Plasma FVII-Activating Protease (FSAP) Antigen and Activity Levels 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 venous thrombosis, but there are no data on plasma FSAP in ischemic stroke (IS). *Objective:* To investigate whether FSAP antigen and activity levels 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 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 three months after index stroke. FSAP was also investigated at the genetic level by genotyping of 33 single nucleotide polymorphisms. *Results:* Increased levels of FSAP antigen 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 measurements, but not with IS. *Conclusions:* Increased plasma levels of FSAP antigen 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.

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. Initial studies showed that FSAP can activate pro-urokinase plasminogen activator (uPA), and coagulation factor VII (FVII) [1,2]. More recent data show that FSAP inhibits tissue factor pathway inhibitor (TFPI), rather than activate FVII [3]. This, coupled with the fact that the enzyme has high homology to fibrinolytic and coagulation enzymes, are 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 the FSAP protein [4]. A few studies have found associations between the MI-SNP, as well as other SNPs in *HABP2*, and venous thrombosis, coronary heart disease, and stroke [5-8]. However, there are conflicting results for the 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, the 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 levels of both FSAP antigen and activity were found in

patients with deep venous 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 or activity levels are associated with ischemic stroke (IS) or any of the etiologic subtypes of IS. A second aim was to explore whether genetic variation in the FSAP gene is associated with (1) variation in FSAP antigen or activity levels, (2) 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 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, or the Swedish Population Register [17]. 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 three 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 sodium citrate. Blood plasma sampling was performed between 8:30 AM and 10:30 AM after overnight fasting. Plasma was isolated within 2 hours by centrifugation 2000 × *g* at 4°C for 20 minutes. 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 were measured by an immunocapture activity test and FSAP-specific ELISA using the buffers and procedures as described [18], with minor modifications. For the activity assay, microtiter plates were coated with 10 μg/ml anti-FSAP mouse mAb 677 (American Diagnostica, Pfungstadt, Germany) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), followed by blocking with standard buffer (20 mM Na-citrate, 150 mM NaCl, 100mM Arginin, pH 6.0) containing 3% BSA (wt/vol). Plasma probes were diluted (1:100) in a standard buffer (see above) containing 0.1% Tween 80 (wt/vol), 1% BSA (wt/vol) and 100 U/ml unfractionated heparin (Liquemin, Roche, Grenzach, Germany) and applied on the plate, incubated for 1 hour at room temperature (RT) and washed three times. Recombinant single-chain uPA (10 μg/ml

Saruplase, Grünenthal, Stolberg, Germany in TBS with 0.1% Tween 80 /wt/vol), 2 mM $CaCl_2$, pH 7.2, was added and incubated for 5 min followed by the addition of the chromogenic substrate S-2444 (2 mM) (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) over 1 hour (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 Ab (5 μg/ml) as above and blocked with 3% (wt/vol) BSA, 0.1% (wt/vol) Tween 20 in TBS. Diluted patient plasma (1:2000) was incubated for 1 hour at RT. After extensive washing, anti-FSAP mAb 570 (2 ug/ml) was added and incubated (1 hour, RT) which was followed by incubation of peroxidase-coupled mouse Ab. The detection step of the ELISA was done using 3,3‘,5,5‘-Tetramethylbenzidin (TMB-Substrate-Kit, Pierce, Rockford, Il, USA) and the optical density was measured at 405 nm.

For both assays, calibration curves were established through a dilution series of Standard Human Plasma (SHP, Siemens Diagnostics, Marburg, Germany). SHP served as reference for the measured FSAP activity, which was defined as 1 plasma equivalent unit (1000 mU/ml). In the ELISA this preparation was assumed to contain approximately 12 μg/ml FSAP as described [18]. The antigen/activity in SHP was similar to a pooled set of five healthy individuals without MI-SNP. One sample was used as quality control throughout the study. The inter- 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 levels

Serum levels of high-sensitivity C-reactive protein (hsCRP) were measured in a solid-phase chemiluminescent immunometric assay as previously described [19].

Genotyping of Single Nucleotide Polymorphisms and Power Calculation

Details on the selection and genotyping of the 32 *HABP2* tagSNPs and the MI-SNP are described in the 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). Due to the low MAF of the 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 and activity levels 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 status, diabetes mellitus, hyperlipidemia, and systolic blood pressure (SBP).

Associations between FSAP levels and overall IS were investigated using 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 thus, an unconditional logistic regression analysis was 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 using linear regression, adjusting for the covariates described above. Associations between SNPs or haplotypes and overall IS or TOAST subtypes were investigated using 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 10000 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 1 standard deviation (SD) in the FSAP activity or logFSAP antigen levels. Statistical softwares used were IBM SPSS Statistics version 19 for Windows (SPSS Inc., Chicago, IL, USA) and HelixTree 6.3 (Golden Helix, Bozeman, MT, USA). 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 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 Ischemic Stroke

Both the FSAP antigen and activity levels were significantly increased in patients with IS, both in the acute phase and at the 3-month follow-up, as compared to controls (Fig. 1). In patients, FSAP activity was significantly higher in the acute phase compared with the follow-up measurement (mean 1274 mU/mL compared to 1214 mU/mL, P<0.001). By contrast, there was no difference between the acute phase and follow-up FSAP antigen levels (geometric mean 13.8 µg/mL compared to 13.7 µg/mL, P=0.90). Excluding those patients who suffered a recurrent stroke within three 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 and activity (Pearson correlation coefficient 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 illustrated 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 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 and activity levels 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 levels, an additional regression analysis was 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 or activity levels at any of the two time-points.

With regard to medication, there were no significant differences in FSAP antigen 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 CE stroke, and in this subtype there were no significant differences in FSAP antigen or activity between patients with (n=61) or without (n=37) anticoagulant therapy in the convalescent phase (P=0.08 and P=0.84, respectively). Furthermore, none of the FSAP measures showed correlation with stroke severity at admission as measured by Scandinavian Stroke Scale, or differed by clinical subtype according to the Oxfordshire Community Stroke Project, or functional outcome at three months or two years after index stroke (Supporting information).

Plasma FSAP Levels in the Etiologic Subtypes of Ischemic Stroke

The levels of FSAP antigen 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 measures of FSAP was sex, and the ANCOVA was adjusted accordingly. Regarding FSAP antigen, the acute phase levels were only significantly increased in the SVD group, as compared to controls (Fig. 1). At the 3-month follow-up, FSAP antigen levels were increased in the LVD, SVD, and CE stroke groups. All subtypes displayed significantly higher FSAP activity than the controls, at both time-points. There were no significant differences in FSAP antigen or activity levels between the subtypes, at any 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 activity of 63.2 and 67.3 mU/mL, 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) between the subtypes in FSAP activity.

In the univariable binary logistic regression, both FSAP antigen and

activity levels 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 and activity levels in controls are illustrated in the Supporting information (Fig S1). The FSAP levels in controls with or without classic vascular risk factors are shown in Table 3. As shown in Table S1 in the Supporting information, 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 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 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 and activity.

FSAP Gene Variation and Plasma FSAP Levels

The genotyping success rate was 98-100%, and genotype frequencies are presented in the Supporting information (Table S2). The 32 tagSNPs were distributed in 8 haplotype blocks. An overview of the FSAP locus (*HABP2*) with the observed linkage disequilibrium pattern is illustrated in Fig. S1.

In controls, 7 and 3 SNPs (including the MI-SNP) were significantly associated with FSAP antigen and activity, respectively (Table 4), and they collectively explained 8% and 21% of the total variance in FSAP antigen and activity, respectively. All genotypes together with vascular risk factors, accounted for 17% and 26% of the total variance in FSAP antigen and activity in controls. Both FSAP antigen and activity were significantly lower in carriers of the A allele (n=43) of the MI-SNP, compared to homozygotes for the wild-type G allele (n=546), (geometric mean 10.3 vs. 12.3 µg/mL, and mean activity 668 vs.1141 mU/mL, respectively, $P<0.001$ for both).

In cases, the percentage of variance collectively explained by the significant SNPs was 4% and 5% for FSAP antigen in the acute and convalescent phases respectively, and for FSAP activity it 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 and activity, respectively, at both time-points.

The haplotype analysis was congruent with the single SNP analysis, i.e. those haplotypes harboring the associated single tagSNPs also explained

the highest percentage of variation in FSAP.

FSAP gene variation and Ischemic Stroke

No tagSNPs or haplotypes were associated with overall IS, in either uni- or multivariable analysis. When analyzing single SNPs in the IS subtypes, 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 the MI-SNP and overall IS (OR of 0.74, 95% confidence interval (CI) 0.48-1.13). Including vascular risk factors in the model did not change the results (multivariable OR of 0.86, 95% CI 0.55-1.37). For the IS subtypes, no association was detected with the MI-SNP.

DISCUSSION

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

In our study, we could also show that the associations for both FSAP measures and overall IS were independent of vascular risk factors, both acutely and after three months. In a study analyzing plasma FSAP antigen and activity in patients with DVT, the associations disappeared after adjustment for CRP levels [10]. In view of this, we also performed a regression analysis including hsCRP levels 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 compared to 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 and activity were increased to a similar extent (approximately 9% higher than in controls), three 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 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, this suggests 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 three 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, as compared to 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 for LVD, it is intriguing to speculate as to 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 rather 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 little part of the variation in FSAP antigen 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 (unpublished data). Moreover, in agreement with previously reported findings, we could confirm the higher systemic FSAP levels in women than in men [18]. We also made a concerted effort to link FSAP antigen and activity levels with the *HABP2* genotype. As expected, the MI-SNP had a prominent effect on FSAP activity, but interestingly we also show 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 the 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 the 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 the MI-SNP has a diminished ability to inhibit TFPI [3]. On account of these findings, a reasonable hypothesis would be that the MI-SNP has a protective effect on 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 the 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 presents 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, since the samples were assayed in triplets 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 in patients with

IS, as well as in a large control sample. Increased FSAP antigen and activity levels 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 only explain a relatively small proportion of the variation in plasma FSAP, with gender and gene variants (particularly the MI-SNP) making the strongest contributions. Altogether, our results add 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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DISCLOSURES OF CONFLICT OF INTEREST

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 and activity in controls.

Figure S2: Linkage disequilibrium (LD) plot of the 32 tagSNPs in *HABP2*.

Table S1: Correlations between FSAP antigen and activity levels 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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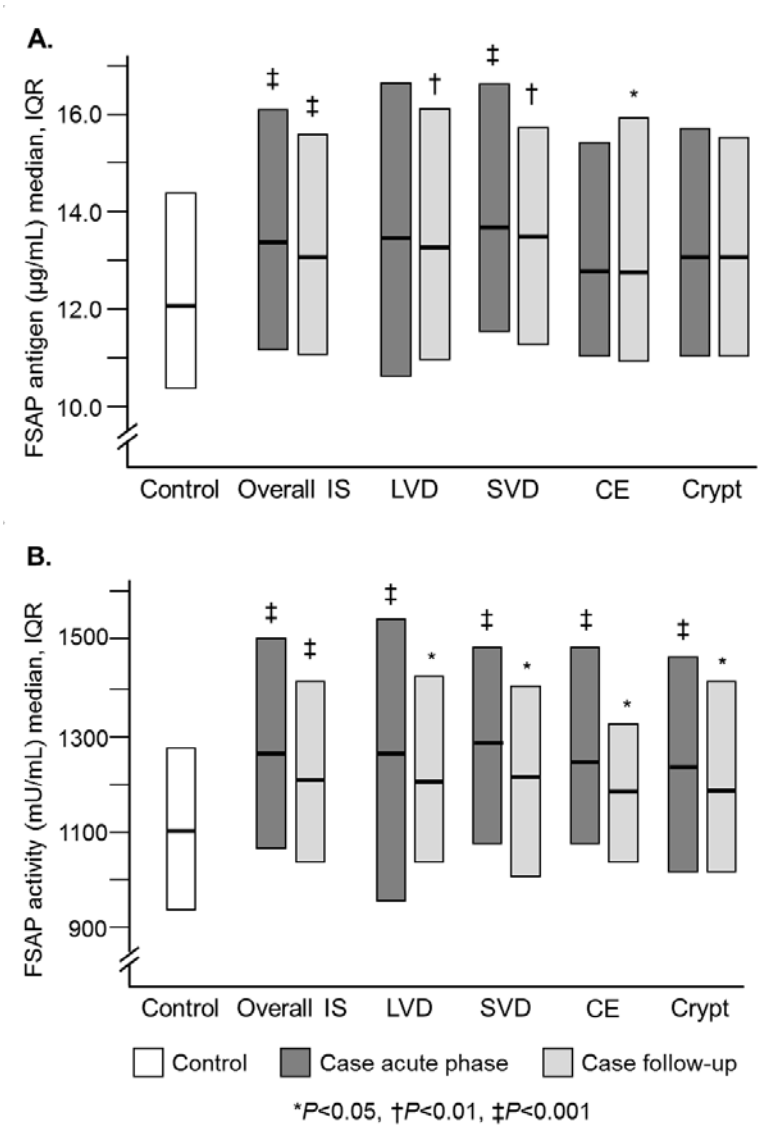

Fig. 1. Median levels and interquartile ranges (IQR) of plasma FSAP antigen (**A**) and activity (**B**) in the acute phase (dark grey) and at the 3-month follow-up (light grey) for overall ischemic stroke (IS) and TOAST subtypes. IS indicates ischemic stroke; LVD, large-vessel disease; SVD, small-vessel disease; CE, cardioembolic stroke; Crypt, cryptogenic stroke. Differences in FSAP activity and log FSAP antigen between overall IS and controls were calculated with Student's t-test, and between TOAST subtypes and controls with ANCOVA adjusting for sex and using Bonferroni correction.

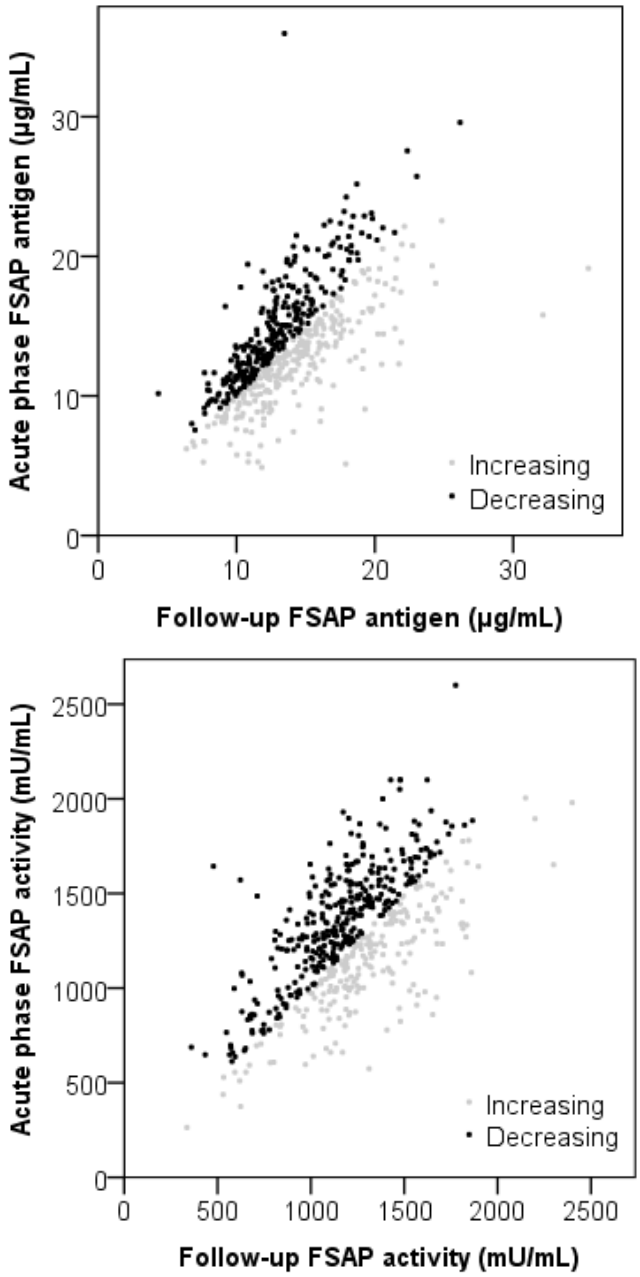

Fig. 2. Individual FSAP antigen (**A**) and activity (**B**) levels for acute phase compared to 3-month follow-up levels. Subjects with decreased levels at the 3-month follow-up, as compared to the acute phase, are indicated in black. Subjects with increased levels at the 3-month follow-up are illustrated in grey.

Table 1. Baseline characteristics of controls, overall ischemic stroke (IS), and the TOAST subtypes.

	Control (n=600)	**IS** (n=600)	**LVD** (n=73)	**SVD** (n=124)	**CE** (n=98)	**Crypt** (n=162)
Mean age, y (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)

*Hypertension was defined by pharmacological treatment for hypertension, systolic blood pressure ≥160 mm Hg, and/or diastolic blood pressure ≥90mm Hg. †Diabetes mellitus was defined by diet or pharmacological treatment, fasting plasma glucose ≥7.0 mmol/L, or fasting blood glucose ≥6.1 mmol/L. ‡Hyperlipidemia was defined as pharmacological treatment, total fasting serum cholesterol level >5.0 mmol/L, and/or LDL >3.0 mmol/L.

Table 2. ORs and 95% CI for overall ischemic stroke (IS) and TOAST subtypes per 1 SD increase in the FSAP activity and log FSAP antigen in the acute phase and at the 3-month follow-up, as compared to the controls.

	Unadjusted OR (95% CI)	**Adjusted, model 1*** OR (95% CI)	**Adjusted, model 2†** OR (95% CI)
Acute phase FSAP antigen			
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	1.4 (1.0-1.7)	1.2 (0.9-1.5)	1.0 (0.7-1.3)
Crypt	1.3 (1.1-1.6)	1.3 (1.1-1.7)	1.3 (1.0-1.6)
Follow-up FSAP antigen			
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	1.4 (1.0-1.7)	1.4 (1.1-1.8)	1.3 (1.0-1.7)
Crypt	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	1.8 (1.4-2.4)	1.7 (1.3-2.2)	1.5 (1.1-2.0)
Crypt	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	1.4 (1.1-1.8)	1.4 (1.0-1.8)	1.3 (1.0-1.8)
Crypt	1.4 (1.1-1.6)	1.5 (1.2-1.8)	1.4 (1.2-1.8)

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 age, sex, and geographic area for subtypes. †Covariates as in model 1, also including hsCRP levels.

Table 3. Mean plasma levels of FSAP antigen and activity in controls with or without classic vascular risk factors, statin therapy or antihypertensive therapy.

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

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 SNPs showing the strongest associations with FSAP antigen and activity in controls (n=600).

	FSAP antigen			**FSAP activity**		
SNP (allele)*	Adjusted R square	*P*-value	P_c	Adjusted R square	*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	24.1%	<0.001	<0.001

Adjusted R square percentages are calculated using linear regression also including age, sex, geographic area, hypertension, systolic blood pressure, diabetes mellitus, smoking, and hyperlipidemia in the model. *The allele associated with increased FSAP levels.