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ORIGINAL ARTICLE

Plasma factor VII-activating protease antigen levels and activity are increased in ischemic stroke

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Summary. *Background:* Factor VII-activating protease (FSAP) is a recently discovered plasma protease with a role in the regulation of hemostasis and vascular remodeling processes. Higher levels and activity of FSAP have been reported in patients with deep vein thrombosis, but there are no data on plasma FSAP in ischemic stroke (IS). *Objective:* To investigate whether FSAP antigen levels and activity are associated with IS and/or etiologic subtypes of IS. *Patients and Methods:* To assess the potential association between FSAP and IS, plasma FSAP antigen levels and activity were measured in 600 consecutive IS patients and 600 population-based controls from the case–control study the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS). Blood sampling was performed in the acute phase and 3 months after the index stroke. FSAP was also investigated at the genetic level by genotyping of 33 single-nucleotide polymorphisms. *Results:* Increased FSAP antigen level and activity, at both time-points, were independently associated with IS. Subtype analysis revealed similar associations for both FSAP measures, at both time-points, in all main IS subtypes. FSAP genotypes showed association with both FSAP plasma measurements, but not with IS. *Conclusions:* Increased plasma FSAP antigen levels and activity were associated with IS and all main etiologic subtypes, suggesting a possible role for FSAP in the pathophysiology of IS, irrespective of the underlying etiology.

Keywords: FSAP, *HABP2*, ischemic stroke, plasma levels, TOAST subtype.

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Introduction

Factor VII-activating protease (FSAP) is a plasma serine protease that is predominantly produced in the liver and circulates in the blood at a concentration of approximately 12 μg mL^{-1}. Initial studies showed that FSAP can activate pro-urokinase plasminogen activator (u-PA), and coagulation factor VII [1,2]. More recent data have shown that FSAP inhibits tissue factor pathway inhibitor (TFPI), rather than activating FVII [3]. This, coupled with the fact that the enzyme has high homology with fibrinolytic and coagulation enzymes, is indicative of a role for FSAP in hemostasis.

The gene encoding FSAP (*HABP2*) is situated on chromosome 10q25, spans approximately 35 kb, and consists of 13 exons. The rare allele of the single-nucleotide polymorphism (SNP) Marburg I (MI-SNP) in *HABP2* is associated with decreased activity of FSAP [4]. A few studies have found associations between MI-SNP, and other SNPs in *HABP2*, and venous thrombosis, coronary heart disease, and stroke [5–8]. However, there are conflicting results regarding MI-SNP and venous thrombosis [9,10].

FSAP has previously been found to be localized and highly expressed in unstable atherosclerotic plaques [11]. In accordance with this observation, MI-SNP has also been associated with progressive carotid stenosis [12]. Moreover, 40 candidate genes involved in inflammation and endothelial function were recently investigated with respect to association with the phenotype carotid plaque [13]. Interestingly, *HABP2* was one of the five genes that were shown to associate with this phenotype, both in the initial study and in the validation study.

To our knowledge, there are only two studies in which plasma FSAP has been determined in clinical samples. In these studies, increased FSAP antigen levels and activity were found in patients with deep vein thrombosis (DVT), and in patients with acute respiratory distress syndrome [10,14]. No previous study has investigated plasma FSAP in patients with arterial thrombotic disease.

Against this background, the primary aim of the present study was to investigate whether plasma FSAP antigen level or

activity is associated with ischemic stroke (IS) or any of the etiologic subtypes of IS. A second aim was to explore whether genetic variation in *HABP2* is associated with: (i) variation in FSAP antigen levels or activity; and (ii) overall IS and/or any of the IS subtypes.

Subjects and methods

Study population

Details of the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS) have been described elsewhere, and additional information is available in the Subjects and Methods section in the online Supporting information [15,16]. Briefly, Caucasian patients ($n = 844$) who presented with first-ever or recurrent acute IS before reaching the age of 70 years were recruited consecutively at four stroke units in western Sweden. Healthy Caucasian community controls ($n = 668$) from the same geographic area and < 70 years of age were randomly selected from participants in a population-based health survey, the GOT-MONICA study [17], or the Swedish Population Register. All patients were classified into etiologic subtypes according to the criteria of the Trial of Org 10172 in Acute Stroke Treatment (TOAST).

Blood sampling

For the first 600 patients included, blood and plasma sampling was performed in the acute phase within 10 days (median, 4 days) of the stroke event, and at follow-up approximately 3 months after the event (median, 101 days; range, 85–125 days). For the first 600 controls, blood and plasma sampling was performed once. For the plasma sample, venous blood was collected in tubes that contained 10% by volume of 0.13 mol L^{-1} sodium citrate. Blood sampling was performed between 8:30 a.m. and 10:30 a.m. after overnight fasting. Plasma was isolated within 2 h by centrifugation at $2000 \times g$ and 4 °C for 20 min. For the additional patients ($n = 244$) and controls ($n = 68$), only whole blood was collected.

Plasma FSAP activity assay and ELISA

Total FSAP activity and antigen levels were measured by an immunocapture activity test and FSAP-specific ELISA, with the buffers and procedures as previously described [18], with minor modifications. For the activity assay, microtiter plates were coated with 10 μg mL^{-1} anti-FSAP mouse mAb 677 (American Diagnostica, Pfungstadt, Germany) in coating buffer (15 mmol L^{-1} Na_2CO_3, 35 mmol L^{-1} $NaHCO_3$, pH 9.6), and this was followed by blocking with standard buffer (20 mmol L^{-1} sodium citrate, 150 mmol L^{-1} NaCl, 100 mmol L^{-1} arginine, pH 6.0) containing 3% bovine serum albumin (BSA) (w/v). Plasma was diluted (1 : 100) in a standard buffer (see above) containing 0.1% Tween-80 (w/v), 1% BSA (w/v), and 100 U mL^{-1} unfractionated heparin (Liquemin; Roche, Grenzach, Germany), and applied to the plate, incubated for 1 h at room temperature, and washed three times. Recombinant single-chain u-PA (10 μg mL^{-1} Saruplase [Grünenthal, Stolberg, Germany] in Tris-buffered saline [TBS] with 0.1% Tween-80 [w/v]) and 2 mmol L^{-1} $CaCl_2$ (pH 7.2), was added and incubated for 5 min; this was followed by the addition of the chromogenic substrate S-2444 (2 mmol L^{-1}) (Haemochrome, Essen, Germany), and further incubation (37 °C). Absorbance at 405 nm was recorded every minute with the microplate reader EL808 (Biotek Instruments, Winooski, OR, USA) for 1 h (37 °C). The maximal velocity (substrate turnover with time) over 8 min was determined, and this was invariably always the initial reaction velocity.

For the ELISA, microtiter plates were coated with a rabbit polyclonal anti-FSAP antibody (5 μg mL^{-1}) as above, and blocked with 3% (w/v) BSA and 0.1% (w/v) Tween-20 in TBS. Diluted patient plasma (1 : 2000) was incubated for 1 h at room temperature. After extensive washing, anti-FSAP mAb 570 (2 μg mL^{-1}) was added and incubated (1 h, room temperature), and this was followed by incubation of peroxidase-coupled mouse antibody. The detection step of the ELISA was performed with 3,3′,5,5′-tetramethylbenzidine (TMB Substrate Kit; Pierce, Rockford, IL, USA), and the optical density was measured at 405 nm.

For both assays, calibration curves were established with a dilution series of standard human plasma (SHP) (Siemens Diagnostics, Marburg, Germany). SHP served as a reference for the measured FSAP activity, which was defined as one plasma equivalent unit (1000 mU mL^{-1}). In the ELISA, this preparation was assumed to contain approximately 12 μg mL^{-1} FSAP, as previously described [18]. The antigen levels and activity in SHP were similar to those in a pooled set of five healthy individuals without MI-SNP. One sample was used for quality control throughout the study. The inter-assay and intra-assay coefficients of variation were 14.7% and 4.7%, respectively, for the activity assay, and 13.2% and 4.1%, respectively, for the ELISA. These values are very similar to what has been reported previously [18].

Serum high-sensitivity C-reactive protein (hsCRP) levels

Serum levels of hsCRP were measured with a solid-phase chemiluminescence immunometric assay, as previously described [19].

Genotyping of SNPs and power calculation

Details of the selection and genotyping of the 32 *HABP2* tagSNPs and MI-SNP are described in the Methods section in the online Supporting information. Assuming a multiplicative genetic model, the odds ratios (ORs) for overall IS ($n = 844$) that can be detected with 80% power at the 5% level are in the range of 1.37–1.24, depending on the minor allele frequency (MAF) (0.35–0.11). Owing to the low MAF of MI-SNP, the power to detect an association for this variant is reduced.

Statistical analyses

FSAP activity was normally distributed, whereas the skewed FSAP antigen levels were logarithmically transformed. Both FSAP measurements were analyzed with parametric tests. Differences in FSAP antigen level and activity between IS subtypes and between IS subtypes and controls were analyzed with ANCOVA, using Bonferroni's correction and adjusting for significant covariates among age, sex, hypertension, smoking, diabetes mellitus, hyperlipidemia, and systolic blood pressure (SBP). Associations between FSAP levels and overall IS were investigated with conditional (cases and controls were matched for age, sex, and geographic area) univariable and multivariable logistic regression, with adjustments for hypertension, smoking status, diabetes mellitus, hyperlipidemia, and SBP. For TOAST subtypes, cases were compared with the whole control population, and an unconditional logistic regression analysis was therefore used, also including age, sex and geographic area as covariates. In a second regression model, hsCRP levels were included together with the above-mentioned covariates. Associations between classic vascular risk factors, SNPs, or haplotypes and plasma FSAP were investigated with linear regression, adjusting for the covariates described above. Associations between SNPs or haplotypes and overall IS or TOAST subtypes were investigated with an additive model in binary logistic regression, adjusting for age, sex, hypertension, smoking, and diabetes mellitus. To correct for multiple testing for single SNPs and haplotypes, permutation tests with 10 000 permutations were performed. Corrected *P*-values are designated as P_c. All ORs for FSAP levels were scaled to estimate the ORs associated with an increase of one standard deviation in the FSAP activity or logFSAP antigen levels. IBM SPSS STATISTICS version 19 for Windows (SPSS, Chicago, IL, USA) and HELIXTREE 6.3 (Golden Helix, Bozeman, MT, USA) were used for statistical analyses. The statistical significance level was 0.05, and *P*-values were two-tailed.

Results

Baseline characteristics of the study subjects have been described elsewhere [15,16,20], and are summarized in Table 1 for the first 1200 cases and controls. There were no significant differences with regard to median time of blood sampling in relation to stroke onset among the TOAST subtypes. Furthermore, there was no correlation between acute-phase FSAP antigen level or activity and the time of the first blood draw ($P = 0.82$ and $P = 0.62$, respectively), or between the 3-month FSAP measurements and the time of the follow-up blood draw ($P = 0.27$ and $P = 0.22$, respectively).

Plasma FSAP levels in overall IS

Both FSAP antigen levels and activity were significantly increased in patients with IS, both in the acute phase and at the 3-month follow-up, as compared with controls (Fig. 1). In patients, FSAP activity was significantly higher in the acute phase than during the follow-up (mean of 1274 mU mL^{-1}, as compared with 1214 mU mL^{-1}; $P < 0.001$). By contrast, there was no difference between the acute-phase and follow-up FSAP antigen levels (geometric mean of 13.8 μg mL^{-1}, as compared with 13.7 μg mL^{-1}; $P = 0.90$). Exclusion of those patients who suffered a recurrent stroke within 3 months of inclusion ($n = 31$) did not alter these results. In addition, the acute-phase and follow-up measures were clearly correlated for both FSAP antigen levels and activity (Pearson correlation coefficients of 0.69 and 0.64, $P < 0.001$ for both). The individual levels for the acute-phase and follow-up measures of FSAP are shown in Fig. 2.

In the univariable regression analysis, associations between overall IS and both FSAP measures, in the acute phase as well as at the 3-month follow-up, were detected (Table 2). In the multivariable analysis, all of these associations remained. After inclusion of hsCRP as a covariate, the associations were attenuated, but remained significant. When men ($n = 770$) and women ($n = 430$) were investigated separately, both FSAP antigen levels and activity were significantly associated with IS at both time-points, and with similar ORs as for the whole sample.

To investigate whether pre-existing vascular disease contributed to the association between overall IS and FSAP antigen level and activity, an additional regression analysis was

Table 1 Baseline characteristics of controls, overall ischemic stroke (IS), and the four main etiologic subtypes

	Control ($n = 600$)	Overall IS ($n = 600$)	LVD ($n = 73$)	SVD ($n = 124$)	CE stroke ($n = 98$)	Cryptogenic stroke ($n = 162$)
Mean age (years) (SD)	56 (10)	56 (10)	59 (8)	58 (7)	57 (10)	53 (12)
Male sex, *n* (%)	385 (64)	385 (64)	54 (74)	77 (62)	66 (67)	95 (59)
Hypertension,* *n* (%)	224 (37)	354 (59)	44 (60)	89 (72)	50 (51)	87 (54)
Diabetes mellitus,† *n* (%)	33 (6)	114 (19)	25 (34)	26 (21)	19 (19)	23 (14)
Current smoking, *n* (%)	109 (18)	233 (39)	39 (53)	54 (44)	34 (35)	60 (37)
Hyperlipidemia,‡ *n* (%)	403 (67)	413 (76)	53 (82)	77 (71)	73 (82)	107 (71)

CE, cardioembolic; LVD, large-vessel disease; SD, standard deviation; SVD, small-vessel disease. *Hypertension was defined by pharmacologic treatment for hypertension, a systolic blood pressure of ≥ 160 mm Hg, and/or a diastolic blood pressure of ≥ 90mm Hg. †Diabetes mellitus was defined by diet or pharmacologic treatment, a fasting plasma glucose level of ≥ 7.0 mmol L^{-1}, or a fasting blood glucose level of ≥ 6.1 mmol L^{-1}. ‡Hyperlipidemia was defined as pharmacologic treatment, a total fasting serum cholesterol level of > 5.0 mmol L^{-1}, and/or a low-density lipoprotein level of > 3.0 mmol L^{-1}.

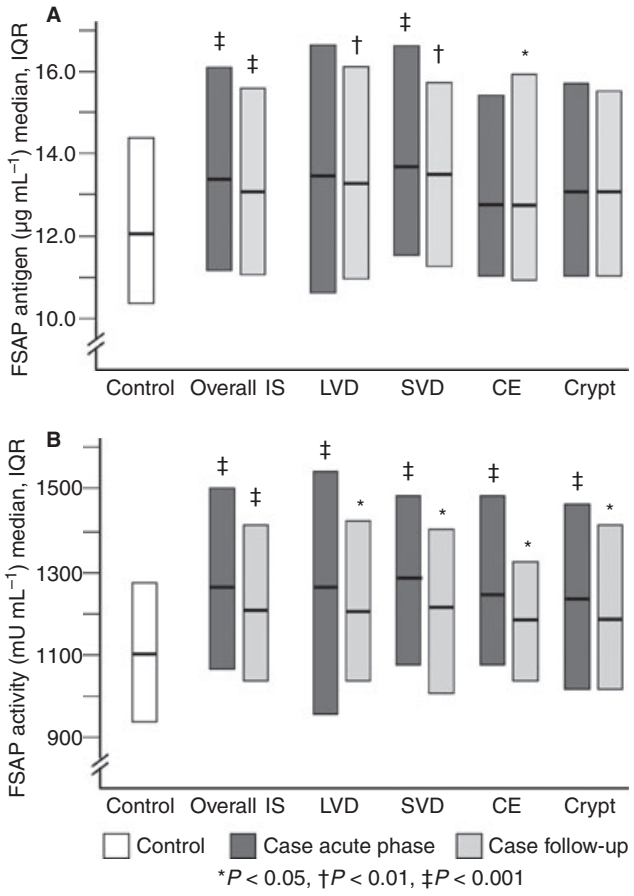

Fig. 1. Median levels and interquartile ranges (IQRs) of plasma factor VII-activating protease (FSAP) antigen (A) and activity (B) in the acute phase (dark gray) and at the 3-month follow-up (light gray) for overall ischemic stroke (IS) and the four main etiologic subtypes. CE, cardioembolic stroke; Crypt, cryptogenic stroke; LVD, large-vessel disease; SVD, small-vessel disease. Differences in FSAP activity and logFSAP antigen level between overall IS and controls were calculated with Student's *t*-test, and differences between TOAST subtypes and controls were calculated with ANCOVA, adjusting for sex and with Bonferroni correction.

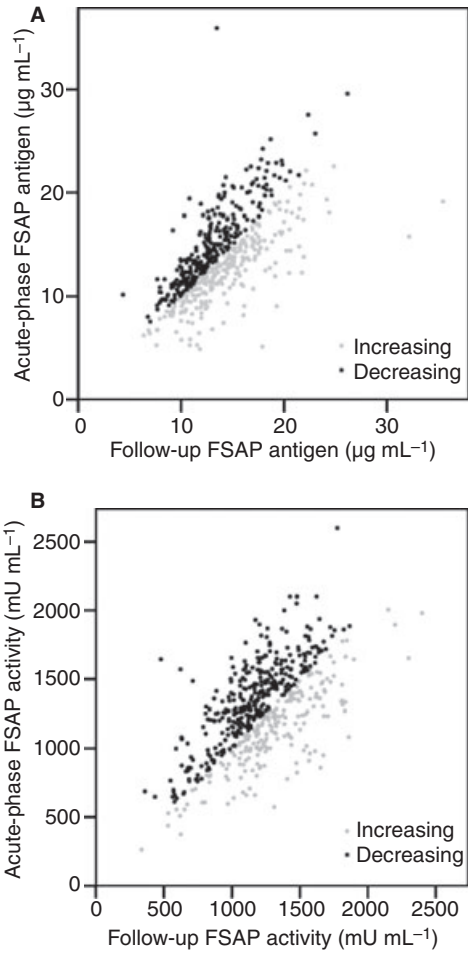

Fig. 2. Individual factor VII-activating protease (FSAP) antigen (A) and activity (B) levels for the acute phase as compared with 3-month follow-up levels. Subjects with decreased levels at the 3-month follow-up, as compared with the acute phase, are indicated in black. Subjects with increased levels at the 3-month follow-up are indicated in gray.

conducted in which patients with a history of stroke, coronary artery disease or peripheral artery disease (n = 196) were excluded. In this analysis, all associations remained. In addition, when patients with or without a history of vascular disease were compared, there were no significant differences in FSAP antigen levels or activity at either of the two time-points.

With regard to medication, there were no significant differences in FSAP antigen levels or activity between patients with (n = 415) or without (n = 185) antiplatelet therapy in the convalescent phase (P = 0.23 and P = 0.21, respectively). The only subtype with a significant proportion (62%) of patients on anticoagulant therapy was cardioembolic (CE) stroke, and in this subtype there were no significant differences in FSAP antigen levels or activity between patients with (n = 61) and without (n = 37) anticoagulant therapy in the convalescent phase (P = 0.08 and P = 0.84, respectively). Furthermore, neither of the FSAP measures showed a correlation with stroke severity at admission as measured by the Scandinavian Stroke Scale, or differed by clinical subtype according to the Oxfordshire Community Stroke Project, or by functional outcome at 3 months or 2 years after the index stroke (Results section in the online Supporting information).

Plasma FSAP antigen levels and activity in the etiologic subtypes of IS

FSAP antigen levels and activity were investigated in the subtypes large-vessel disease (LVD), small-vessel disease (SVD), cardioembolic (CE) stroke, and cryptogenic stroke. The only covariate that significantly differed between subtypes with respect to the FSAP measures was sex, and the ANCOVA was adjusted accordingly. Regarding FSAP antigen levels, the acute-phase levels were only significantly increased in the SVD group as compared with controls (Fig. 1). At the 3-month follow-up, FSAP antigen levels were increased in the LVD, SVD and CE groups. All subtypes displayed significantly higher FSAP activity than the controls, at both time-points. There were no significant differences in FSAP antigen levels or

Table 2 Odds ratios (ORs) and 95% confidence intervals (CIs) for overall ischemic stroke (IS) and the four main etiologic subtypes per one standard deviation increase in factor VII-activating protease (FSAP) activity and log FSAP antigen level in the acute phase and at the 3-month follow-up, as compared with controls

	Unadjusted OR (95% CI)	Adjusted, model 1 OR (95% CI)*	Adjusted, model 2 OR (95% CI)†
Acute-phase FSAP antigen level			
Overall IS	1.9 (1.6–2.3)	1.5 (1.2–1.9)	1.3 (1.1–1.7)
LVD	1.3 (1.0–1.8)	1.1 (0.8–1.6)	1.0 (0.7–1.5)
SVD	1.6 (1.3–2.0)	1.4 (1.0–1.8)	1.3 (1.0–1.7)
CE stroke	1.4 (1.0–1.7)	1.2 (0.9–1.5)	1.0 (0.7–1.3)
Cryptogenic stroke	1.3 (1.1–1.6)	1.3 (1.1–1.7)	1.3 (1.0–1.6)
Follow-up FSAP antigen level			
Overall IS	2.1 (1.7–2.5)	1.8 (1.4–2.3)	1.7 (1.3–2.1)
LVD	1.6 (1.2–2.0)	1.5 (1.1–2.0)	1.3 (0.9–1.9)
SVD	1.5 (1.2–1.8)	1.5 (1.1–1.9)	1.5 (1.1–1.9)
CE stroke	1.4 (1.0–1.7)	1.4 (1.1–1.8)	1.3 (1.0–1.7)
Cryptogenic stroke	1.3 (1.1–1.6)	1.3 (1.1–1.6)	1.3 (1.0–1.6)
Acute-phase FSAP activity			
Overall IS	2.1 (1.8–2.5)	1.8 (1.5–2.2)	1.8 (1.5–2.2)
LVD	1.8 (1.4–2.3)	1.6 (1.1–2.3)	1.5 (1.0–2.1)
SVD	1.7 (1.4–2.2)	1.6 (1.2-2.0)	1.5 (1.2–2.0)
CE stroke	1.8 (1.4–2.4)	1.7 (1.3–2.2)	1.5 (1.1–2.0)
Cryptogenic stroke	1.7 (1.4–2.0)	1.9 (1.5–2.4)	1.8 (1.5–2.3)
Follow-up FSAP activity			
Overall IS	1.7 (1.5–2.0)	1.6 (1.3–1.9)	1.5 (1.2–1.8)
LVD	1.5 (1.1–1.9)	1.5 (1.1–2.1)	1.4 (1.0–1.9)
SVD	1.3 (1.0–1.6)	1.3 (1.1–1.7)	1.3 (1.0–1.7)
CE stroke	1.4 (1.1–1.8)	1.4 (1.0–1.8)	1.3 (1.0–1.8)
Cryptogenic stroke	1.4 (1.1–1.6)	1.5 (1.2–1.8)	1.4 (1.2–1.8)

CE, cardioembolic; LVD, large-vessel disease; SVD, small-vessel disease. Conditional and unconditional regression analysis was used for overall IS and for the subtypes, respectively. *Adjusted for smoking, diabetes, hypertension, systolic blood pressure and hyperlipidemia for overall IS, as well as for age, sex and geographic area for subtypes. †Covariates as in model 1, also including high-sensitivity C-reactive protein levels.

activity between the subtypes, at either of the two time-points ($P > 0.78$).

The reductions in FSAP activity between the acute-phase and 3-month follow-up measures were significant in the subtypes SVD and cryptogenic stroke (mean ΔFSAP activities of 63.2 and 67.3 mU mL^{-1}, respectively, $P < 0.01$ for both). There were no significant differences either in the absolute reduction ($P > 0.85$ throughout) or in the relative reduction ($P > 0.85$ throughout) in FSAP activity between the subtypes.

In the univariable binary logistic regression, both FSAP antigen levels and activity showed significant associations with all subtypes at both time-points (Table 2). After adjustment for vascular risk factors, all associations remained, except for LVD and CE stroke for acute-phase FSAP antigen levels. The results remained essentially the same when hsCRP was included in the regression model, except for the subtype LVD (Table 2).

Classic risk factors and plasma FSAP levels

The distribution of FSAP antigen levels and activity in controls are shown in Fig. S1. The FSAP levels in controls with or without classic vascular risk factors are shown in Table 3. As shown in Table S1, the FSAP measures were only weakly correlated with age, blood pressure, anthropometric measures, metabolic variables, hsCRP, fibrinogen, and fibrinolytic variables. Sex was the risk factor explaining most of the variation in FSAP antigen levels and activity, but, in general, risk factors explained a very small part of the variation in plasma FSAP. In fact, age, sex, geographic area, hypertension, diabetes mellitus, smoking status, hyperlipidemia and SBP explained only 8.9% and 4.7% of the variation in FSAP antigen levels and FSAP activity in controls, respectively. The corresponding figures were 8.1% and 3.3% for patients in the acute phase, and 5.3% and 2.0% for patients at follow-up, respectively. In a subset of controls who did not receive any treatment for hypertension, diabetes, or hyperlipidemia ($n = 485$), categorical variables were replaced with the corresponding continuous variables. In this group, classic risk factors accounted for 12.0% and 6.4% of the variance in FSAP antigen levels and activity.

FSAP gene variation and plasma FSAP levels

The genotyping success rate was 98–100%, and genotype frequencies are shown in Table S2. The 32 tagSNPs were distributed in eight haplotype blocks. An overview of the FSAP locus (*HABP2*) with the observed linkage disequilibrium pattern is shown in Fig. S2.

In controls, seven and three SNPs (including MI-SNP) were significantly associated with FSAP antigen levels and activity, respectively (Table 4), and they collectively explained 8% and 21% of the total variance in FSAP antigen levels and activity, respectively. All genotypes together with vascular risk factors accounted for 17% and 26% of the total variance in FSAP antigen levels and activity in controls. Both FSAP antigen levels and activity were significantly lower in carriers of the A allele ($n = 43$) of MI-SNP than in homozygotes for the wild-type G allele ($n = 546$) (geometric mean 10.3 vs. 12.3 μg mL^{-1}, and mean activity 668 vs. 1141 mU mL^{-1}, respectively, $P < 0.001$ for both).

In cases, the percentages of variance collectively explained by the significant SNPs were 4% and 5% for FSAP antigen levels in the acute and convalescent phases, respectively, and for FSAP activity the percentage was 19% at both time-points. All significant genotypes together with vascular risk factors explained approximately 15% and 20% of the variance in FSAP antigen levels and activity, respectively, at both time-points.

The haplotype analysis was congruent with the single SNP analysis; that is, those haplotypes harboring the associated

Table 3 Mean plasma factor VII-activating protease (FSAP) antigen levels and activity in controls with or without classic vascular risk factors, statin therapy, or antihypertensive therapy

	FSAP antigen level*			FSAP activity		
	n	Geometric mean (μg mL^{-1})	*P*-value	*n*	Mean (mU mL^{-1})	*P*-value
Sex						
Women	213	13.2	0.002	213	1153	< 0.001
Men	383	11.6		384	1078	
Smoking						
Current smoker	107	12.2	0.92	107	1081	0.35
Non-smoker	489	12.1		490	1110	
Hypertension†						
Hypertensive	221	12.3	0.32	221	1146	0.006
Normotensive	374	12.0		375	1080	
Diabetes mellitus†						
Diabetes	32	12.8	0.21	32	1155	0.31
No diabetes	562	12.1		563	1102	
Hyperlipidemia†						
Hyperlipidemic	400	12.3	0.03	400	1125	0.01
Normolipidemic	195	11.7		196	1062	
Statin therapy						
Statins	31	12.6	0.44	31	1138	0.50
No statins	565	12.1		566	1102	
Antihypertensive drugs						
Drugs	87	12.1	0.98	87	1137	0.25
No drugs	509	12.1		510	1099	

Differences between groups were calculated with Student's *t*-test. *Levels were logarithmically transformed. †Hypertension, diabetes mellitus and hyperlipidemia were defined as stated in Table 1.

Table 4 Seven single-nucleotide polymorphisms (SNPs) showing the strongest associations with factor VII-activating protease (FSAP) antigen levels and activity in controls ($n = 600$)

	FSAP antigen level			FSAP activity		
SNP (allele)*	Adjusted R^2 (%)	*P*-value	P_c	Adjusted R^2 (%)	*P*-value	P_c
rs10509980 (A)	11.7	< 0.001	< 0.001	5.7	0.004	0.13
rs10509981 (A)	11.7	< 0.001	0.003	5.7	0.008	0.28
rs2286744 (A)	10.1	0.003	0.04	6.3	0.001	0.04
rs2286745 (G)	11.2	< 0.001	0.002	5.7	0.009	0.31
rs3850688 (A)	10.4	< 0.001	0.02	6.2	0.001	0.05
rs3862015 (A)	12.7	< 0.001	< 0.001	5.6	0.01	0.40
rs7080536, MI-SNP (G)	12.2	< 0.001	< 0.001	24.	< 0.001	< 0.001

MI-SNP, Marburg I SNP. Adjusted R^2 percentages were calculated with linear regression, with age, sex, geographic area, hypertension, systolic blood pressure, diabetes mellitus, smoking and hyperlipidemia included in the model. *The allele associated with increased FSAP antigen levels and activity.

single tagSNPs also explained the highest percentage of variation in FSAP.

FSAP gene variation and IS

No tagSNPs or haplotypes were associated with overall IS, in either univariable or multivariable analysis. When single SNPs in the IS subtypes were analyzed, weak associations were observed for 11 SNPs with different subtypes, but none of them remained significant after correction for multiple testing. Haplotype analysis did not add any further information.

No association was found for MI-SNP and overall IS (OR 0.74, 95% confidence interval [CI] 0.48–1.13). Inclusion of vascular risk factors in the model did not change the results (multivariable OR 0.86, 95% CI 0.55–1.37). For the IS subtypes, no association with MI-SNP was detected.

Discussion

Here, we describe for the first time a large and comprehensive investigation of FSAP antigen levels and activity, as well as genetic variation in the gene encoding FSAP, in patients with IS and controls. Our major finding is that both FSAP antigen levels and activity are increased in patients with IS, in both the acute and convalescent phases of stroke.

In our study, we also showed that the associations for both FSAP measures and overall IS were independent of vascular risk factors, both acutely and after 3 months. In a study analyzing plasma FSAP antigen levels and activity in patients with DVT, the associations disappeared after adjustment for C-reactive protein levels [10]. In view of this, we also performed a regression analysis including hsCRP level as a covariate, and we found that all associations with overall IS remained. This suggests that our finding of increased plasma FSAP in patients with IS does not only reflect an inflammatory response.

In patients with IS, the increase in FSAP activity was most pronounced in the acute phase (13% increase as compared with controls). FSAP is an inactive circulating zymogen activated by an autocatalytic mechanism that is enhanced by factors released from apoptotic or dead cells, such as nucleic acids, nucleosomes, and histones [21,22]. Elevated levels of nucleic acids and nucleosomes have been detected in the circulation after IS [23,24]. Hence, it is plausible that tissue injury contributes to the increase in FSAP activity in the acute phase of stroke.

On the other hand, both FSAP antigen levels and activity were increased to a similar extent (approximately 9% higher than in controls) 3 months after IS. Therefore, an interpretation of our results could be that the increased FSAP levels, at least in part, preceded the stroke. Plausible mechanisms involve both increased synthesis and decreased clearance. One could speculate that atherosclerosis may lead to cell death and the release of factors that activate FSAP. Previous studies have indicated that FSAP is prothrombotic through activation of FVII [1,25] or through TFPI inactivation [3]. Altogether, these findings suggest that FSAP, through prothrombotic mechanisms, may contribute to an increased risk of IS.

In the present study, we also analyzed plasma FSAP in the etiologic subtypes of IS. At follow-up 3 months after the index stroke, we found independent associations for both measures of FSAP with all four major subtypes. After adjustment for hsCRP levels, similar results were obtained, with the exception of LVD, where the associations disappeared. This is in line with our previous observations for LVD, indicating that inflammation plays a larger role in this subtype than in the other etiologic subtypes [19]. Considering that previous studies have associated FSAP with unstable atherosclerotic plaques and carotid stenosis [11–13], and that atherosclerosis is the pathophysiologic mechanism of LVD, it is intriguing to speculate why we do not find the highest FSAP levels in this subtype. A possibility could be that, in LVD, FSAP mainly plays a role locally in the arterial wall, whereas in the other major subtypes it is involved in systemic prothrombotic mechanisms. It is also worth noting that, in the present study, the subtype LVD included the smallest number of patients ($n = 73$).

FSAP in plasma has not been assessed in large clinical studies before. Here, we report an extensive analysis of FSAP in relation to classic vascular risk factors in a large sample of healthy controls and patients with IS. We could clearly see that clinical covariates explained a very small part of the variation in FSAP antigen levels and activity, e.g. 8.9% and 4.7%, respectively, in controls. In comparison, these risk factors account for 24% of the variation in tissue-type plasminogen activator antigen levels in the same controls (adjusted R^2 percentage, $P < 0.001$). Moreover, in agreement with previously reported findings, we confirmed the higher systemic FSAP levels in women than in men [18]. We also made a concerted effort to link FSAP antigen levels and activity with the *HABP2* genotype. As expected, MI-SNP had a prominent effect on FSAP activity, but, interestingly, we also showed an effect on FSAP antigen. We also found a few tagSNPs that influenced the variation in plasma FSAP.

We could not find any association between tagSNPs, haplotypes, or MI-SNP and IS, unlike two previous studies on stroke [7,8]. The tagSNP rs4918851 included in the present study was recently associated with IS risk in a study by Zakai *et al.* [8]. Regarding MI-SNP, it cannot be excluded that our study is underpowered to detect a significant association for this SNP, as the MAF of this SNP is low. We have recently reported that MI-SNP FSAP has a diminished ability to inhibit TFPI [3]. Given these findings, a reasonable hypothesis would be that MI-SNP has a protective effect against IS. This seems plausible, as we found increased FSAP activity in IS patients. In contrast, an increased risk for all-cause stroke was found for MI-SNP in a study of older subjects [7].

The strengths of the present study are a large sample size, a comprehensive classification of the etiologic IS subtypes, and standardized blood sampling at two different time-points. There are also some limitations that should be considered. First, the case–control design represents a limitation to interpretations regarding plasma FSAP and IS risk, and our results need to be confirmed in large prospective studies. Second, a drawback in our analysis is the generally higher inter-assay variability. However, as the samples were assayed in triplicate, with the acute-phase, follow-up and control samples on the same plate, the measurements are less susceptible to inter-assay influences.

In conclusion, this is the first study to investigate plasma FSAP both in patients with IS and in a large control sample. Increased FSAP antigen levels and activity were independently associated with all main etiologic subtypes, indicating a role for FSAP in IS, irrespective of the underlying etiology. We also show that vascular risk factors and FSAP gene variation explain only a relatively small proportion of the variation in plasma FSAP, with sex and gene variants (particularly MI-SNP) making the strongest contributions. Altogether, our results provide new knowledge to enhance our understanding of the role of FSAP in IS, and the influence of vascular risk factors and FSAP gene variation on plasma FSAP.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Histogram of FSAP antigen levels and activity in controls.
Figure S2. Linkage disequilibrium (LD) plot of the 32 tagSNPs in *HABP2*.
Table S1. Correlations between FSAP antigen levels and activity and age, blood pressure, anthropometric measures, metabolic variables, hsCRP, fibrinogen, and fibrinolytic variables, in controls.
Table S2. Genotype frequencies for 33 SNPs in *HABP2* in controls, ischemic stroke, and etiologic subtypes.

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