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Factor VII activating protease (FSAP) is not essential in the pathophysiology of angioedema in patients with C1 inhibitor deficiency

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ABSTRACT

Background: Excessive bradykinin (BK) generation from high molecular weight kininogen (HK) by plasma kallikrein (PK) due to lack of protease inhibition is central to the pathophysiology of hereditary angioedema (HAE). Inadequate protease inhibition may contribute to HAE through a number of plasma proteases including factor VII activating protease (FSAP) that can also cleave HK.

Objective: To investigate the interaction between FSAP and C1 inhibitor (C1Inh) and evaluate the potential role of FSAP in HAE with C1Inh deficiency.

Materials and methods: Plasma samples from 20 persons with HAE types 1 or 2 in remission were studied and compared to healthy controls. We measured and compared antigenic FSAP levels, spontaneous FSAP activity, FSAP generation potential, activation of plasma pre-kallikrein (PPK) by FSAP, and the formation of FSAP-C1Inh and FSAP-alpha2-antiplasmin (FSAP-α2AP) complexes. Furthermore, we measured HK cleavage and PK activation after activation of endogenous pro-FSAP and after addition of exogenous FSAP.

Results: In plasma from HAE patients, there is increased basal FSAP activity compared to healthy volunteers. HAE plasma exhibits decreased formation of FSAP-C1Inh complexes and increased formation of FSAP-α2AP complexes in histone-activated plasma. Although exogenous FSAP can cleave HK in plasma, this was not seen when endogenous plasma pro-FSAP was activated with histones in either group. PK was also not activated by FSAP in plasma.

Conclusion: In this study, we established that FSAP activity is increased and the pattern of FSAP-inhibitor complexes is altered in HAE patients. However, we did not find evidence suggesting that FSAP contributes directly to HAE attacks.

1. Introduction

Hereditary angioedema (HAE) is a rare condition estimated to affect 1 in 50,000 people (Maurer et al., 2018). The classical types of this condition, HAE types 1 and 2, are characterised by an inherited lack of functional C1 inhibitor (C1Inh) and spontaneous and triggered subcutaneous and submucosal oedema (Donaldson and Evans, 1963). In type 1, the plasma levels of functional and antigenic C1Inh are low. In type 2, the antigenic levels of C1Inh are normal or elevated but due to a dysfunctional C1Inh protein, the functional levels are low (Rosen et al., 1965). HAE without C1Inh deficiency also exists. In these cases, the underlying aetiology is often unknown, although there have been discoveries of a number of novel pathophysiological mechanisms in recent years (Maas and López-Lera, 2019; Bork et al., 2021). Recognised triggers for episodes of angioedema include physical trauma in addition to physical exertion, mental stress, infection (Zotter et al., 2014), and last

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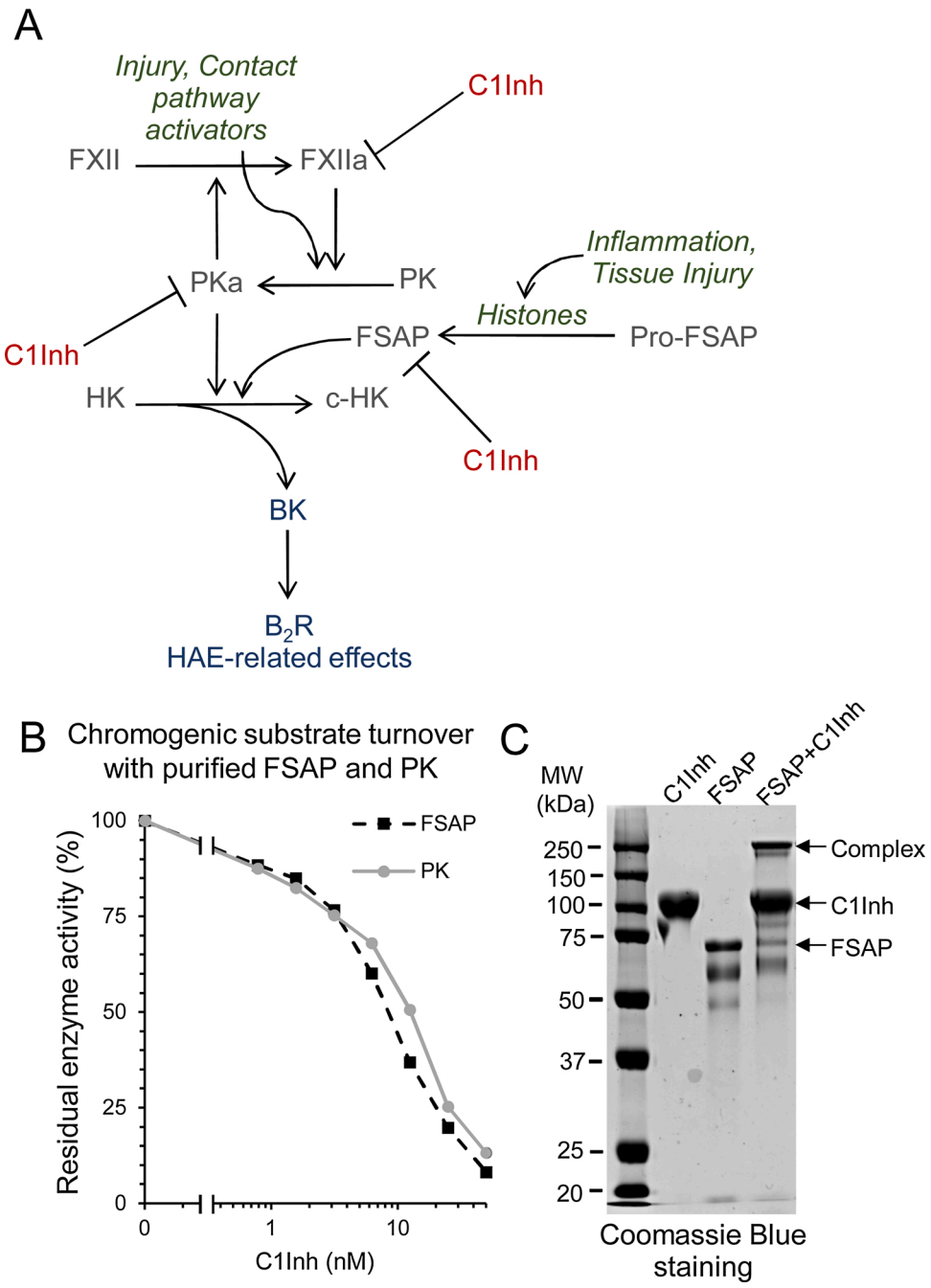

Fig. 1. Characterization of the inhibition of FSAP by C1Inh: (A) The flowchart represents our hypothesised model in which tissue injury leads to histone release and the activation of FSAP and FSAP-catalysed cleavage of BK from HK. Also included, is a simplified graphical representation of the proposed mechanisms of trauma-induced contact system activation .3435 In the latter, tissue injury generates PK via either FXII activation on endothelial cells or via action of heat shock protein 90 (HSP-90). PK then cleaves BK from HK and activates the B_2 receptor. C1Inh is a known inhibitor of both plasma kallikrein and FSAP. (B) Purified plasma FSAP and PK (10-20 nM each) was incubated with increasing concentration of C1Inh for 30 min at RT and residual FSAP and PK activity was measured with the chromogenic substrate S-2288 and CS31, respectively (n = 5 independent experiments). (C) FSAP (1 μg) and C1Inh (4 μg) were incubated for 60 min at RT and then run on non-reduced SDS-PAGE to visualize formation of SDS-stable high MW complex. This experiment was independently replicated three times.

Table 1
Second order rate constants of major plasma serpins for target proteases and for FSAP.

Inhibitor	Plasma concentr.	Major target proteases	Second order rate constant target proteases (M^{-1} s^{-1})	Second order rate constant FSAP (M^{-1} s^{-1})
α2AP	1.0 μM	Plasmin	3.8x 10^{7}*	4.2 x 10^{4}
		C1r	2.8 x 10^{3}*	
C1Inh	1.7 μM	C1s	1.2–5 x 10^{4}*	2.2 x 10^{4}
		PK	1.7 x 10^{4}*	
		FXIIa	3.1 x 10^{3}†	

* Data from Gettins (2002).
† Data from Wuillemin et al. (1996).

but not least oestrogens (Maas and López-Lera, 2019).

C1Inh is a serine protease inhibitor (SERPIN) with broad specificity. It was named for its ability to inhibit C1r and C1s of the classical pathway of the complement system (Pensky et al., 1961; Ratnoff et al., 1969) and C1Inh also inhibits MASP2 in the lectin pathway of the complement system (Presanis et al., 2004). However, C1Inh inactivates proteases in other physiological systems as well, among these are plasma kallikrein (PK) (Schapira et al., 1982) and factor XIIa (de Agostini et al., 1984) in the contact system, plasmin (Harpel and Cooper, 1975) and tissue plasminogen activator (tPA) (Booth et al., 1987) in the fibrinolytic system, and factor XIa (Wuillemin et al., 1995) and thrombin (Cugno et al., 2001) in the coagulation system. Its inhibitory effects also extend to other proteases such as factor VII activating protease (FSAP) (Choi--Miura et al., 2001) (Fig. 1A).

Oedema formation in HAE patients is mediated by the nonapeptide

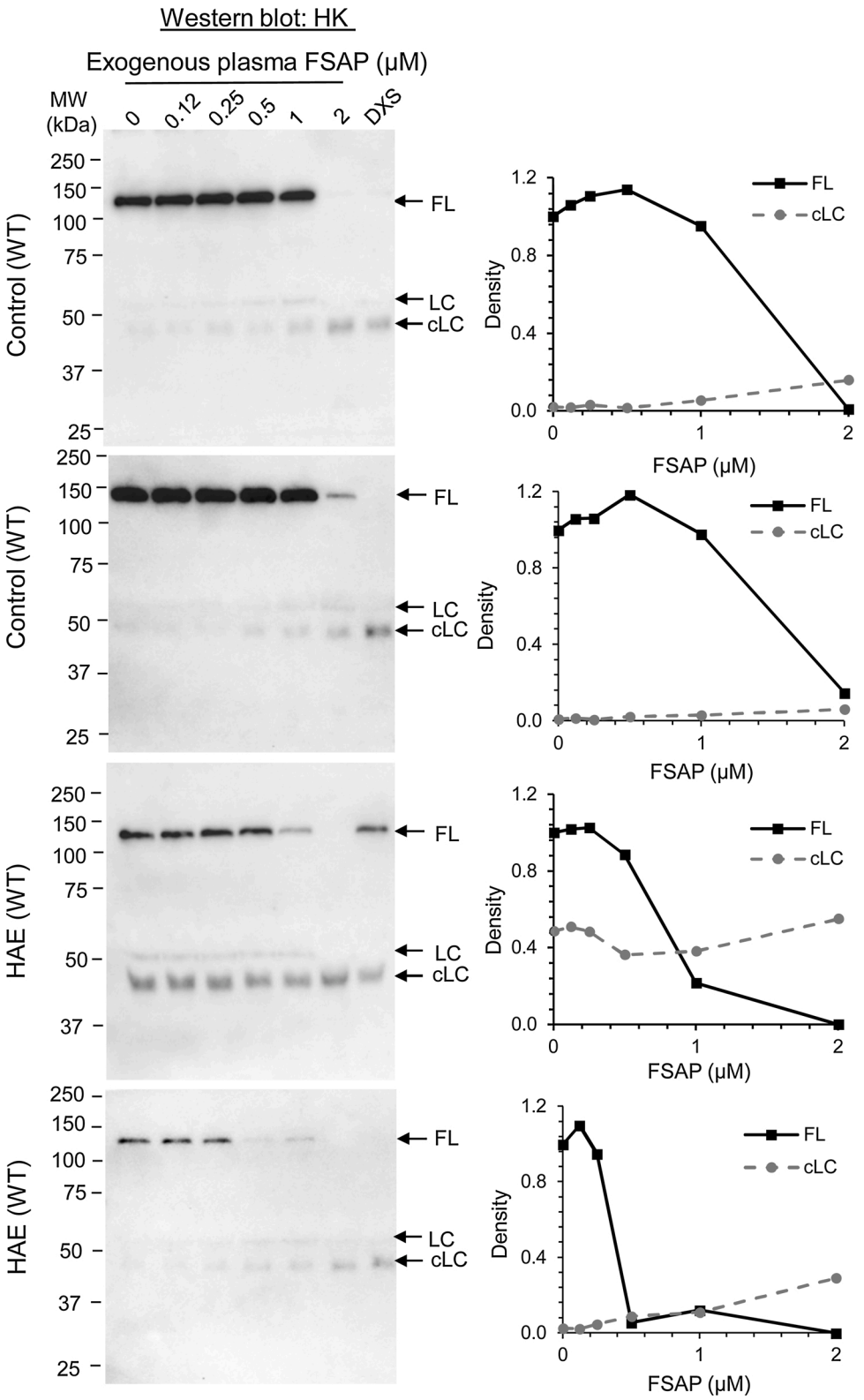

Fig. 2. Effect of exogenously added purified FSAP on the cleavage of HK in plasma of HAE patients and control donors: Increasing concentration of FSAP was added to plasma and incubated for 60 min at RT and the samples analysed by Western blotting with an antibody against HK. The uncleaved full-length HK band (MW 120 kDA) and the cleaved light chain (cLC) band (MW 46 kDa) were quantified by densiometry. DXS (2.5 μg/mL) was added to plasma and incubated for 15 min on ice to prevent complete autolysis and was used as a positive control for HK activation. Similar results were obtained with two additional samples from HAE patients and control donors.

bradykinin (BK) (Fields et al., 1983) which via agonism on B_2 receptor on endothelial cells promotes vascular permeability (Bouillet et al., 2011). In plasma, BK is generated by proteolytic cleavage from its precursor protein high molecular weight kininogen (HK) by action of PK (Thompson et al., 1978). The lack of functional C1Inh thus leads to loss of PK inhibition, increased cleavage of HK and overproduction of BK (Fig. 1A).

FSAP is a serine protease produced in the liver and released into circulation as an inactive single-chain zymogen (pro-FSAP). On contact with extracellular histone, it is converted into the two-chain active protease (referred to as FSAP in the rest of the article) by auto proteolysis (Etscheid et al., 2000; Yamamichi et al., 2011). Histone release, and subsequent pro-FSAP activation, may occur with tissue injury, infectious disease, apoptosis and necrosis (Zeerleder et al., 2008; Kanse

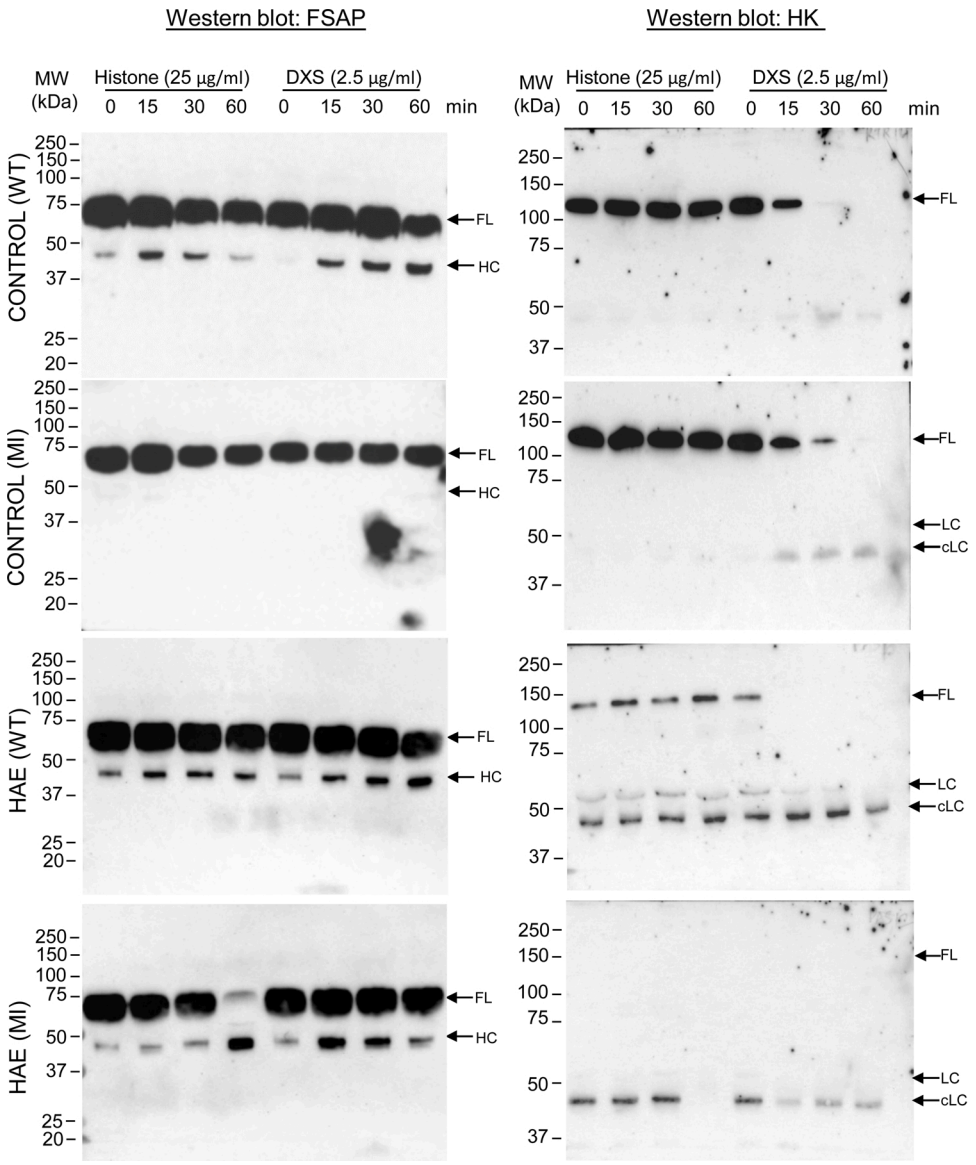

Fig. 3. Effect of activation of endogenous pro-FSAP on HK cleavage in plasma of HAE patients and control donors: Plasma was incubated with histones (25 µg/mL) and DXS (2.5 µg/mL) at RT for 0-60 min. Samples were analysed by Western blotting with an antibody against FSAP (left panels). Full-length (FL) FSAP (MW 64 kDa) and heavy chain (HC) of FSAP (45 kDa) are indicated with arrows. One plasma each, from an HAE patient with and without the FSAP-MI-SNP and similarly from control donors with and without MI-SNP were analysed. The same samples were also analysed by Western blotting with an anti-HK antibody and full-length (FL) HK (120 kDa) and the light chain (LC) (56 kDa) and cleaved light chain (cLC) (46 kDa) HK are indicated by arrows (right panels). Similar results were obtained in an additional set of fours plasma samples with the same diagnosis and genotype.

et al., 2012a, 2012b). Negatively charged polyanions such as heparin, nucleic acids and dextran sulphate also activate pro-FSAP (Zeerleder, 2018).

FSAP acts proteolytically on a number of substrates, among them HK from which it cleaves BK (Etscheid et al., 2002). On endothelial cells, FSAP was found to trigger intracellular calcium flux after BK release from cell-bound HK and subsequent B_2 receptor signalling (Kress et al., 2006). FSAP is also an activator of pro-urokinase plasminogen activator (pro-uPA) (Römisch et al., 1999) and inactivates tissue factor pathway inhibitor (TFPI) rather than activating factor VII (Kanse et al., 2012a, 2012b). The proteolytic activity of FSAP is significantly reduced in carriers of the Marburg I single nucleotide polymorphism (MI-SNP) in the FSAP gene (Etscheid et al., 2012). The MI-SNP is associated with carotid stenosis (Willeit et al., 2003) and increased risk of stroke and stroke-related mortality (Trompet et al., 2011). FSAP inhibitors include, in addition to C1Inh, alpha2-antiplasmin (α2AP) (Hunfeld et al., 1999) plasminogen activator inhibitor-1 (PAI-1) (Wygrecka et al., 2007) and TFPI (Stephan et al., 2012). Thus, alterations in inhibitor activity could impact FSAP activity and functions *in vivo*.

Trauma and inflammation are triggers associated with HAE attacks. Proposed mechanisms include activation of plasma pre-kallikrein via FXIIa on exposure to endothelium and/or via the release of heat-shock protein 90 (HSP-90) in response to stress (Kaplan, 2010; de Maat et al., 2021). However, the possible consequences of histone release from trauma and inflammation have not been considered in HAE attack pathogenesis. Here, we hypothesize that injury and/or inflammation leads to the release of histones and the activation of pro-FSAP, which in turn cleaves HK and releases BK and contributes to HAE attacks. We have tested this hypothesis by characterizing the inhibition of FSAP with C1Inh and the activation of HK by FSAP. We then compared the activation of FSAP, HK and PK in plasma from a cohort of HAE patients and controls using Western blotting and fluorogenic substrate assays. We also measured FSAP antigen, activity and FSAP-inhibitor complexes in these samples to characterize the effect of FSAP on HK activation in HAE patients and to characterize how deficiency of C1Inh affects FSAP function.

2. Materials and methods

2.1. Patients

HAE type 1 or 2 was diagnosed based on low antigenic or functional C1Inh either due to angioedema episodes or by HAE screening in persons with family history for the condition. Patient and control samples were

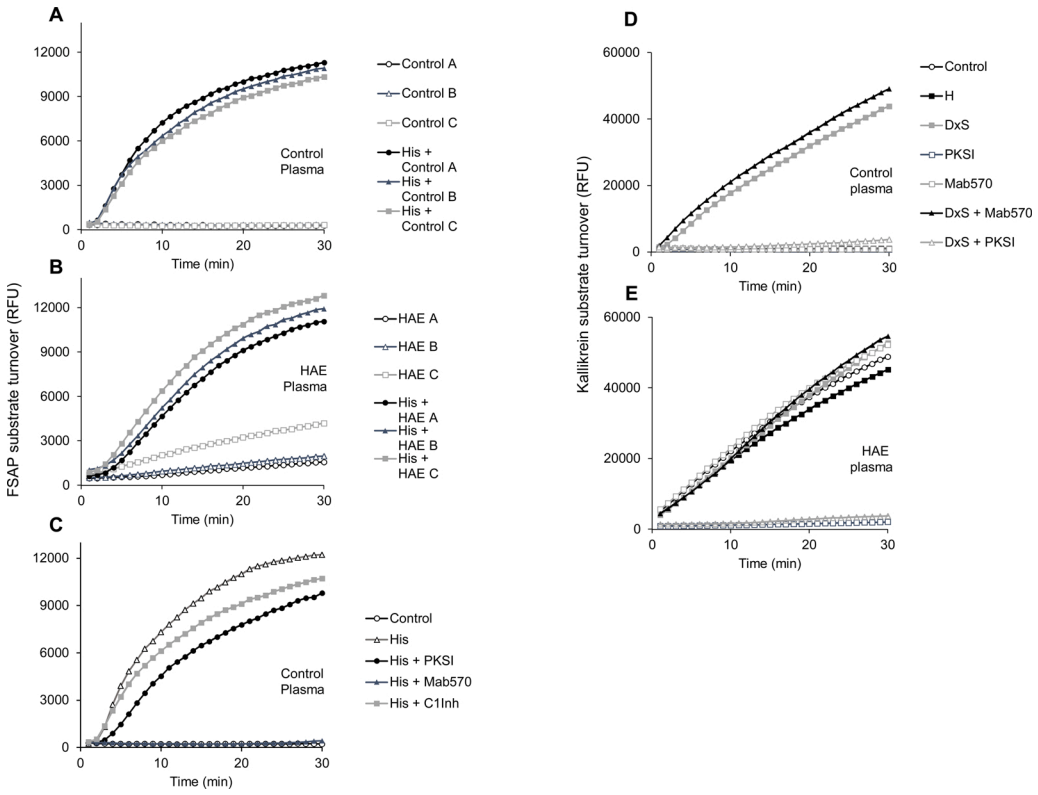

Fig. 4. Effect of activation of endogenous pro-FSAP on PK activation in plasma of HAE patients and control donors: For the analysis of pro-FSAP activation, plasma was activated with histones (25 μg/mL). Cleavage of the FSAP fluorescence substrate was detected as relative fluorescence units (RFU). Results from (A) 3 control donor plasma and (B) 3 HAE patients are shown. (C) In plasma from control donors, the activation of pro-FSAP with histones was tested in the presence of a blocking antibody against FSAP (Mab570) (25 μg/mL), a PK inhibitor (PKSI-527, 5 μM) or C1Inh (100 μg/mL). (D) Plasma from control donors was activated with histones (25 μg/mL) and DXS (25 μg/mL) and the turnover of PK substrate was measured in the presence of PKSI-527 (5 μM) and Mab570 (25 μg/mL). (E) The same was also done for HAE plasma. Results from a single well, from duplicate wells, are shown. All results were replicated in 3 control and 3 HAE plasmas with similar results.

collected with informed written consent. Sampling was performed between January 2019 and September 2021. The study was approved by the Regional Ethical Committee (REC) of South East Norway (reference no. 2018/1289/REK Sør-Øst C).

2.2. Sample collection

All HAE patients were in remission at the time of sampling, and none had taken C1Inh concentrate within the last 48 h preceding the sampling. As controls, we used samples from healthy volunteers that were matched by sex and age with the patients. Patient (n = 20) and control (n = 20) had a mean age of 45.8 (range 25–66) and 45.8 (range 24–68), respectively. In each group there were 8 males and 12 females. Samples were also collected from three female patients during an HAE attack. Citrated blood was stored on ice immediately after sampling, and centrifuged within 10 min at 1200 x *g* for 15 min at 4 °C. Plasma was aliquoted immediately thereafter and stored at -70 °C. The samples we used had not been thawed and re-frozen prior to the analyses.

2.3. FSAP-antigen concentration and pro-uPA activation of immobilised FSAP

FSAP antigen and activity (pro-uPA activation) in plasma samples was determined exactly as described before by Hanson et al. (2012). We classified donors with about 50 % of the normal activity/antigen ratio as being MI-SNP carriers as described before by Römisch et al. (2002). The low activity was also confirmed in activity assays with the fluorescent substrate described below but was not further substantiated by genotyping.

2.4. FSAP-α2AP -complexes and FSAP-C1Inh-complexes

Undiluted plasma was activated with histones (Calf thymus, Sigma Aldrich, Oslo, Norway) (25 μg/mL) for 60 min at room temperature (RT) to generate active FSAP and to form complexes with inhibitors. The ELISAs were performed as described for the antigen assay except that the second antibody was either directed against α2AP (Mab 7AP, Technoclone, Vienna, Austria) or against C1Inh (Mab KOK12, Sanquin, Amsterdam, The Netherlands).

2.5. Western blot analysis of HK and FSAP cleavage in plasma

FSAP was purified from human plasma as described by Hunfeld et al. (1999). Dextran sulphate (DXS) (500 kDa, Sigma Aldrich, Oslo, Norway) (2.5 μg/mL), histones (25 μg/mL) or purified FSAP was added to plasma and incubated for the indicated time. Plasma was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (10 % v/v mercaptoethanol) conditions. Proteins were transferred onto nitrocellulose membrane (Millipore, Burlington, MA, USA) and probed with primary antibodies. As primary antibody, we used the anti-HK light chain monoclonal antibody MAB15691 from R&D Systems, Inc. (Minneapolis, MN, USA) and FSAP was detected with a polyclonal rabbit antibody. Bound antibodies were detected using horseradish peroxidase conjugated secondary antibodies (Dako, Nestved, Denmark) and the enhanced chemiluminescence detection system (Amersham-Pharmacia, GE Healthcare, Solingen, Germany). All blots were analysed by densiometric analysis using ImageJ software (NIH, Bethesda, MD, USA).

2.6. Fluorogenic substrate assays for FSAP and PK in plasma

Plasma was diluted 1:12 with TBS with 2 mM $CaCl_2$ and mixed with histones or DXS at the indicated concentrations. Ac-Pro-*D*Tyr-Lys-Arg-AMC (AMC = amino-methyl-coumarin) (custom synthesis) was used as a sensitive and specific substrate for FSAP (Rut et al., 2019). Hydrolysis of the fluorogenic substrates was measured every min using a Synergy HI plate reader (BioTek Instruments, Winooski, VT, USA) with excitation at 320 nm and emission at 460 nm (at 37 °C for 30 min). PK activity was measured in a similar fashion with the substrate H-Pro-Phe-Arg-AMC (Bachem, Bubendorf, Switzerland) except that the plasma was diluted

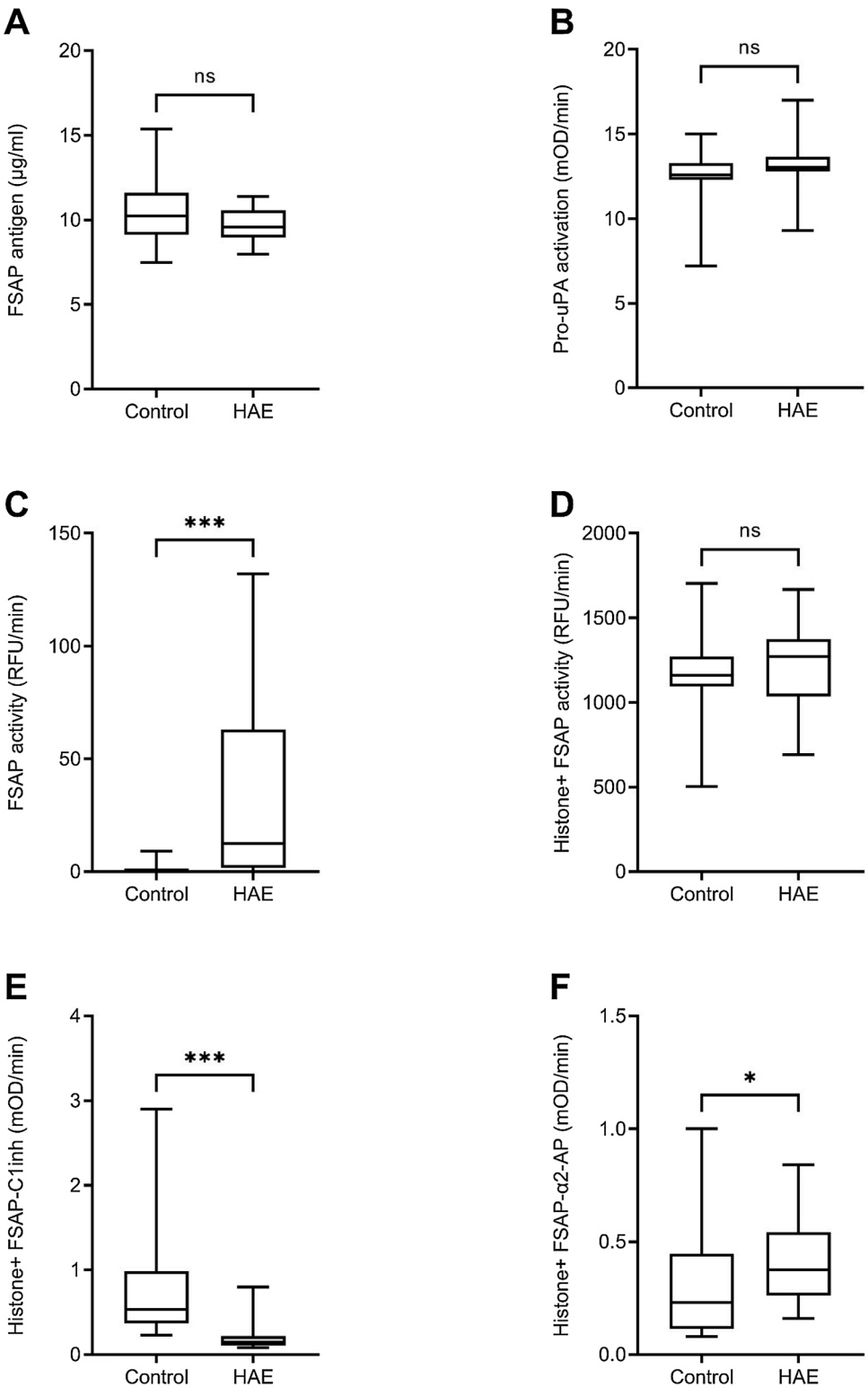

Fig. 5. Characteristics of FSAP in plasma of a cohort of HAE patients and control donors: All graphs compare HAE patients with healthy controls (n = 20 samples each). We measured FSAP antigen concentrations µg/mL (A), pro-uPA activation (mOD/min) (B), spontaneous FSAP proteolytic activity given in relative fluorescence units (RFU/min) (C), FSAP proteolytic activity in histone activated plasma (RFU/min) (D), formation of FSAP-C1Inh complexes in histone-activated plasma given as absorbance (E) and formation of FSAP-α2AP complexes in histone-activated plasma given in absorbance (F). Box-and-whiskers plot: Extreme top and bottom lines ("whiskers") mark the maximal and minimal reading, respectively. The top and bottom margins of the box mark the 75th and 25th percentiles, respectively. The horizontal line inside the box marks the median, ns = $p > 0.05$, * = $p < 0.05$, *** = $p < 0.001$.

1:4. Mab570 (Clone 1102−570) was obtained from the predecessor company of Imbiomed (Pfungstadt, Germany). PKSI-527 was obtained from Cayman Chemicals (Ann Arbor, MI).

2.7. Assays with plasma purified FSAP and PK and recombinant C1Inh

The FSAP preparation was first active site-titrated with aprotinin as described by Hunfeld et al. (1999), and commercially available recombinant C1 inhibitor (Conestat alpha, Pharming Group NV, The Netherlands) was used. Both were incubated in TBS with 2 mM $CaCl_2$ and 0.01 % Tween-20 for 30 min at RT. Thereafter the percentage residual FSAP activity was measured using the chromogenic substrate S-2288, H_2N-DIle-Pro-Arg-pNA, as described by Rut et al. (2019). PK (Haemochrom Diagnostica, Essen, Germany) activity was measured with the chromogenic substrate CS31, H_2N-DPro-Phe-Arg-pNA. All chromogenic substrates were from Hyphen Biomed (Neuville Sur Oise,

Table 2
Comparison of plasma samples during an HAE attack (n = 3) with controls (n = 8).

Sample	FSAP antigen (ug/mL)	Pro-uPA activation (mOD/min)	Histone-stimulated fluorescence substrate turnover (RFU/min)
HAE-attack 1	14.88	11.06	980
HAE-attack 2	14.55	14.17	690
HAE-attack 3	20.66	6.56	523
Controls mean (range)	11.98 (7.36–13.55)	8.81 (9.92–8.08)	702.5 (483–1030)

France). IC_{50} values were calculated using GraphPad Prism 8.3.0 from GraphPad Software, LLC

Second order rate constants for the major FSAP-binding plasma SERPINs C1Inh and α2AP were determined in slow binding inhibition studies in the presence of chromogenic substrate S-2288 using C1Inh (CSL Behring, Marburg, Germany) and α2AP (Merck Biosciences, Schwalbach, Germany). To prevent auto-degradation of FSAP, slow binding inhibition experiments were performed at very low enzyme concentration at [I]>>[E0]. Briefly, 64 pM FSAP was added to a dilution series of 2.5–160 nM inhibitor in assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.02 % acetylated BSA (Promega, Madison, WI, USA), 0.1 % Tween 20, pH 7.5) containing 0.25 mM substrate S-2288 (end concentration) in a total volume of 1.7 mL in acryl cuvettes (Brand, Wertheim, Germany). Substrate hydrolysis was recorded at 405 nm and 25 °C for up to 540 min in a spectrophotometer (GE Healthcare, Freiburg, Germany). The inhibition curve data were fitted by nonlinear regression analysis to the slow binding inhibition equation $P = v_i*t+(v_0-v_i)*(1-e^{-kapp*t})/k_{app}$. (Morrison and Walsh, 1988). From the slope of a plot of k_{app} versus [I] the second order rate constant k_{on} was calculated using the equation: $k_{app} = k_{on}*[I] / (1+ [S]/K_m)$ (Schechter and Plotnick, 2004) using a K_m value of FSAP for S-2288 of 0.071 mM.

For the formation of SDS-stable complexes between FSAP and C1Inh for mobility shift assays, both were incubated at RT for 30 min and then the samples analysed by SDS-PAGE under non-reducing conditions followed by Coomassie Blue staining.

2.8. Statistical analysis

Statistics was performed on data in Fig. 5 using GraphPad Prism version 8.3.1 from GraphPad Software, LLC. Only FSAP antigen concentration followed a normal distribution pattern, and the P value for this analysis was thus calculated using an unpaired t test. The other P values (for FSAP activity on fluorescent substrate (with and without histone activation), pro-uPA activation, FSAP-C1Inh complexes after histone activation and FSAP-α2AP complexes after histone activation) were calculated using the non-parametric Mann-Whitney *U* test All p-values are two-tailed. Statistical significance was set as a P value <0.05.

3. Results

First, we examined the inhibition of FSAP and PK by C1Inh in parallel. C1Inh inhibits plasma FSAP and PK with an IC_{50} of 7.04 +/- 0.64 nM and 11.45 +/- 1.59 nM (mean/ SD, n = 5), respectively (Fig. 1B). SDS-PAGE confirmed the formation of SDS-stable complexes between C1Inh and FSAP with a size range between 200−250 kDa (Fig. 1C). The second order rate constants between FSAP and C1Inh and α2AP, respectively, were in the same range as for the best-known target proteases, C1s and PK (Table 1). All these results confirm the previously published observations that C1Inh is an effective inhibitor of FSAP (Choi-Miura et al., 2001; Stephan et al., 2011). We then tested how C1Inh deficiency influences the characteristics of FSAP activation and HK cleavage in plasma.

The effects of exogenously added purified FSAP on HK cleavage in plasma were studied first. We chose Western blotting to investigate HK cleavage since direct BK measurements are problematic due to its short plasma half-life (Blais et al., 1999). After BK release, the cleaved HK consists of a 66 kDa heavy chain linked with a 56 kDa light chain (LC). The latter is then further degraded by PK to a 46 kDa LC (Mori and Nagasawa, 1981). HK cleavage was observed at lower concentrations of exogenous FSAP in HAE plasma (EC_{50} 0.51 ± 0.08 μg/mL, mean/ SD, n = 3) compared to control plasma (EC_{50} 1.46 ± 0.48 μg/mL, mean/ SD, n = 4) (Fig. 2). This confirms that endogenous C1Inh levels can influence the activity of exogenously added FSAP with respect to HK cleavage.

Next, we tested if C1Inh deficiency influences the cleavage and activation of endogenous pro-FSAP by cell-free histones and DXS. Addition of histone or DXS to wild type (WT)-control donor plasma led to a decrease in full-length (FL) FSAP and the appearance of the heavy chain (HC) of FSAP, indicating that plasma pro-FSAP was converted into its two-chain active form by both histones and DXS (Fig. 3 left panels). MI-SNP is associated with decreased proteolytic activity of FSAP, and it was therefore of interest to investigate if this would also influence the results. We selected, in addition to one control with WT FSAP, one HAE patient and one control with MI-SNP and one HAE patient with WT FSAP. The same pattern, as in WT control plasma, was also observed in MI-SNP samples and this pattern was reproducible across another set of 4 samples. In all 4 HAE patients, the activation of FSAP by histones and DXS was not overtly different than in control donors but the presence of basal HC-FSAP was more sustained (Fig. 3 left panels).

The same samples were probed for HK cleavage. Histones had no influence on the band pattern in any of the samples. DXS activated HK and led to the consumption of full-length HK with the appearance of the cleaved light chain (cLC), indicating BK release. In the WT-HAE patient most of the HK was already in cleaved form, whereas in the MI-HAE patient no FL—HK was present at all (Fig. 3, right panels). A nearly identical pattern was observed in another set of 4 plasma samples. Taken together, HAE plasma showed a higher baseline consumption of HK than plasma from control donors, and the addition of cell-free histones elicited no further cleavage of HK.

PK activity is a key determinant of BK generation, so we measured the cleavage of a fluorogenic substrate for PK and compared it to the cleavage of a fluorogenic FSAP substrate. Using a FSAP-specific substrate we found very similar kinetics of pro-FSAP activation by histones in HAE and control donor plasma (Fig. 4). The basal level of FSAP activity was higher in HAE plasma (Fig. 4B) compared to control plasma (Fig. 4A). In control donor plasma, the activation by histones was completely inhibited by the FSAP-blocking antibody Mab570. PKSI-527, a specific small molecular weight inhibitor of PK, or exogenously added C1Inh (100 μg/mL) had moderate inhibitory effects (Fig. 4C).

Using a PK-specific fluorogenic substrate, we found no activation at all with histones in control donor plasma but a strong activation with DXS (Fig. 4D). In HAE plasma, there was high PK activity in the basal state that was not altered in the presence of either histone of DXS. PK activity in both groups was not affected by Mab570 and completely inhibited with PKSI-527 (Fig. 4E). All these experiments were performed with plasma from three HAE patients and control donors with similar results. Hence, the lack of endogenous C1Inh does influence basal FSAP activity but not the activation of pro-FSAP in the presence of histones. In contrast, PK activity is strongly influenced by a lack of endogenous C1Inh but not at all by exogenously added histones.

We also tested if a deficiency in C1Inh would influence FSAP antigen and activity in a cohort of 20 HAE patients and control donors. FSAP antigen concentrations were comparable in both groups (Fig. 5A). While there was seemingly increased pro-uPA activation in the HAE group, the difference did not reach statistical significance (Fig. 5B). There was, however, a significantly higher FSAP activity in the HAE group when we compared activity of FSAP per unit of FSAP antigen (p = 0.03). Using an

FSAP specific substrate, we found that spontaneous FSAP activity in HAE patients is indeed increased significantly (Fig. 5C). FSAP generation in the presence of histones was similar among HAE patients and healthy controls (Fig. 5D). A deficiency of C1Inh in plasma of HAE patients led to a significant decrease in FSAP-C1Inh complexes in histone-activated plasma (Fig. 5E) and there was a significant increase in FSAP-α2AP complexes (Fig. 5F). Hence, measuring FSAP activity using different assays demonstrates that the lack of endogenous C1Inh does have a marginal influence of FSAP activity in the plasma against the fluorogenic substrate but other markers of FSAP activity are not significantly elevated.

We hypothesized that FSAP activity was likely to be higher during an HAE attack than during remission. In a small subset of 3 samples, we found no significant differences to control in terms of FSAP activity, FSAP activity per unit antigen or histone activated turnover of FSAP substrate (Table 2).

4. Discussion

Exaggerated BK formation due to excessive PK activity is central in HAE. However, the full nature of the aetiology of these attacks is not yet fully understood (Bova et al., 2018). We therefore investigated the possible contributory role of FSAP in PK-independent BK generation in HAE patients. FSAP is a plasma protease with a probable role in haemostasis, fibrinolysis and the complement system (Kanse et al., 2012a, 2012b). Its direct ability to cleave HK and to generate BK and the propensity of cell free-histones, released after injury, to activate pro-FSAP provide a rationale for its involvement in the genesis of HAE attacks (Etscheid et al., 2002; Yamamichi et al., 2011). Through a systematic analysis of HK cleavage by FSAP through exogenously added purified FSAP, activation of endogenous FSAP and measurement of FSAP activity in HAE patients and control donors we conclude that FSAP is unlikely to be involved in C1Inh deficiency-dependent HAE through direct HK cleavage and BK generation in plasma.

The plasma concentration of C1Inh is 125−250 μg/mL or 1700–3500 nM, and the association constant between FSAP and C1Inh is comparable to the one for PK. Therefore, it seems FSAP and PK are equally well inhibited by C1Inh, but C1Inh deficiency leads to higher PK activation and consumption which is not the case for FSAP. One explanation could be that other plasma inhibitors compensate for the missing C1Inh quite well in relation to FSAP. A major candidate inhibitor is α2AP, a relatively abundant plasma SERPIN (1 μM) with a relatively high association rate for FSAP. Indeed, in HAE patients upon histone addition we observed more FSAP-α2AP complexes compared to healthy controls. FSAP-α2AP complexes have also been reported in patients suffering from sepsis (Stephan et al., 2011). FSAP-C1Inh complexes in plasma are observed during trauma (Kanse et al., 2012a, 2012b), sepsis (Stephan et al., 2011) or by exogenously added cell-free histones (Yamamichi et al., 2011), and depletion of C1Inh may exhibit other FSAP-related consequences. For instance, FSAP can activate protease-activated receptor-1 (PAR-1) (Byskov et al., 2020), which in turn is known to regulate microvascular permeability (Kondreddy et al., 2020). Thus, a lack of C1Inh might influence other completely different functions of FSAP and can influence HAE through other pathways.

In HAE plasma, basal FSAP activity against a fluorogenic substrate was slightly elevated. A caveat to this observation is that the FSAP fluorogenic substrate is cleaved to a small extent by PK and FXIIa, and in the absence of C1Inh in HAE plasma their basal activity is elevated (Rut et al., 2019). A notable observation we made was that in the basal HAE samples, we could detect the heavy chain of FSAP but this was not the case in controls, suggesting that deficiency of C1Inh is likely to influence the clearance of active FSAP from the plasma. In fact, we have shown previously that FSAP-inhibitor complexes are cleared by cells via the low-density lipoprotein-receptor-related protein (LRP-1) (Muhl et al., 2007).

We could demonstrate HK cleavage by lower concentrations of FSAP in HAE plasma than control plasma but the activation of endogenous FSAP by cell-free histones did not lead to increased HK cleavage in HAE plasma. These observations were made with fluorogenic FSAP substrates as well as by Western blotting. Hence, we conclude that although HK is a substrate for FSAP, cleavage of HK and BK generation requires high FSAP concentrations. FSAP has been shown to generate BK on HK bound to endothelial cells (Kress et al., 2006). In contrast to plasma where HK and PK circulate as a complex and may be less susceptible to activation by FSAP, on the endothelial cell surface the situation could be different and needs examination. Another important factor could be that C1Inh-FSAP interaction may lead to consumption of the limited amount of C1Inh present and thus lower the threshold for an HAE attack.

In a small subset of 3 plasma samples from HAE patients during an attack we found no significant changes in FSAP activity in any of the assays used. A limitation of this experiment is the small samples size but we expect similar results even with a larger group size. This would suggest no systemic activation of FSAP during an HAE attack.

This study is the first to characterize the role of FSAP in C1Inh deficiency patients and brings new insights into the interactions between FSAP and C1Inh in the pathogenesis of HAE attacks. These results are also relevant for understanding other effects of FSAP in relation to haemostasis and cellular regulation as well as its clearance.

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Authorship

All authors have contributed substantially to the conception of the study and its design and/or the acquisition and analysis of data. All authors have edited the manuscript for important intellectual content. All authors have seen, reviewed and approved the final version of the manuscript and accept their accountability towards its accuracy.

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Declaration of Competing Interest

None of the authors have relations to for-profit or not-for-profit third parties whose interests may be affected by the contents of the manuscript.

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