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Research Article

Circulating Factor Seven Activating Protease (FSAP) in the Hyperacute Phase of Stroke

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Background. Factor VII activating protease (FSAP) is a circulating serine protease that could be involved in the pathophysiology of stroke. We analyzed the temporal changes in FSAP antigen and FSAP activity after acute cerebral ischemia (ACI) and tested if FSAP could be used to differentiate between stroke subtypes in the hyperacute phase (<4.5 hours after symptom onset). *Methods*. Of the 118 suspected stroke patients enrolled, 76 had ACI; of which 20 suffered from large vessel occlusion (LVO), 19 had intracerebral hemorrhage (ICH), and 23 had stroke mimics. Median time from symptom onset to the two plasma sample collections, <4.5 hours, were 66 and 107 minutes for the entire study population. Additional samples were collected up to 90 days post stroke in a subset of ACI patients ($n = 19$). FSAP antigen, FSAP activity, FSAP-α2-antiplasmin-complex (FSAP-AP complex), and nucleosomes were measured by activity assays or ELISA. *Results*. ACI patients treated with tissue plasminogen activator (tPA) had elevated FSAP activity < 4.5 hours ($p = 0.016$) that subsequently normalized after 6 hours. FSAP-AP complex levels decreased significantly from <4.5 hours ($p = 0.015$) to 6 hours after symptom onset. tPA did not increase FSAP activity significantly in plasma *in vitro*. FSAP antigen significantly decreased < 4.5 hours after symptom onset in LVO ($p = 0.008$) and ICH ($p = 0.017$) patients. FSAP could not differentiate ACI from ICH or strokes (ACI and ICH) from stroke mimics. FSAP did not correlate with stroke severity. *Conclusion*. LVO and ICH seem to influence FSAP levels in the hyperacute phase of stroke, but FSAP does not differentiate between stroke subtypes in a hyperacute setting.

1. Introduction

Factor VII activating protease (FSAP), a serine protease, is produced by hepatocytes as a zymogen (pro-FSAP) [1]. Circulating pro-FSAP is activated by histones [2] released from nucleosomes by plasma DNAses [3]. FSAP can degrade histones and neutralize their toxicity [3], cleave fibrinogen and thereby promote fibrinolysis by tissue plasminogen activator (tPA)/plasminogen [4]. The activity of FSAP is blocked by circulating protease inhibitors such as α2 antiplasmin (AP), C1 inhibitor [5], and tissue factor pathway inhibitor (TFPI) [6].

The involvement of FSAP in the pathophysiology of stroke is unclear. Increased FSAP antigen and FSAP activity levels were reported after AIS in plasma samples collected within 10 days (median 4 days) after symptom onset [7], and lower FSAP antigen levels were associated with recanalization after tPA treatment [8]. In plasma collected up to 7 days post stroke, elevated FSAP antigen correlates with outcome in AIS caused by large vessel occlusion (LVO) [9]. Modelling of AIS in FSAP-knockout mice shows increased infarct size [10], and treatment with recombinant serine protease domain (SPD) of FSAP (FSAP-SPD) improved the outcome after large vessel thromboembolic strokes in mice [11].

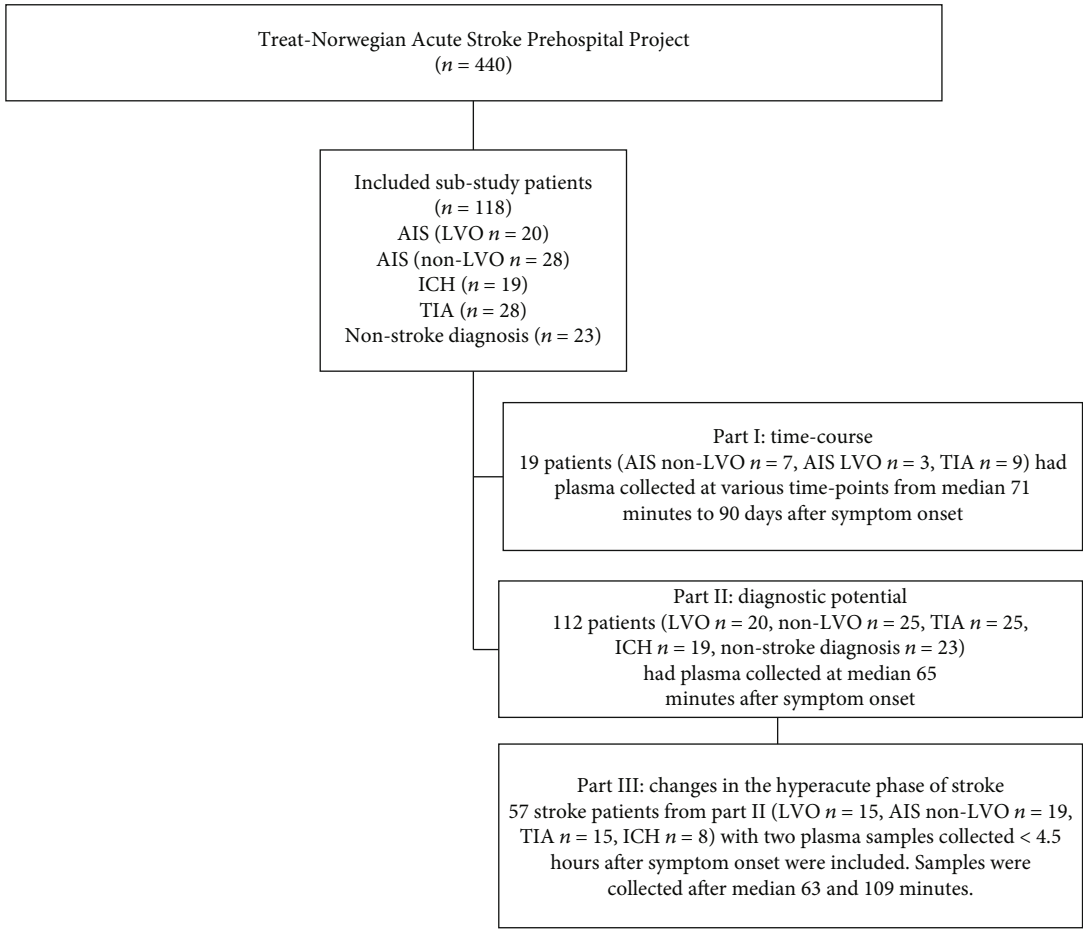

Figure 1: Flowchart. FSAP and nucleosome levels were quantified in the plasma of a subgroup of suspected stroke patients enrolled in the Treat-Norwegian Acute Prehospital Project (Treat-NASPP). In part I, all eligible ACI patients with multiple samples collected in the hyperacute phase, during hospital stay, 30 and 90 days post stroke were included. In part II, plasma from patients with ongoing stroke symptoms was used to investigate the differential diagnostic potential of FSAP. All samples were collected prior to tPA therapy. In part III, stroke patients included in part II, but with an additional plasma sample collected in the hyperacute phase (in the prehospital environment and at hospital admission), were included to characterize the dynamics of FSAP and nucleosome levels.

To date, there is no information about changes in FSAP in the first 4.5 hours after symptom onset, which is the treatment window of tPA and hereafter called the hyperacute phase of stroke. The diagnostic potential of FSAP in stroke subtyping has also not been investigated previously.

Evidence from human genetic studies [12], experiments in mice, and that FSAP is activated by histones suggests that FSAP antigen and/or activity is likely to change in the hyperacute phase of stroke. We aimed to investigate how FSAP and nucleosome levels change after symptom onset in stroke patients, and the utility of FSAP and nucleosomes as diagnostic biomarkers in acute stroke.

2. Materials and Methods

2.1. Study Design and Inclusion of Patients. This study used data collected in the Treat-Norwegian Acute Stroke Prehospital Project (Treat-NASPP). Treat-NASPP enrolled nonpregnant, suspected stroke patients ≥ 18 years met by a mobile stroke unit or ordinary ambulance within 4 hours after symptom onset [13]. Blood sample collection and stroke severity assessment, by the National Institutes of Health Stroke Scale (NIHSS), were performed in the prehospital environment and at hospital arrival. Patients that received tPA therapy had blood samples collected during hospital stay and after 30 and 90 days post stroke. Patients with one or multiple plasma samples were included in this substudy.

For part I, all eligible patients with a final diagnosis AIS (I63) or TIA (G45) with multiple samples collected in the hyperacute phase, during hospital stay and up to 90 days post stroke, were analyzed. For part II, the number of patients in each group was determined by the number of eligible patients diagnosed with ICH (I61) and AIS caused by LVO with plasma sample collected. Up to 25 patients with the final diagnosis of TIA, AIS (caused by non-LVO), and other nonstroke diagnoses were picked out to make the groups as equal in age and gender composition as possible. All stroke patients included in part II with an additional plasma sample collected in the hyperacute phase were included in part III (Figure 1).

2.2. Sample Collection. Plasma was collected in EDTA tubes (BD Vacutainer, USA) in the prehospital environment and/or at the hospital. The blood samples were centrifuged (1500 × g, 10 minutes, room temperature) immediately upon laboratory arrival. Plasmas were aliquoted and frozen down at -20°C for up to 3 days and then moved to -80°C for long-term storage.

2.3. Stroke Diagnosis. Suspected stroke patients were admitted to primary stroke center, comprehensive stroke center, or neurosurgical department. Final diagnosis was obtained by clinical examination and radiological cerebral imaging according to in-hospital procedures for suspected stroke patients. Patients with a final ICD diagnosis other than I61 (ICH), I63 (AIS), and G45 (TIA) were classified as stroke mimics. For LVO, the definition by Rennert et al. was used, including occluded vessels in the posterior circulation [14]. As it is not possible to differentiate AIS and TIA in the hyperacute phase, AIS and TIA were analyzed as one group called acute cerebral ischemia (ACI), as previously done by Pihlasviita et al. [15].

2.4. Ethical Considerations. Written informed consent was obtained from the patient or a legal representative in the hyperacute phase, during hospital stay or after hospital discharge. The Regional Committees for Medical and Health Research Ethics approved The Biomarker Study (Document ID 2014/1161) and Treat-NASPP (Document ID 2016/974). Clinical information was collected from prehospital and in-hospital medical journals including baseline characteristics, comorbidities, and final diagnosis at discharge.

2.5. Outcomes. The primary outcome was to determine the differences in FSAP and nucleosome levels at several time points: in the hyperacute phase of stroke, during hospital stay, and up to 90 days after the onset of symptoms. Secondary outcomes were to determine the diagnostic potential of FSAP and nucleosome levels and investigate the association between FSAP, nucleosome levels, and stroke severity as well as functional outcome after 90 days.

Modified Rankin Scale (mRS) was used as a measure of functional outcome. mRS was evaluated in patients, treated with tPA, through a telephone interview at 90 days ± 1 week after symptom onset.

2.6. Laboratory Measurements

2.6.1. FSAP Antigen and FSAP Activity Assays. FSAP antigen and FSAP activity measurements were performed by enzyme-linked immunosorbent assays (ELISAs) as previously described [7]. All samples and standard (standard human plasma, Siemens Healthineers, Erlangen, Germany, or Normal Reference plasma (Precision Biologic, Dartmouth, Canada)) were analyzed in duplicates. Internal controls were run on each plate. The analysis was performed by a person blinded to the clinical information. Laboratory measurements on the samples in part I were performed separately from part II and III, with a 15-month interval.

2.6.2. FSAP-α2-Antiplasmin (AP) Complex. Complex formation between active FSAP and inhibitors in plasma was determined by using AP as a reference inhibitor. Anti-FSAP antibody (rabbit polyclonal) was used to coat 96-well MaxiSorp ELISA plates (NUC, Denmark). The detection antibody was a mouse monoclonal antibody against AP (clone 7AP, Technoclone, Vienna, Austria). Normal healthy plasma pooled from 3 individuals was treated with histones (20 μg/ml) for 60 minutes at 37°C to activate pro-FSAP. This was then used as a standard with the assumption that all pro-FSAP (12 μg/ml) is activated and forms complexes with AP. Thus, the values obtained are relative and not absolute.

2.6.3. Nucleosome ELISA. Nucleosomes were measured using the cell death detection ELISA PLUS kit (No. 11774 425 001) (Roche/Merck, Oslo, Norway). Since there are no absolute standards for this measurement, the results are given in absorbance units in assays performed identically. An internal control, provided in the kit, was analyzed on each plate.

2.6.4. Effect of tPA on Activation of FSAP in Plasma. It is estimated that plasma concentration of tPA during thrombolytic therapy is less than 2 μg/ml [16]. tPA (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) was diluted in buffer (25 mM Tris-HCL, pH 7.4, 140 mM NaCl, 2 mM $CaCl_2$), added to plasma in two different concentrations (2 μg/ml and a 10-fold higher concentration of 20 μg/ml), and incubated at 37°C for 60 minutes. Plasma anticoagulated with citrate, EDTA, and hirudin-diluted 1 : 10 was tested. Pro-uPA activity was measured, and FSAP-AP ELISA were performed as described above. Histones (20 μg/ml) were used as a positive control for the activation of pro-FSAP.

2.7. Statistical Analysis and Data Representation. IMB SPSS Statistics Data Editor 27 and R version 4.2.1 were used for statistical analysis. p values < 0.05 were considered statistically significant. Categorical variables are presented as sums (n) and percent (%), and continuous variables are presented as median with interquartile range (IQR) or mean with standard deviation (SD). The distribution and density of the data are shown as violin plots with box plots displaying median and IQR. For skewed distributions, the Mann–Whitney U test or Wilcoxon Signed Ranks Test were used for univariate analysis of independent and dependent samples. Correlation analyses were performed using Spearman's rank test. mRS scores were dichotomized where mRS 0-2 was defined as a good outcome and mRS > 2 as a poor outcome. One-way repeated measure ANOVA was performed to test the between-group variation in the *in vitro* experiments. To identify differences in FSAP activity and FSAP-AP among groups, pairwise paired t-tests were used. Significant p values were adjusted with the Bonferroni multiple testing correction method.

3. Results

In total, 118 patients were included. Mean (SD) age was 69 (±14) years, and 44 (39%) were females. The diagnoses were 76 (64%) ACIs, including 20 LVOs, 19 (16%) ICHs, and 23 (20%) stroke mimics. The median (IQR) time from symptom onset to the first sample collection was 66 (47-116)

Table 1: Minutes from symptom onset to blood collection.

	Onset to first blood sample, minutes (IQR)	Onset to second blood sample, minutes (IQR)	Difference in median minutes
Entire study population ($n = 118$)	66 (47-116)	107 (79-155)	41
Part I			
ACI ($n = 16$)	71 (60-99)	119 (92-154)	48
Part II			
ACI ($n = 70$)	80 (56-123)	—	
ICH ($n = 19$)	70 (41-127)	—	
SM ($n = 23$)	90 (42-165)	—	
Overall study population part II ($n = 112$)	79 (50-132)		
Part III			
LVO ($n = 15$)	56 (48-80)	109 (92-147)	53
ACI ($n = 28$)	82 (59-140)	117 (83-169)	35
ICH ($n = 8$)	55 (26-129)	88 (52-184)	33
Overall study population part III ($n = 57$)	63 (48-105)	109 (84-157)	46

Abbreviations: ACI: acute cerebral ischemia; ICH: intracerebral hemorrhage; SM: stroke mimics; LVO: large vessel occlusion; IQR: interquartile range.

minutes and 107 (79-155) minutes for the second sample collection. Blood sampling times are listed in Table 1. Diagnoses represented among patients with stroke mimics were unspecified sensory deficits, unspecified paresis, migraine, headache, epilepsy, Bell's palsy, vertigo, and syncope.

3.1. Part I: Time Course in ACI. We first determined the changes in FSAP antigen, FSAP activity, and FSAP-AP complex (collectively called FSAP measures) and nucleosomes levels in a subset of ACI with plasma collected from the median (IQR) 71 (60-99) minutes after symptom onset to 90 days post stroke. Of the 19 ACI patients in part I, 18 were treated with tPA. Baseline characteristics, comorbidities, and blood sampling times are listed in Supplementary Table 1–2. FSAP antigen levels showed no statistically significant changes when comparing the different time points (Figure 2(a)). FSAP antigen levels were significantly lower in samples from ACI patients with mRS > 2 ($n = 3$) on day 90 compared to mRS 0-2 ($n = 13$) in samples collected from the median 71 minutes ($p = 0.039$), after 12 hours ($p = 0.016$), and 24 hours ($p = 0.034$) after symptom onset. There was a significant increase in FSAP activity in the samples collected from the median (IQR) 119 (92-154) minutes compared to the initial sample ($p = 0.016$). After 6 hours, this reverted to the lower levels ($p = 0.008$) and did not change at other sampling times (Figure 2(b)). FSAP-AP complex, a surrogate marker of FSAP activation, significantly decreased from the median 119 minutes to the 6-hour time point ($p = 0.015$) (Figure 2(c)). No differences in FSAP activity or FSAP-AP complex were observed between patients with mRS 0-2 and mRS > 2 at any time point.

Nucleosomes showed no statistically relevant changes throughout (Figure 2(d)). The nucleosome levels in samples collected after 71 minutes were not significantly different in ACI with mRS 0-2 and mRS > 2 90 days post stroke ($p = 0.313$). Two patients had very high nucleosome levels at all time points, but there was nothing different about these patients.

3.2. tPA Treatment Might Interfere with FSAP Activity Measurements. We tested if tPA could interfere with FSAP activity measurements in an *in vitro* experiment. As with plasma of stroke patients, we used two different assays to assess FSAP activation in plasma by tPA in comparison to histones as a positive control. No significant effect of 2 μg/ml of tPA on plasma FSAP activity was observed. But a significant increase in FSAP activity, pro-uPA activation assay, was observed in all three anticoagulated plasmas with the higher concentration (20 μg/ml) of tPA (Figure 3(a)). Measurement of the FSAP-AP complex showed no activation of FSAP with any concentration of tPA in any of the three anticoagulated plasmas tested (Figure 3(b)).

3.3. Part II: Does FSAP Differentiate between Stroke Subtypes or Correlate with Stroke Severity? We tested the diagnostic potential of FSAP measures and nucleosomes in suspected stroke patients ($n = 112$). Baseline characteristics, blood sampling times, and comorbidities are listed in Table 2. Blood samples were collected early in the hyperacute phase with 68 (58%) patients included within 90 minutes, 35 (30%) patients included between 91 and 180 minutes, and 15 (13%) patients included between 181 and 270 minutes after symptom onset.

FSAP measures or nucleosomes could not differentiate between ACI and ICH, nor between stroke (ACI and ICH) and stroke mimics (Figures 4(a)–4(d) and Supplementary Tables 3-4). The differences in FSAP antigen levels among ACI patients with mRS > 2 and mRS 0-2 were retested in this larger cohort and were found not to be statistically significant ($p = 0.869$).

No correlations between FSAP measures, nucleosomes, and NIHSS were observed for neither ACI nor ICH. FSAP measures correlated with each other in ACI patients. FSAP antigen correlates with FSAP activity ($\rho = 0.344$, $p = 0.004$, $n = 70$), and FSAP activity correlates with FSAP-AP-complex levels ($\rho = 0.429$, $p < 0.001$, $n = 70$),

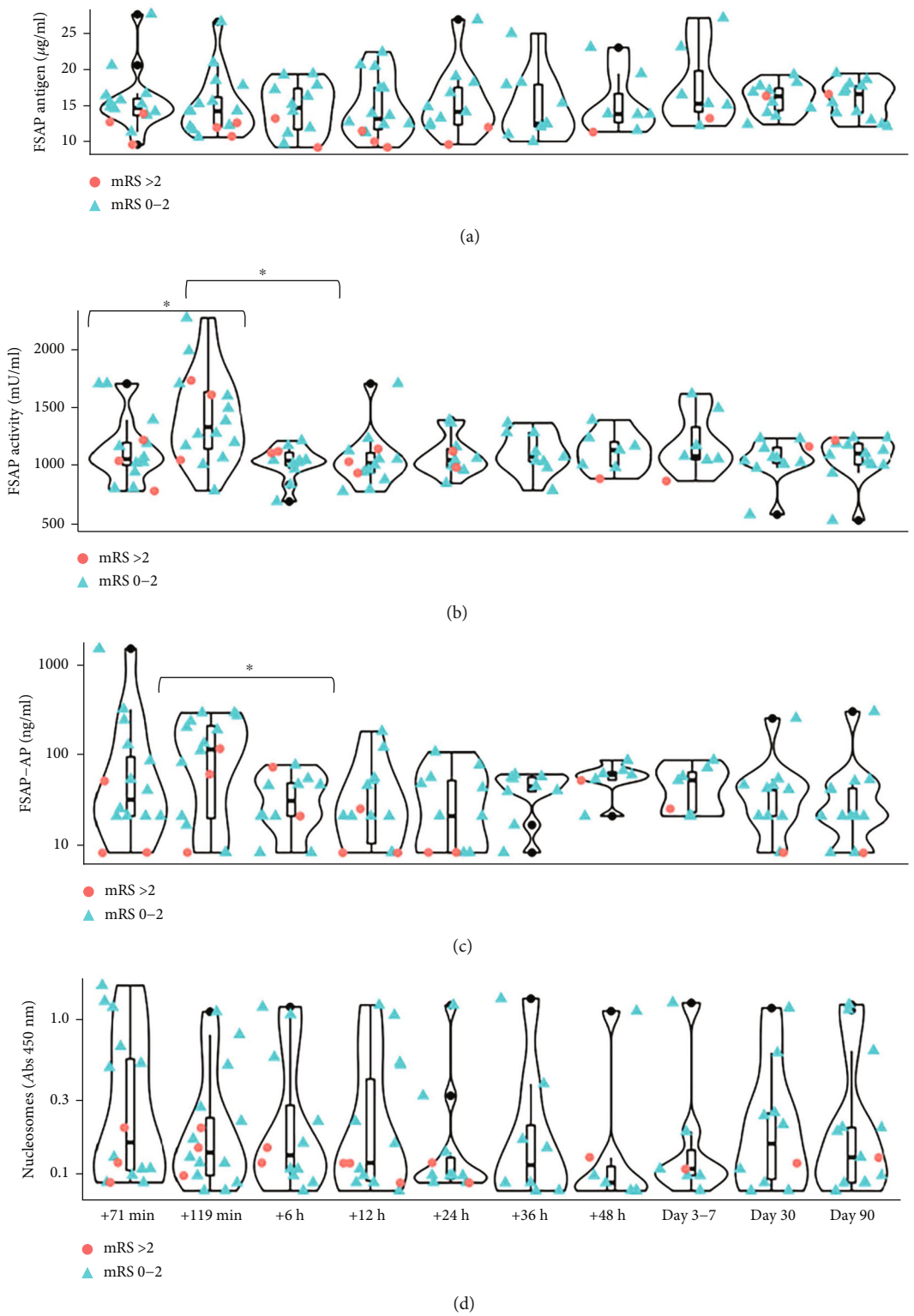

FIGURE 2: FSAP measures and nucleosome levels in a time-course study of ACI patients. FSAP measures and nucleosome levels were quantified in plasma collected in the hyperacute phase, during hospital stay, and at day 30 and day 90 post stroke. (a) FSAP antigen, (b) FSAP activity and (c) FSAP-AP complex, and (d) nucleosomes. Results are shown as violin plots with box plots. The box marks the 25th and 75th percentile, and the line in the middle indicates the median. The whiskers above and below the box represent the 90th and 10th percentiles, and outliers are marked as black dots. Red circles represent patients with mRS > 2 (poor outcome), and turquoise triangles represent patients with mRS 0-2 (good outcome) after 90 days. The significance of changes in FSAP measures and nucleosome levels was determined using the Wilcoxon Signed Rank Test, $^{*}p < 0.05$.

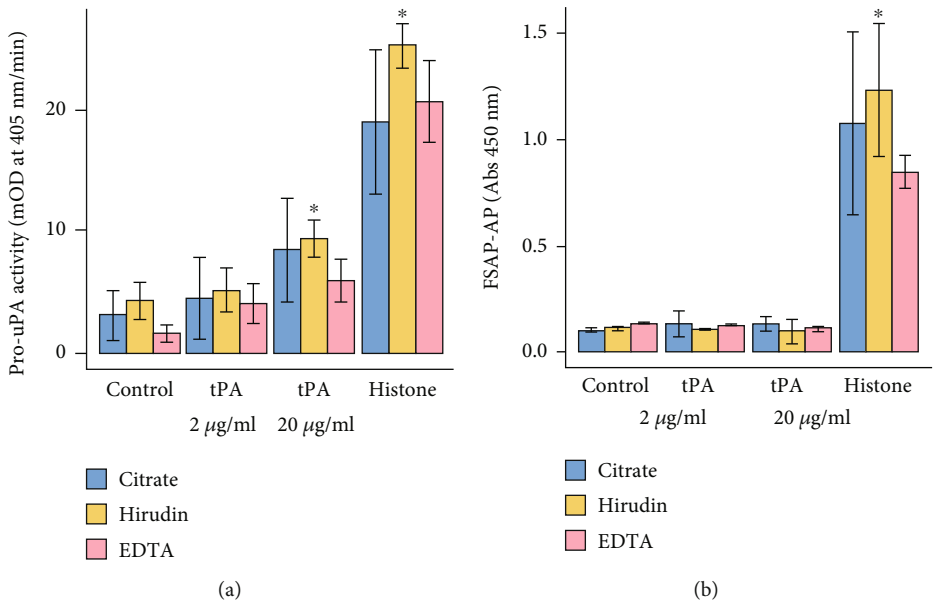

Figure 3: Effect of tPA on FSAP activity measurements *in vitro*: Plasma anticoagulated with citrate, hirudin, or EDTA was incubated with tPA (2 μg/ml and 20 μg/ml) at 37°C for 60 minutes. Histones (20 μg/ml) were used as a positive control. FSAP activity (measured by the ability of FSAP to activate pro-uPA) and FSAP-AP complex levels were measured. The bars represent the mean value, and the error bars represent the standard deviation of a minimum of 6 measurements. One-way repeated measure ANOVA showed significant differences among the conditions (control, tPA2 μg/ml, tPA 20 μg/ml, and histones). Pairwise paired t-tests were used to compare tPA 2 μg/ml, tPA 20 μg/ml, and histones to control. Significant p values were adjusted by the Bonferroni multiple testing correction method. *padj < 0.05.

Table 2: Baseline characteristics in part II.

Final diagnosis	ACI	ICH	SM
Number of patients (n)	70	19	23
Females (%) (n)	36 (25)	32 (6)	57 (13)
Age, years, median (IQR)	72 (64-77)	69 (58-83)	68 (49-83)
NIHSS at sample collection, median (IQR)	5 (2-8)	12 (6-15)	2 (0-3)
LVO (%) (n)	44 (20)		
Atrial fibrillation (%) (n)	10 (6)	21 (4)	4 (1)
Cerebrovascular disease (%) (n)	18 (11)	32 (6)	35 (8)
Diabetes (%) (n)	10 (7)	21 (4)	4 (1)
Heart disease (%) (n)	34 (24)	32 (6)	13 (3)
Hyperlipidemia (%) (n)	31 (22)	21 (4)	13 (3)
Hypertension (%) (n)	56 (39)	79 (15)	48 (11)

Abbreviations: ACI: acute cerebral ischemia; ICH: intracerebral hemorrhage; SM: stroke mimics; LVO: large vessel occlusion; NIHSS: National Institutes of Health Stroke Scale; IQR: interquartile range.

but no significant correlation between FSAP activity and nucleosomes ($\rho = 0.121$, $p = 0.322$, $n = 70$) was observed (Supplementary Figure 1). Strong correlations were observed between FSAP activity and nucleosome levels ($\rho = 0.677$, $p = 0.001$, $n = 19$), FSAP activity, and FSAP-AP complexes ($\rho = 0.616$, $p = 0.005$, $n = 19$), and a moderate correlation between FSAP antigen and FSAP activity ($\rho = 0.511$, $p = 0.026$, $n = 19$) was observed for ICH patients (Figures 5(a)–5(c)).

3.4. Part III: Change in FSAP Measures < 4.5 Hours after Symptom Onset. To characterize how FSAP measures change in the hyperacute phase, 57 stroke patients were included in this arm of the study. LVO patients ($n = 15$) were analyzed as a separate group with ACI ($n = 34$) and ICH ($n = 8$) patients. Baseline characteristics for LVO patients are listed in Supplementary Table 5. FSAP antigen levels significantly decrease in LVO ($p = 0.008$) and ICH ($p = 0.017$) patients (Figure 6(a) and Supplementary Table 6). No significant changes were observed for FSAP activity, FSAP-AP complex, and nucleosomes (Figures 6(b)–6(d)). For ICH patients, the first sample was collected at a median of 55 minutes and the second at 88 minutes after symptom onset. For LVO, the first sample was collected after

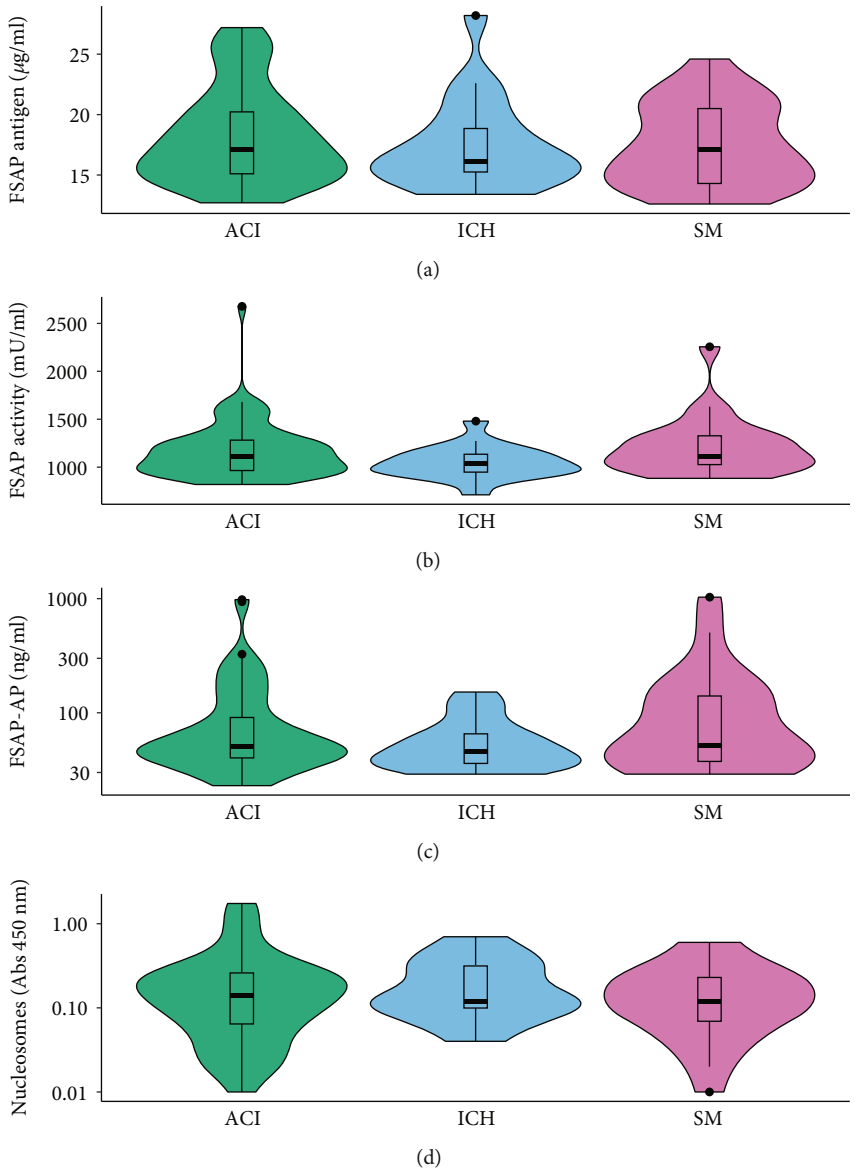

Figure 4: FSAP and nucleosomes in suspected stroke patients. Plasma from 112 suspected stroke patients (ACI ($n = 70$), ICH ($n = 19$), and stroke mimics (SM) ($n = 23$)) were investigated to determine the diagnostic potential of FSAP in the hyperacute phase. No significant differences in FSAP measures and nucleosomes among ACI and ICH patients were observed, nor between stroke patients (ACI and ICH) and patients suffering from stroke mimics. (a) FSAP antigen, (b) FSAP activity, (c) FSAP-AP complex, and (d) nucleosomes. Results are shown as violin plots with box plots. The box marks the 25th and 75th percentile, and the line in the middle indicates the median. The whiskers above and below the box represent the 90th and 10th percentiles, and outliers are marked as black dots. Significance $^{*}p < 0.05$ was determined using the Mann–Whitney U test.

56 minutes and the second sample after 109 minutes after symptom onset (Table 1).

4. Discussion

This is the first study to investigate the changes in FSAP measures and nucleosome levels in the hyperacute phase of suspected stroke and to test the differential diagnostic potential of these biomarkers. The prehospital blood collection provided samples gathered close to symptom onset, with a median time from onset of symptoms to first sample collection of 66 minutes for the entire study population. We found a significant reduction in FSAP antigen for ICH and LVO patients, and an increase in FSAP activity and FSAP-AP complex in ACI patients receiving tPA therapy.

An explanation for an increase in FSAP activity in ACI patients observed in part I could be that tPA, or plasmin, activates pro-FSAP or increases FSAP activity. This possibility was tested *in vitro* with the concentration of tPA expected in patients undergoing thrombolysis [16]. The results from the *in vitro* experiments did not support the hypothesis that the increased FSAP activity observed in ACI could be related to the treatment with the expected plasma concentration of tPA.

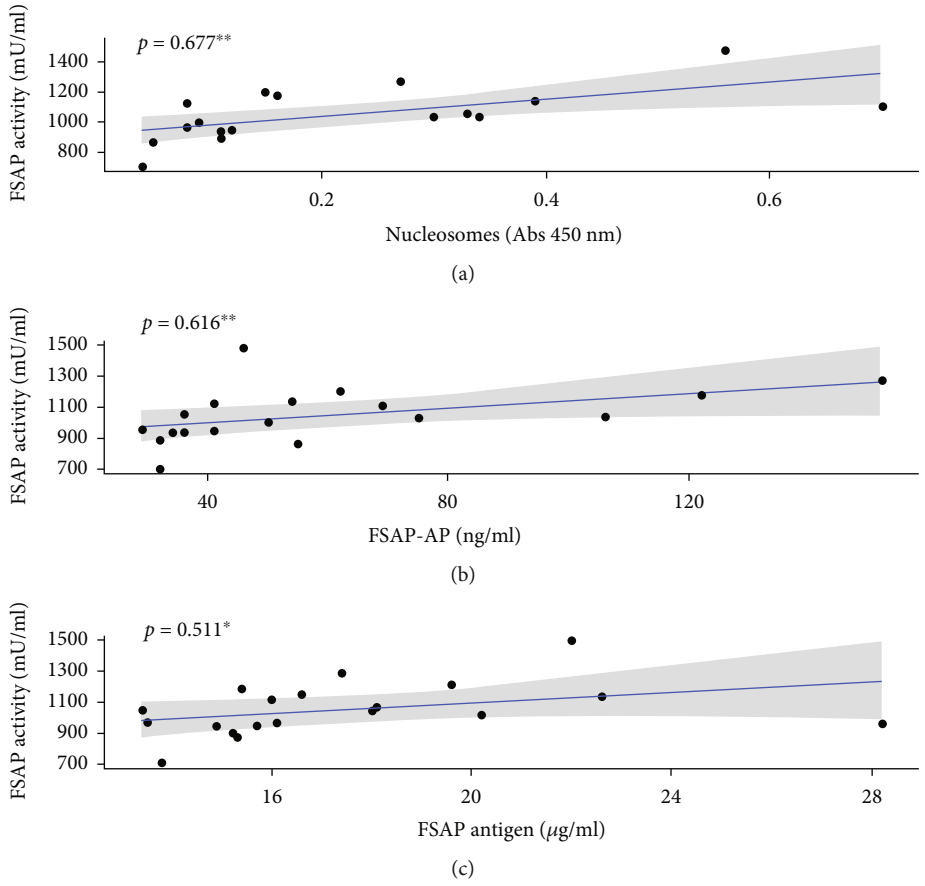

Figure 5: Correlations between FSAP measures and nucleosomes in ICH patients. FSAP measures and nucleosome levels positively correlate with each other in ICH patients ($n = 19$). (a) Correlations between FSAP activity and nucleosomes, (b) FSAP activity and FSAP-AP complex, and (c) FSAP activity and FSAP antigen levels are shown as scatterplots with the correlation coefficient ρ. Confidence intervals are marked with grey, and the regression line is blue. The correlations were determined by Spearman's rank test, and significant correlations are marked with $^{*}p < 0.05$ and $^{**}p < 0.01$.

The tissue damage caused by ICH and LVO could be the reason for the observed significant reduction in FSAP antigen levels in the hyperacute phase. The immediate tissue damage after ICH explains the rapid release of astroglial proteins like GFAP into the circulation [17], and why GFAP can differentiate ICH from ACI and stroke mimics [18]. The changes in FSAP antigen levels are most likely due to activation of pro-FSAP and the formation of inhibitor complexes that are rapidly taken up by cells and removed from the circulation. The short half-life of active FSAP [11] and the time span between the two sample collections (median 53 minutes for LVO patients and median 33 minutes for ICH patients) might be the reason why we do not observe a corresponding increase in FSAP activity and FSAP-AP complex in these groups.

Changes in circulating FSAP measures in the hyperacute phase of stroke could provide important clues to the role of FSAP in stroke pathophysiology. Lower FSAP antigen levels at a median of 175 minutes after symptom onset are associated with recanalization after tPA therapy in a population of AIS patients with a median NIHSS score of 16 [8]. Combined with the reduction in FSAP antigen that we observe for LVO patients, the ability of FSAP to cleave fibrinogen and therefore promote fibrinolysis [4] indicates a role of FSAP in the pathophysiological mechanisms initiated after ACI/LVO. In addition, mice treated with FSAP-SPD in combination with tPA had improved outcomes caused by better clot lysis and had reduced side effects of tPA treatment after large vessel thromboembolic stroke [11]. Taken together, these findings suggest that FSAP plays a significant role in the pathophysiology of ACI and that further research is needed to explore the precise mechanisms involved and the potential of FSAP-SPD as a future therapeutic agent.

The tissue damage caused by ICH also affects FSAP measures and nucleosomes. A strong correlation between FSAP activity and nucleosomes was observed for ICH but not for ACI, probably caused by the different kinetics of cell destruction in ACI and ICH [17]. FSAP can have a procoagulant effect by the inhibition of TFPI [6]. The ability of FSAP to neutralize histones and the consumption of FSAP that we observe in ICH suggest FSAP to be a part of the pathophysiological mechanisms initiated after ICH as well as ACI/LVO.

Although there was a correlation between FSAP activity and nucleosomes in trauma [19] and sepsis [20], this does not seem to be the case in ACI early after symptom onset.

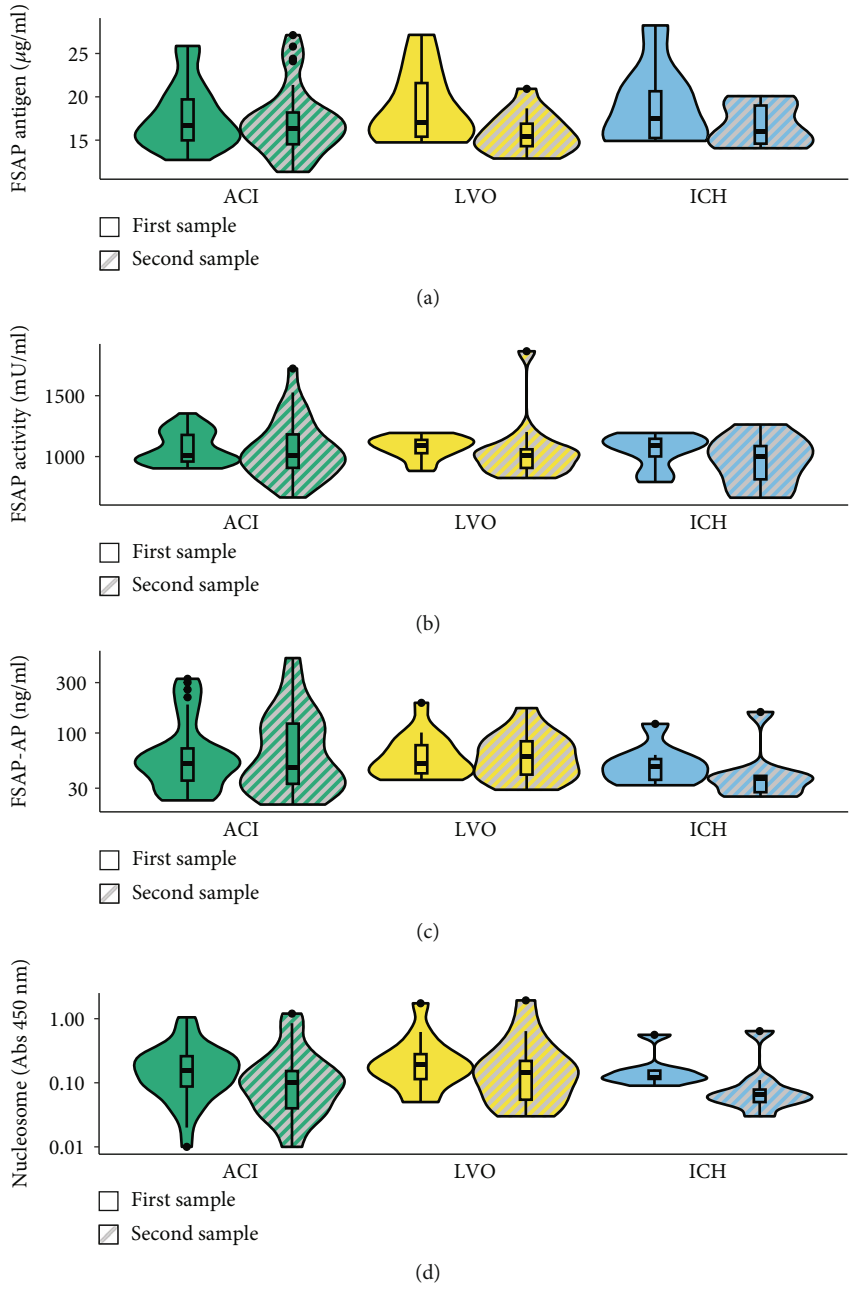

Figure 6: Changes in FSAP and nucleosomes in the hyperacute phase of stroke. Plasma samples collected twice, within the hyperacute phase, were investigated. LVO patients were analyzed as a separate group with ACI and ICH. Plasma from 57 stroke patients (ACI $n = 34$, LVO $n = 15$, and ICH $n = 8$) was analyzed. (a) Significant reductions in FSAP antigen levels were observed for ICH and LVO patients. No significant changes were observed for (b) FSAP activity, (c) FSAP-AP complex, and (d) nucleosomes. Results are shown as violin plots with box plots. The box marks the 25th and 75th percentile, and the line within the box indicates the median. The whiskers above and below the box represent the 90th and 10th percentile. Outliers are marked as black dots. Significance $^{*}p < 0.05$ was determined using the Wilcoxon Signed Rank Test.

A reason for this could be the lower extent of cell death in the early phase of ACI compared to trauma and sepsis. Furthermore, cell death in ACI may occur with a delay and reach its maximum at a later time point [17]. Progressive brain injury with increasing time and/or the heightened inflammatory state in patients in the later phase of stroke has been demonstrated in many studies, e.g., the increase in the (circulating) concentration of neurofilament over days after stroke [21]. Thus, if nucleosomes are not involved in pro-FSAP activation after ACI, it remains to be investigated exactly how this activation occurs.

Earlier studies have investigated FSAP antigen in LVO caused by occluded intracranial carotid artery (ICA) and the MI segment of the middle cerebral artery at later time points (up to 7 days post stroke). They report FSAP antigen levels to be higher in LVO with poor collaterals compared to

LVO with good collaterals and AIS caused by non-LVO [9]. Here, we have used a different definition of LVO and, unfortunately, do not have any data on collaterals. These results indicate that changes in FSAP antigen levels evolve over days, not just in the hyperacute phase of LVO patients.

A blood-based rapid point-of-care diagnostic test of stroke subtypes would be of immense value for timely treatment and triage of acute stroke patients, especially in out-of-hospital settings. FSAP is unlikely to be a candidate for early subtyping of stroke, as FSAP measures could not differentiate ACI and ICH, and could not differentiate stroke from stroke mimics. We identified no significant correlation with FSAP measures and stroke severity. Our results are comparable to another study which found no correlation between stroke severity measured by the Scandinavian Stroke Scale and FSAP antigen and FSAP activity [7].

Blood samples collected in the prehospital environment are centrifuged with a delay compared to samples collected at hospital arrival. In an earlier study, we noted that a 3-hour delay in the centrifugation of blood has no effect on FSAP activity [22]. These 3 hours are exceeded for a small number of patients (triaged directly to a comprehensive stroke center or neurosurgical department). The blood samples from these patients were centrifuged within 4 hours, and we do not think that this extra hour in delay influenced FSAP activity measurements.

A limitation of this study is the small sample size and large variations in the values of the different FSAP parameters. Other factors such as sex and hormonal status are also known to influence plasma FSAP and could be potential confounders [23]. Due to the small sample size, we were not able to adjust for this. tPA treatment in ACI patients may confound the FSAP measurements, but we did not find any evidence for this from our *in vitro* experiments. The strength of this study is that we have analyzed samples from the hyperacute phase of stroke. Furthermore, we used stroke mimics whose blood samples were obtained in the same manner, as internal controls to avoid any artefacts due to differences in how the sampling is performed.

5. Conclusion

The information provided here about the changes in FSAP measures and nucleosomes in the hyperacute phase is important for understanding the role of FSAP in stroke subtypes. Even though FSAP measures could not differentiate between stroke subtypes or discriminate stroke from stroke mimics in the hyperacute phase, we found evidence for a transient elevation in FSAP activity after ACI, and a reduction in FSAP antigen levels in LVO and ICH. Our results support the role of FSAP in the pathophysiological mechanisms of both ischemic and hemorrhagic stroke.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

KGB and SMK designed the study and obtained the funding. KGB, KL, and HSJ organized the sample collection. SMK supervised all the laboratory measurements. HSJ performed the statistical analysis. HSJ, KL, KGB, CF, and SMK analyzed and interpreted the data. HSJ and SMK wrote the manuscript. All authors read and edited the final version of the manuscript and provided critical intellectual input.

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Supplementary Materials

Additional information about the included patients and results that support the findings of this study are provided in the Supplementary Materials. *(Supplementary Materials)*

References

[1] J. Römisch, "Factor VII activating protease (FSAP): a novel protease in hemostasis," *Journal Biological Chemistry*, vol. 383, no. 7-8, pp. 1119–1124, 2002.

[2] S. Yamamichi, Y. Fujiwara, T. Kikuchi, M. Nishitani, Y. Matsushita, and K. Hasumi, "Extracellular histone induces plasma hyaluronan-binding protein (factor VII activating protease) activation *in vivo*," *Biochemical and Biophysical Research Communications*, vol. 409, no. 3, pp. 483–488, 2011.

[3] G. Marsman, H. von Richthofen, I. Bulder et al., "DNA and factor VII-activating protease protect against the cytotoxicity of histones," *Blood Advances*, vol. 1, no. 26, pp. 2491–2502, 2017.

[4] M. Etscheid, S. Subramaniam, G. Lochnit et al., "Altered structure and function of fibrinogen after cleavage by factor VII activating protease (FSAP)," *Biochimica et Biophysica Acta - Molecular Basis of Disease*, vol. 1864, no. 10, pp. 3397–3406, 2018.

[5] O. R. Gramstad, S. P. S. Kandanur, M. Etscheid, E. W. Nielsen, and S. M. Kanse, "Factor VII activating protease (FSAP) is not essential in the pathophysiology of angioedema in patients with C1 inhibitor deficiency," *Molecular Immunology*, vol. 142, pp. 95–104, 2022.

[6] F. Stephan, I. Dienava-Verdoold, I. Bulder et al., "Tissue factor pathway inhibitor is an inhibitor of factor VII-activating protease," *Journal of Thrombosis and Haemostasis*, vol. 10, no. 6, pp. 1165–1171, 2012.

[7] E. Hanson, S. M. Kanse, A. Joshi et al., "Plasma factor VII-activating protease antigen levels and activity are increased in ischemic stroke," *Journal of Thrombosis and Haemostasis*, vol. 10, no. 5, pp. 848–856, 2012.

[8] A. Bustamante, B. Díaz-Fernández, D. Giralt et al., "Factor seven activating protease (FSAP) predicts response to intravenous thrombolysis in acute ischemic stroke," *International Journal of Stroke*, vol. 11, no. 6, pp. 646–655, 2016.

[9] D. S. Tian, C. Qin, L. Q. Zhou et al., "FSAP aggravated endothelial dysfunction and neurological deficits in acute ischemic stroke due to large vessel occlusion," *Signal Transduction and Targeted Therapy*, vol. 7, no. 1, p. 6, 2022.

[10] A. U. Joshi, C. Orset, B. Engelhardt et al., "Deficiency of factor VII activating protease alters the outcome of ischemic stroke in mice," *The European Journal of Neuroscience*, vol. 41, no. 7, pp. 965–975, 2015.

[11] J. Y. Kim, D. Manna, M. Etscheid, T. B. Leergaard, and S. M. Kanse, "Factor VII activating protease (FSAP) inhibits the outcome of ischemic stroke in mouse models," *The FASEB Journal*, vol. 36, no. 10, article e22564, 2022.

[12] S. Trompet, D. Pons, S. M. Kanse et al., "Factor VII Activating Protease Polymorphism (G534E) Is Associated with Increased Risk for Stroke and Mortality," *Stroke Research and Treatment*, vol. 2011, Article ID 424759, 6 pages, 2011.

[13] K. Larsen, H. S. Jæger, L. H. Tveit et al., "Ultraearly thrombolysis by an anesthesiologist in a mobile stroke unit a prospective, controlled intervention study," *European Journal of Neurology*, vol. 28, no. 8, pp. 2488–2496, 2021.

[14] R. C. Rennert, A. R. Wali, J. A. Steinberg et al., "Epidemiology, natural history, and clinical presentation of large vessel ischemic stroke," *Neurosurgery*, vol. 85, supplement_1, pp. S4–S8, 2019.

[15] S. Pihlasviita, O. S. Mattila, J. Ritvonen et al., "Diagnosing cerebral ischemia with door-to-thrombolysis times below 20 minutes," *Neurology*, vol. 91, no. 6, pp. e498–e508, 2018.

[16] C. Smith, Y. al-Nuaimi, J. Wainwright et al., "The influence of bolus to infusion delays on plasma tissue plasminogen activator levels," *International Journal of Stroke*, vol. 9, no. 7, pp. 939–942, 2014.

[17] R. Brunkhorst, W. Pfeilschifter, and C. Foerch, "Astroglial proteins as diagnostic markers of acute intracerebral hemorrhage-pathophysiological background and clinical findings," *Translational Stroke Research*, vol. 1, no. 4, pp. 246–251, 2010.

[18] O. S. Mattila, N. J. Ashton, K. Blennow et al., "Ultra-early differential diagnosis of acute cerebral ischemia and hemorrhagic stroke by measuring the prehospital release rate of GFAP," *Clinical Chemistry*, vol. 67, no. 10, pp. 1361–1372, 2021.

[19] S. M. Kanse, A. Gallenmueller, S. Zeerleder et al., "Factor VII-activating protease is activated in multiple trauma patients and generates anaphylatoxin C5a," *Journal of Immunology*, vol. 188, no. 6, pp. 2858–2865, 2012.

[20] F. Stephan, J. A. Hazelzet, I. Bulder et al., "Activation of factor VII-activating protease in human inflammation: a sensor for cell death," *Critical Care*, vol. 15, no. 2, p. R110, 2011.

[21] T. F. Gendron, M. K. Badi, M. G. Heckman et al., "Plasma neurofilament light predicts mortality in patients with stroke," *Science Translational Medicine*, vol. 12, no. 569, 2020.

[22] M. Etscheid, K. M. Hanschmann, P. M. Sandset, and S. M. Kanse, "Development of a factor VII activating protease (FSAP) generation assay and its application in studying FSAP in venous thrombosis," *Thrombosis Research*, vol. 220, pp. 24–34, 2022.

[23] M. S. Parahuleva, H. Hölschermann, A. Erdogan et al., "Factor seven activating protease (FSAP) levels during normal pregnancy and in women using oral contraceptives," *Thrombosis Research*, vol. 126, no. 1, pp. e36–e40, 2010.