Ultra-Early Differential Diagnosis of Acute Cerebral Ischemia and Hemorrhagic Stroke by Measuring the Prehospital Release Rate of GFAP

Olli S. Mattila,[a,*] Nicholas J. Ashton,[b,c,d,e] Kaj Blennow,[b,f] Henrik Zetterberg ,[b,f,g,h] Heini Harve-Rytsälä,[i] Saana Pihlasviita,[a] Juhani Ritvonen,[a] Gerli Sibolt,[a] Tiina Nukarinen,[a] Sami Curtze,[a] Daniel Strbian ,[a] Mikko Pystynen,[i] Turgut Tatlisumak,[j,k] Markku Kuisma,[i] and Perttu J. Lindsberg [a]

BACKGROUND: Plasma glial fibrillary acidic protein (GFAP) and tau are promising markers for differentiating acute cerebral ischemia (ACI) and hemorrhagic stroke (HS), but their prehospital dynamics and usefulness are unknown.

METHODS: We performed ultra-sensitivite single-molecule array (Simoa®) measurements of plasma GFAP and total tau in a stroke code patient cohort with cardinal stroke symptoms [National Institutes of Health Stroke Scale (NIHSS) ≥3]. Sequential sampling included 2 ultra-early samples, and a follow-up sample on the next morning.

RESULTS: We included 272 cases (203 ACI, 60 HS, and 9 stroke mimics). Median (IQR) last-known-well to sampling time was 53 (35–90) minutes for initial prehospital samples, 90 (67–130) minutes for secondary acute samples, and 21 (16–24) hours for next morning samples. Plasma GFAP was significantly higher in patients with HS than ACI ($P < 0.001$ for <1 hour and <3 hour prehospital samples, and <3 hour secondary samples), while total tau showed no intergroup difference. The prehospital GFAP release rate (pg/mL/minute) occurring between the 2 very early samples was significantly higher in patients with HS than ACI [2.4 (0.6–14.1)] versus 0.3 (−0.3–0.9) pg/mL/minute, $P < 0.001$. For cases with <3 hour prehospital sampling (ACI $n = 178$, HS $n = 59$), a combined rule (prehospital GFAP >410 pg/mL, or prehospital GFAP 90–410 pg/mL together with GFAP release >0.6 pg/mL/minute) enabled ruling out HS with high certainty (NPV 98.4%) in 68% of patients with ACI (sensitivity for HS 96.6%, specificity 68%, PPV 50%).

CONCLUSIONS: In comparison to single-point measurement, monitoring the prehospital GFAP release rate improves ultra-early differentiation of stroke subtypes. With serial measurement GFAP has potential to improve future prehospital stroke diagnostics.

Introduction

Emergency medical services (EMS) encountering a patient with acute stroke symptoms are challenged with the difficult task of rapidly triaging the patient to the appropriate hospital with sufficient therapeutic capabilities, a challenge that has become increasingly complicated after the advent of thrombectomy and its expanding time window (1). Therefore, novel methods for on-scene diagnosis of acute stroke and differentiation of ischemic and hemorrhagic subtypes are needed, and such methods could also facilitate selection of patients for future prehospital therapeutic studies (2).

Blood-based biomarkers are an attractive approach, since they have potential for rapid cost-effective point-of-care (POC) measurement, as is feasible for cardiac biomarkers such as troponins (3, 4). Importantly, the prospect of rapid and sensitive analysis of brain biomarkers in blood has recently become less elusive

[a]Neurology and Clinical Neurosciences, University of Helsinki and Helsinki University Hospital, Helsinki, Finland; [b]Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, the Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden; [c]Wallenberg Centre for Molecular and Translational Medicine, University of Gothenburg, Gothenburg, Sweden; [d]Department of Old Age Psychiatry, Institute of Psychiatry, Psychology and Neuroscience, King's College London, London, UK; [e]NIHR Biomedical Research Centre for Mental Health and Biomedical Research Unit for Dementia at South London and Maudsley NHS Foundation, London, UK; [f]Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden; [g]Department of Neurodegenerative Disease, UCL Queen Square Institute of Neurology, Queen Square, London, UK; [h]UK Dementia Research Institute at UCL, London, UK; [i]Emergency Medicine and Services, Department of Emergency Care, University of Helsinki and Helsinki University Hospital, Helsinki, Finland; [j]Department of Clinical Neuroscience/Neurology, Institute of Neuroscience and Physiology, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden; [k]Department of Neurology, Sahlgrenska University Hospital, Gothenburg, Sweden.

*Address correspondence to this author at: Biomedicum Helsinki, Haartmaninkatu 8, 00290 Helsinki, Finland. E-mail olli.s.mattila@helsinki.fi.

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through novel analytical methods (5–7), while the relevant prehospital time window for using these measurements has increased (1, 8).

Glial fibrillary acidic protein (GFAP) and tau are differentially released in ischemic and hemorrhagic stroke (HS), but their very early release dynamics are unknown, as previous studies have focused on single-timepoint measurements after hospital admission (9, 10). Circulating GFAP was increased in only 36% of patients with intracerebral hemorrhage (ICH) in a small proof-of-concept prehospital study (11), casting doubts on its practical usefulness. We set out to explore the early dynamics of GFAP and tau, utilizing the highly streamlined stroke pathway of our hospital district to collect very early sequential samples. We hypothesized that measuring early change in these biomarkers using highly sensitive measurement technology could provide novel diagnostic benefit, analogous to monitoring the release rate of troponins in acute myocardial infarction.

Materials and Methods

STANDARD PROTOCOL APPROVALS, REGISTRATIONS, AND PATIENT CONSENTS

The Helsinki Ultra-acute Stroke Biomarker Study was approved by the relevant local institutional review board (397/13/03/01/2012), the institutional ethics committee, and registered as clinical trial NCT02145663 (clinicaltrials.gov). Written informed consent was collected from each patient or next of kin (12, 13).

STUDY DESIGN

The hospital district of Helsinki and Uusimaa has regional EMS operating under centralized management, serving a population of 1.6 million. To identify acute stroke, EMS use the Face Arm Speech Test, including testing for lower limb weakness, and, if needed, consult an on-call neurologist or EMS physician over phone. Candidates for recanalization therapies are transported with a high-priority stroke code (SC) together with prenotification to the Helsinki University Hospital, which manages all SC patients from the district. A separate neurosurgical unit receives all emergency cases with suspected acute subarachnoid hemorrhage.

The Helsinki Ultra-acute Stroke Biomarker Study is an observational project aiming to improve stroke diagnostics through optimization of treatment pathways and investigation into blood-based biomarkers in a large cohort of SC patients (12, 13). Study recruitment was carried out between May 20, 2013, and November 19, 2015, and the inclusion criteria were primary SC transport to our hospital, age ≥18 years, and successful collection and processing of prehospital blood samples. Transportation from another emergency department or a hospital ward were predetermined exclusion criteria.

During the recruitment period a total of 2392 SC patients were transported to the Helsinki University Hospital. Training for study recruitment and blood sampling was organized in all EMS divisions in the region, with recruitment relying on voluntary participation of EMS personnel. Thus, the cohort represents a convenience sample of all transported SC patients (Fig. 1). A total of 1079 patients fulfilled recruitment criteria. Final written informed consent was not received for 61 patients, and 3 patients had missing EMS or hospital records, which left 1015 patients in the final study cohort. Data collection for the study was performed through chart review after conclusion of all follow-up investigations. The final diagnosis group [ischemic stroke (IS), transient ischemic attack (TIA), HS or stroke mimic] was defined based on all available patient records, and cohort representativeness has been discussed previously (12, 13).

In addition to initial prehospital blood samples, secondary acute samples were collected immediately on hospital admission, and for patients with ACI and HS still available in hospital, also the next morning. To establish the acute evolution of the studied biomarkers, this proof-of-concept study included patients with plasma samples from all 3 timepoints ($n = 369$), and with a clear neurological deficit of ≥3 points on the admission National Institutes of Health Stroke Scale (NIHSS); 91 cases with admission NIHSS 0–2, and 3 with admission NIHSS missing were excluded giving a net 275 cases. An additional 3 patients were excluded due to missing last-known-well (LKW) times, leaving a final cohort of 272 patients. Patients who had a final diagnosis of IS or TIA were ascribed to an ACI group, as all of them had persistent ACI symptoms in the prehospital and admission phases. Likewise, patients with ICH and with subarachnoid hemorrhage (SAH) were ascribed to a HS group, as a practical diagnostic biomarker needs to differentiate both of these HS subtypes from ACI. The cohort included 9 additional cases in which ACI had been initially suspected and next morning samples collected, but the final determined diagnosis was stroke mimic.

ICH VOLUME MEASUREMENTS

To achieve highly detailed measurement of ICH volume we used 3D-Slicer software (http://www.slicer.org) (14, 15). Hematoma area was identified in each horizontal section and total hematoma volume was then automatically calculated by the software. Intraventricular and subarachnoid hemorrhage components were not included in the volume measurements, but were documented separately.

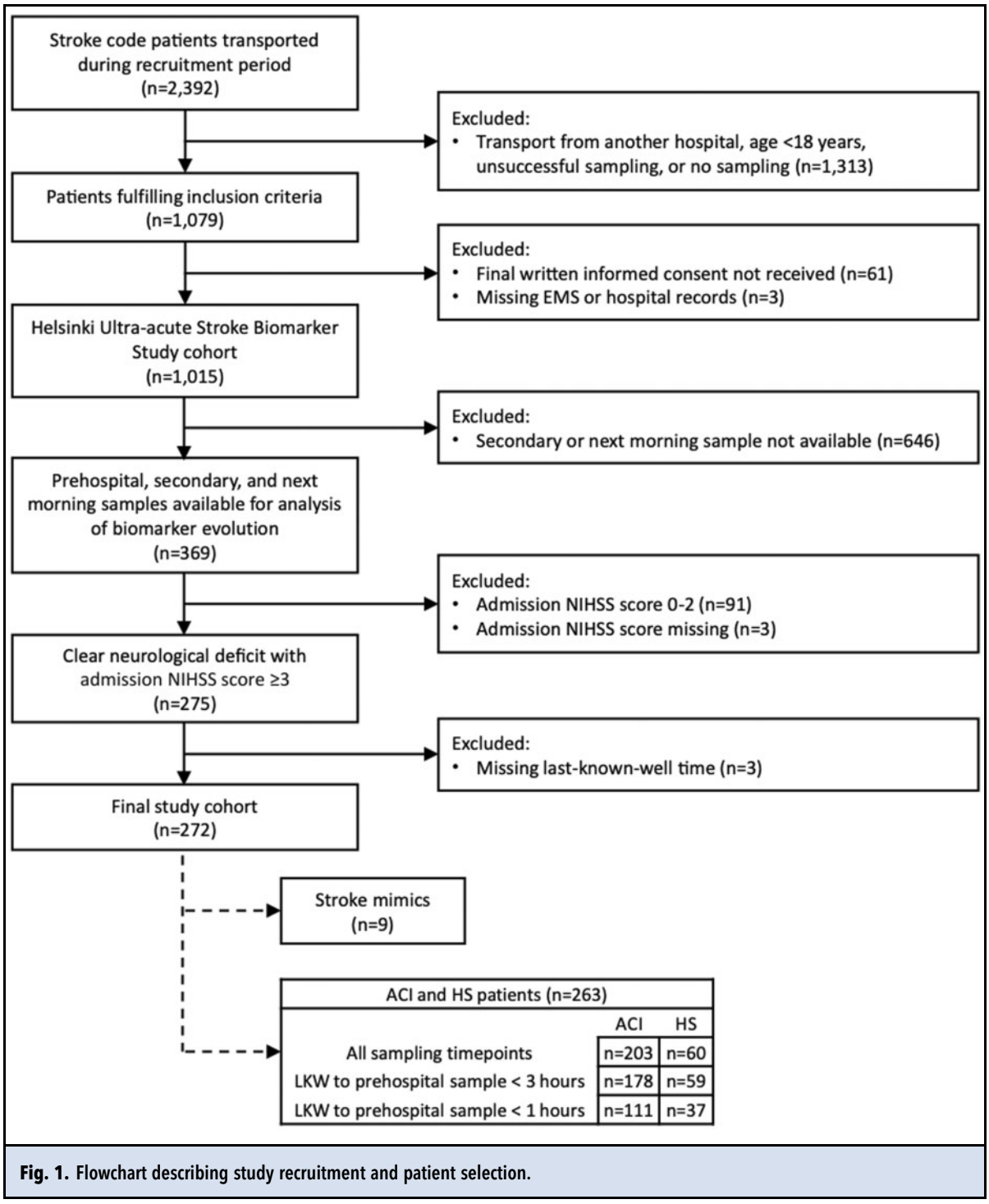

Fig. 1. Flowchart describing study recruitment and patient selection.

BLOOD SAMPLING

By protocol a standard large-bore antecubital cannula is routinely placed by EMS before transport of SC patients. For the purpose of our study, initial prehospital blood samples were collected through the cannula immediately after its placement, before administering any fluids or medications through the cannula. We used vacuum tubes with a draw volume of 5.5 mL (serum and potassium EDTA, Venosafe®, Terumo) and a cannula adapter (Vacutainer® Luer-LokTM adaptor, BD),

and sampling time was registered. Immediately on hospital admission, prehospital samples were passed on to laboratory staff, and secondary acute samples were collected per protocol into equivalent 10-mL vacuum tubes using venipuncture. Finally, study samples were directly processed in the hospital laboratory with centrifugation at 2000*g* for 10 minutes at 20 °C, and final plasma and serum samples were aliquoted into cryotubes for storage at −80°C. Next morning follow-up samples were collected as described for secondary samples.

MEASUREMENT OF PLASMA GFAP AND TOTAL TAU

The principles of single-molecule array methodology have been described previously (6). We performed plasma GFAP and total tau (T-tau) measurements using commercially available GFAP Discovery and Tau 2.0 kits on the Simoa® HD-1 Analyzer, according to kit inserts (Quanterix, Billerica, MA) unless otherwise stated below. For T-tau, all samples measured above the reported lower limit of quantification (LLOQ) (0.061 pg/mL). Internal quality controls for low concentrations (6.8 pg/mL) gave an intra-assay coefficient of variation (CV) of 6.9% and interassay CV was 9.5%). For the high-concentration quality control sample (25.1 pg/mL), the corresponding CVs were 6.8% and 9.8%, respectively. For GFAP, samples were analyzed at a 8-fold dilution and all samples measured above the reported LLOQ (0.686 pg/mL). Samples with GFAP measurements above the highest point on the standard curve were reanalyzed at a 100-fold dilution. Internal quality controls for low concentrations (82.9 pg/mL) gave an intraassay CV% of 5.6% and interassay CV was 11.9%. For the high-concentration quality control sample (241.1 pg/mL), the corresponding CVs were 8.6% and 14.5%, respectively. All measurements were performed without knowledge of the clinical characteristics of the patients. The measurements were performed in June 2019 at the Clinical Neuroschemistry Laboratory at the University of Gothenburg, Mölndal, Sweden.

STATISTICAL ANALYSIS

Continuous variables are summarized as medians with interquartile range (IQR), and categorical variables as absolute counts and percentages. For univariate analyses we used the Mann–Whitney *U*-test. NIHSS scores were categorized as mild (0–8), moderate (9–15), or severe (>15) (16). The nonparametric Spearman rank test measured correlation. Following cohort description, we explored the acute phase dynamics of the studied biomarkers by calculating per minute changes in concentration between acute phase sampling timepoints, and comparing groups. The ability of plasma GFAP measures to differentiate patients with acute ACI and HS was explored in area under the receiver operating characteristic curve (AUC) analyses. AUC of less than 0.7 have been considered poor, 0.7–0.8 fair, 0.8–0.9 good, and 0.9–1.0 excellent (7). We then evaluated the diagnostic benefits of combining initial prehospital GFAP concentrations and the GFAP release rate. Diagnostic measures were calculated from cross-tabulations, and suggestions for optimal cutoff values for the biomarkers were derived from AUC analysis and plotting. Further analyses and plotting of diagnostic performance was performed in patients with moderate to severe stroke, for differentiation of both ACI and HS, and large-vessel occlusion (LVO) and HS. Finally, biomarker concentrations were analyzed in stroke mimic (SM) patients. Significance was considered at $P \leq 0.05$ for all comparisons. Analyses were performed with SPSS (v.25, IBM).

Results

Of 272 cases, the final diagnosis was acute cerebral ischemia (ACI) in 203 patients [74.6%, including IS ($n = 195$) and TIA ($n = 8$)], HS in 60 patients [22.1%, including ICH ($n = 50$), subarachnoid hemorrhage (SAH, $n = 2$), or both ($n = 8$)], and a SM in 9 patients (3.3%) (Table 1). Main comparisons were performed between the ACI and HS groups ($n = 263$). For these patients, median (IQR) time to sampling was 53 (35–90) minutes for initial prehospital samples, 89 (67–130) minutes for secondary samples, and 21 (16–24) hours for next morning samples. "Golden hour" prehospital samples (<1 hour from LKW) were available for 148 cases (ACI $n = 111$, HS $n = 37$), and <3 hour prehospital samples for 237 cases (ACI $n = 178$, HS $n = 59$). Supplementary comparisons of cohort representativeness are provided in Table 1 in the online Data Supplement.

We first explored sequential changes in plasma GFAP and T-tau (Fig. 2). On the group level, patients with HS had significantly higher plasma GFAP concentrations compared to patients with ACI at all sampling timepoints ($P < 0.001$, Table 2). No significant intergroup differences were seen for median plasma T-tau concentrations.

Using ultra-early serial sampling, we calculated the prehospital GFAP release rate as the change in plasma GFAP between the 2 ultra-early samples, dividing by the exact time between sampling (pg/mL/minute). Notably, plasma GFAP increased in 82% of patients with HS, and the median (IQR) prehospital GFAP release rate was significantly greater in patients with HS compared to ACI [2.4 (0.6–14.1) versus 0.3 (−0.3 to 0.9) pg/mL/minute, $P < 0.001$, $n = 263$]. The median (IQR) rate of T-tau change calculated based on the 2 early-phase samples was found to be decreasing in both groups, with no significant intergroup difference

Table 1. Demographic characteristics.

Variable	Final diagnosis			
	Total ($n = 272$)	ACI ($n = 203$)	HS ($n = 60$)	SM ($n = 9$)
Age, years, median (IQR)	69 (60–79)	70 (61–80)	67 (57–76)	61 (46–66)
Sex, Male, *n* (%)	152 (55.9)	111 (54.7)	38 (63.3)	3 (33.3)
Medical history, *n* (%)				
Hypertension	158 (58.1)	119 (58.6)	35 (58.3)	4 (44.4)
Hyperlipidemia	103 (37.9)	83 (40.9)	18 (30.0)	2 (22.2)
Diabetes	36 (13.2)	25 (12.3)	9 (15.0)	2 (22.2)
Atrial fibrillation	50 (18.4)	42 (20.7)	8 (13.3)	0 (0)
Ischemic heart disease	44 (16.2)	39 (19.2)	4 (6.7)	1 (11.1)
Previous IS	31 (11.4)	25 (12.3)	5 (8.3)	1 (11.1)
Previous TIA	11 (4.0)	6 (3.0)	4 (6.7)	1 (11.1)
Previous ICH/SAH	5 (1.8)	4 (2.0)	1 (1.7)	0 (0)
NIHSS on hospital arrival				
Median (IQR)	8 (5–14)	7 (5–13)	13 (7–16)	4 (4–9)
Subgroups, *n* (%)				
3–5	89 (32.7)	73 (36.0)	11 (18.3)	5 (55.6)
6–10	74 (27.2)	59 (29.1)	12 (20.0)	3 (33.3)
11–15	55 (20.2)	35 (17.2)	19 (31.7)	1 (11.1)
16–21	39 (14.3)	27 (13.3)	12 (20.0)	0 (0)
≥22	15 (5.5)	9 (4.4)	6 (10.0)	0 (0)
LKW to sample time				
Prehospital samples, minutes, median (IQR)	53 (35–90)	53 (35–110)	50 (38–74)	70 (40–80)
Secondary samples, minutes, median (IQR)	90 (67–130)	89 (66–146)	90 (71–113)	101 (70–117)
Next morning samples, hours, median (IQR)	21 (16–24)	21 (16–24)	20 (16–23)	18 (17–24)
Onset during sleep, *n* (%)	17 (6.3)	15 (7.4)	1 (1.7)	1 (11.1)

Abbreviations: ACI, acute cerebral ischemia; HS, hemorrhagic stroke; SM, stroke mimic; IQR, interquartile range; IS, ischemic stroke; TIA, transient ischemic attack; ICH/SAH, intracerebral hemorrhage/subarachnoid hemorrhage; LKW, last known well.

[−0.017 (−0.046 to −0.002) pg/mL/minute for patients with ACI and −0.019 (−0.047 to −0.003) pg/mL/minute for patients with HS, $P = 0.732$, including cases with <3 hour prehospital samples].

Admission hematoma volume correlated with prehospital GFAP ($\rho = 0.549$, $P < 0.001$, $n = 58$), and with prehospital GFAP release rate ($\rho = 0.450$, $P < 0.001$, $n = 58$). Patients with HS also showed a correlation between admission NIHSS and prehospital GFAP ($\rho = 0.362$, $P = 0.005$, $n = 60$), and with prehospital GFAP release rate ($\rho = 0.393$, $P = 0.002$, $n = 60$). No significant correlation with admission NIHSS was seen in patients with ACI ($\rho = 0.133$, $P = 0.059$ for prehospital GFAP, and $\rho = -0.073$, $P = 0.298$ for prehospital GFAP release rate, $n = 203$).

The AUC for differentiating patients with ACI and HS with <3 hour prehospital sampling was 0.781 (95% CI 0.712–0.850) for initial prehospital GFAP, 0.850 (95% CI 0.795–0.905) for secondary acute samples, and 0.740 (95% CI 0.651–0.829) for prehospital GFAP release rate (Fig. 3, A). Notably, as symptom severity correlated with high prehospital GFAP concentrations in patients with HS but not ACI, we found that AUCs improved when considering only patients with moderate or severe stroke (<3 hour prehospital sampling and admission NIHSS >8, ACI $n = 79$, HS = 41, Fig. 3, B).

Plotting prehospital GFAP and prehospital GFAP release rate together revealed that these measures have independent capability to differentiate patients with ACI and HS (Fig. 4, A). Notably, analyzing cases with <3 hour prehospital samples, patients with HS generally had either high initial prehospital GFAP concentrations >410 pg/mL, or more moderate initial prehospital

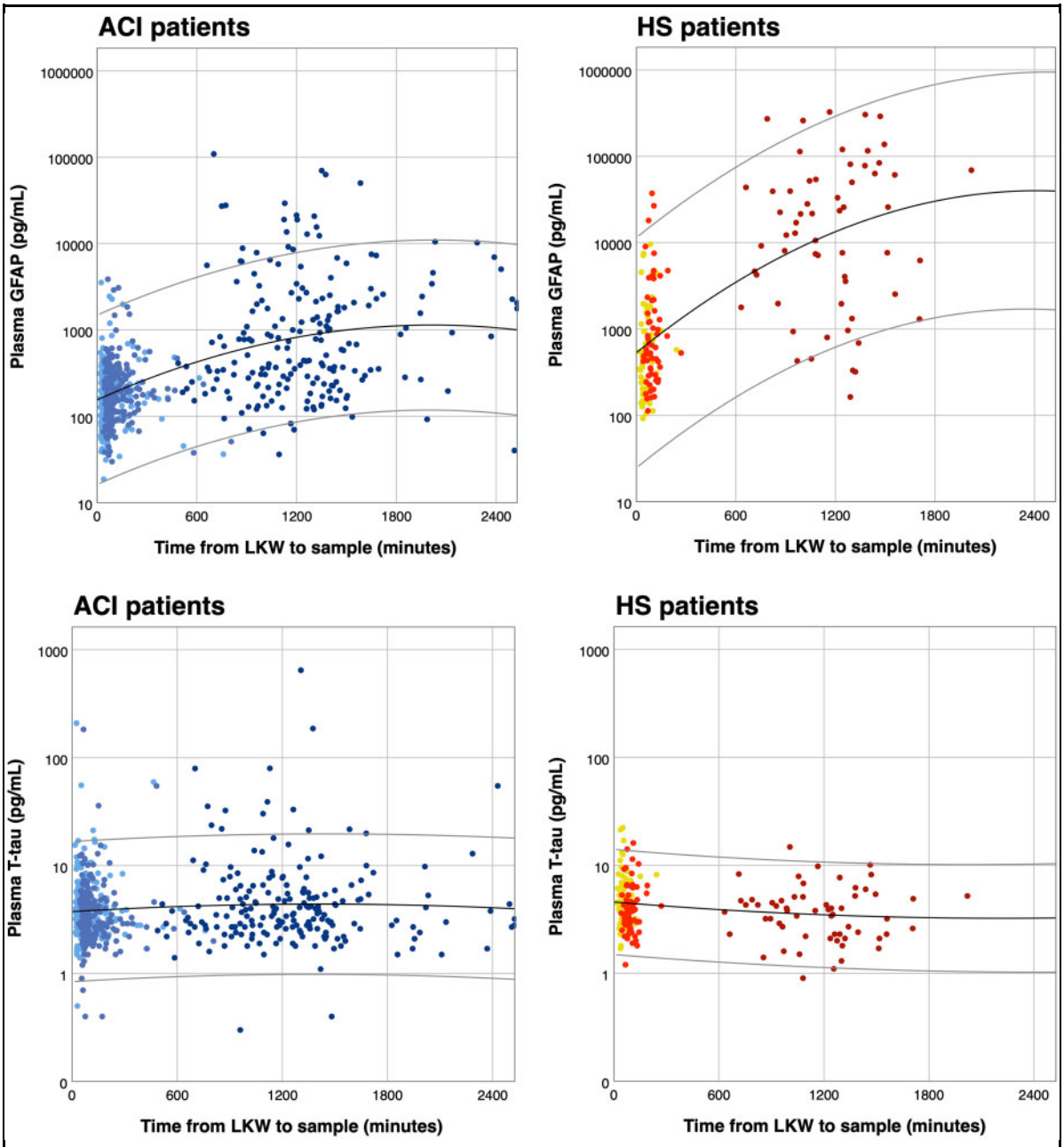

Fig. 2. Evolution of plasma GFAP and T-tau after acute stroke. Measurements from different sampling time points are shown in different colors (prehospital, secondary sample, and next morning samples). A quadratic trend line was fitted with 95% confidence intervals. Note the logarithmic *y* axis used in all graphs.

concentrations between 90 and 410 pg/mL together with active release of GFAP, with a rate >0.6 pg/mL/minute. These cutoff values enabled detecting HS with high sensitivity (96.6%), providing a high negative predictive value (98.4%), i.e., a high certainty of ruling out HS in over two-thirds of patients with ACI (specificity 68%, PPV 50%, Fig. 4, A). For patients with HS and only SAH ($n = 2$), the initial prehospital GFAP concentration was 164.8 and 1669.5 pg/mL, and the respective GFAP release rates were 1.1 pg/mL/min and 16.3 pg/mL/min, thus both being test positive. Diagnostic performance of these selected cutoffs was equivalent in

Table 2. Intergroup differences in plasma GFAP and total tau.

				Diagnosis group						
				Acute cerebral ischemia (ACI)			Hemorrhagic stroke (HS)			
Biomarker	Sampling timepoint	*n* (ACI)	*n* (HS)	Median concentration (pg/mL)	IQR		Median concentration (pg/mL)	IQR		*P* value
GFAP	Initial prehospital sample, <1 hour	111	37	156.4	97.3	294.7	361.7	189.4	604.1	<0.001
	Initial prehospital sample, <3 hour	178	59	178.2	99.7	295.1	468.9	197.7	1188.2	<0.001
	Secondary sample, <3 hour	166	57	189.6	110.1	310.2	563.1	301.3	1625.3	<0.001
	Next morning	203	60	622.7	241.3	2297.4	19 300.7	3055.0	57 593.7	<0.001
TAU	Initial prehospital sample, <1 hour	111	37	4.0	3.1	5.2	4.5	3.2	6.9	0.146
	Initial prehospital sample, <3 hour	178	59	4.0	3.0	5.4	4.6	3.2	7.2	0.088
	Secondary sample, <3 hour	166	57	3.2	2.4	4.5	3.8	2.8	5.3	0.058
	Next morning	203	60	3.6	2.6	5.7	3.8	2.3	5.0	0.316

patients with golden hour prehospital sampling (sensitivity 94.6%, NPV 97.5%, specificity 71.2%, PPV 52.2%).

Diagnostic performance improved significantly (Fig. 4, B) when only considering patients with moderate to severe stroke (admission NIHSS >8). Adjusted cutoffs (prehospital GFAP >410 pg/mL, or prehospital GFAP 110–410 pg/mL together with GFAP release >2 pg/mL/min) enabled relatively specific differentiation of patients with ACI and HS (sensitivity 92.7%, specificity 83.5%, NPV 95.7%, PPV 74.5%, in patients with <3 hour prehospital sampling). In our study cohort recruited between 2013 and 2015, admission computed tomography (CT) angiography was performed in 50% ($n = 89$) of patients with ACI with <3 hour prehospital sampling, of which 34 cases had LVO [ICA, the first segment of the middle cerebral artery (M1), ICA+M1, or basilar artery occlusion]. For patients who had moderate to severe symptoms with LVO ($n = 31$) or HS ($n = 41$), GFAP showed good capability to differentiate the groups (Fig. 4, C) when using the same adjusted cutoffs (sensitivity 92.7%, specificity 90.3%, NPV 90.3%, PPV 92.7%).

For SM patients ($n = 9$) the median (IQR) GFAP concentration was 214.9 (72.1–236.2, range 38.1–281.9) pg/mL for prehospital samples and 212.5 (73.0–235.0, range 33.8–299.6) pg/mL for secondary acute samples, with a low median rate of change of 0.028 (−0.14 to 0.28, range −0.59–1.49) pg/mL/minute. For patients with <3 hour prehospital sampling we found no significant differences in initial prehospital GFAP or GFAP release rate between ACI and SM groups ($P = 0.846$ and $P = 0.436$, respectively, ACI $n = 178$, SM $n = 8$), but found significantly higher initial prehospital GFAP concentrations and GFAP release rates in HS compared to SM patients ($P = 0.009$ and $P = 0.007$, respectively, HS $n = 59$, SM $n = 8$).

Discussion

This study demonstrates that while initial prehospital GFAP plasma concentrations (pg/mL) differentiate patients with ACI and HS with only moderate capability, measuring the prehospital GFAP release rate (pg/mL/minute) significantly improves the early diagnostic utility of this biomarker, analogous to the routine diagnostic use of monitoring the realease of cardiac troponins in acute myocardial infarction. Applying both initial prehospital GFAP and the prehospital GFAP release rate provided a high certainty (98.4%) of ruling out HS in two-thirds of patients with ACI. Even though our second sequential plasma sample was collected on hospital admission, due to the highly optimized speed of SC referral in our hospital district, our 2 very early sequential samples are comparable to prehospital timepoints in many other hospital districts (median 90 minutes to secondary sampling). Our data reveal that when suffient POC measurement sensitivity can be achieved, GFAP-based diagnostics hold promise to advance prehospital stroke management. These findings may also have applicability in other acute neurological presentations, such as nonalleviating acute headaches,

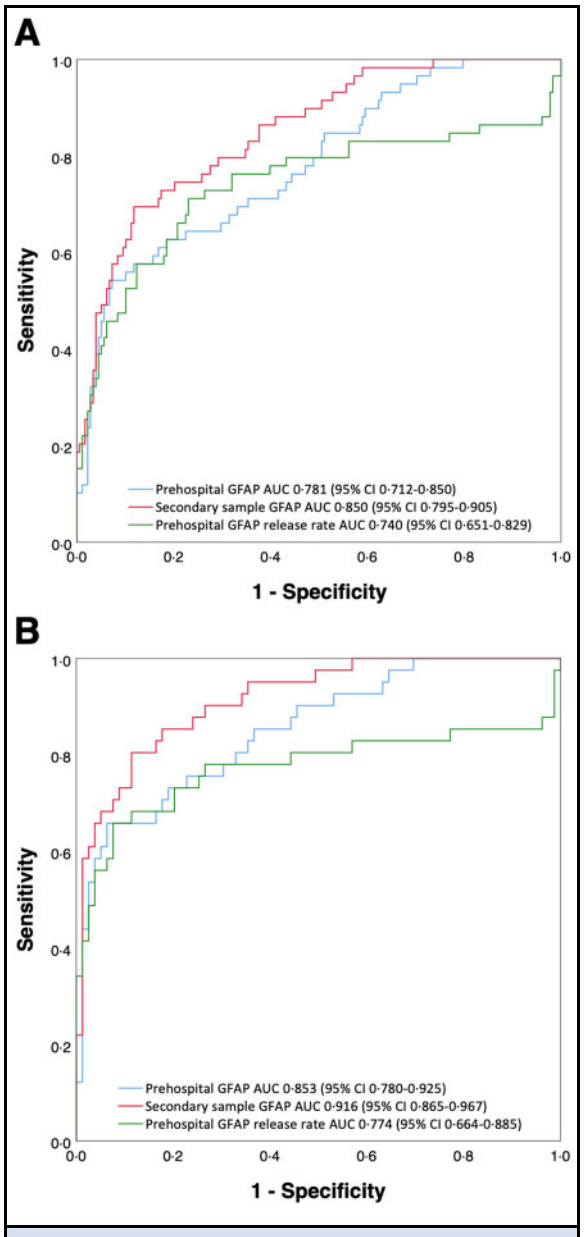

Fig. 3. Receiver operating characteristic curves for differentiating patients with HS and ACI with the different plasma GFAP measures, including patients with prehospital samples collected within 3 hours. AUCs are shown separately for all NIHSS subroups (A) and cases with moderate to severe stroke (B) (NIHSS > 8, ACI $n = 79$, HS $n = 41$).

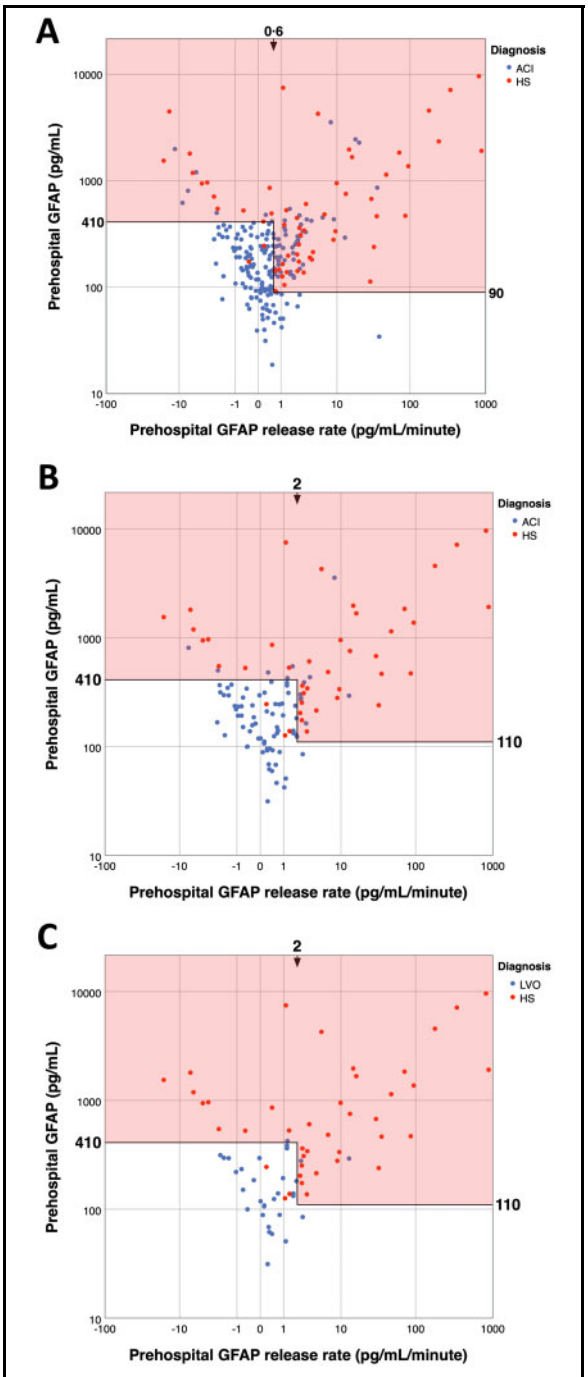

Fig. 4. Plots demonstrating differentiation of ACI and HS by utilizing both initial prehospital GFAP concentrations (*y* axis, pg/mL) and the prehospital GFAP release rate (*x* axis, pg/mL/min), with test-positivity in the area highlighted red. (A), Patients with prehospital samples collected within 3 hours (ACI $n = 178$, HS $n = 59$). (B), Moderate/severe stroke (admission NIHSS > 8, ACI $n = 79$, HS $n = 41$), (C), Moderate/severe stroke and LVO ($n = 31$) or HS ($n = 41$).

where serial GFAP measurement could prove useful to select patients for neuroimaging to rule out hemorrhage.

GFAP is a leading biomarker candidate for identifying acute HS (10). Using emergency department samples, previous studies have shown GFAP to differentiate between patients with HS and ACI in the initial hours

after stroke, with equivalent AUC values for admission samples as seen for secondary samples in our study (10, 17–19). However, as modern emergency departments have good availability of highly accurate neuroimaging, the greatest practical benefits of GFAP would be achieved earlier, in the prehospital setting, where differential stroke diagnosis is currently achievable only with costly CT-equipped mobile stroke units (20).

In the first prehospital GFAP study, Rozanski et al. found a sensitivity of only 36% for identifying HS with high certainty, with sensitivity decreasing to 24% when only <1 hour samples were considered. This suggested that GFAP increases may occur too slowly to be useful in the very early phase (11). Notably, the study used a GFAP assay with limited sensitivity, providing measurements near the limit of quantification in many patients with HS, and mostly below this limit for patients with ACI. Our study builds on these initial findings, providing a larger sample size, serial blood sampling, and highly sensitive GFAP measurements, with no measured GFAP values under the LLOQ.

For now, very little is known of the cellular and tissue level mechanisms of cerebral GFAP release in ACI and HS, an area requiring further experimental investigation. Most notably, it is still unclear to what extent GFAP, a highly hydrophilic protein liberated from the cytosol and cytoskeleton of disrupted astrocytes, travels into circulation through the disrupted blood brain barrier, versus into the glymphatic system, cerebrospinal fluid, and its outflow tracts. The diagnostic value of GFAP is thought to arise from different release mechanisms and dynamics, early in HS and slower in ACI (17–19). Importantly, in a hospital-phase cohort, Foerch and collaborators found GFAP concentrations to correlate with both symptom severity (admission NIHSS) and hematoma volume in patients with HS, while no correlation was seen between GFAP and symptom severity in patients with ACI (18). Our report replicates these correlations in the prehospital phase, also for GFAP release rate. This suggests that early plasma GFAP release is highly dependent on the extent of hemorrhagic tissue damage in HS, while it does not depend on the extent of tissue ischemia and symptom severity in ACI. Thus, further inquiry is needed to determine the mechanisms of early GFAP release seen in a portion of patients with ACI in our study (Fig. 4).

While no POC GFAP assays are currently commercially available, and thus could not be used in our study, the future prehospital application of GFAP would require a sufficiently sensitive, rapid, and hand-held POC assay optimized for EMS use, to perform 2 successive prehospital measurements. Notably, use of a rapid GFAP assay based on the i-STAT® platform was recently reported in patients with traumatic brain injury, with only 15-minute measurement times on a laboratory platform (7). As suitable POC-assays are still under development, future studies will be needed to demonstrate feasibility of prehospital GFAP diagnostics, and define the required time between serial samples that provides sufficient diagnostic accuracy without unnecessary delay, which will depend on assay accuracy. Of note, the median (IQR) time between prehospital and admission samples was only 31 (24–42) minutes for patients with <3-hour prehospital sampling in our study and based on the tendency of GFAP to rise rapidly and exponentially in HS, this interval can likely be further shortened.

Combining initial prehospital GFAP and GFAP release rate was able to rule out HS with high certainty in over two-thirds of patients with ACI. In a real-life setting such a prehospital diagnostic tool would steer transportation to a primary or even tertiary neurovascular management unit, expedite preparations for thrombolytic or endovascular therapy, and triage for future prehospital therapeutic studies necessitating exclusion of patients with HS from therapies involving augmentation of collateral circulation or antithrombotic or thrombolytic approaches. However, based on our findings early GFAP monitoring alone cannot satisfactorily confirm HS, a diagnostic challenge that may require combining GFAP with other biomarkers or clinical scores.

In subgroup analyses, we found that the diagnostic performance of GFAP is improved when focusing on patients with moderate to severe symptoms. One such group is patients with suspected LVO identified using prehospital LVO-scales that currently have no capability to rule out HS. Although the rate of CT-angiography imaging was low in our study (recruitment performed between 2013 and 2015), in our explorative analysis prehospital GFAP measures showed good performance for differentiating LVO and HS cases, warranting further study into use of GFAP for LVO-triage.

Our study is so far the largest to explore plasma GFAP in very early stroke with prehospital sampling, and the first to apply ultra-sensitive Simoa technology at this early time point. As acknowledged reference calibrators for GFAP assays do not exist, Simoa measurements are not directly comparable with the plethora of different GFAP assays used in previous stroke biomarker studies. Due to this, we could not use prespecified GFAP cutoffs, but rather provide suggestions of cutoffs for future confirmatory studies. Unfortunately, to our knowledge, no suitable cohort with early serial sampling currently exists for the direct validation of our results. Importantly, recent reports have proposed additional novel biomarkers to be combined with GFAP as a diagnostic stroke biomarker panel (21), which may further improve the diagnostic performance seen in our study. Our findings also indicate that the early kinetics of these other biomarker candidates may be worth exploring.

Our analysis focused on ACI and HS cases with clear neurological deficits (≥3 NIHSS points), limiting the applicability of our results in patients presenting with milder symptoms, thus necessitating future studies to determine whether the diagnostic performance of GFAP pertains to HS cases with very low bleeding volumes. If this is not the case, prehospital use of GFAP may have to be targeted to patients with clear neurological deficits on a prehospital stroke severity scale, e.g., a LVO-scale (22). At the outset of this study, we did not have previous knowledge of the time windows when deflections in GFAP or tau concentrations would provide the best diagnostic performance, which led us to target this analysis to patients with all 3 serial sampling timepoints. While this is a proof-of-principle study with patients with well-defined ACI and HS demonstrating the novel diagnostic usefulness of measuring GFAP release dynamics, further replication of the findings in larger and demographically different SC patient cohorts is warranted to evaluate and tailor this approach for different hospital districts. Based on the results presented herein, we will conduct a larger replicative study with our whole study cohort focusing on the very early phase, to be performed with a more pratical and rapid assay when one becomes available. This will also provide further important information on early plasma GFAP in stroke mimics, although previous data have shown increases to be minimal (23). Based on the very small SM group in our study and previous GFAP studies, it seems unlikely that GFAP alone would be useful to differentiate patients with ACI and SM, a separate diagnostic challenge that may require biomarker panels and clinical scores (21, 24). Finally, as all EMS units from our district contributed to recruitment in our study, it was not practically feasible to achieve fully consecutive recruitment of SC patients. However, our study proves the wide applicability of a prehospital stroke biomarker approach, demonstrating that samples collected by first-line professionals in the heat of real-life prehospital care are of sufficient quality to allow highly sensitive measurement.

In conclusion, this proof-of-concept study demonstrates that when sufficiently sensitive assay technology is applied, the very early prehospital GFAP plasma concentration and its prehospital release rate can in combination provide improved differential diagnosis of patients with ACI and HS. GFAP ruled out HS in two-thirds of patients with ACI with high certainty, with improved performance in cases with moderate to severe stroke symptoms. In comparison, T-tau measurements did not show clinical value. Our findings encourage further study into rapid high-sensitivity POC assay technology and the clinical application of GFAP as an ultra-early stroke biomarker, the clinical utility of which may also have applicability in other on-scene neurological emergencies where cerebral hemorrhage is a relevant clinical suspicion worth ruling out.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations ACI, acute cerebral ischemia; HS, hemorrhagic stroke; NIHSS, National Institutes of Health Stroke Scale; NPV, negative predictive value; PPV, positive predictive value; EMS, emergency medical services; POC, point-of-care; ICH, Intracerebral hemorrhage; SC, stroke code; IS, ischemic stroke; TIA, transient ischemic attack; LKW, last-known-well; SAH, subarachnoid hemorrhage; T-tau, total tau; LLOQ, lower limit of quantification; AUC, area under the receiver operating characteristics curve; LVO, large-vessel occlusion; SM, stroke mimic; ICA, internal carotid artery; CT, computed tomography; M1, the first segment of the middle cerebral artery.

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