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Genome-wide analysis of genetic determinants of circulating factor VII-activating protease (FSAP) activity

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Essentials

- Knowledge of genetic regulators of plasma factor VII activating protease (FSAP) levels is limited.
- We performed a genome-wide analysis of variants influencing FSAP activity in Scandinavian cohorts.
- We replicated an association for Marburg-1 and identified an association for a *HABP2* stop variant.
- We identified a novel locus near *ADCY2* as a potential additional regulator of FSAP activity.

Summary. *Background:* Factor VII-activating protease (FSAP) has roles in both coagulation and fibrinolysis. Recent data indicate its involvement in several other processes, such as vascular remodeling and inflammation. Plasma FSAP activity is highly variable among healthy individuals and, apart from the low-frequency missense variant Marburg-I (rs7080536) in the FSAP-encoding gene *HABP2*, determinants of this variation are unclear. *Objectives:* To identify novel genetic variants within and outside of the *HABP2* locus that influence circulating FSAP activity. *Patients/Methods:* We performed an exploratory genome-wide association study (GWAS) on plasma FSAP activity amongst 3230 Swedish subjects. Directly genotyped rare variants were also analyzed with gene-based tests. Using GWAS, we confirmed the strong association between the Marburg-I variant and FSAP activity. *HABP2* was also significant in the gene-based analysis, and remained significant after exclusion of Marburg-I carriers. This was attributable to a rare *HABP2* stop variant (rs41292628). Carriers of this stop variant showed a similar reduction in FSAP activity as Marburg-I carriers, and this finding was replicated. A secondary genome-wide significant locus was identified at a 5p15 locus (rs35510613), and this finding requires future replication. This common variant is located upstream of *ADCY2*, which encodes a protein catalyzing the formation of cAMP. *Results and Conclusions:* This study verified the Marburg-I variant to be a strong regulator of FSAP activity, and identified an *HABP2* stop variant with a similar impact on FSAP activity. A novel locus near *ADCY2* was identified as a potential additional regulator of FSAP activity.

Keywords: blood coagulation factors; epidemiology; genetic variation; hemostasis; plasma.

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Introduction

Factor VII-activating protease (FSAP) is a plasma serine protease targeting various substrates. FSAP is mainly produced by the liver, and is present in the circulation as an inactive proenzyme. Activation is triggered by histones released from apoptotic or necrotic

cells [1]. The protein was initially separately shown to be involved in fibrinolysis, as an activator of single-chain pro-urokinase, and as an activator of FVII [2–4]. More recently, FVII was recognized as a poor FSAP substrate [5,6], and tissue factor pathway inhibitor (TFPI) was identified as a novel substrate. In line with this, impaired FSAP modulation of TFPI levels was suggested as an explanation for the defective thrombus formation observed in mice made deficient for FSAP (FSAP$^{-/-}$) [7]. Other studies have shown that FSAP has a role not only in vascular compartments [8–10], but also in liver fibrosis [11,12], inflammation [13–16], and cancer [17,18]. *In vivo* experiments in wild-type and FSAP$^{-/-}$ mice support a role for FSAP in vascular remodeling, liver fibrosis, neointima formation, and arteriogenesis [11,19–21].

Epidemiological studies have shown that circulating FSAP activity is increased in women as compared with men, and is further enhanced by pregnancy or the use of oral contraceptives [22–24]. FSAP activity is also increased in subjects with deep vein thrombosis [25] or with coronary heart disease [26] as compared with controls. We have found that traditional vascular risk factors explain very little of the variation in plasma FSAP activity, i.e. < 10% in healthy individuals [27]. We have also reported on increased FSAP activity in ischemic stroke cases as compared with controls [27]. Furthermore, a region near the FSAP-encoding gene hyaluronan-binding protein 2 (*HABP2*) was recently identified as being associated with young-onset stroke [28].

An early study of interindividual plasma levels of FSAP in healthy subjects discovered individuals with markedly reduced FSAP activity that was not related to antigen levels [23]. Low levels of FSAP activity were found to be associated with the minor allele of the so-called Marburg-I (MI) single-nucleotide polymorphism (SNP) (rs7080536), which introduces an amino acid change, Gly534Glu (NP_004123.1), in the FSAP protein [29]. The MI-SNP has been associated with several disease processes, such as carotid stenosis [30], stroke [31], and liver fibrosis [12]. Associations between the MI-SNP and venous thrombosis have also been reported [32,33], but inconsistent results do exist [25,34–36].

To our knowledge, only three studies have searched for genetic variants associated with FSAP activity, and these studies were restricted to variants within *HABP2* [16,27,29]. We hypothesized that there are additional genetic variants that contribute to the variation in circulating FSAP activity. Hence, we set out to test this hypothesis by conducting the first genome-wide association study (GWAS) of this quantitative trait. We used a genotyping platform that is enriched with exome content, and searched for both common and rare genetic variants associated with FSAP activity.

Methods

Study design

This study included 3230 participants from the Malmö Diet and Cancer (MDC) study and the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS).

The MDC study is a population-based prospective study that has been described in detail elsewhere [37]. In brief, all men and women living in the Malmö area in southern Sweden and born in 1923–1950 were invited to participate. The participation rate was 41% [38]. The present study is based on the MDC Cardiovascular Cohort, which randomly selected participant from the MDC study [39]. We included subjects without both prevalent (i.e. at baseline) and incident cardiovascular disease (CVD) with biobanked plasma available for FSAP activity measurements (*n* = 2030).

The SAHLSIS is a case–control study for which FSAP activity has been reported [27]. In brief, 600 patients with ischemic stroke at ages 18–69 years were consecutively recruited at stroke units in western Sweden [40]. Controls (*n* = 600) were selected from population-based health surveys or registers to match the cases with regard to age, sex, and geographical region [40]. Only control subjects without a history of CVD or signs of ischemic heart disease on electrocardiogram were included [41].

Sample sizes and baseline characteristics for the two studies are summarized in Table 1. The studies were approved by the ethics committee at the respective universities. All participants or their next of kin gave informed consent.

FSAP activity measurement

Venous blood samples were collected in tubes containing 10% by volume of 0.13 mol L^{-1} sodium citrate. Aliquots of plasma were stored at − 80 °C. For the prospective MDC study, blood samples were drawn during the baseline examinations in 1991–1996. For the SAHLSIS, blood sampling was performed in 1998–2003; at enrollment for controls, and at 3-month follow-up for cases [27,40]. Plasma levels of FSAP activity were measured with an immunocapture activity test, as previously described [23,27]. These measurements were performed in 2010 and 2014 for the SAHLSIS and the MDC study, respectively. The interassay and intra-assay coefficients of variation (CVs) for FSAP activity were 14.7% and 4.7%, respectively, in the SAHLSIS, as reported in [27], and the interassay CV was 8.2% in the MDC study. FSAP activity values followed a normal distribution, and were not transformed for analysis.

Genotyping, imputation, and quality control (QC)

DNA was extracted from whole blood, and genotyping was performed with either the HumanOmniExpress Exome BeadChip v1.0 at the Broad Institute or the HumanOmni

Table 1 Characteristics of participants

	MDC study (n = 2030)	SAHLSIS (n = 1200)	Total (n = 3230)
Ischemic stroke cases, n (%)	0 (0)	600 (50)	600 (18)
Age (years), median (IQR)	58 (53–63)	59 (52–65)	58 (52–63)
Male sex, n (%)	797 (39)	770 (64)	1567 (49)
Hypertension*, n (%)	1227 (60)	578 (48)	1805 (56)
Diabetes mellitus†, n (%)	170 (8)	147 (12)	317 (10)
Current smoking, n (%)	429 (21)	342 (29)	771 (24)
Hyperlipidemia‡, n (%)	1822 (90)	816 (68)	2638 (82)
BMI (kg m^{-2}), median (IQR)	25.3 (23.1–27.7)	26.0 (23.8–28.7)	25.5 (23.4–28.2)
hsCRP§ (mg L^{-1}), median (IQR)	1.2 (0.6–2.7)	1.9 (1.0–4.1)	1.5 (0.7–3.2)
FSAP activity (mU ml^{-1}), median (IQR)	938 (778–1100)	1152 (981–1334)	1008 (822–1192)
Genotyping platform	HumanOmniExpress Exome BeadChip v1.0	HumanOmniExpress Exome BeadChip v1.0, and HumanOmni 5M Exome v1.0¶	Imputed to the UK10K + 1000 Genomes Phase 3

BMI, body mass index; FSAP, factor VII-activating protease; hsCRP, high-sensitivity C-reactive protein; IQR, interquartile range; MDC, Malmö Diet and Cancer; SAHLSIS, Sahlgrenska Academy Study on Ischemic Stroke. *Hypertension was defined as pharmacological treatment for hypertension and/or a systolic blood pressure of ≥ 160 mm Hg and/or a diastolic blood pressure of ≥ 90 mm Hg. †Diabetes mellitus was defined as dietary or pharmacological treatment for diabetes and/or a fasting glucose level of ≥ 7.0 mmol L−1 or a fasting blood glucose level of ≥ 6.1 mmol L−1. ‡Hyperlipidemia was defined as pharmacological treatment for hyperlipidemia and/or a total fasting serum cholesterol level of > 5.0 mmol L−1 and/or an LDL level of > 3.0 mmol L−1. §hsCRP levels in the two studies were determined as described previously [61,62]. ¶Included in the NINDS Stroke Genetic Network study, n = 444 ischemic stroke cases.

5M Exome v1.0 as part of the Stroke Genetics Network (SiGN) study (Illumina, San Diego, CA, USA), and is described in detail elsewhere [42,43]. These arrays have an overlap of the Omni content (coverage of common genome-wide variation) and of the exome content (240k probes). The QC criteria were filtering for sex mismatch, call rate of < 0.95, identical by descent sharing (> 0.375), population outliers, excess autosomal heterozygosity, and deviation from Hardy–Weinberg equilibrium ($P < 10^{-3}$). After separate QC for each dataset had been performed, genotypes available on both platforms were merged and subjected to repeat QC according to the above criteria. Chromosomes were phased by the use of SHAPEIT2 (v2.r790 [44]) and imputed against the UK10K + 1000 Genomes Phase 3 reference panel at the Sanger Imputation Service mapped to hg19 [45]. Approximately 10×10^6 variants with a minor allele frequency (MAF) of > 0.01 and an information score of > 0.3 were brought forward for analysis. After QC, the total number of subjects included in the analyses was 3126.

Genome-wide association analyses

Genotype–phenotype association analyses were performed according to a prespecified analysis plan on variants with an MAF of > 0.01. The association between variants and FSAP activity was assessed with a linear model, on the assumption of an additive effect of each risk allele, with PLINK version 1.9. All analyses were adjusted for age, sex, study (i.e. the MDC study or the SAHLSIS to adjust for study-associated biases such as variations in year of sample collection, plasma storage time, and batch effects on FSAP activity measurements), and ischemic stroke case–control status. Selected MDC study participants were free of both prevalent and incident stroke, so all MDC study subjects were assigned control status. The thresholds of genome-wide significance and suggestive association were set at the conventional levels of $P = 5 \times 10^{-8}$ and $P = 1 \times 10^{-5}$, respectively. On the basis of our sample size and on the assumption of an additive model, the minimal effect size (β-coefficient) per allele that is detectable with 80% power is 0.27 for a MAF of 10%, 0.37 for a MAF of 5%, and 0.78 for a MAF of 1%. Conditional analyses were performed on the lead SNPs for their respective chromosomal regions.

Gene-based analyses

All directly genotyped functional variants (missense, nonsense and splice variants) with a MAF of < 0.05 that passed QC were included in the gene-based tests. Genes were required to contain at least two variants to be included in the analysis (71 134 variants in 11 425 genes in total). Gene-based tests were performed with the optimal combination of the sequence kernel association test and the burden test (SKAT-O) [46] in R package SKAT v.1.0.7, with adjustment for age, sex, case–control status, and study. A Bonferroni-corrected P-value threshold of 4.4×10^{-6} was used (0.05/11 425 genes).

Owing to the association of multiple rare variants in HAPB2 with FSAP activity, we removed one rare variant at a time and repeated SKAT-O to determine the impact of each variant on the gene-based association.

Genotyping of variants associated with FSAP activity in additional cohorts

The MI-SNP, rs35510613 and rs41292628 were genotyped in 665 subjects from the Venous Thromboembolism in Pregnancy (VIP) study from Norway [47,48] and in 276 healthy subjects from the Danish Risk Score (DanRisk) study [49], which have measured FSAP activity with the same assay as was used in the present study. In brief, the VIP study included 313 women with pregnancy-related venous thromboembolism and 353 controls. The DanRisk study included 155 women and 121 men born in either 1949 or 1959. Genotyping was performed at the University of Oslo (Norway) and at LGC genomics (UK) with KASPar genotyping chemistry. The studies were approved by the respective Norwegian and Danish regional committees on medical health research ethics, and all participants gave their written informed consent to participate.

Annotation and functional prediction of variants

Genetic variants of interest were visualized in the UCSC Genome Browser, with regional association plots [50], in HaploReg v4.1 [51], in the Genbank SNP database, and in the Exome Aggregation Consortium (ExAC) [52]. Prediction of functional effects of SNPs (PolyPhen and SIFT) were retrieved from the ExAC. Genetic variants associated with gene expression levels were identified in the Genotype-Tissue Expression Project (GTEx), and expression levels were analyzed in the GTEx and in BioGPS [53]. Genetic variants with a correlation with lead SNPs ($r^2 > 0.6$) were identified in HaploReg v4.1, and additional functional predictions were assessed with RegulomeDB [54].

Cell culture

Mouse primary hepatocytes were isolated from BALB/c mice by collagenase perfusion as described previously [55]. Hepatocytes were cultivated in Dulbecco's modified Eagle's medium High Glucose/F12 (1 : 1) supplemented with 5 mM sodium pyruvate (Thermo Fisher Scientific, Fermentas, Rockford, IL, USA), 10 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 1 mM L-glutamine (Thermo Fisher Scientific), 0.05% (v/v) NaH_2CO_3 (Sigma-Aldrich), 10 mM glucose (Sigma-Aldrich), 10% fetal bovine serum (Thermo Fisher Scientific), 10 units mL^{-1} penicillin, and 10 µg mL^{-1} streptomycin (Invitrogen, Darmstadt,

Germany). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C, and treated with 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium (8-CPT) or forskolin (both from Sigma-Aldrich).

RNA isolation and quantitative PCR analysis

Total RNA was extracted from hepatocytes with the total RNA Miniprep Kit (Sigma-Aldrich). Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). For real-time PCR, the SensiMix SYBR Kit (Bioline, Luckenwalde, Germany) was used. *Habp2* transcript levels were analyzed as described previously, and normalized against the reference gene *Gusb* [55]. Primer sequences are shown in Table 2.

Statistical analysis

We used linear regression to determine the amount of variance in FSAP activity explained by the covariates included in the GWAS model, i.e. age, sex, case–control status, and study. We then performed a model conditioned on these covariates to determine the remaining variance explained by a particular SNP or SNP combination, or by traditional vascular risk factors (hypertension, diabetes mellitus, smoking, and hyperlipidemia), body mass index, and high-sensitivity C-reactive protein (hsCRP, log-transformed). Associations between FSAP activity and genotypes in the VIP and DanRisk studies were analyzed with linear regression adjusting for age, sex, and case–control status, or with Student's *t*-test, as appropriate. Changes in *Habp2* transcript levels in response to treatment as compared with control were analyzed with Student's *t*-test.

Results

We conducted a GWAS and evaluated rare and low-frequency functional variants by using gene-based tests in 3230 individuals of predominantly northern European ancestry to identify genetic loci influencing circulating FSAP activity. Characteristics of participants and genotyping arrays are summarized in Table 1. The majority of the participants (i.e. > 80%) were population-based and free of CVD. Subjects homozygous for the minor allele of the MI-SNP had approximately five-fold lower FSAP activity than subjects homozygous for the major allele

Table 2 List of PCR primers used for real-time PCR of *Habp2*

Target gene	Forward primer sequence	Reverse primer sequence
Gusb	AAAATGGAGTGCGTGTTGGGT	CCACAGTCCGTCCAGCGCCTT
Habp2	TTCCCGACACAGACGGAGA	GTCGTCCGGACCTATTTCA

Gusb, the gene encoding β-glucuronidase.

Table 3 Plasma factor VII-activating protease (FSAP) activity for different Marburg-I genotypes

Genotype	Median FSAP activity (mU mL^{-1})	FSAP activity IQR (mU mL^{-1})	No.
MI:GG	1032	862–1208	2898
MI:AG	592	510–712	224
MI:AA	207	49–366	4

IQR, interquartile range; MI, Marburg-I (rs7080536).

(Table 3), which is in line with previous reports [56]. Above the variance in FSAP activity explained by the covariates adjusted for in the GWAS (i.e. age, sex ischemic stroke case-control status, and study), cardiovascular risk factors, anthropometrics and hsCRP explained only 3.6% of the variance in FSAP activity.

GWAS

After adjustment for age, sex, study, and ischemic stroke case–control status, 164 variants were genome-wide significant, and 239 additional variants were suggestively associated with FSAP activity (Fig. 1; Table S1). The quantile–quantile plot for the GWAS revealed more variants with lower observed *P*-values than expected (Fig. 1B). The genomic control (lambda) was 1.016. Associated loci with $P < 1 \times 10^{-6}$ are shown in Table 4 and outlined below.

The majority of the genome-wide associations ($n = 163$) were located at the 10q25 locus near *HABP2*. The strongest association was found for the MI-SNP rs7080536 ($P = 7.0 \times 10^{-142}$; Fig. 1C).

At the 5p15 locus, rs35510613 was genome-wide significant ($P = 1.3 \times 10^{-8}$; Fig. S1). This variant is located 19 kbp upstream of the adenylate cyclase 2 (*ADCY2*) gene. Two SNPs, rs12652415 and rs1609428, located in *ADCY2* introns were in linkage disequilibrium (LD) with rs35510613 (r^2, 0.74 and 0.64; and D', 0.89 and 0.96, respectively) and showed suggestive associations with FSAP activity ($P = 1.1 \times 10^{-6}$ and $P = 4.3 \times 10^{-6}$, respectively).

SNPs with suggestive associations with $P < 1 \times 10^{-6}$ were also located at 12q21 (rs75809015), 17q25 (rs62073440), 7p11 (rs373067567) and 16q24 (rs61613787) loci (Table 4).
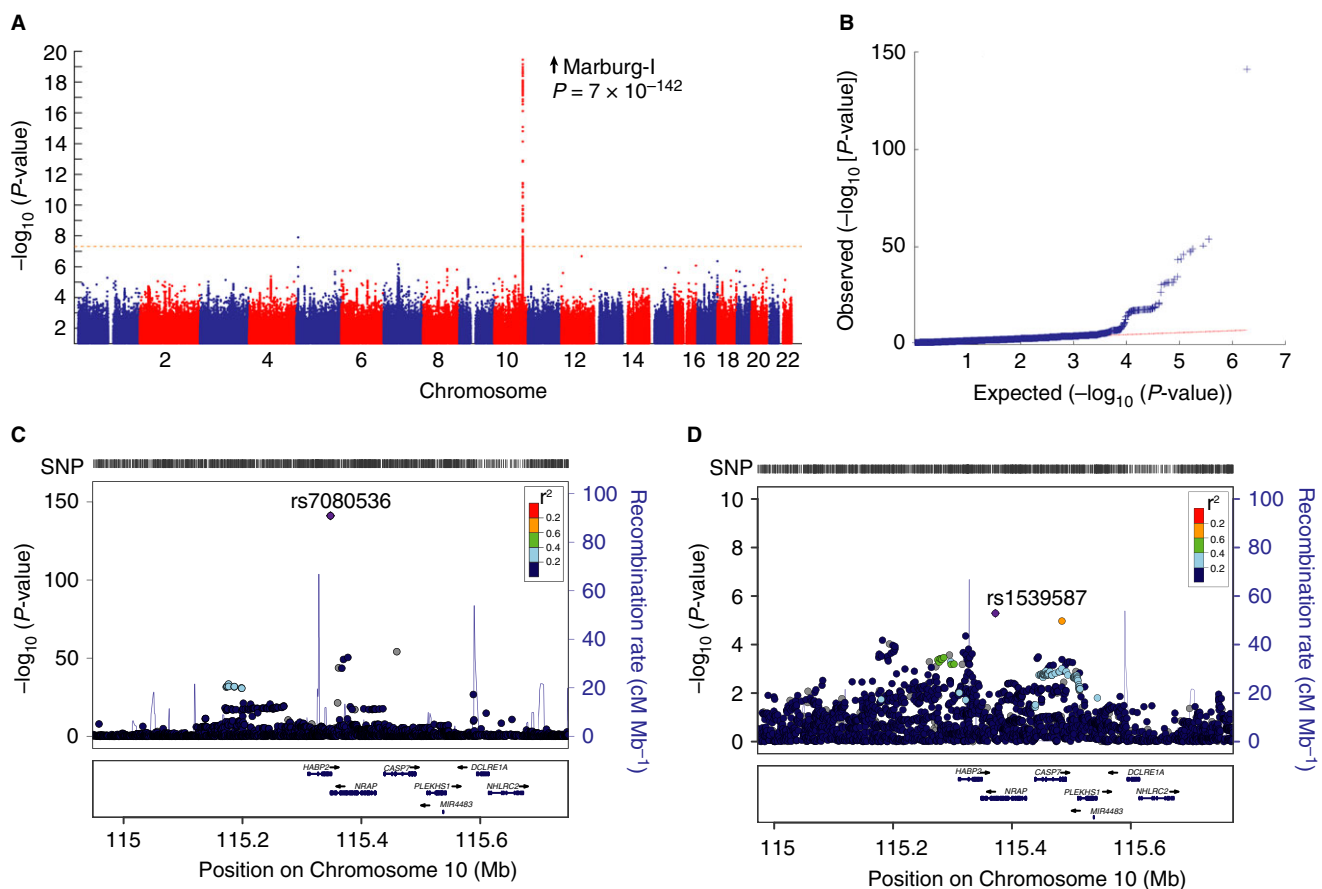

Fig. 1. Genome-wide association analyses of factor VII-activating protease (FSAP) activity. (A) Manhattan plot of associations for FSAP activity. The dotted line shows genome-wide significance (5×10^{-8}). The plot is truncated at a *P*-value of 10^{-20}. (B) Quantile–quantile plot for associations. (C, D) Regional association plots of the Marburg-I (MI)-single-nucleotide polymorphism (SNP) (rs7080536) (C) and of rs1579587 (D), which showed suggestive association ($P = 5.1 \times 10^{-6}$) when the 10q25 region was adjusted for MI-SNP. Linkage disequilibrium (r^2) is indicated by the color scale. [Color figure can be viewed at wileyonlinelibrary.com]

Table 4 Lead associated genetic loci ($P < 1 \times 10^{-6}$) for factor VII-activating protease activity

Locus	Lead variant	Chromosome: position of lead variant in hg19	Gene-related position	Alleles (A1/A2)	Frequency	β	P-value
10q25.3	rs7080536	10: 115 348 046	Missense in *HABP2*	A/G	0.037	−429	7.0×10^{-142}
5p15.31	rs35510613	5: 7 377 210	Intergenic, upstream of *ADCY2*	–/G	0.77	−45	1.3×10^{-8}
12q21.31	rs75809015	12: 82 823 136	Intron in *METTL25*	G/A	0.012	194	2.1×10^{-7}
17q25.3	rs62073440	17: 81 005 762	Intron in *B3GNTL1*	C/T	0.11	59	4.6×10^{-7}
7p11.2	rs373067567	7: 57 749 605	Intergenic	CAT/C	0.61	−37	7.0×10^{-7}
16q24.1	rs61613787	16: 84 728 954	Intergenic, upstream of *USP10*	C/T	0.014	169	8.6×10^{-7}

ADCY2, adenylate cyclase 2; *B3GNTL1*, UDP-GlcNAc:beta Gal β-1,3-*N*-acetylglucosaminyltransferase-like 1; *HABP2*, hyaluronan-binding protein 2; *METTL25*, methyltransferase-like 25; *USP10*, ubiquitin-specific peptidase 10.

Above the variance in FSAP activity explained by the covariates adjusted for in the GWAS (i.e. age, sex, ischemic stroke case-control status, and study), rs7080536 explained 18.6% of the remaining variance in FSAP activity, whereas rs35510613 explained 1%. Associations for these variants were also evaluated in a stratified analysis of study participants without stroke or CVD (i.e. all MDC study subjects and SAHLSIS controls). The effect sizes for both rs7080536 and rs35510613 were similar to those in the whole sample, and the same was true when MDC subjects only were analyzed (Table S2).

Conditional analyses on lead SNPs

To identify independent signals within each locus, conditional analyses were performed on the lead variants with $P < 1 \times 10^{-6}$. At the 10q25 locus, rs1539587 remained suggestively associated after adjustment for rs7080536 ($P = 5.1 \times 10^{-6}$). This variant is located in the neighboring nebulin-related anchoring (*NRAP*) gene (Fig. 1D). No other suggestive associations remained.

Gene-based analyses

Next, we performed gene-based association tests by using SKAT-O. Two genes were significantly associated with FSAP activity after Bonferroni correction. *HABP2* showed the strongest association ($P = 8.2 \times 10^{-120}$), and was represented by 10 variants. *DCLRE1A* ($P = 3.0 \times 10^{-16}$), which is also located on chromosome 10q25, was represented by nine variants. The gene-based analysis was repeated for chromosome 10 without the carriers of the MI-SNP, and, in this analysis, only *HABP2* remained significant ($P = 8.9 \times 10^{-9}$). *NRAP* ($P = 1.5 \times 10^{-5}$, represented by 22 variants) was ranked as the second most associated gene.

On testing of the contribution of each rare variant in *HABP2* to the SKAT-O P-value, rs41292628 (MAF of 0.002), which encodes a stop codon at amino acid position 203, was identified as a determinant of FSAP activity (Fig. 2). The FSAP activity in 13 carriers of this stop variant was reduced as compared with that in non-carriers ($IQR_{rs41292628:C/T} = 404–592$ mU mL^{-1},
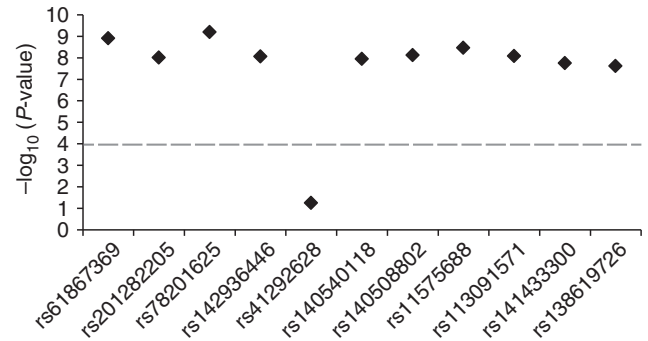

Fig. 2. Analysis for factor VII-activating protease activity associations of rare variants (minor allele frequency of < 5%) in *HABP2*. The $-\log_{10}(P$-value) is shown from optimal combination of the sequence kernel association test and the burden test (SKAT-O) of *HABP2*. For each rare variant presented in the graph, the variant was removed from the test and the P-value for the gene-based SKAT-O analysis was determined. The analyses were performed in individuals homozygous for the major allele of the Marburg-I single-nucleotide polymorphism (MI-SNP:GG). The dashed line shows the Bonferroni-corrected threshold P-value of 0.00011 (0.05/447 genes) for gene-based tests on chromosome 10 among MI-SNP:GG-carrying subjects.

$IQR_{rs41292628:C/C} = 825–1190$ mU mL^{-1}, $P_{t\text{-test}} < 0.001$). The association for rs41292628 was also independent of the *NRAP* rs1539587 variant ($P = 2.5 \times 10^{-16}$) after adjustment for both the MI-SNP and rs1539587 in a linear model. In subjects homozygous for the MI major allele (MI:GG), after adjustment for GWAS covariates, 3.5% of the remaining variance in FSAP activity was explained by rs41292628, rs35510613, and rs1539587.

Genotypes for top associated variants in the VIP and DanRisk studies

The genotyping success rate was > 95% for all variants. The known association between the MI-SNP and FSAP activity was detected in both studies ($P < 0.001$). One carrier of the stop variant was identified in the VIP study ($MAF_{rs41292628} = 0.0009$) and six in the DanRisk study ($MAF_{rs41292628} = 0.011$). Similarly to the discovery cohort, carriers of the stop variant had reduced FSAP activity (VIP, $FSAP_{rs41292628:C/T} = 770$ mU mL^{-1} versus

interquartile range $[IQR]_{rs41292628:C/C}$ = 910–1210 mU mL^{-1}; and DanRisk, $IQR_{rs41292628:C/T}$ = 14–62% of reference plasma versus $IQR_{rs41292628:C/C}$ = 60–85% of reference plasma, $P_{t\text{-test}}$ < 0.001). In contrast, the association for rs35510613 on the 5p15 locus was not confirmed in either the Norwegian or the Danish study (P > 0.2 for both).

Annotation and functional prediction of top associated variants

For the top associated variants (Table 4), we also evaluated expression quantitative trait locus (eQTL) presence, and the putative regulatory function of variants in LD (Tables S3 and S4). There were, in total, 12 SNPs with r^2 > 0.6 with top associated variants (P < 1 × 10^{-6}) in the CEU population dataset in the HaploReg database (Table S3). Of these, 11 were included in the GWAS. None of the top associated variants had a RegulomeDB score of < 5. For rs62073440 and for 5p15 variants in LD with the lead SNP in this region (rs1265241 and rs1609428), eQTLs were identified towards *B3GNTL1* and non-protein-coding *CTD-2296D1.5* (Table S4), respectively.

The missense MI-SNP is known to influence FSAP activity, and was thus not evaluated further here, apart from the *in silico* annotation provided in Table S3. The *NRAP* rs1539587 is also a missense mutation. The estimated effect of the minor allele on NRAP protein function is deleterious and benign according to PolyPhen and SIFT, respectively. This variant is also predicted to alter a putative regulatory motif sequence (Table S3). In BioGPS, the *NRAP* transcript was solely expressed in heart tissue, whereas GTEx indicated high gene expression also in skeletal muscle (Fig. S2). However, except for the genomic proximity, we could not identify any clear biological or functional link between FSAP and *NRAP*.

In contrast, the 5p15 locus, containing *ADCY2*, may be of functional relevance. Adenylate cyclase catalyzes the conversion of ATP to the secondary messenger molecule cAMP. Therefore, we investigated whether cAMP modifiers influence *Habp2* expression *in vitro*, as described below. Given that circulating FSAP is mainly produced in the liver [2], hepatocytes were investigated.

Habp2 expression in hepatocytes in response to cAMP modifiers

In order to determine whether *Habp2* mRNA levels were affected by cAMP modifiers, primary mouse hepatocytes were incubated with 8-CPT, an activator of the downstream cAMP activator-dependent kinase (protein kinase A [PKA]), or with forskolin, which stimulates cAMP production. Increases in *Habp2* transcript levels were found after 48 h of incubation with 8-CPT (P < 0.05 for both 50 μM and 200 μM) and after 24 h of forskolin treatment (P < 0.05 for both 50 μM and 200 μM) relative to control (Fig. 3).

Discussion

We searched for genetic factors contributing to the variation in circulating FSAP activity by using GWAS and gene-based analysis. We identified several signals at the 10q25 locus: (i) the lead SNP in the GWAS was the well-known missense variant in the FSAP-encoding *HABP2* gene, i.e. MI (P = 7.0 × 10^{-142}); (ii) suggestive significance remained for an SNP at the 10q25 locus in *NRAP* after conditioning for this MI variant; and (iii) in gene-based analysis, *HABP2* was genome-wide significant, an *HABP2* stop variant was identified as an additional determinant of FSAP activity, and *NRAP* showed suggestive association. In the GWAS, we also identified a novel genome-wide significant locus on chromosome 5 with the
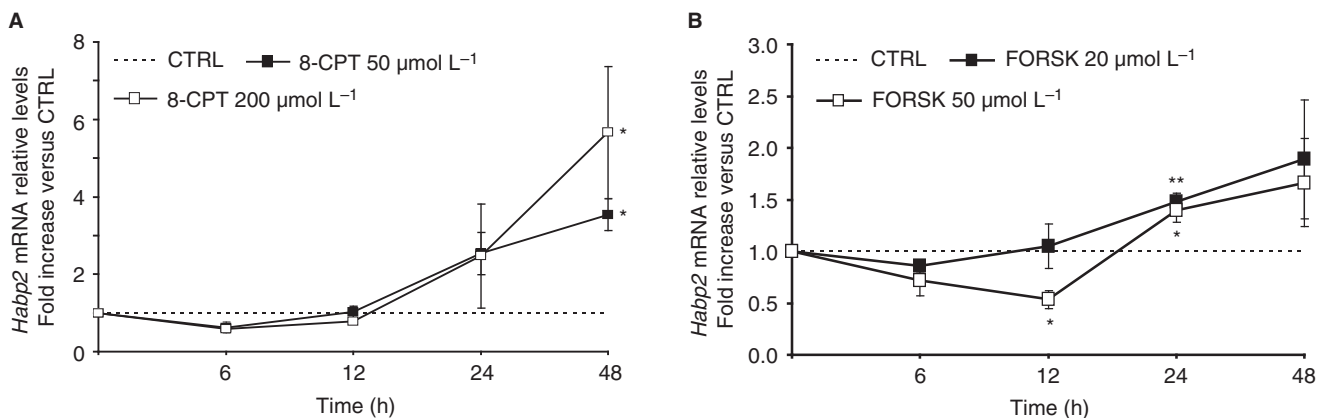

Fig. 3. *Habp2* expression in primary mouse hepatocytes in response to cAMP modifiers. (A) Cells were stimulated with 50 μM or 200 μM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium (8-CPT) in n = 4 biological replicates (n = 2 technical replicates). (B) Cells were stimulated with 20 μM or 50 μM forskolin in n = 5 biological replicates (n = 2 technical replicates). Data are presented as mean ± standard error of the mean. Significance is compared with control (CTRL) at each time point *P < 0.05; **P < 0.005. FORSK, forskolin.

lead SNP located upstream of *ADCY2*. Taken together, variants within *HABP2*, *NRAP* and *ADCY2* were found to explain 21.4% of the variance in FSAP activity, above the variance explained by the covariates in the GWAS (i.e. age, sex, ischemic stroke case-control status, and study). In comparison, cardiovascular risk factors, including hsCRP, explained only 3.6% of the variance in FSAP activity above the variance explained by the covariates included in the GWAS.

In the GWAS, we identified a high number of SNPs with genome-wide significance at the 10q25 locus. The lead MI-SNP was reported to be associated with FSAP activity in 2002 [29], and we confirmed here that homozygous carriers of the MI variant have approximately five-fold lower plasma FSAP activity than non-carriers [56]. We also identified additional signals at the 10q25 locus that were independent of the MI-SNP, indicating a complex genomic architecture at this locus. After conditioning on the MI-SNP, rs1539587 in the neighboring *NRAP* gene remained suggestively associated with FSAP activity. After adjustment for GWAS covariates, the amounts of FSAP activity variance explained by the MI-SNP alone and together with rs1539587 were 18.6% and 19.2%, respectively.

HABP2 was also significantly associated with FSAP activity in the gene-based analysis. When we omitted all MI carriers from this analysis, the association with *HABP2* remained. This association was mainly attributable to a newly identified rare variant (rs41292628), which is located in the coding region and introduces a premature stop codon at amino acid position 203 (NP_004123.1). This position is before the serine protease domain of FSAP, and thus total loss of function can be expected to result from the introduction of this stop codon. Carriers of the stop variant had FSAP activity in the same range as MI variant carriers. In line with our results, a study from the Netherlands reported on an anonymous serum donor with deficient FSAP protein who was homozygous for the stop variant [16]. As a replication effort, we genotyped 941 subjects from Norway and Denmark. We identified seven heterozygous carriers of the stop variant for whom the FSAP activities were in the same range as for MI variant carriers. Our results also indicated a north-to-south gradient of increasing MAF for the stop variant. As FSAP activity has been implicated in several pathophysiological processes (recently reviewed in [57]), and the mechanisms remain elusive, future genetic studies that take into account both the *HAPB2* MI and stop variant would be of interest. However, given the low frequency of these variants, this will require very large datasets genotyped with rare variant information (e.g. exome chips or exome sequencing).

Using the GWAS approach, we also identified a novel genome-wide significance locus at 5p15. The lead SNP (rs35510613) is located upstream of *ADCY2* and is in high LD with two SNPs in *ADCY2* introns. The protein encoded by *ADCY2* is one of 10 adenylate cyclase isoforms that catalyzes the formation of cAMP. We have shown that

there is a significant correlation between plasma levels of FSAP antigen and FSAP activity [27], suggesting that, apart from variants affecting the FSAP protein itself, such as the MI and stop variants, gene variants influencing FSAP expression may influence FSAP activity. There are also data indicating that *Habp2* mRNA and FSAP protein levels change in parallel [22,58,59], and experimental data from studies on hepatic rodent cells indicating that cAMP may be involved in regulating *Habp2* expression [55].

In order to obtain more direct information on the potential involvement of the cAMP-dependent pathway in regulating *Habp2* expression, we therefore performed *in vitro* studies on primary mouse hepatocytes. Using either forskolin or 8-CPT, we found increased mRNA levels of *Habp2* in these cells. Although the results from both *in vitro* manipulations agree with a model in which increased cAMP levels may affect *Habp2* transcription through PKA activation, direct functional characterization is needed to support a functional role for *ADCY2* in regulating FSAP.

It is also of note that we were not able to replicate the association between rs35510613 and FSAP activity in the two other, smaller, Scandinavian studies. However, the replication effort was mainly targeted at the *HABP2* stop variant, and we did not have sufficient statistical power to replicate the effect size for rs35510613. Thus, we believe that future independent replications in larger samples are warranted before further studies are performed to functionally characterize this variant.

The major strength of this study is that we analyzed ~ 10 million markers spread throughout the genome, covering both common variants (assessed via GWAS) and rare variants (assessed via gene-based testing) in 3230 well-characterized individuals who were ethnically relatively homogeneous [43], and the majority were ascertained by population-based recruitment methods. This is thus the most comprehensive genetic study of circulating FSAP activity to date. However, in the context of genome-wide association analysis, the sample size is still modest, and we thus have limited power to detect relatively small effect sizes, even for common variants. Another limitation is that we included participants with ischemic stroke, who, as a group, have increased FSAP activity as compared with controls. However, in a sensitivity analysis excluding stroke cases, we found similar effect sizes for the lead variants in *HABP2* and *ADCY2*. Another strength of the study is the use of a standardized protocol for blood sampling in both cohorts, and the fact that all samples were analyzed with the same FSAP activity assay. A limitation is that blood sampling and FSAP activity measurements were performed on different occasions. However, analysis of the largest cohort (MDC study) alone provided similar effect sizes for the top findings. We used a well-characterized FSAP activity assay, but a limitation of this assay is that FSAP hydrolyzes single-chain pro-urokinase, generating urokinase plasminogen activator, the activity of which is used as a proxy for FSAP activity. It is not known whether this

covers all aspects of FSAP activity. It is thus plausible that an assay with another substrate may generate somewhat different findings. However, we have shown that the MI FSAP protein has reduced activity as compared with wild-type FSAP towards all identified substrates that we have tested so far [21,56]. With regard to the rare variant analysis, we used an exome array, so rare variants that impact on FSAP activity may remain to be discovered with exome sequence analysis. Additionally, it should be emphasized that the results were generated in a population of predominantly European ancestry, and our findings may not be generalizable to populations of different ethnicity.

We confirmed that the *HABP2* MI variant is a strong regulator of FSAP activity, and we identified and replicated a *HABP2* stop variant with a similar impact on FSAP activity. We also identified a novel locus near *ADCY2* as an additional potential regulator of circulating FSAP activity that requires future independent replication. Interestingly, the identified genetic variants explained ≈ 20% of the variation in FSAP activity, whereas cardiovascular risk factors explained < 4%. Additional larger genetic studies, potentially employing future novel assays for the measurement of FSAP activity [60], may provide further insights into the genetic regulation of this plasma protease, and thus pave the way for future studies on its role in different pathophysiological pathways.

Addendum

C. Jern, S. M. Kanse, O. Melander, M. Olsson, and T. M. Stanne conceived the research design of the present study. C. Jern and A. Pedersen were responsible for the SAHLSIS (sample and phenotype contribution). G. Engström and O. Melander were responsible for the MDC study (sample and phenotype contribution). P.M. Sandset and A. F. Jacobsen were responsible for the VIP study (sample and phenotype contribution). J. J. Sidelmann and N. P. Rønnow Sand were responsible for the DanRisk study (sample and phenotype contribution). A. Martinez-Palacian and E. Kara performed *in vitro* experiments and measurements of plasma FSAP activity, respectively. S. M. Kanse supervised *in vitro* experiments and measurements of plasma FSAP activity. E. Lorentzen, M. Olsson, and A. Martinez-Palacian performed statistical analyses. M. Olsson, T. M. Stanne, C. Jern and S. M. Kanse interpreted the data. M. Olsson and C. Jern: drafted the manuscript. S. M. Kanse, T. M. Stanne, O. Melander, G. Engström, P. M. Sandset, and J. J. Sidelmann intellectually reviewed the manuscript. All authors contributed to the last revision process and approved the version to be published.

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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 online in the Supporting Information section at the end of the article.

Fig. S1. Regional association plot of rs35510613, which showed significant association for FSAP activity.
Fig. S2. Tissue distributions of *NRAP* gene expression.
Table S1. Genome-wide ($P < 5 \times 10^{-8}$) and suggestive significant loci ($P < 1 \times 10^{-5}$) for FSAP activity.
Table S2. Association of rs7080536, and rs35510613 with FSAP activity in study participants without stroke or CVD.
Table S3. Functional annotations from HaploReg 4v1, level of association with FSAP activity in present GWAS, and RegulomeDB score for top associated lead genetic variants ($P < 1 \times 10^{-6}$), of rs1539587, and variants in linkage disequilibrium (LD; $r^2 \geq 0.6$ in EUR population in HaploReg 4v1) with these variants.
Table S4. Expression quantitative trait loci (eQTLs) from the GTEx Portal identified for top associated lead variants ($P < 1 \times 10^{-6}$) for FSAP activity, and variants highlighted in this study.
Data S1. STROBE Statement – checklist of items that should be included in reports of observational studies

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