plasma (SHP) at various concentrations. The raw data of a single experiment are shown as relative fluorescence units (RFU, left). Substrate turnover rate per min (dF/min; right) was calculated from the raw data curves in A. The inlet figure shows the maximum rate as function of plasma concentration. **B)** FSAP generation in 25 % SHP as function of histone concentration. **C)** Time curve of FSAP generation, triggered with 25µg/ml histones. Unlike the experiments in Figures A and B, in this sub-sampling experiment substrate was added freshly at each time point. **D)** Calcium-dependence of histone-mediated FSAP generation in 25 % plasma shown as raw data and as dF/min of a single experiment. Histones were added at t = 15 min. Inlet: Maximum rate as function of calcium chloride concentration. Results are representative of 3 independent experiments or are the mean of 3 ± S.D.

Calibrator

A reference standard for FSAP, labelled in activity units, does not exist. Therefore, several alternatives were considered: a plasma-based calibrator, purified tc-FSAP or tc-FSAP/α2-macroglobulin (A2M) complexes as internal calibrator. Preparation of FSAP/A2M complexes was not successful due to instability of the active protease during prolonged incubation with A2M (data not shown). A calibrator based on a normal plasma has a limited range and would require extrapolation and results are not given in active enzyme being generated. We opted for an active-site-titrated purified tc-FSAP (in nM) as a standard (Supplementary data 2 B), in a buffer with a similar dose/response curve of the AMC fluorophore as seen in 25 % plasma (cf. Supplementary data 2 A).

Assay precision

Commercially available citrated pooled plasma (SHP and NRP) served as internal controls on each plate of the FGA (Supplementary data 2 C). Intra-and inter-assay variation of FGA parameter was evaluated based on results of these pooled plasma controls (Table 1). For a graphical description of the different output parameters see Figure 4 A. The overall coefficient of variation was below 14 % and 8 % for the most important parameters peak FSAP and EFP, respectively.

Variation	Lag phase (min)		TtPeak (min)		Velocity (nM/min)		Peak FSAP (nM)		EFP (nM x min)	
	SHP	*NRP*	*SHP*	*NRP*	*SHP*	*NRP*	*SHP*	*NRP*	*SHP*	*NRP*
mean	2.7	2.2	7.4	6.4	6.5	8.2	30.1	33.5	229.5	238.4
Intra-assay % CV	7.8	18.1	7.6	9.0	13.0	13.7	4.5	6.7	2.6	5.5
Inter-assay % CV	7.4	0.0	5.4	7.6	17.6	20.6	12.6	12.0	6.1	4.9
total % CV	**10.8**	**18.1**	**9.3**	**11.8**	**21.9**	**24.8**	**13.4**	**13.8**	**6.7**	**7.4**

Table 1: Intra- and inter-assay variation. Mean results, intra- and inter-assay variability and total variation coefficient in % were determined for each FGA output parameter. Two pooled plasma used as internal controls, SHP and NRP, were measured in 7 independent FGA runs with 8 replicates per run.

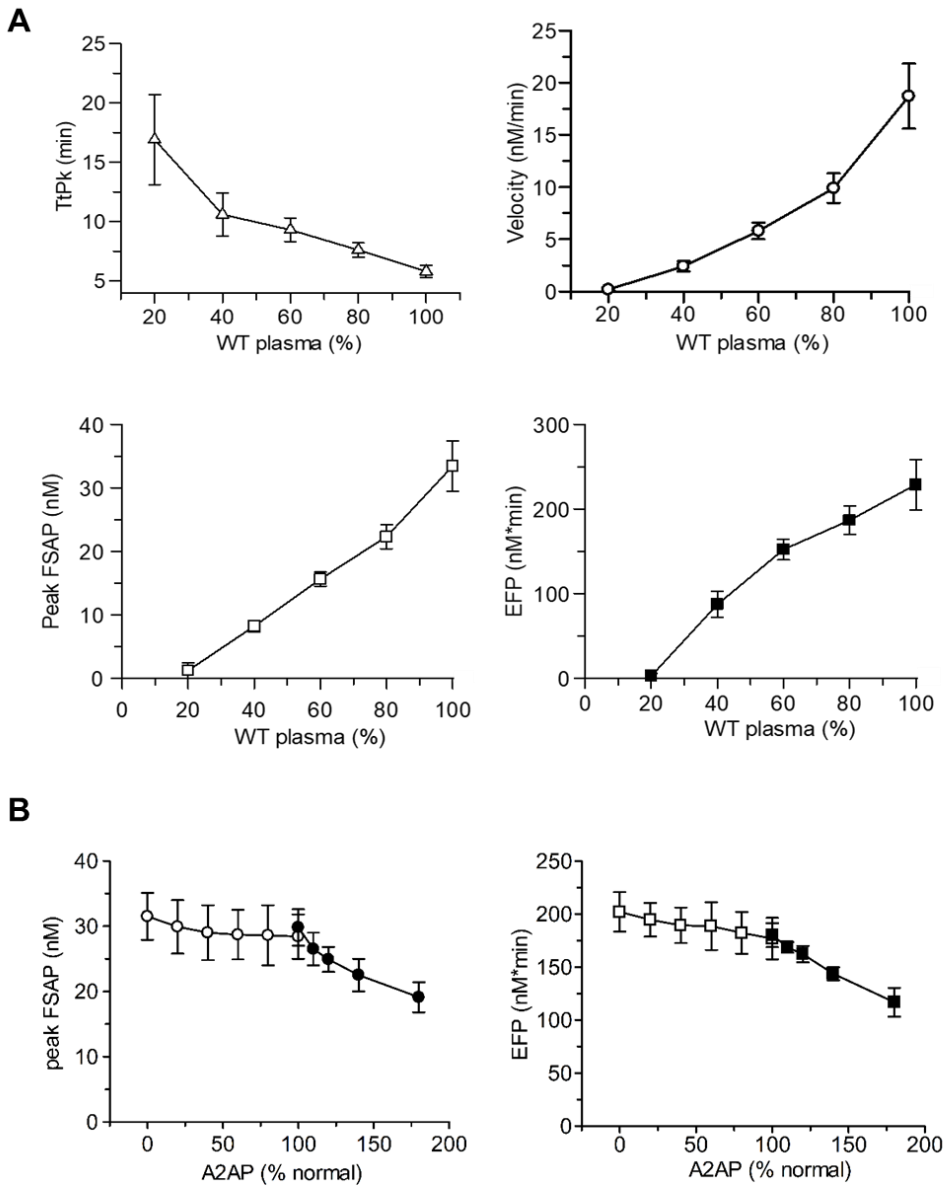


Figure 2: Assay linearity and responsiveness to plasma inhibitors. A) Linearity: Plasma with normal FSAP activity ("wildtype", WT) was diluted into a plasma lacking any functional FSAP mimicking a zymogen dilution series. **B) Responsiveness**: Pooled plasma (SHP) was mixed with α2 antiplasmin depleted plasma (A2AP-DP) (white symbols) in different ratios to attain mixtures with 0-100 % A2AP. SHP was spiked with 0-50 µg/ml A2AP (= 80 % normal) (black symbols) to vary its concentration from 100-180 %. Results for peak FSAP (left panel) and EFP (right panel) are shown as function of A2AP level. Data in A) and B) are the mean ± S.D. of 3 experiments.

Assay linearity

To test assay linearity, plasma with normal FSAP activity was diluted into a plasma devoid of any detectable FSAP activity (Figure 2 A). The time to peak decreased linearly between 40 % and 100 %. Peak FSAP and EFP showed a fairly good linearity between 20 % and 100 % WT ($R^2 > 0.99$ and $R^2 > 0.98$, respectively). Velocity followed rather an exponential function ($R^2 > 0.99$).

Influence of plasma inhibitors

We tested whether the assay would respond to differences in the inhibitor environment, using α2 antiplasmin (A2AP) as example, a high abundant inhibitor of FSAP in plasma (Figure 2 B). Between 0 % and 180 % a continous reduction in peak FSAP and EFP could be observed, more clearly seen at level above 100 % A2AP. This result confirmed the assumption that plasma inhibitor levels will affect FGA results, and peak FSAP results will most likely differ from results in an immune-capture assay.

Assay robustness

The robustness of the FGA with respect to the blood collection and handling procedures was studied on plasma collected from two donors with known high (WT) or low (MI) FSAP activity level (Figure 3 A). Plasma that was frozen at -80°C immediately after blood donation and centrifugation served as control (=100 %). Other conditions tested were freshly donated blood or plasma kept at RT for 3 h before centrifugation and freezing, plasma subjected to two centrifugation steps or undergoing multiple freeze/thaw cycles, and plasma from the same donors stored frozen for more than 1 year. The mean recovery for peak FSAP was 97.3 % ± 6.9 % of control and 100.7 % ± 6.2 % for EFP. This indicated that the FGA is relatively insensitive to various preanalytical conditions, but prolonged storage of plasma at RT before freezing should be avoided.

Next, we tested the assay with respect to heparinized plasma (Figure 3 B). Unfractionated heparin (up to 0.8 IU/ml) increased the histone-triggered FSAP activity by 30-50 % in a concentration-dependent manner in pool plasma (SHP) as well as in plasma with high or low activity (left). Heparin at 1 IU/ml was, however, effectively neutralized in SHP by 6.25 µg/ml polybrene, without detectable effect of polybrene on the FGA in the heparin-free plasma (right).

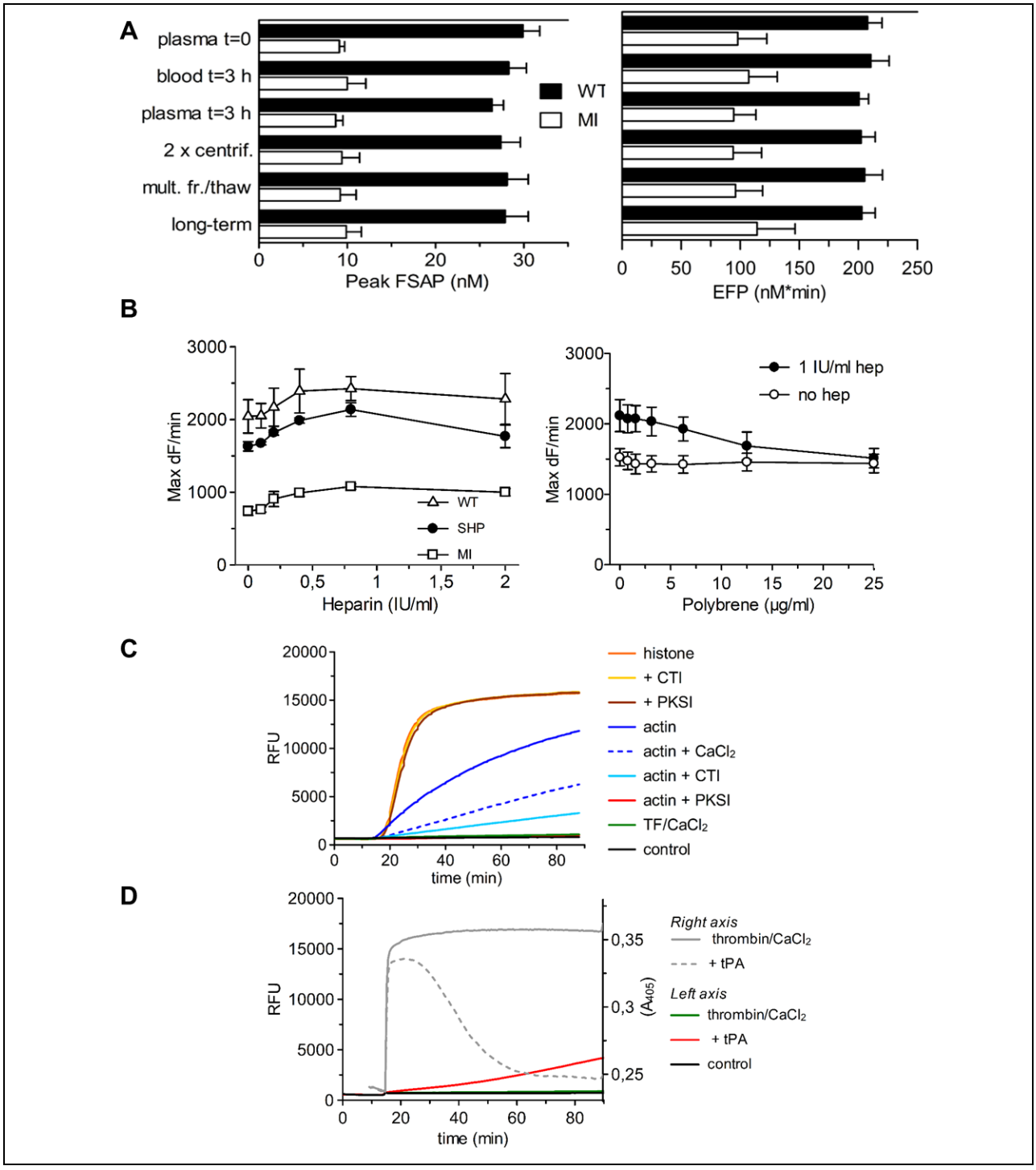


Figure 3: Assay robustness. A) Impact of collection and handling of blood and plasma samples on the FGA. Peak FSAP and EFP data of individual plasma samples collected from a WT (black) and a MI carrier (white) analysed after handling as indicated. Results are the mean ± SD of at least 4 measurements. **B)** FSAP activity in heparinized plasma. Left: The influence of unfractionated heparin (concentration in undiluted plasma) on FG was studied in a pooled plasma (SHP), plasma of a heterozygous MI carrier and a homozygous WT carrier. Right: Neutralisation of 1 IU/ml heparin in SHP by polybrene. Data are the mean ± SD of 3 experiments.
C) Fluorescence formation after activation of intrinsic and extrinsic coagulation. Cleavage of substrate Ac-DTyr-Lys-Arg-AMC in 25 % plasma during contact activation by ellagic acid/phospholipids (1:20 aPTT reagent Actin SL) with/without 10 mM calcium chloride, 1 µg/ml FXIIa inhibitor CTI or 150 µM of plasma kallikrein inhibitor PKSI-527. Extrinsic coagulation was triggered by tissue factor/$CaCl_2$ (1:20 Thromborel S). Histone-triggered FSAP generation is shown in the absence and presence of CTI and PKSI-527, respectively. **D)** Fluorescence formation during fibrinolysis. Thrombin (1.5 IU/ml) and 10 mM $CaCl_2$ with or without 0.68 nM tPA was added to 25 % SHP. An overlay figure of fluorescence (left axis, coloured lines) and turbidity in a clot lysis assay, performed as described in [8], is shown (right axis, grey lines), measured in parallel plates as RFU and absorbance, respectively. Raw data curves in C) and D) are shown as mean curves of at least 3 independent experiments.

Further we investigated how procoagulant or fibrinolytic activity in a test plasma could affect the outcome of the FGA. First, histones were replaced by an aPTT reagent (ActinSL, with and without $CaCl_2$) to trigger intrinsic coagulation, or by tissue factor/$CaCl_2$ (Thromborel) to initiate the extrinsic pathway (Figure 3 C). The aPTT reagent in the absence of $CaCl_2$ caused an increase in RFU, which was strongly reduced by the FXIIa inhibitor corn trypsin inhibitor (CTI) and completely prevented by the plasma kallikrein inhibitor PKSI-527. In the presence of 10 mM $CaCl_2$ the effect of aPTT reagent was weaker and was not seen anymore in the presence of CTI and PKSI-527, respectively (not shown). These results verify earlier reports that plasma kallikrein and FXIIa have some specificity for the fluorogenic substrate [32]. In contrast, CTI and PKSI-527 had no impact on the fluorescence curve in the

histone-triggered FSAP generation, confirming that no background FXIIa or plasma kallikrein activity interfere with the assay. Activation of extrinsic coagulation with TF/$CaCl_2$ (Thromborel) caused only a very weak cleavage of the fluorogenic substrate.

Next, we studied the effect of fibrinolytic enzymes on the FGA (Figure 3 D). Thrombin/$CaCl_2$ generated only a minor fluorescence signal, similar to TF/$CaCl_2$ above, and caused immediate coagulation in a clot lysis assay performed in parallel (solid grey line). When exogenous tPA (680 pM) was added simultaneously, clot lysis (dotted grey line) and increasing fluorescence was seen. Most likely fluorescence was generated by both tPA and plasmin, because both show some cleavage of the substrate [32]. Taken together, in the histone-triggered FGA, fluorescence formation by activation of coagulation or fibrinolysis is unlikely and underlines the high specificity of this assay.

Marburg I polymorphism and FSAP generation

Plasma from genotyped donors served to verify the assay. A clear difference between genotypes Marburg I (MI) and "wild type" (WT) could already be seen in the raw data curves (RFU) and in the 1st derivative dF/min (Supplementary data 3). The different FGA analytical parameters are explained in Figure 4 A. In all output parameters, the MI plasma was clearly different to WT plasma, with a prolonged lag phase and TtPk, a much lower velocity in the "burst phase" and very low peak FSAP and EFP (Figure 4 B-F). In plasma with a known non-functional FSAP [8] (sample "MI 2" in Supplementary data 3), or in FSAP-depleted plasma (data not shown), no background fluorescence was generated, underlining the high selectivity of the assay.

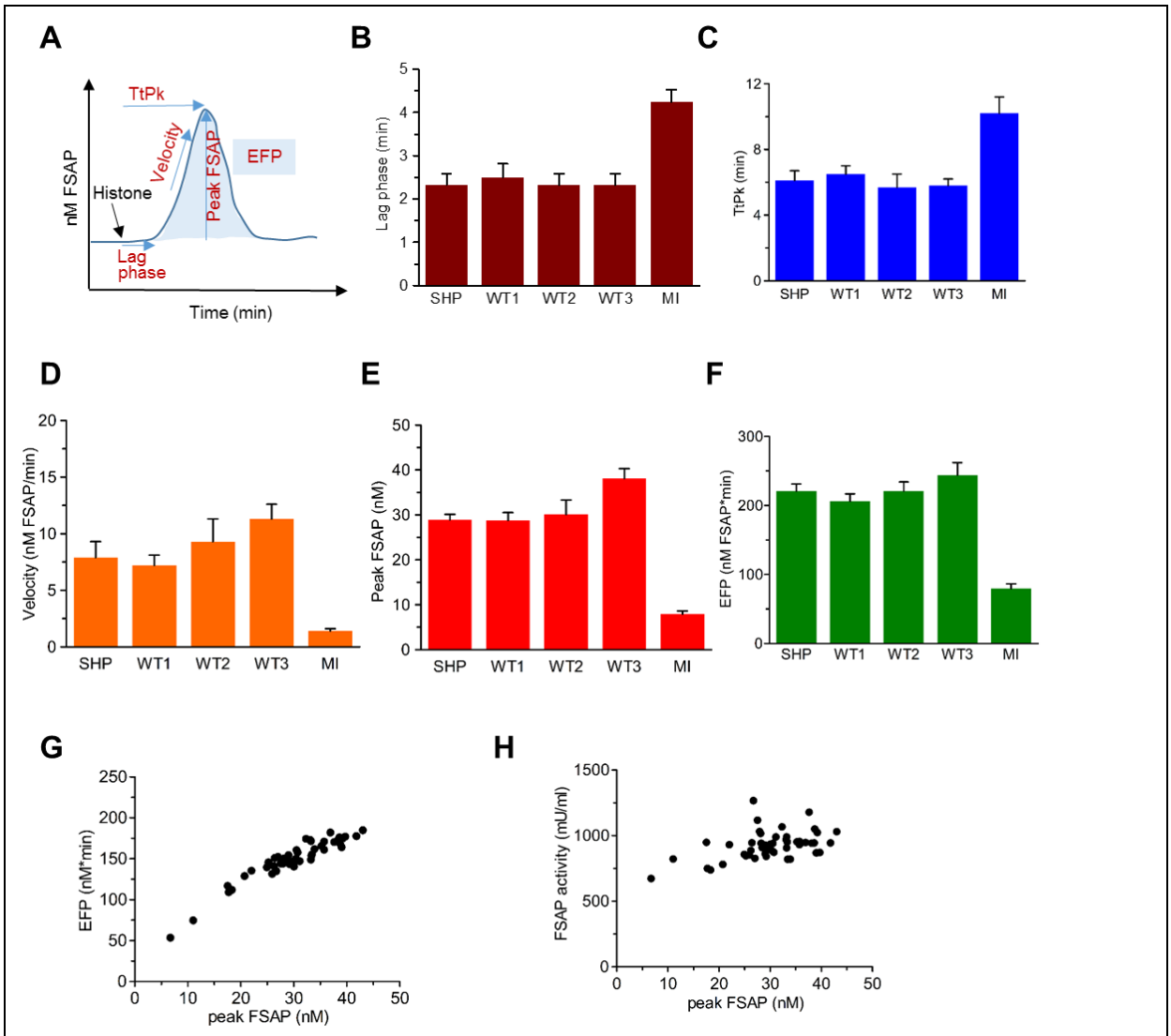


Figure 4: FGA results of individual genotyped plasma and of normal donors. A) Graphic presentation of individual FGA parameters. **B)-F)** The lag phase, the time to peak (TtPk), the velocity of FSAP generation during the exponential phase, peak FSAP and accumulated FSAP activity over time (endogenous FSAP potential, EFP; "area under the curve") of a citrated pooled plasma (SHP) and of plasma from genotyped individuals. Data are the mean ± S.D. of 3 independent experiments. **G) Peak FSAP and FSAP potential in healthy donors.** The results of peak FSAP and EFP in plasma from 25 male and 25 female healthy donors (age 18-66) were plotted against each other. Data are the mean of at least 4 measurements in duplicate. **H) FSAP activity by standard assay plotted versus peak FSAP.** FSAP activity in normal donors was measured in an immunocapture/pro-urokinase activation assay [39], using NRP as reference (with an activity of 1000 mU/ml per definition). Activity results were plotted against the peak FSAP results shown in Figure 4 G. FSAP activity was measured in duplicate.

Normal reference interval

Peak FSAP and EFP results as well as FSAP activity results by a standard immune-capture-based assay were determined in normal healthy donors and reference intervals (5 %-95 % percentile) were calculated (Table 2). Considering that a normal pooled plasma contains 12 µg/ml or 200 nM pro-FSAP zymogen [4], the median peak FSAP result of 30 nM indicated that in average about 60 % of the zymogen becomes activated by histones. Comparing male and female donors (Table 2), earlier reports of higher FSAP activity level in female donors [4] is also seen in this healthy donor set using the FGA.The individual level of peak FSAP and accumulated activity (EFP) were highly correlated (R=0.95) (Figure 4 G). Comparing peak FSAP to activity measured in an immune-capture based assay (Figure 4 H and Table 2), some correlation was seen (R=0.47), but a much wider reference interval was found for peak FSAP. This reflects most likely the impact of the individual plasma environment in the FGA that is not seen in a standard immune-capture based assay.

Normal donors	N	Median			Reference Interval (5%-95% percentile)		
		Peak FSAP (nM)	EFP (nM*min)	FSAP activity (mU/ml)	Peak FSAP (nM)	EFP (nM*min)	FSAP activity (mU/ml)
female	25	31,2	149,5	944,4	22,1 - 43,1	134,5 - 184,4	852,8 - 1175,1
male	25	29,3	150,5	902,7	17,6 - 38,8	108,7 - 175,6	746,7 - 1046,7
all	50	30,3	150,3	929,1	17,6 - 41,9	108,7 - 181,5	746,7 - 1113,5

Table 2: Reference interval in adult healthy donors. Peak FSAP, EFP and FSAP activity by standard assay were determined in 25 female and 25 male healthy donors. Peak FSAP and EFP are derived from 4 independent measurements in duplicate wells, FSAP activity data are based on a duplicate measurement.

FSAP generation in venous thrombosis samples

The suitability of the FGA was tested in a pilot study on clinical specimen. In plasma samples of female patients with a history of venous thrombosis (VT) and a female healthy control group we evaluated the differences between groups. The FGA results showed a wide

distribution in all parameters in both groups (Figure 5 A-E), and there were only weak differences in FGA parameter between both groups. The lag phase and the TtPk seem to be slightly delayed in the samples from the venous thrombosis patients and the velocity was lower. The peak FSAP and EFP values are slightly higher in the control group compared to venous thrombosis patients, but these differences were all non-significant (Table 3, left). Normalization of the FGA test results of individual samples to the results of the control plasma SHP run on the same plate (and calculated as % control) reduced variability in some parameter but did not alter the outcome between groups (data not shown).

Plotting EFP versus peak FSAP for all tested samples (Figure 5 F) showed a high correlation (R=0.92), as also seen in normal donors. Eleven samples (VT 6, control 5) were clearly separated due to a very low peak FSAP (circled). Most likely, these were plasma samples from carrier of the MI polymorphism, although their genotype was not determined during this study. These samples with low peak FSAP were less clearly separated by their EFP. Possibly, FSAP activity at lower level may still accumulate over time, leading to a relatively higher EFP.

Since participants with very low FSAP activity were evenly distributed between groups, this may influence the significance of difference between groups. After excluding in both groups participants with very low peak FSAP, the difference between the VT and control group (Table 3, right) became significant for the parameters TtPk and velocity, indicating a delayed activation of FSAP in the VT group, also reflected in peak FSAP, which was lower in VT samples, borderline to significant.

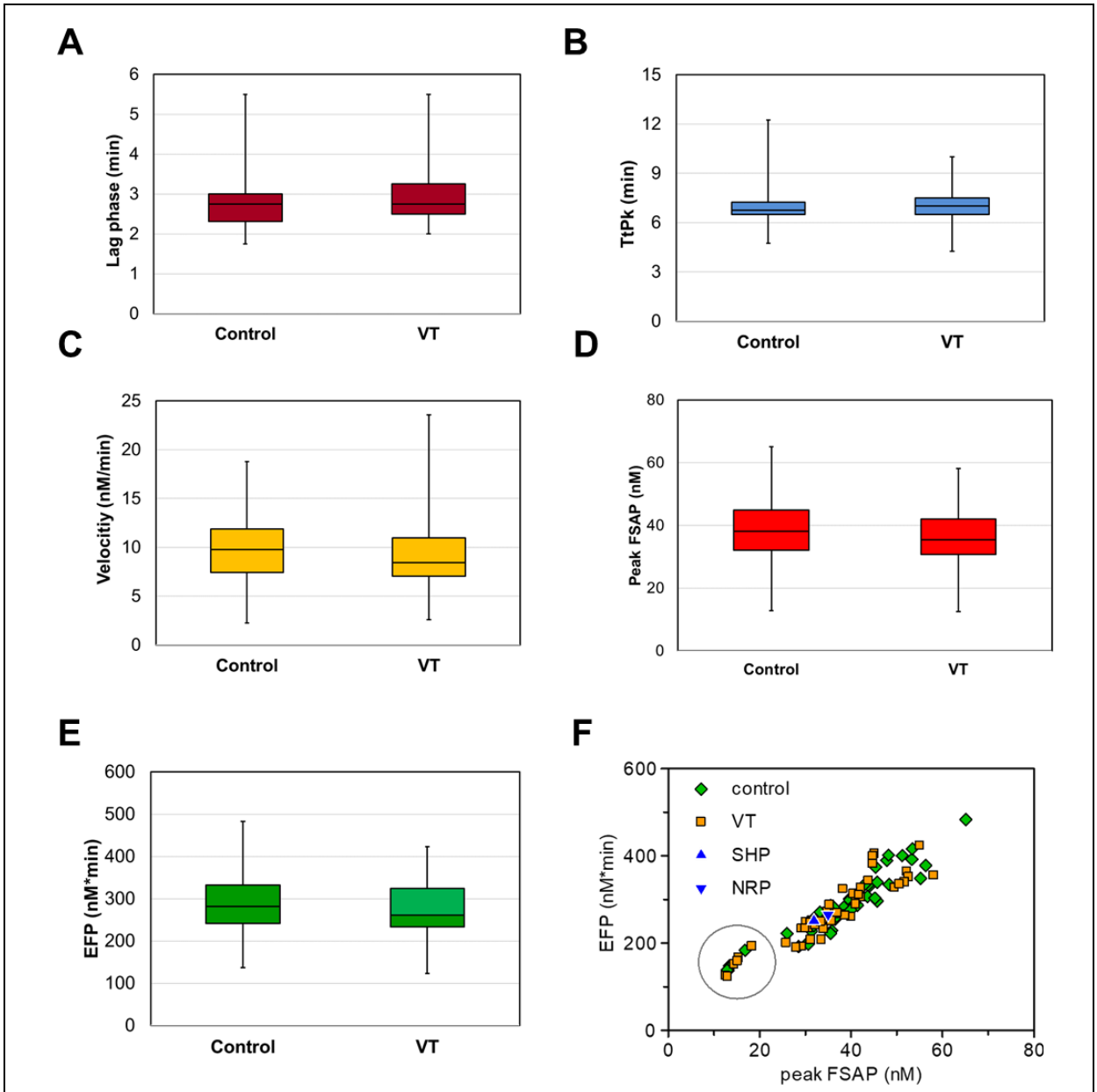


Figure 5: Comparison of FSAP generation in plasma from VT patients and healthy controls.

A-E) Boxplot presentation of all FGA parameters of 50 female VT patients and 50 female healthy controls. For all test parameters, the difference between groups was not significant. **F)** EFP was plotted versus peak FSAP for all tested plasma samples and for the two pooled plasma controls. Putative MI-carrier with low peak FSAP are encircled.

	including carrier of Marburg I Mutation					**excluding** carrier of Marburg I Mutation				
Parameter	N[1]	Median difference[2]	95% Confidence Interval[2]		p value[3]	N[1]	Median difference[2]	95% Confidence Interval[2]		p value[3]
lag phase	50 / 50	0.00	-0.25	0.25	**0.7237**	44 / 45	0.25	0.00	0.50	**0.0987**
TtPK	50 / 50	0.13	-0.25	0.50	**0.2917**	44 / 45	0.38	0.00	0.75	**0.0259***
velocity	50 / 50	-0.87	-2.21	0.46	**0.2412**	44 / 45	-1.51	-2.81	-0.21	**0.0270***
peak FSAP	50 / 50	-2.04	-5.94	1.86	**0.2916**	44 / 45	-3.98	-7.96	0.01	**0.0530**
EFP	50 / 50	-13.35	-42.18	15.48	**0.3782**	44 / 45	-24.78	-53.31	3.75	**0.0861**

1 = Sample size VT / Control, 2 = Hodges-Lehmann Estimate and 95% Confidence Interval,
3 = Wilcoxon Rank Sum test

Table 3: Comparison between groups. Data were analysed by including or excluding individuals with very low level of FSAP (presumably carrier of the MI-SNP).* p values < 0.05 were considered significant.

Discussion

Current assays to measure FSAP activity in plasma have a number of limitations and are cumbersome to perform. This has hampered the measurement of FSAP activity in various pathophysiological conditions to determine the functions of FSAP. To better understand the life cycle of active FSAP in plasma, a new assay was developed to cover the process from activation to inactivation in the solution phase. We adapted the principle of thrombin generation in an FSAP generation assay, using a highly selective fluorogenic substrate, histones as activators and an active site-titrated purified tc-FSAP preparation as calibrator. This assay will enable measurements of FSAP activity in plasma in the wider scientific community and speed up its analysis in different disease conditions.

The assay requires purified FSAP as calibrator but no antibodies or any other speciality reagents apart from commercially available histones and fluorogenic substrate. With this FGA, in a one-step assay design, the contribution of FSAP antigen, sequence variations and plasma anti-FSAP activities are taken into account and various aspects of FSAP activity in plasma are captured in a robust and reproducible manner. Putative carrier of the MI SNP can be easily identified. Some limitations may apply to this assay, e.g. under conditions where endogenous histones are released and lead to activation of pro-FSAP [5]. The histone-triggered FGA may only detect residual non-activated FSAP, and measuring, in addition, FSAP-inhibitor complexes in plasma may be a useful complementary approach.

General limitations of fluorescence-based thrombin generation assays are a non-linear dose-response curve of the fluorescence signal due to substrate consumption and fluorescence quenching at higher fluorescence signals and by the plasma matrix itself (inner filter effect) [34]. To overcome these limitations during thrombin generation, complex mathematical correction models and/or an internal alpha2-macroglobulin/thrombin calibrator are used [44]. Here, a higher diluted plasma and a lower substrate concentration were chosen that limited substantial fluorescence formation, with low risk of underestimation of the real FSAP activity

level. The accumulated FSAP activity over time, measured as EFP, may somewhat underestimate the total level of FSAP being generated, due to fluorescence quenching. The development of an FSAP- A2M complex as calibrator could help to account for this and may improve the assay in future studies.

For a larger number of individual donors, peak FSAP and EFP values were well above 100 % relative to pooled plasma. This most likely reflects the fact that a standard pool plasma is collected from qualified healthy donors of both sexes and of a wide age group and is industrially manufactured, including freeze-drying in the case of SHP. In the VT study only post-menopausal women were included, and FSAP antigen and activity level are higher in female compared to male donors [4]. Slightly higher peak FSAP levels in females were also seen in the normal donor group.

Current data on the role of the Marburg I polymorphism in venous thrombosis are inconclusive. Using the FGA on clinical samples, a high variability in peak FSAP and EFP was seen among individuals, with only a weak, but non-significant overall difference between groups. Our results suggest that in postmenopausal women a very low FSAP potential, as caused e.g. by the MI SNP, is not a predeterminant for a thrombotic risk. However, when excluding putative MI carrier from both study groups, a direct link of low FSAP generation was seen in plasma of post-menopausal women with a history of venous thrombosis. Whether the observed difference is clinically relevant is uncertain, but may provide an explanation why it has been difficult in the past to find a clear consensus on the role of the Marburg I genotype of FSAP in venous thrombosis.

Acknowledgements

We would like to thank Nicole Beer for her excellent technical assistance and Gerrit Praefke for providing reagents.

Author contributions

ME supervised all the experiments, KMH performed the statistical analysis. PMS provided the samples from patients with venous thrombosis and respective controls and analysed the data. ME and SMK designed the study, analysed the data and co-wrote the manuscript. All authors edited the final version of the manuscript.

Disclosure of conflict

The authors declare that they have no conflicts of interest with the contents of this article.

Funding

This work was supported in part by grants from Helse Sør-Øst, Norway [201311] and the Research Council of Norway [251239].

References

[1] N.H. Choi-Miura, T. Tobe, J. Sumiya, Y. Nakano, Y. Sano, T. Mazda, M. Tomita, Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a kringle and a serine protease domain, similar to hepatocyte growth factor activator, Journal of Biochemistry 119 (1996) 1157–1165.

[2] A. Hunfeld, M. Etscheid, H. König, R. Seitz, J. Dodt, Detection of a novel plasma serine protease during purification of vitamin K-dependent coagulation factors, FEBS Letters 456 (1999) 290–294.

[3] J. Römisch, A. Feußner, S. Vermöhlen, H.-A. Stöhr, A protease isolated from human plasma activating factor VII independent of tissue factor, Blood Coagulation and Fibrinolysis 10 (1999) 471–479.

[4] J. Römisch, A. Feussner, H.-A. Stöhr, Quantification of the factor VII-and single chain plasminogen activator-activating protease in plasmas of healthy subjects, Blood Coagulation and Fibrinolysis 12 (2001) 375–383.

[5] S. Yamamichi, Y. Fujiwara, T. Kikuchi, M. Nishitani, Y. Matsushita, K. Hasumi, Extracellular histone induces plasma hyaluronan-binding protein (factor VII activating protease) activation in vivo, Biochem.Biophys.Res.Commun. 409 (2011) 483–488.

[6] J. Römisch, S. Vermöhlen, A. Feußner, H.-A. Stöhr, The FVII activating protease cleaves single-chain plasminogen activators, Haemostasis 29 (2000) 292–299.

[7] S.M. Kanse, P.J. Declerck, W. Ruf, G. Broze, M. Etscheid, Factor VII-Activating Protease Promotes the Proteolysis and Inhibition of Tissue Factor Pathway Inhibitor, Arterioscler.Thromb.Vasc.Biol. 32 (2012) 427–433.

[8] M. Etscheid, S. Subramaniam, G. Lochnit, M. Zabczyk, A. Undas, I.M. Lang, K.-M. Hanschmann, S.M. Kanse, Altered structure and function of fibrinogen after cleavage by Factor VII Activating Protease (FSAP), Biochim. Biophys. Acta Mol. Basis Dis. 1864 (2018) 3397–3406. https://doi.org/10.1016/j.bbadis.2018.07.030.

[9] S.M. Kanse, A. Gallenmueller, S. Zeerleder, F. Stephan, O. Rannou, S. Denk, M. Etscheid, G. Lochnit, M. Krueger, M. Huber-Lang, Factor VII-activating protease is activated in multiple trauma patients and generates anaphylatoxin C5a, J. Immunol. 188 (2012) 2858–2865. https://doi.org/10.4049/jimmunol.1103029.

[10] J. Römisch, A. Feussner, C. Nerlich, H.A. Stoehr, T. Weimer, The frequentMarburg I polymorphism impairs the pro-urokinase activating potency of the factor VII activating protease (FSAP), Blood Coagul.Fibrinolysis 13 (2002) 433–441.

[11] M. Etscheid, L. Muhl, D. Pons, J.W. Jukema, H. König, S.M. Kanse, The Marburg I polymorphism of factor VII activating protease is associated with low proteolytic and low pro-coagulant activity, Thrombosis Research 130 (2012) 935–941. https://doi.org/10.1016/j.thromres.2012.07.023.

[12] J. Willeit, S. Kiechl, T. Weimer, A. Mair, P. Santer, C.J. Wiedermann, J. Roemisch, Marburg I polymorphism of factor VII--activating protease: a prominent risk predictor of carotid stenosis, Circulation 107 (2003) 667–670.

[13] S. Trompet, D. Pons, S.M. Kanse, A.J. de Craen, M.A. Ikram, J.J. Verschuren, A.H. Zwinderman, P.A. Doevendans, R.A. Tio, R.J. de Winter, P.E. Slagboom, R.G. Westendorp, J.W. Jukema, Factor VII Activating Protease Polymorphism (G534E) Is Associated with Increased Risk for Stroke and Mortality, Stroke Res.Treat. 2011 (2011) 424759.

[14] H. Ireland, G.J. Miller, K.E. Webb, J.A. Cooper, S.E. Humphries, The factor VII activating protease G511E (Marburg) variant and cardiovascular risk, Thromb.Haemost. 92 (2004) 986–992.

[15] H.E. Wasmuth, C.G. Tag, L.E. van de, C. Hellerbrand, T. Mueller, T. Berg, G. Puhl, P. Neuhaus, D. Samuel, C. Trautwein, S.M. Kanse, R. Weiskirchen, The Marburg I variant (G534E) of the factor VII-activating protease determines liver fibrosis in hepatitis C

infection by reduced proteolysis of platelet-derived growth factor BB, Hepatology 49 (2009) 775–780.

[16] P. Ahmad-Nejad, C.E. Dempfle, C. Weiss, P. Bugert, M. Borggrefe, M. Neumaier, The G534E-polymorphism of the gene encoding the Factor VII-activating protease is a risk factor for venous thrombosis and recurrent events, Thromb.Res. 130 (2012) 441–444.

[17] B. Hoppe, F. Tolou, H. Radtke, H. Kiesewetter, T. Dorner, A. Salama, Marburg I polymorphism of factor VII-activating protease is associated with idiopathic venous thromboembolism, Blood 105 (2005) 1549–1551.

[18] R. van Minkelen, M.C. de Visser, H.L. Vos, R.M. Bertina, F.R. Rosendaal, The Marburg I polymorphism of factor VII-activating protease is not associated with venous thrombosis, Blood 105 (2005) 4898.

[19] F. Franchi, I. Martinelli, E. Biguzzi, P. Bucciarelli, P.M. Mannucci, Marburg I polymorphism of factor VII-activating protease and risk of venous thromboembolism, Blood 107 (2006) 1731. https://doi.org/10.1182/blood-2005-09-3603.

[20] T. Gulesserian, G. Hron, G. Endler, S. Eichinger, O. Wagner, P.A. Kyrle, Marburg I polymorphism of factor VII-activating protease and risk of recurrent venous thromboembolism, Thromb.Haemost. 95 (2006) 65–67.

[21] V. Weisbach, R. Ruppel, R. Eckstein, The Marburg I polymorphism of factor VII-activating protease and the risk of venous thromboembolism, Thromb.Haemost. 97 (2007) 870–872.

[22] N.M. Pecheniuk, D.J. Elias, X. Xu, J.H. Griffin, Failure to validate association of gene polymorphisms in EPCR, PAR-1, FSAP and protein S Tokushima with venous thromboembolism among Californians of European ancestry, Thromb.Haemost. 99 (2008) 453–455.

[23] J.J. Sidelmann, F. Vitzthum, E. Funding, A.M. Munster, J. Gram, J. Jespersen, Factor VII-activating protease in patients with acute deep venous thrombosis, Thromb.Res. 122 (2008) 848–853.

[24] M. Olsson, T.M. Stanne, A. Pedersen, E. Lorentzen, E. Kara, A. Martinez-Palacian, N.P. Rønnow Sand, A.F. Jacobsen, P.M. Sandset, J.J. Sidelmann, G. Engström, O. Melander, S.M. Kanse, C. Jern, Genome-wide analysis of genetic determinants of circulating factor VII-activating protease (FSAP) activity, J. Thromb. Haemost. 16 (2018) 2024–2034. https://doi.org/10.1111/jth.14258.

[25] A.U. Joshi, C. Orset, B. Engelhardt, E. Baumgart-Vogt, T. Gerriets, D. Vivien, S.M. Kanse, Deficiency of Factor VII activating protease alters the outcome of ischemic stroke in mice, European Journal of Neuroscience (2015) 965–975. https://doi.org/10.1111/ejn.12830.

[26] D.-S. Tian, C. Qin, L.-Q. Zhou, S. Yang, M. Chen, J. Xiao, K. Shang, D.B. Bosco, L.-J. Wu, W. Wang, FSAP aggravated endothelial dysfunction and neurological deficits in acute ischemic stroke due to large vessel occlusion, Signal Transduct. Target. Ther. 7 (2022) 6. https://doi.org/10.1038/s41392-021-00802-1.

[27] M.S. Parahuleva, N. Ball, B. Parviz, D. Zandt, Y. Abdallah, H. Tillmanns, H. Hoelschermann, S.M. Kanse, Factor seven activating protease (FSAP) expression in human placenta and its role in trophoblast migration, Eur. J. Obstet. Gynecol. Reprod. Biol. 167 (2013) 34–40. https://doi.org/10.1016/j.ejogrb.2012.10.035.

[28] F. Stephan, J.A. Hazelzet, I. Bulder, M.A. Boermeester, J.O. van Till, T. van der Poll, W.A. Wuillemin, L.A. Aarden, S. Zeerleder, Activation of factor VII-activating protease in human inflammation: a sensor for cell death, Crit Care 15 (2011) R110.

[29] S. Stephan, H. Schwarz, A. Borchert, D. Bussfeld, E. Quak, B. Simshaeuser-Knaub, S. Teigelkamp, F. Behrens, F. Vitzthum, Tests for the measurement of factor VII-activating protease (FSAP) activity and antigen levels in citrated plasma, their correlation to PCR

testing, and utility for the detection of the Marburg I-polymorphism of FSAP, Clin.Chem.Lab Med. 46 (2008) 1109–1116.

[30] S. Stephan, H. Schwarz, A. Haude-Barten, J.J. Sidelmann, B. Fischer, H. Althaus, M. Hahn, A. Kappel, M. Ehm, F. Vitzthum, Direct chromogenic substrate immuno-capture activity assay for testing of factor VII-activating protease, Clin. Chem. Lab. Med. 49 (2011) 1199–1204. https://doi.org/10.1515/CCLM.2011.199.

[31] O.R. Gramstad, S.P.S. Kandanur, M. Etscheid, E.W. Nielsen, S.M. Kanse, Factor VII activating protease (FSAP) is not essential in the pathophysiology of angioedema in patients with C1 inhibitor deficiency, Mol. Immunol. 142 (2022) 95–104. https://doi.org/10.1016/j.molimm.2021.11.019.

[32] W. Rut, N.V. Nielsen, J. Czarna, M. Poreba, S.M. Kanse, M. Drag, Fluorescent activity-based probe for the selective detection of Factor VII activating protease (FSAP) in human plasma, Thrombosis Research 182 (2019) 124–132. https://doi.org/10.1016/j.thromres.2019.08.016.

[33] J. Kintigh, P. Monagle, V. Ignjatovic, A review of commercially available thrombin generation assays, Res. Pract. Thromb. Haemost. 2 (2018) 42–48. https://doi.org/10.1002/rth2.12048.

[34] H.C. Hemker, P. Giesen, R. Al Dieri, V. Regnault, E. de Smedt, R. Wagenvoord, T. Lecompte, S. Béguin, Calibrated automated thrombin generation measurement in clotting plasma, Pathophysiol. Haemost. Thromb. 33 (2003) 4–15. https://doi.org/10.1159/000071636.

[35] E. Høibraaten, E. Qvigstad, H. Arnesen, S. Larsen, E. Wickstrøm, P.M. Sandset, Increased risk of recurrent venous thromboembolism during hormone replacement therapy--results of the randomized, double-blind, placebo-controlled estrogen in venous thromboembolism trial (EVTET), Thrombosis and Haemostasis 84 (2000) 961–967.

[36] A.L. Eilertsen, L. Sandvik, M.C. Mowinckel, T.O. Andersen, E. Qvigstad, P.M. Sandset, Differential effects of conventional and low dose oral hormone therapy (HT), tibolone, and raloxifene on coagulation and fibrinolysis, Thrombosis Research 120 (2007) 371–379. https://doi.org/10.1016/j.thromres.2006.10.013.

[37] J.F. Morrison, Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors, Biochim.Biophys.Acta 185 (1969) 269–286. https://doi.org/10.1016/0005-2744(69)90420-3.

[38] R.J. Leatherbarrow, GraFit Version 7, Erithacus Software, Horley, U.K., 2009.

[39] E. Hanson, S.M. Kanse, A. Joshi, K. Jood, S. Nilsson, C. Blomstrand, C. Jern, Plasma factor VII-activating protease antigen levels and activity are increased in ischemic stroke, J. Thromb. Haemost. 10 (2012) 848–856. https://doi.org/10.1111/j.1538-7836.2012.04692.x.

[40] H.J. Tapp, C. Grundmann, M. Kusch, H. König, Calibrating thrombin generation in different samples: Less effort with a less efficient substrate., The Open Atherosclerosis & Thrombosis Journal (2009) 6–11.

[41] W.L. Chandler, M. Roshal, Optimization of plasma fluorogenic thrombin-generation assays, Am. J. Clin. Pathol. 132 (2009) 169–179. https://doi.org/10.1309/AJCP6AY4HTRAAJFQ.

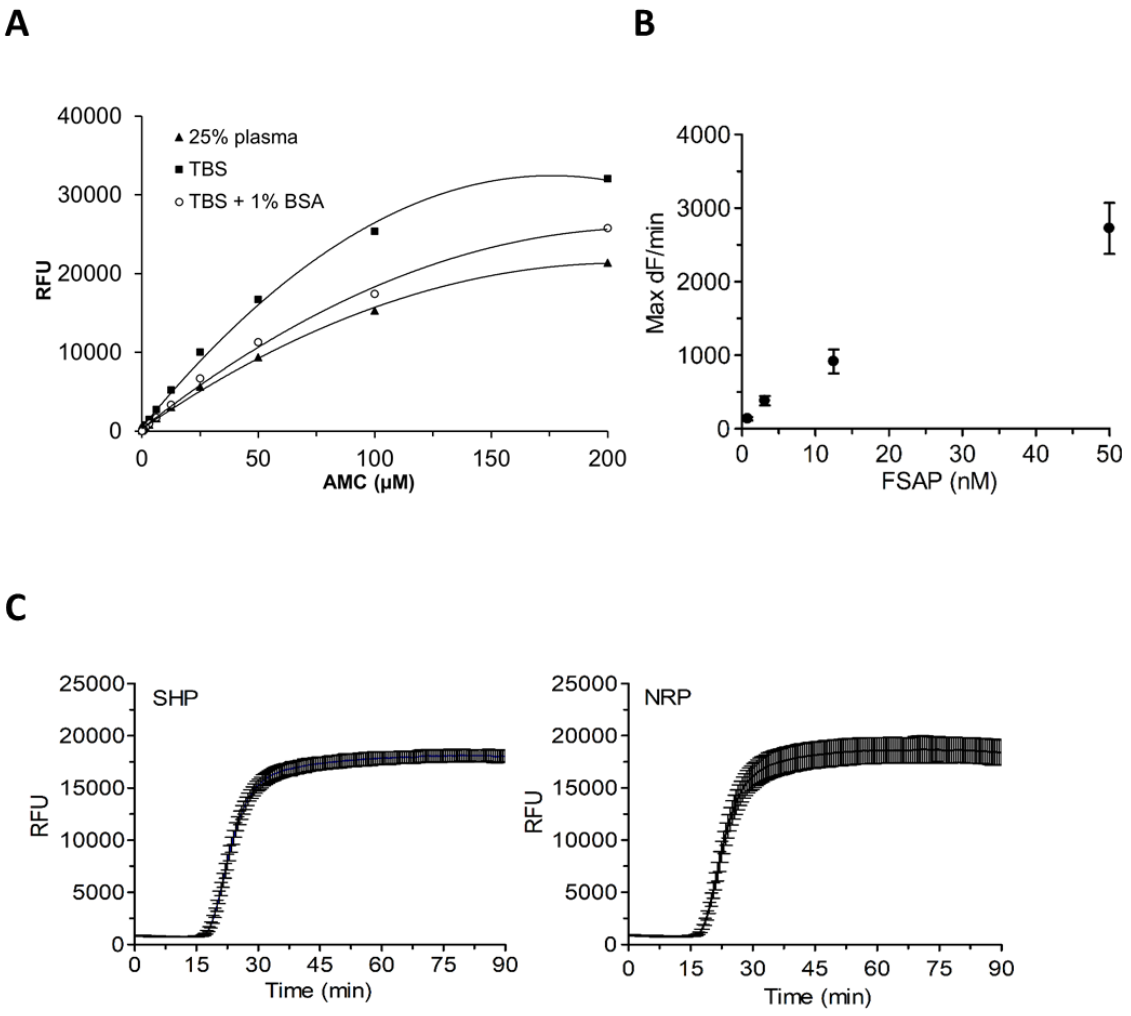
[42] N.V. Nielsen, E. Roedel, D. Manna, M. Etscheid, J.P. Morth, S.M. Kanse, Characterization of the enzymatic activity of the serine protease domain of Factor VII activating protease (FSAP), Sci. Rep. 9 (2019) 18990. https://doi.org/10.1038/s41598-019-55531-x.

[43] F. Stavenuiter, I. Dienava-Verdoold, M.G. Boon-Spijker, H.J. Brinkman, A.B. Meijer, K. Mertens, Factor Seven Activating Protease (FSAP): does it Activate Factor VII?, J. Thromb. Haemost. 10 (2012) 859–866.

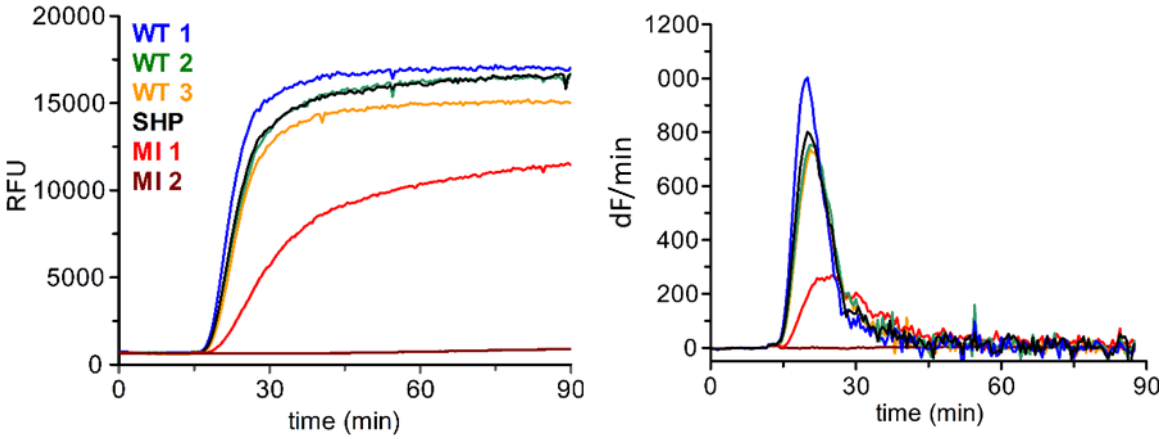
[44] H.C. Hemker, R. Kremers, Data management in thrombin generation, Thrombosis Research 131 (2013) 3–11. https://doi.org/10.1016/j.thromres.2012.10.011.

Sample	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}*s^{-1}$)
FSAP in buffer (n=5)	10.0 ± 2.1	45.1 ± 1.4	4.6 ± 0.7*10^6
Histone-activated FSAP in 25 % plasma (n=4)	37.1 ± 2.6	3.9 ± 0.2	1.1 ± 0.7 *10^5

Supplementary Data 1. Kinetic parameters of FSAP and substrate Ac-Pro-DTyr-Lys-Arg-AMC. Data are the mean ± S.D. of 4-5 independent experiments.



Supplememtary Data 2: Calibration of the FGA. A) Dose-response curve of the fluorophore AMC in buffer and plasma. The RFU as function of increasing amounts of AMC in 25 % pooled plasma (SHP) and in Tris-NaCl (TN) with or without 1 % BSA is shown. Up to a concentration of 50 µM AMC or ~ 9000 RFU, the fluorescence signal increases almost linearly and very similar in 25 % plasma and in TN + 1% BSA. Data are the mean ± S.D. of 3 independent measurements. **B)** FSAP calibrator. Active site titrated two chain FSAP was used as 4-point calibrator in the FGA (data are the mean ± S.D. of n=11 independent FGA runs). The RSD was between 13 % (at 50 nM FSAP) and 19 % (at 0.8 nM FSAP). **C)** Raw data curves of pooled plasma controls (SHP and NRP). Curves are the mean ± S.D of 11 independent measurements in duplicate. The mean % CV of the SHP and NRP curve are 4.7 %, and 7.5 %, respectively.



Supplementary Data 3: Raw data and 1st derivative (peak FSAP) of plasma from individuals genotyped as heterozygous carrier of the MI polymorphism and homozygous WT carriers. A single experiment is shown. SHP: pooled standard human plasma. The MI 2 proband had no activity in plasma as described before [8].

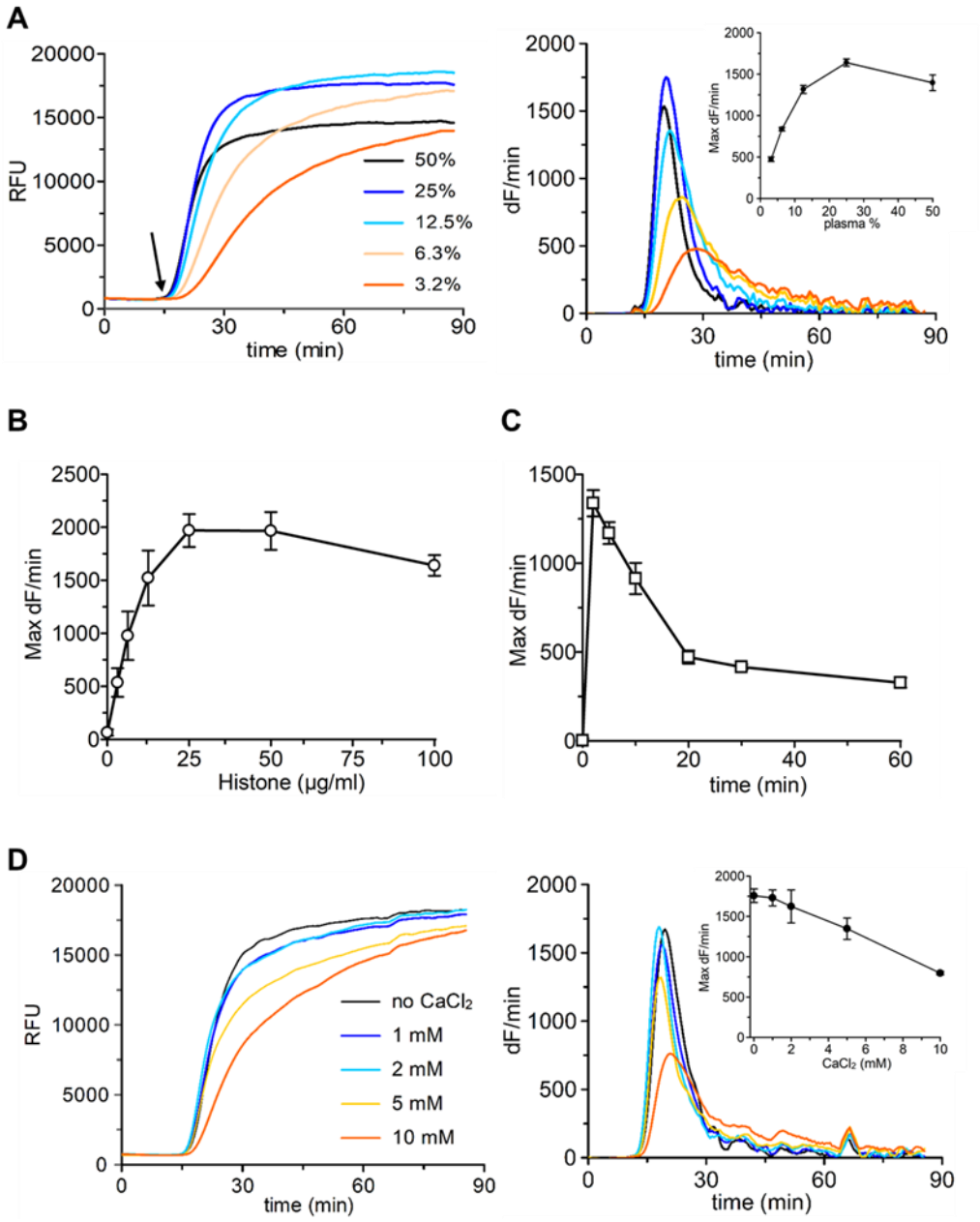


Figure 1

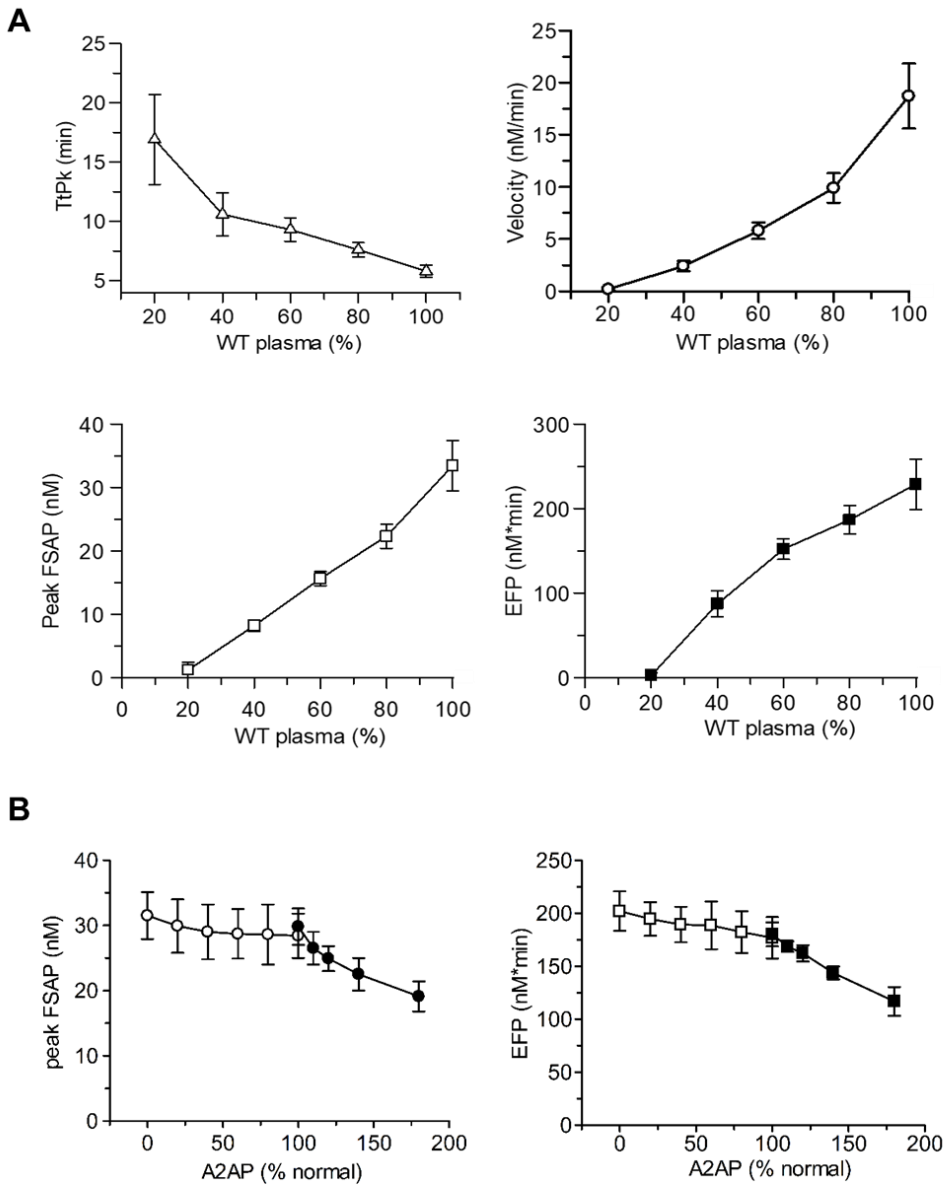

A
TtPk (min)
WT plasma (%)
Velocity (nM/min)
WT plasma (%)
Peak FSAP (nM)
WT plasma (%)
EFP (nM*min)
WT plasma (%)
B
peak FSAP (nM)
A2AP (% normal)
EFP (nM*min)
A2AP (% normal)

Figure 2

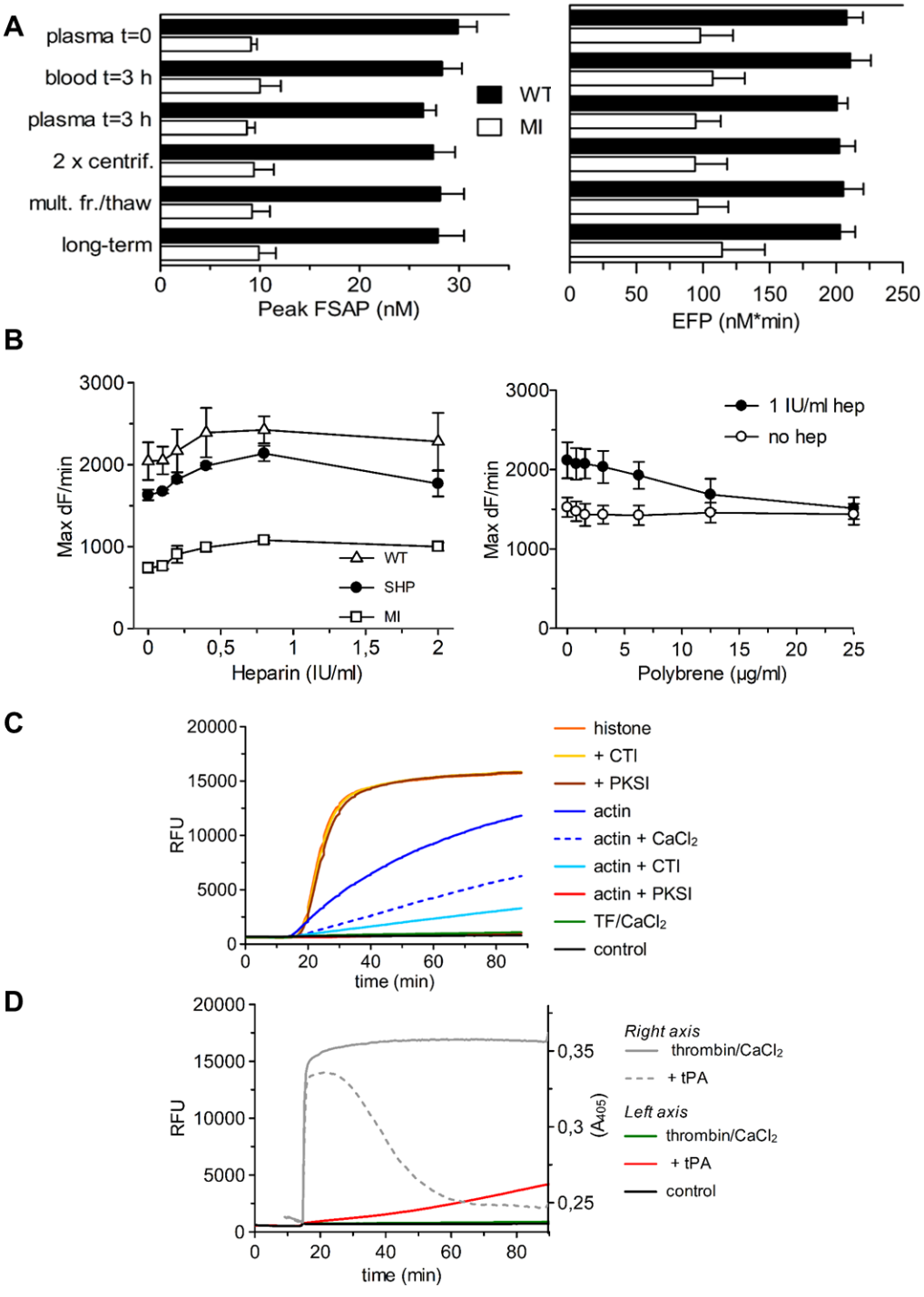

A
plasma t=0
blood t=3 h
plasma t=3 h
2 x centrif.
mult. fr./thaw
long-term
WT
MI
Peak FSAP (nM)
EFP (nM*min)
B
Max dF/min
WT
SHP
MI
Heparin (IU/ml)
1 IU/ml hep
no hep
Polybrene (µg/ml)
C
RFU
time (min)
histone
+ CTI
+ PKSI
actin
actin + CaCl2
actin + CTI
actin + PKSI
TF/CaCl2
control
D
RFU
time (min)
Right axis
thrombin/CaCl2
+ tPA
Left axis
thrombin/CaCl2
+ tPA
control
(A405)

Figure 3

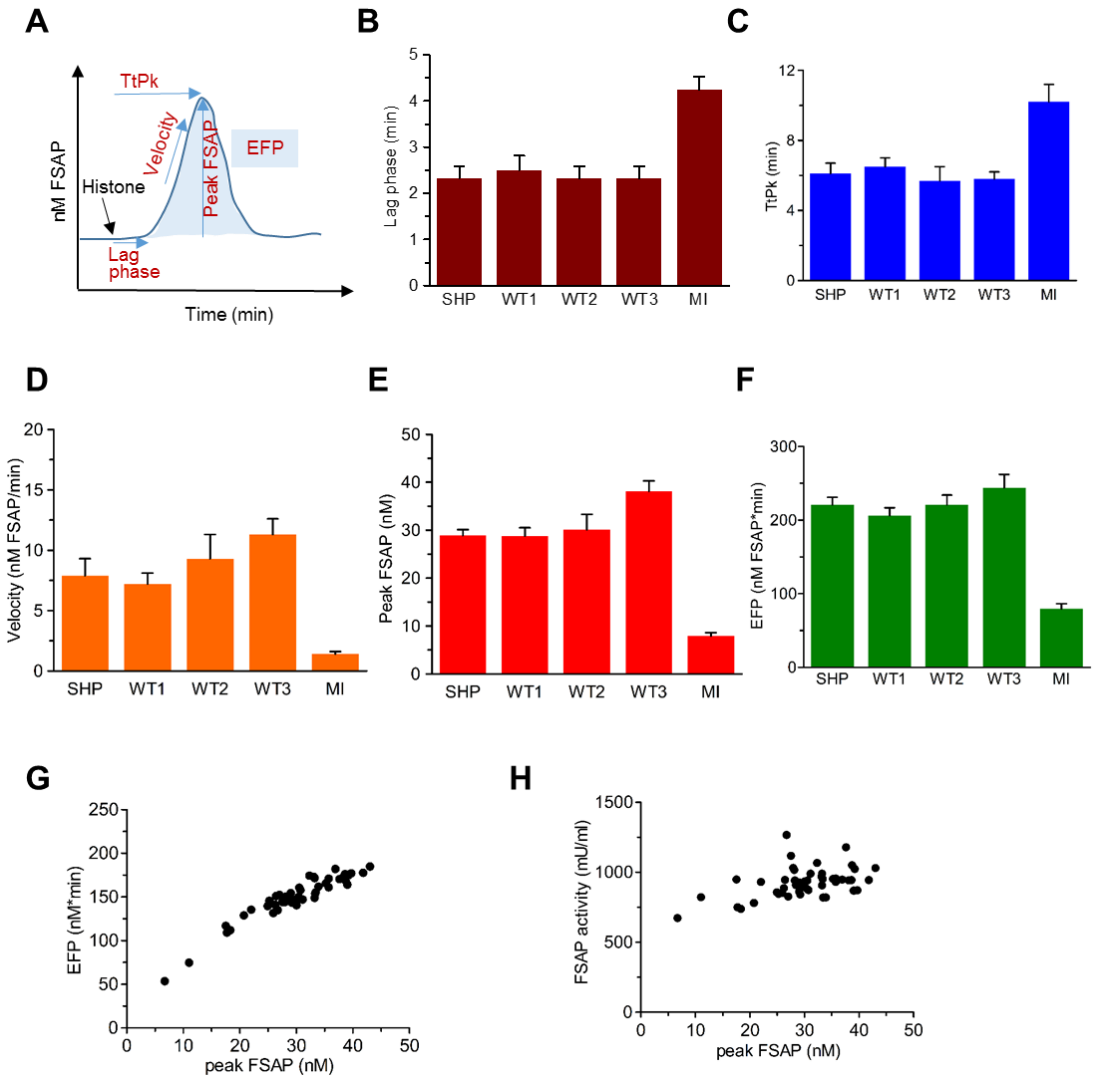

A
TtPk
Velocity
Peak FSAP
EFP
Histone
Lag phase
nM FSAP
Time (min)
B
Lag phase (min)
SHP
WT1
WT2
WT3
MI
C
TtPk (min)
D
Velocity (nM FSAP/min)
E
Peak FSAP (nM)
F
EFP (nM FSAP*min)
G
EFP (nM*min)
peak FSAP (nM)
H
FSAP activity (mU/ml)
peak FSAP (nM)

Figure 4

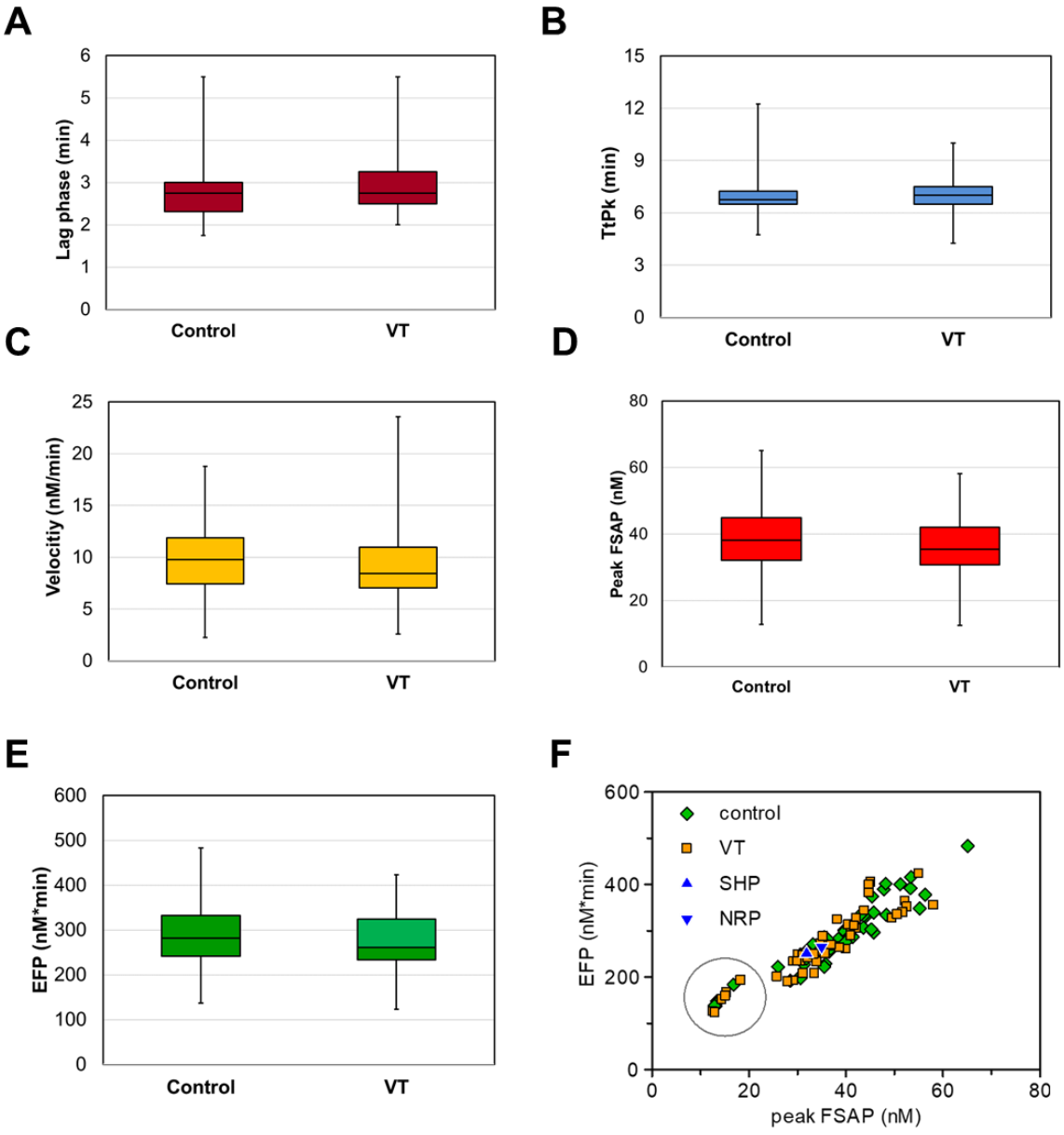

A
Lag phase (min)
0
1
2
3
4
5
6
Control
VT
B
TtPk (min)
0
3
6
9
12
15
Control
VT
C
Velocitiy (nM/min)
0
5
10
15
20
25
Control
VT
D
Peak FSAP (nM)
0
20
40
60
80
Control
VT
E
EFP (nM*min)
0
100
200
300
400
500
600
Control
VT
F
EFP (nM*min)
0
200
400
600
0
20
40
60
80
peak FSAP (nM)
control
VT
SHP
NRP

Figure 5

Variation	Lag phase (min)		TtPeak (min)		Velocity (nM/min)		Peak FSAP (nM)		EFP (nM x min)	
	SHP	*NRP*	*SHP*	*NRP*	*SHP*	*NRP*	*SHP*	*NRP*	*SHP*	*NRP*
mean	2.7	2.2	7.4	6.4	6.5	8.2	30.1	33.5	229.5	238.4
Intra-assay % CV	7.8	18.1	7.6	9.0	13.0	13.7	4.5	6.7	2.6	5.5
Inter-assay % CV	7.4	0.0	5.4	7.6	17.6	20.6	12.6	12.0	6.1	4.9
total % CV	**10.8**	**18.1**	**9.3**	**11.8**	**21.9**	**24.8**	**13.4**	**13.8**	**6.7**	**7.4**

Table 1: Intra- and inter-assay variation. Mean results, intra- and inter-assay variability and total variation coefficient in % were determined for each FGA output parameter. Two pooled plasma used as internal controls, SHP and NRP, were measured in 7 independent FGA runs with 8 replicates per run.

Normal donors	N	*Median*			*Reference Interval (5%-95% percentile)*		
		Peak FSAP (nM)	*EFP (nM*min)*	*FSAP activity (mU/ml)*	*Peak FSAP (nM)*	*EFP (nM*min)*	*FSAP activity (mU/ml)*
female	25	31,2	149,5	944,4	22,1 - 43,1	134,5 - 184,4	852,8 - 1175,1
male	25	29,3	150,5	902,7	17,6 - 38,8	108,7 - 175,6	746,7 - 1046,7
all	50	30,3	150,3	929,1	17,6 - 41,9	108,7 - 181,5	746,7 - 1113,5

Table 2: Reference interval in adult healthy donors. Peak FSAP, EFP and FSAP activity by standard assay were determined in 25 female and 25 male healthy donors. Peak FSAP and EFP are derived from 4 independent measurements in duplicate wells, FSAP activity data are based on a duplicate measurement.

	including carrier of Marburg I Mutation					**excluding** carrier of Marburg I Mutation				
Parameter	N[1]	Median difference[2]	95% Confidence Interval[2]		p value[3]	N[1]	Median difference[2]	95% Confidence Interval[2]		p value[3]
lag phase	50 / 50	0.00	-0.25	0.25	**0.7237**	44 / 45	0.25	0.00	0.50	**0.0987**
TtPK	50 / 50	0.13	-0.25	0.50	**0.2917**	44 / 45	0.38	0.00	0.75	**0.0259***
velocity	50 / 50	-0.87	-2.21	0.46	**0.2412**	44 / 45	-1.51	-2.81	-0.21	**0.0270***
peak FSAP	50 / 50	-2.04	-5.94	1.86	**0.2916**	44 / 45	-3.98	-7.96	0.01	**0.0530**
EFP	50 / 50	-13.35	-42.18	15.48	**0.3782**	44 / 45	-24.78	-53.31	3.75	**0.0861**

1 = Sample size VT / Control, 2 = Hodges-Lehmann Estimate and 95% Confidence Interval,
3 = Wilcoxon Rank Sum test

Table 3: Comparison between groups. Data were analysed by including or excluding individuals with very low level of FSAP (presumably carrier of the MI-SNP).* p values < 0.05 were considered significant.