Early plasma biomarker dynamic profiles are associated with acute ischemic stroke outcomes

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Running title: Early Biomarker Dynamics in AIS

Abstract
Background: Early outcome prediction after acute ischemic stroke (AIS) might be improved with blood-based biomarkers. We investigated whether the longitudinal profile of a multi-marker panel could predict the outcome of successfully recanalized AIS patients.

Methods: We used ultrasensitive single-molecule array (Simoa) to measure glial fibrillary acidic protein (GFAP), neurofilament light chain (NfL), total-tau (t-tau) and ELISA for brevican in a prospective study of AIS patients with anterior circulation large vessel occlusion successfully submitted to thrombectomy. Plasma was obtained at admission, upon treatment, 24 h and 72 h after treatment. Clinical and neuroimaging outcomes were assessed independently.

Results: Thirty-five patients (64.8%) had good early clinical or neuroimaging outcome. Baseline biomarker levels did not distinguish between outcomes. However, longitudinal intra-individual biomarker changes followed different dynamic profiles with time and according to outcome. GFAP levels exhibited an early and prominent increase between admission and just after treatment. NfL increase was less pronounced between admission and up to 24 h. T-tau increased between treatment and 24 h. Interestingly, GFAP rate-of-change (pg/ml/h) between admission and immediately after recanalization had a good discriminative capacity between clinical outcomes (AUC = 0.88, $p < 0.001$), which was higher than admission CT-ASPECTS (AUC = 0.75, $p < 0.01$). T-tau rate-of-change provided moderate discriminative capacity (AUC = 0.71, $p < 0.05$). Moreover, in AIS patients with admission CT-ASPECTS <9 both GFAP and NfL rate-of-change were good outcome predictors (AUC = 0.82 and 0.77, $p < 0.05$).

Conclusion: Early GFAP, t-tau and NfL rate-of-change in plasma can predict AIS clinical and neuroimaging outcome after successful recanalization. Such dynamic measures match and anticipate neuroimaging predictive capacity, potentially improving AIS patient stratification for treatment, and targeting individualized stroke care.

Keywords: GFAP; NfL; outcome; plasma biomarkers; stroke; thrombectomy; total-tau.

INTRODUCTION

Stroke is a major cause of death and disability worldwide[1]. Since 2015, mechanical thrombectomy (MT) is being used for large vessel occlusion (LVO) in acute ischemic stroke (AIS) and dramatically changed AIS treatment landscape[2]. Further demonstration that patients are admissible for treatment up to 24 hours since "last known well" (LKW), expanded even more the population eligible for treatments[3]. However, even in optimal randomized clinical trial conditions, only 44-48% of the patients submitted to MT achieve functional independence[4]. In the real-world scenario functional independence drops to 37% and mortality is higher (29%)[5]. This is also true in analysis restricted to successfully recanalized patients (TICI 2B/3) where a pooled analysis of the results of five randomized clinical trials presented futile reperfusion rates of 54%[4]. Such negative observations hint that clinical-neuroimaging tools are insufficient for patient selection for treatment because they hold limitations in distinguishing penumbral infarct tissue salvable by reperfusion from unviable tissue[6]. This is probably due to individual endurance to brain ischemia. Multiple prognostic scales and approaches have been attempted but hold limitations to accurately predict AIS outcome[7].

In this setting, fluid biomarkers that reflect different dimensions of brain damage may provide easy assessable for a more personalized approach to improve patient selection for treatment and anticipate short- and long-term outcome allowing to hierarchize treatment priority and define post-treatment unit level of care.

Glial fibrillary acidic protein (GFAP) is brain-specific intermediate filament protein marker of astrogliosis. Plasma GFAP has previously been shown to be released rapidly out of damaged brain[8,9] in hemorrhagic stroke and to lesser extent in AIS patients[10–12]. Neurofilament light chain (NfL) is one of the neuronal scaffolding proteins that is released into the extracellular space upon neuroaxonal damage[13]. Serum NfL levels increase during aging and are elevated in multiple neurologic conditions including AIS[14–17] with potential applications both for patient-monitoring and for interventional studies. T-Tau is a brain-enriched protein that may be expected to leak from brain interstitial fluid to the plasma compartment upon neuronal injury. Plasma t-Tau concentration increases in neurodegenerative diseases and is also recognized elevated in the post-acute stage of AIS[18]. Brevican is a brain-specific extracellular matrix (ECM) chondroitin sulfate proteoglycan (CSPG), thought to regulate axonal guidance and modulate

synaptic connections. CSF brevican levels have been shown to decrease in TBI and vascular dementia compared to Alzheimer's disease (AD)[19,20]. However longitudinal analysis of these biomarkers at very early AIS pretreatment stages and in response to treatment are largely missing.

We hypothesized that very early dynamic changes of these biomarkers of brain damage in AIS patients could reflect distinctive clinical outcome. To address this question, we took advantage of a prospective stroke cohort of AIS patients admitted for acute stroke treatment and used ultrasensitive assays for Kinetic biomarker profiling in LVO patients successfully recanalized. We further established the discriminative value of fluid biomarkers variation in AIS short-term outcome prediction.

METHODS

BioStroke Study Population

We conducted a prospective cohort study of all patients presenting in the emergency room up to 24 hours since symptoms onset (leading to stroke code activation) in two academic Stroke Centers, between January 2019 and March 2020 (figure 1). For the present study, we selected patients with an anterior circulation large vessel occlusion stroke (LVO-S) eligible for MT either isolated or combined with intravenous thrombolysis (IVT) in whom successful vessel recanalization was achieved (TICI 2B or higher) and blood plasma from at least three timepoints had been obtained. All MT procedures were performed using endovascular devices comprising stent retrievers or aspiration catheters, or both. After MT, all patients were admitted to a stroke unit or intermediate care unit and received standard of care treatment.

Clinical assessment

AIS Patients were evaluated upon admission (T_0), immediately after thrombectomy (T_1), 24 hours after treatment (T_2) and 72 hours after treatment (T_3). All demographic (age, sex) and clinical variables [previous functional status (modified Rankin scale), time since symptom onset or last known well (LKW), previous vascular risk factors (hypertension, dyslipidemia, diabetes mellitus, smoking, atrial fibrillation] were registered systematically. Stroke severity measured by admission National Institutes of Health Stroke Scale (NIHSS) in all timepoints. Functional status was measured by modified Rankin Scale (mRS) score upon admission (pre-stroke status), at discharge, 3- and 12-months. Hospitals stroke registry was reviewed for any missing data. Ischemic stroke etiology according to the trial of ORG 10172 in acute stroke treatment (TOAST) classification based on information available at discharge and modified if new data was obtained on follow-up.

Clinical outcome was defined by stroke severity reassessment with NIHSS at 24 hours: good outcome – minimum of 4 points reduction in the NIHSS score or NIHSS at 24hours <2; bad outcome – increased, stabilized, or less than 4 points improvement from initial score.

Neuroimaging Assessment

Imaging acquisition

On admission (T_0), approximately at the time of plasma sampling, non-contrast CT (NCCT), CT angiography (CTA) and CT perfusion (CTP) studies were performed as part of the routine clinical stroke workup and on the basis of clinical need. At 24 hours (T_2), NCCT was repeated.

CTA consisted of a single-phase study with coverage ranging from the aortic arch to vertex. CTP was performed on a multidetector 64 section scanner as a 45-second cine series, beginning 8 to 12 seconds after power injection of 50 mL of contrast at 4 mL/s. The obtained slab consisted of eight sections, 5 mm thick, from the level of the basal ganglia to a level above the lateral ventricles. CTP maps (cerebral blood volume and mean transit time) were post-processed by using a standard deconvolution software package (CTP3 "Std," GE Healthcare).

Imaging analysis

ASPECTS score[21], as a surrogate of the volume of established infarction, was determined independently by two observers blinded to the patient outcome at admission (T_0) and at 24h (T_2) NCCT. When discordant a consensus classification was done. Hemorrhagic transformation at 24 NCCT was classified accordingly to European Co-operative Acute Stroke Study-II (ECASS-II) criteria[22]. CTA was analyzed to determine the site of intracranial occlusion, the existence of tandem lesions (i.e., simultaneous extracranial occlusion/high grade stenosis and intracranial occlusion in the same arterial territory) and to assess intracranial collateral status[23] (0 - absent collateral supply; 1 - collateral supply filling 0-50% of the occluded territory; 2 - collateral supply filling 50 to 99% of the occluded territory; 3 - fully patent collaterals). All these evaluations were performed by a single observer, also blinded to the patient outcome, with more than 10 year-experience in reading NCCT, CTA and CTP studies.

Final reperfusion status after thrombectomy was graded according to the modified TICI score[24], in which mTICI 2b, 2c or 3 were accepted as successful reperfusion.

Good neuroradiologic outcome was defined as 24h CT-ASPECTS of at least 8.

Blood Plasma Collection

Blood samples were collected into EDTA containing tubes (Vacuette®) in all timepoints (T_0, T_1, T_2 and T_3) and promptly processed. Samples were centrifuged at 2000 rpm at room temperature for 10 min. Plasma was transferred to polypropylene tubes

(Nalgene®), stored at -20ºC and transferred to -80ºC within 3-7 days until measurements. There was one freeze-thaw cycle prior to biomarker measurements.

Biomarker Measurement

We measured GFAP, NfL and t-Tau concentrations in plasma using the Quanterix Simoa 4-Plex assay on the Simoa HD1 Analyzer following the manufacturer's protocol (Quanterix Corp, Billerica, MA, USA). In brief, plasma samples were thawed on wet ice, centrifuged at 500 × g for 5 min at 4°C. Calibrators (neat) and samples (plasma: 1:4 dilution) were measured in duplicates. Samples were randomly assigned to different plates using the same batch of reagents. A four-parameter logistic curve fit data reduction method was used to generate a calibration curve. Two internal control samples of known concentration of the protein of interest (high-ctrl and low-ctrl) were included as quality control. Individual measurements fulfilling acceptance criteria were included in the analysis (accuracy = 80–120%, coefficient of variation of duplicate determination ≤20%). Three samples were measured at a distinct date using the same experimental approach. Assay performance was as follows: GFAP: For a quality control (QC) sample with a concentration of 80.9 pg/mL, repeatability was 4.4% and intermediate precision was 7.9%. For a QC sample with a concentration of 102.1 pg/mL, repeatability was 2.9% and intermediate precision was 6.2%. NfL: For a QC sample with a concentration of 12.3 pg/mL, repeatability was 5.9% and intermediate precision was 10.0%. For a QC sample with a concentration of 454 pg/mL, repeatability was 4.0% and intermediate precision was 6.3%. t-Tau: For a QC sample with a concentration of 2.2 pg/mL, repeatability was 3.8% and intermediate precision was 6.4%. For a QC sample with a concentration of 6.7 pg/mL, repeatability was 1.8% and intermediate precision was 5.0%. Plasma brevican (ng/mL) was measured using a commercially available validated ELISA using manufacturer`s instructions (RayBio), as previously described in detail.

All measurements were conducted by personal blinded to the patient outcome at the biomarker lab at the UGOT, Mölndal, Sweden.

Statistical analysis

Characteristics of patients, stroke, treatment and relevant time points of observation and data collection are described and compared according to clinical and neuroradiological outcomes using standard tests (t-test or Mann-Whitney test for continuous variables and Chi-square or Fisher's exact test for categorical variables). We studied the trajectories overtime of the four biomarkers according to the clinical/neuroradiological outcomes by fitting linear mixed effects models (LMM) using restricted maximum likelihood estimation. Besides the fixed effect of outcome, we included as covariate hours since symptoms onset/wake up to admission (T0) along with the 2-way interaction between outcome and time. This interaction term is the most relevant, indicating whether changes in mean biomarkers values over time differ across outcome. Whenever a significant interaction was detected, we compared piecewise changes in mean values across adjacent time points (T_1-T_0, T_2-T_1, T_3-T_2) among outcome groups. In all models GFAP, NfL, t-Tau and brevican values were modeled as continuous measures using the natural logarithm transformation to reduce skewness. We used model estimates to represent graphically changes in mean values and respective 95% confidence intervals over time. Models fitted data well as judged by agreement between observed and expected values and residuals followed approximately a normal distribution.

Based on early biomarker dynamic profiles, receiver-operator characteristics (ROC) curves were used to establish discriminative capacity in anticipating clinical and neuroradiological outcome using R software, version 4.0.3. For this purpose, a logistic regression model was fitted. An initial natural logarithmic transformation was applied to the biomarkers' variation features (ln(x+1)). The model was then fitted with 10-fold cross validation to avoid overfitting, which was repeated 100 times to reduce the bias and variance of the estimations. Specifically, data was randomly split into 10 folds, and for each subset, one-fold was used to validate the model, and the remaining to train it. Statistical significance was set at $p<0.05$. All the statistical analyses, except where otherwise noted, were performed using IBM SPSS Statistics Software version 21, (NY).

Standard protocol approvals, registrations, and patient consents.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethical review boards at CHUP (123-DEFI/122-CES) and CHUSJ ().

All participants or legal representatives have provided written informed consent to participate in the study.

Data Availability Statement.

All de-identified participant data requests should be submitted to the corresponding author.

RESULTS

Clinical characteristic of the BioStroke patient cohort.

During the study period, 540 patients were admitted to the emergency department with Acute Stroke Activation Code within the first 24 hours after symptom onset. We excluded 271 patients with no relevant diagnosis (including 117 stroke mimics). Out of 269 AIS, we excluded 118 patients not submitted to thrombectomy, and further 37 patients for whom no recanalization was achieved (TICI 0-2a). From the remaining 114 patients 60 were excluded because of inadequate blood sampling and/or logistic problems. Therefore, 54 AIS patients successfully recanalized by MT were included in the study. (Fig. 1)

AIS patients successfully recanalized

Thirty-five patients (64.8%) presented early neurological clinical and good neuroradiological outcome. (Table 1; Supplemental. Table 1) In 42 patients (78%) clinical outcome matched the neuroradiological outcome (29 with good and 13 with bad outcome). Discrepancies in outcome classification were evenly spread due to a worst clinical outcome (clinical benefit was below NIHSS difference cut-off of 4) or due to initial ASPECTS score <8.

Patients with high blood pressure ($p<0.05$), longer times from symptom onset to admission ($p<0.05$) and with symptoms noted upon awakening (wake-up stroke) ($p<0.05$) had significantly worse clinical outcome. Regardless of outcome definition, distribution according to age, sex, symptomatic arterial segment, AIS etiology and stroke severity (admission NIHSS) were similar. Noteworthy, patients with early neurological recovery and good neuroradiological outcome presented a higher median admission ASPECTS (9 vs. 8, $p<0.01$ and 9 vs. 7, $p<0.001$, respectively) and had higher collateral score ($p<0.01$). In addition, patients submitted to thrombolysis with rt-PA before MT had better clinical outcome ($p<0.05$). There were no significant differences regarding blood sampling intervals between both groups, namely from last known well time to admission (T_0), time from admission to recanalization (T_0-T_1) and from recanalization to 24 hours since last known well. Three cases with symptomatic brain hemorrhage were diagnosed at 24 hours (ECASS score=4).

Plasma biomarker dynamic profiles

Intra-individual biomarker panel (GFAP, NfL, t-Tau and brevican) levels at the four pre-specified sampling time points were plotted according to clinical outcome after a successful recanalization. (Fig.2) Globally, we observed that patients with bad clinical outcome showed a higher increase in biomarker levels (GFAP, NfL, t-Tau) compared to good clinical outcome, with the exception of brevican that showed no obvious pattern of fluctuation over the first 72 hours. The biomarker profile according to neuroradiological outcome was similar. (Supplemental Fig.1)

The results of all LMM including clinical or neuroradiological outcome for the four biomarkers are summarized in Table 2 and supplementary table 2 (see supplemental Table 3 for descriptive statistics of biomarkers levels at distinct time points). In LMM models including clinical outcome, the covariate time from stroke onset was significant only when modelling GFAP. However, in LMM models including neuroradiological outcome significant associations were observed for GFAP, NfL and t-Tau. Brevican levels did not depend on any variable included in the model. There was a significant interaction between outcome and time in all LMM models, indicating a different longitudinal change in biomarker levels according to clinical or neuroradiological outcome, with the exception of brevican. Observed and estimated biomarker dynamic profiles according to clinical outcome are depicted in Fig. 3 and according to neuroradiological outcome in Supplemental Fig. 2. GFAP levels presented a different profile according to clinical and neuroradiological outcome definitions; a higher increase in patients with bad clinical or neuroradiological outcome between admission and just after treatment (T_0-T_1) followed by successive increases not dependent on clinical outcome, while the increase between T_1 and 24h or 72h post-treatment was always higher in patients with bad compared with good neuroradiological outcome. NfL presented an identical profile according to clinical and neuroradiological outcome, a higher increase between T_0 and T_1 and 24h post-treatment in patients with bad outcome, followed by an identical increase between 24h and 72h post-treatment. The increase in t-Tau levels in patients with bad or good clinical outcome was almost identical, only slightly higher in those with bad outcome between T_1 and 24h post-treatment, but in patients with bad neuroradiological outcome there was a higher increase between T_0 and T_1, that persists between T_1 and 24h post-treatment.

AIS outcome prediction based on early plasma biomarker rate of change

Considering the observed biomarker dynamic profiles, admission to post-treatment is the more relevant time period, since estimated differences between good and bad outcome are more consistent and also bearing in mind the clinical relevance of the pre-treatment period in stroke care treatment decision. Therefore, we studied the discriminative capacity of biomarker rate of change per hour between these time points in anticipating clinical and neuroradiological outcome. GFAP rate of change was higher in bad outcome patients in both clinical and neuroradiological outcome (305 pg/mL/h vs. 34 pg/mL/hour; p<0.001)). (Supplemental fig. 3 and fig. 4 and Supplemental Table 3). This observation did not change if patients that latter suffered a symptomatic hemorrhagic transformation at 24 hours (n=3) were excluded from the analysis. NfL rate of change was increased in bad outcome patients but did not reach statistical significance (p=0.056). T-Tau rate of change was increased in bad outcome patients in both clinical and neuroradiological outcome (0.39 pg/mL/h vs. 0.09 pg/mL/h; p<0.001). No variation in brevican rate of change was observed. (Supplemental table 3 and supplemental fig. 3 and fig. 4)

We used ROC curves to assess the discriminative outcome capacity of the rate of change of the different biomarkers. We observed that GFAP rate of change had a good discriminative capacity in anticipating good and bad clinical or neuroradiological outcome with and an area under curve (AUC) of 0.88 (p<0.001) and 0.83 (p<0.001), respectively (Fig.4 and Supplemental Fig.5). To further validate these findings, we generated ROC curves based on a logistic regression model that disclosed a similar performance (AUC = 0.86, p<0.001). The cut-off point for clinical outcome prediction based on the Youden index was 72.8 pg/mL/h and if we maximize specificity (specificity 100% and sensitivity 42%) the estimated cut-off to be 880.8 pg/mL/h. For the same patients, observed admission CT-ASPECTS AUC was 0.75 (p<0.01). (Fig. 4B)

We observed that t-Tau rate of change had a moderate discriminative capacity between good and bad clinical or neuroradiological outcome with an AUC of 0.71 (p<0.05) and 0.76 (p<0.01), respectively. ROC curves based on a logistic regression model significance only held for the neuroradiological outcome (AUC=0.75, p<0.05). Regarding NfL and brevican ROC analyses, neither of them showed statistically significant results after logistic regression modeling.

In patients with lower admission CT-ASPECTS, there is some uncertainty about the clinical benefit of MT[25]. Consistently, after restricting the analysis for patients with admission CT ASPECTS <9 (n=28), admission CT-ASPECTS did not discriminate between good and bad clinical outcome (AUC=0.56; p>0.05). (Fig. 4D) To evaluate the biomarker panel potential in such cases, we performed the same analysis and found that GFAP rate of change and NfL rate of change had a good and moderate discriminative capacity between good and bad clinical outcome with AUCs of 0.82 (p<0.01) and 0.77 (p<0.05), respectively. These results were supported by ROC curves based on a logistic regression models (Fig. 4C). The cut-off point based on the Youden index for GFAP was 219.3 pg/mL/h and for NfL was 2.8 pg/mL/h. When we maximized specificity, we estimated the cut-off to be 1134.6 pg/mL/h for GFAP (specificity 100%; sensitivity 47%) and 2.8pg/mL/h for NfL (specificity 100%; sensitivity 47%). Neither t-Tau nor brevican rate of change were significant in logistic regression analyses.

DISCUSSION

The present study was designed to explore the early dynamic profile of blood-based biomarkers in the acute-phase of AIS of the anterior circulation due to LVO. The main findings of this study were that GFAP, t-Tau and, in cases with early ischemic changes, NfL rates of change between admission and MT treatment are significantly higher in patients with bad outcome despite successful recanalization. Notably, this was true considering clinical or neuroradiological definitions of outcome, supporting that these biomarkers anticipate brain tissue damage and neurological dysfunction. Therefore, they hold relevance for clinical decision.

To the best of our knowledge, this is one of the first studies showing the potential of brain-derived proteins measured in peripheral blood as biomarkers to predict early neurological outcome in recanalized AIS patients. Our results indicate that GFAP and t-Tau rates of change in plasma correlate well with the extent of brain injury and infarct progression, but this was not the case for initial single timepoint measurements. In fact, when single timepoints measurements are compared, clinical outcome discriminative capacity was only good at time of successful recanalization and highest at 24h after symptom(s) onset or last known well supporting the use of dynamic over a static measurement. This observation parallels longitudinal plasma NfL dynamics in familial AD, though in that case the timeline is in years and in the stroke setting, changes occur in a much shorter time frame[26].

Remarkably, early biomarker rate of change performance as outcome predictor outstands CT-ASPECTS at admission in the all cohort. This is particularly notorious for GFAP rate of change and, to a lesser extent, t-Tau rate of change. GFAP showed an overall better discriminative capability than admission CT-ASPECTS score and the best overall accuracy for clinical outcome prediction. No further improvement in discriminative capacity was obtained when biomarkers were combined. Importantly, in the case of patients with lower ASPECTS score, known to have poorer outcome after treatment, GFAP and NfL rate of change clearly outperformed CT-ASPECTS in outcome prediction (AUC=0.5, $p>0.05$)[4,25]. Therefore, the use of sensitive and specific blood-based biomarkers of stroke could significantly impact AIS treatment by providing and objective assessment tool. Such biomarkers could complement the currently used neuroimaging modalities in assessment of brain tissue at risk, particularly for patients

with established lesions at admission, to avoid futile interventions and, eventually, offer treatment to otherwise excluded patients. In our exploratory study we intended to identify biomarkers that could confirm the potential benefit of recanalization. For that purpose, by maximizing the test specificity we were able to establish cut-offs to identify true-positives. Having done so, using GFAP and NfL rate of change with a specificity of 100%, we could identify AIS patients that would not benefit from recanalization with a sensitivity of 42% to 47%, respectively. If our findings are validated in other cohorts, this concept of biomarker-based AIS patient stratification for treatment may provide more personalized approaches in AIS acute patient care, minimizing futile treatments and maximizing good candidates. This concept would constitute a major gain to optimize patient selection to direct patients to comprehensive stroke centers, particularly in the "Drip and Ship" model of care.

The observed biomarker longitudinal profiles suggest that the ischemic pathological cascade can be detected in the blood, early in the disease course. Our data depicts prominent and sequential changes in GFAP, NfL and t-Tau that follow distinct profiles between good and bad clinical outcomes. Consistently, in patients with bad neuroradiological outcome, that have increased tissue damage (ASPECTS 6-8), the estimated biomarker profiles are even more distinctive from good outcome patients supporting that GFAP, NfL and t-Tau can also monitor AIS associated brain damage at early disease stages. Available evidence from literature on treated and untreated AIS patients is consistent with our findings[11,14,18]. Specifically, glial and neuronal damage biomarkers may provide diagnostic information, such as time of onset, severity, discrimination between ischemic or hemorrhagic stroke and long-term outcomes[27]. Our ultrasensitive methodology may have disclosed more subtle changes in GFAP levels early in AIS, that were probably missed in more seminal works that measured GFAP in AIS using less sensitive approaches.[11] In fact, the measurement of plasma GFAP using SIMOA has recently been shown of relevance for distinguishing AIS from intracerebral hemorrhage[10]. Total-Tau is known to increase in the CSF as well as in the blood following stroke[28,29]. Conversely to what was previously found[28], our results support that elevation of t-Tau is associated with AIS early stroke outcome after successful recanalization. NfL release dynamics follows a less pronounced but prolonged increasing trend assuming relevance for the prognosis of patients with more brain parenchyma at risk (ASPECTS

<9). Brevican is a CNS extracellular matrix constituent overexpressed in the late phase of stroke[30] and involved in brain injury repair, still our results do not support a role of this protein as an early outcome predictor.

The main strengths of our study are the prospective design, as well as the consistency of the results considering different definitions of outcome. In addition, our selection criteria included only recanalized patients, which allowed us to explore the predictive value of plasma biomarkers in early AIS outcome. Still, the main limitation of our study is the low sample size and lack of replication cohort. This may be compensated by balance of risk factors, clinical and neuroimaging characteristics such as admission NIHSS, admission CT-ASPECTS, time to treatment and recanalization outcome. Other limitation includes the timing of the second sample (T_1), as it was obtained after MT treatment, we cannot exclude that the observed biomarker changes and rates of change may be partially due to effects of recanalization and do not provide evidence of definite pre-treatment variation.

In the future, the rate of plasma GFAP, NfL and t-Tau concentration change in patients with AIS, with a first sample taken in the prehospital setting, could help stroke physicians to identify fast progressing AIS patients and assist in determining infarct extension by complementing neuroimaging modalities. For this, biomarker assessment needs to be quick, inexpensive and deliver a readily available result for interpretation, like point of care devices that are under evaluation for GFAP in traumatic brain injury patients to avoid CT scanning[31,32].

Conclusion

Our study characterizes the longitudinal dynamic profile of four brain-specific biomarkers and their early clinical outcome discriminative capacity based on their rate of change in the first hours after AIS. We found that plasma GFAP and t-Tau rates of change and that plasma NfL rate of change in cases with some degree of ischemic tissue damage, might be good predictors of early clinical outcome. If confirmed by future studies, this would significantly add to the field of AIS treatment and personalized medical care.

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HZ has served at scientific advisory boards for Alector, Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies and CogRx, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

The other authors have declared that no competing interest exist.

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Table

Table 1. Demographic and clinical Characteristics of the baseline cohort according to clinical outcome

			Clinical outcome				
Characteristic	All (n=54)		Bad (n=19)		Good (n=35)		*P*
Mean age (SD), years	73.0	(12.2)	70.3	(12.7)	74.5	(11.8)	0.227
Men, n %	37	68.5	13	68.4	24	68.6	0.991
Vascular risk factors, n %							
High blood pressure	45	83.3	13	68.4	32	91.4	**0.030**
Diabetes	12	22.2	6	31.6	6	17.1	0.223
Dyslipidemia	35	64.8	13	68.4	22	62.9	0.683
Atrial fibrillation	14	25.9	3	15.8	11	31.4	0.210
Left side Stroke n (%)	25	46.3	10	52.6	15	42.9	0.492
Blood vessel, n %							0.605
ACI	8	14.8	4	21.1	4	11.4	
M1	38	70.4	12	63.2	26	74.3	
M2	8	14.8	3	15.8	5	14.3	
Aetiology, n %							0.434
Large-artery atherosclerosis	8	14.8	3	15.8	5	14.3	
Cardioembolism	34	63.0	10	52.6	24	68.6	
Unknown/others	12	22.2	6	31.6	6	17.1	
Wake-up stroke, n %	24	44.4	12	63.2	12	34.3	**0.041**
At admission (T_0)							
Median NIHSS (IQR)	13	(9-19)	13	(10-19)	13	(8-19)	0.964*
Median ASPECTS (IQR)	8	(7-10)	8	(7-8)	9	(8-10)	**0.002***
Collateral status							**0.011**
0	0		-		-		
1	4	7.4	4	21.1	0	-	
2	21	38.9	8	42.1	13	37.1	
3	29	53.7	7	36.8	22	62.9	
Thrombolysis (rt-PA), n %	11	20.4	1	5.3	10	28.6	**0.042**
Median time (IQR), hours							
Symptom/LKW – Admission (T_0)	6.1	(3.5-12.5)	9.8	(5.8-12.8)	4.8	(2.9-8.8)	**0.046**
Symptom/wake up hour – T_0	3.9	(2.2-6.3)	3.5	(2.3-8.2)	4.2	(1.9-6.3)	0.393
Admission – Recanalization	2.0	(1.6-2.5)	1.9	(1.7-2.8)	2.0	(1.5-2.5)	0.776
Recanalization – T_1	3.8	(1.8-5.8)	3.9	(2.3-7.1)	3.7	(1.7-5.4)	0.697

IQR - interquartile range; LKW – Last known well; SD – Standard Deviation. * Mann-Whitney test.

Table 2. Linear Mixed Model analysis for biomarkers levels

	Models including Clinical Outcome		
	Coef.	95% CI	P value
ln(GFAP)			
Time from onset*	0.074	0.003 to 0.145	**0.042**
Bad vs. Good outcome			
T_1 vs. T_0	1.537	0.878 to 2.196	**<0.001**
T_2 vs. T_1	0.365	-0.387 to 1.116	0.335
T_3 vs. T_2	-0.342	-0.813 to 0.129	0.147
ln(NfL)			
Time from onset*	0.051	-0.013 to 0.116	0.115
Bad vs. Good outcome			
T_1 vs. T_0	0.382	0.139 to 0.624	**0.003**
T_2 vs. T_1	0.312	0.005 to 0.619	**0.047**
T_3 vs. T_2	0.167	-0.211 to 0.545	0.372
ln(t-Tau)			
Time from onset*	0.062	-0.013 to 0.137	0.104
Bad vs. Good outcome			
T_1 vs. T_0	0.358	-0.279 to 0.996	0.264
T_2 vs. T_1	0.555	0.039 to 1.071	**0.036**
T_3 vs. T_2	0.613	-0.129 to 1.356	0.102

*Time from symptoms/wake-up hour to admission (T_0); for ln(Brevican) model coefficients are not shown because both main effects and interaction did not reach statistical significance (p>0.05)

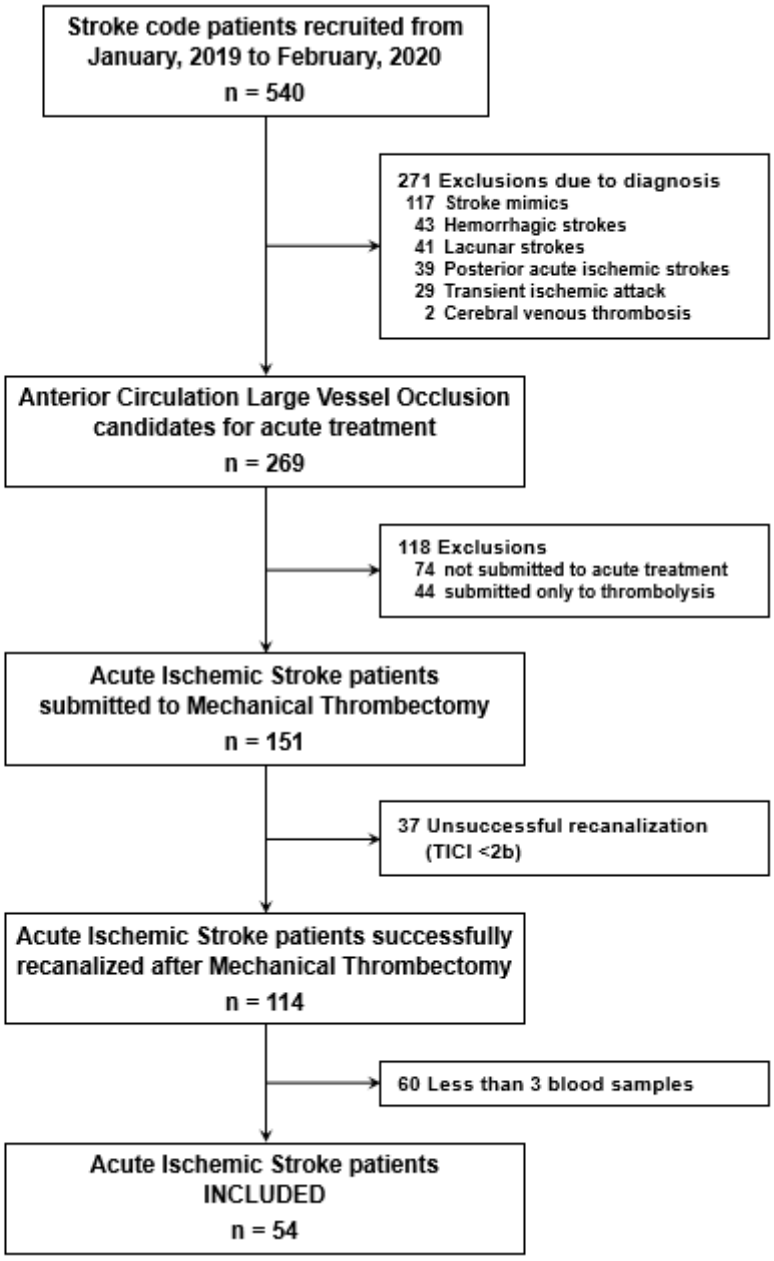


Figure 1. Flow chart of study population selection

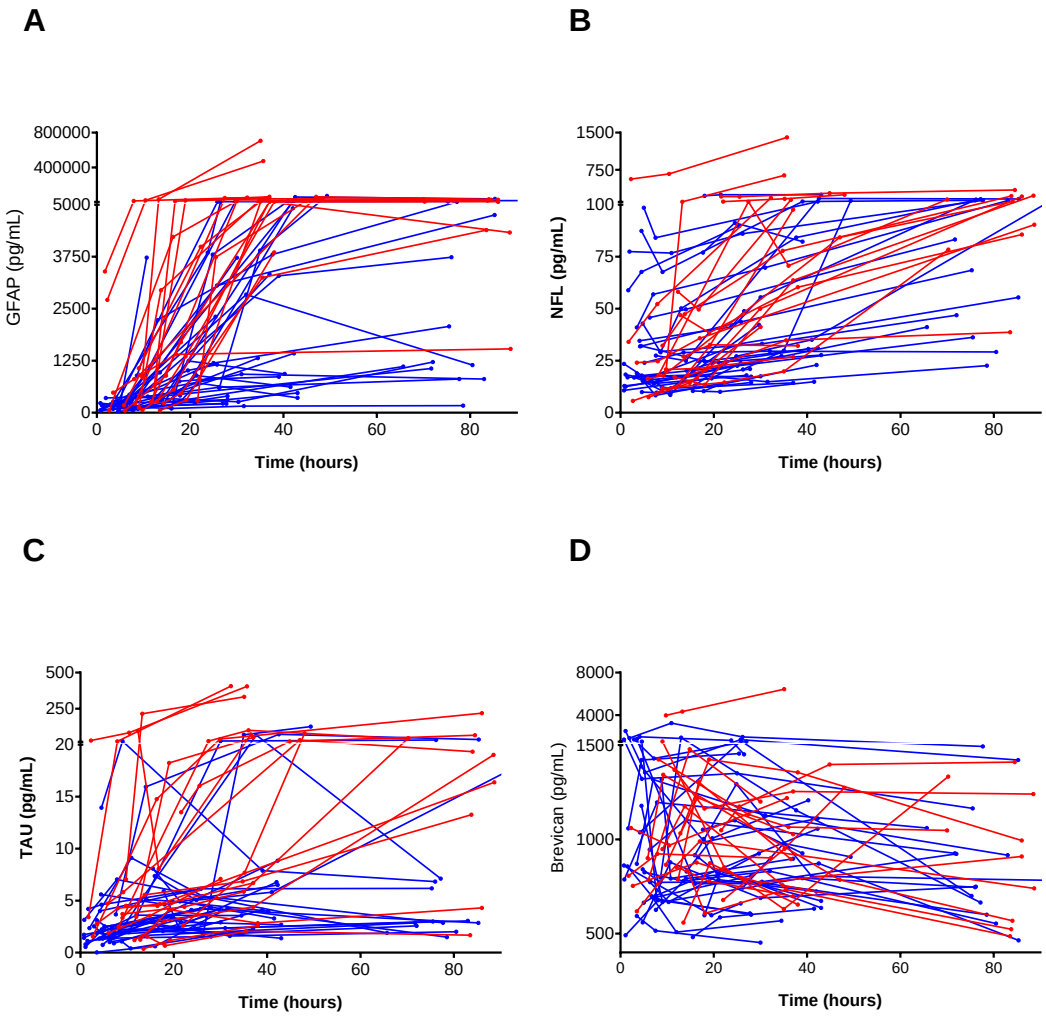


Figure 2. Longitudinal biomarker panel profile in the acute phase of stroke patient care. A to **D** Spaghetti plot showing longitudinal plasma GFAP(**A**), NfL(**B**), t-Tau(**C**) and brevican (**D**) from individual AIS patients with good clinical outcome (blue, n=35) and bad clinical outcome (red, n=19). In the *x axis* time is represented in hours and "0" is the time of symptom onset or wake-up time. The Y axis is divided in 2 segments to improve visualization and fit all sample values.

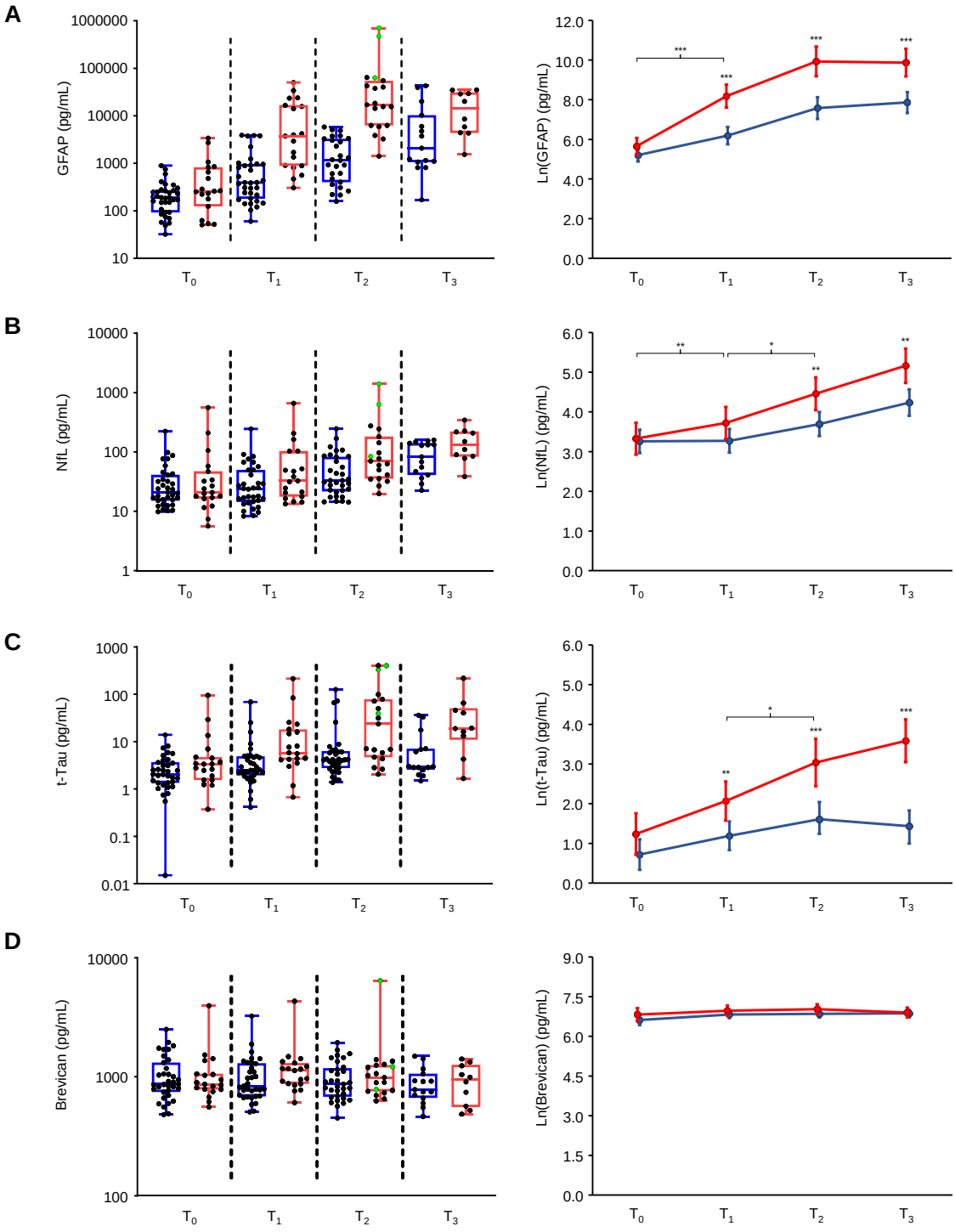


Figure 3. Biomarker levels in AIS patients with good and bad clinical outcome with time. Left panels Boxplots represent the median and max and minimal values of each biomarker at predetermined time points (T_0 admission; T_1 after treatment; T_2 at 24 hours; T_3 at 72hours) GFAP(**A**), NfL(**B**), t-Tau(**C**) and brevican (**D**). AIS patients with good clinical outcome (n=35) are represented in blue and bad clinical outcome (n=19) in red. Three patients with a symptomatic intracranial hemorrhage at 24 hours are highlighted in green. Y axis is in logarithmic scale and units were selected to fit all sample values.

Right panel Estimated means and 95% confidence levels based on the LMM model (**A**) GFAP (**B**) NfL (**C**) t-Tau and (**D**) brevican. *P<0.05, **P<0.01, ***P<0.001.

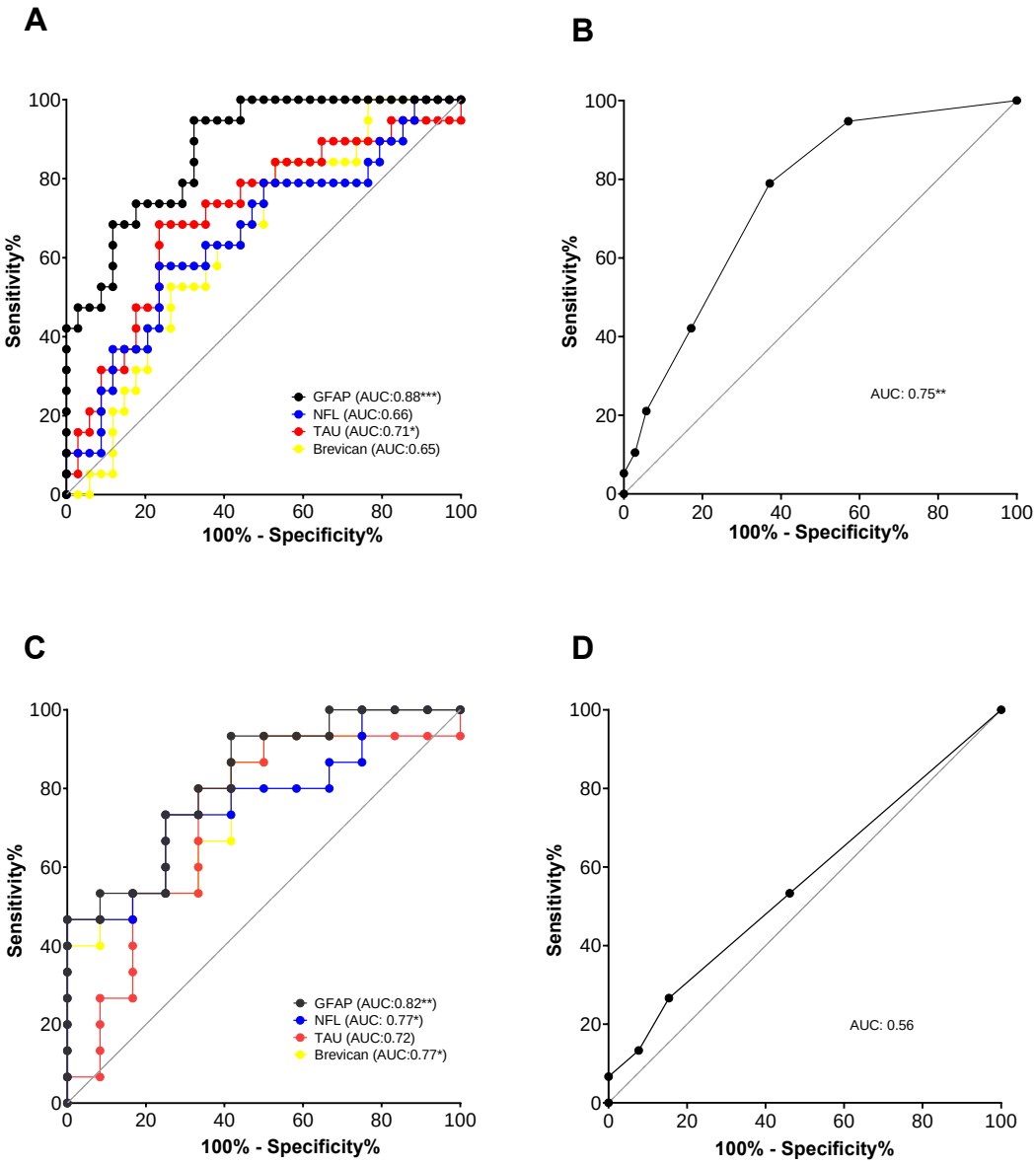


Figure 4. Biomarker panel performance in early clinical outcome prediction Receiver-operator characteristics (ROC) curves to differentiate AIS patients with good (n=35) and bad (n=19) clinical outcome based on observed individual biomarkers rate of change (panel **A**) and admission CT-ASPECTS (panel **B**). In panels **C** and **D**, we show the same analysis in the group of patients that had an admission CT-ASPECTS <9 (n=28). **(A)** GFAP rate of change exhibits the best performance in early outcome anticipation. Logistic

regression derived ROC confirmed this observation with area under of curve (AUC) = 0.86*** [95% CI 0.76-0.96]. The observed t-Tau rate of change performance was not confirmed after logistic regression modeling. Both NfL and brevican did not differentiate between clinical outcomes (B) CT-ASPECTS performance in early outcome anticipation was 0.75** [95% CI 0.62-0.88]. (C) GFAP and NfL rate of change had the best performance in early outcome anticipation of patients with admission CT-ASPECTS<9. GFAP (black) had an AUC=0.79* [95% CI = 0.61-0.97], with an optimal cutoff point of 219.3 pg/mL/hour; NfL (in blue) had an AUC = 0.77* [95% CI = 0.58-0.95], with an optimal cutoff point of 2.8 pg/mL/hour; t-Tau (red) and brevican (in yellow) performance was not confirmed after logistic regression modeling. (D) Admission CT-ASPECTS could not differentiate clinical outcomes in this subset of patients. *P<0.05, **P<0.01, ***P<0.001.

Supplementary Material

Supplemental Table 1. Demographic and clinical characteristics of baseline cohort according to neuroradiological outcome

Characteristic	All (n=54)		Bad (n=19)		Good (n=35)		*P*
Mean age (SD), years	73.0	(12.2)	69.2	(12.2)	75.1	(11.8)	0.088
Men, n %	37	68.5	15	78.9	22	62.9	0.224
Vascular risk factors, n %							
High blood pressure	45	83.3	15	78.9	30	85.7	0.524
Diabetes	12	22.2	2	10.5	10	28.6	0.128
Dislipidemia	35	64.8	12	63.2	23	65.7	0.851
Atrial fibrillation	14	25.9	2	10.5	12	34.3	0.057
Left side Stroke n (%)	25	46.3	11	57.9	14	40.0	0.208
Blood vessel, n %							0.605
ACI	8	14.8	4	21.1	4	11.4	
M1	38	70.4	12	63.2	26	74.3	
M2	8	14.8	3	15.8	5	14.3	
Aetiology, n %							0.137
Large-artery atherosclerosis	8	14.8	5	26.3	3	8.6	
Cardioembolism	34	63.0	9	47.4	25	71.4	
Unknown/others	12	22.2	5	26.3	7	20.0	
Wake-up stroke, n %	24	44.4	12	63.2	12	34.3	0.041
At admission (T_0)							
Median NIHSS (IQR)	13	(9-19)	14	(12-20)	12	(7-18)	0.160*
Median ASPECTS (IQR)	8	(7-10)	7	(6-8)	9	(8-10)	**<0.001***
Collateral status							**0.002**
1	4	7.4	4	21.1	0	-	
2	21	38.9	10	52.6	11	31.4	
3	29	53.7	5	26.3	24	68.6	
Thrombolysis (rt-PA), n %	11	20.4	2	10.5	9	25.7	0.186
Median time (IQR), hours							
Symptom/LKW – Admission (T_0)	8.1	(6.3)	9.3	(6.7)	7.5	(6.0)	0,342
Symptom/wake up – T_0	4.7	(3.6)	4.2	(3.8)	4.9	(3.4)	0.487
Admission – Recanalization	2.1	(0.8)	2.2	(0.8)	2.1	(0.8)	0.635
Recanalization – T_1	4.8	(4.2)	5.4	(4.3)	4.4	(4.3)	0.444
$T_2 – T_1$	19.7	(6.2)	19.6	(8.3)	19.7	(4.7)	0.931
$T_3 – T_2$	47.0	(8.5)	49.5	(9.5)	45.3	(7.5)	0.252

IQR, interquartile range; LKW, Last known well; SD, Standard Deviation; *Mann-Whitney test

Supplemental Table 2. Linear Mixed Model analysis for biomarkers levels

	Model including Neuroradiological Outcome		
	Coef.	95% CI	P value
ln(GFAP)			
Time from onset*	0.095	0.023 to 0.166	0.011
Bad vs. Good outcome			
T_1 vs. T_0	1.442	0.765 to 2.119	<0.001
T_2 vs. T_1	1.064	0.366 to 1.762	0.003
T_3 vs. T_2	-0.489	-0.952 to -0.026	0.039
ln(NfL)			
Time from onset*	0.073	0.011 to 0.134	0.023
Bad vs. Good outcome			
T_1 vs. T_0	0.256	0.002 to 0.511	0.048
T_2 vs. T_1	0.626	0.358 to 0.894	<0.001
T_3 vs. T_2	0.297	-0.058 to 0.652	0.097
ln(t-Tau)			
Time from onset*	0.110	0.031 to 0.188	0.007
Bad vs. Good outcome			
T_1 vs. T_0	0.934	0.334 to 1.535	0.003
T_2 vs. T_1	0.574	0.068 to 1.081	0.027
T_3 vs. T_2	0.425	-0.34 to 1.191	0.264

*Time from symptoms/wake-up hour to admission (T_0); for ln(brevican) model coefficients are not shown because both main effects and interaction did not reach statistical significance (p>0.05)

Supplemental Table 3. Biomarker panel levels at predefined time points according to clinical and neuroradiological outcome

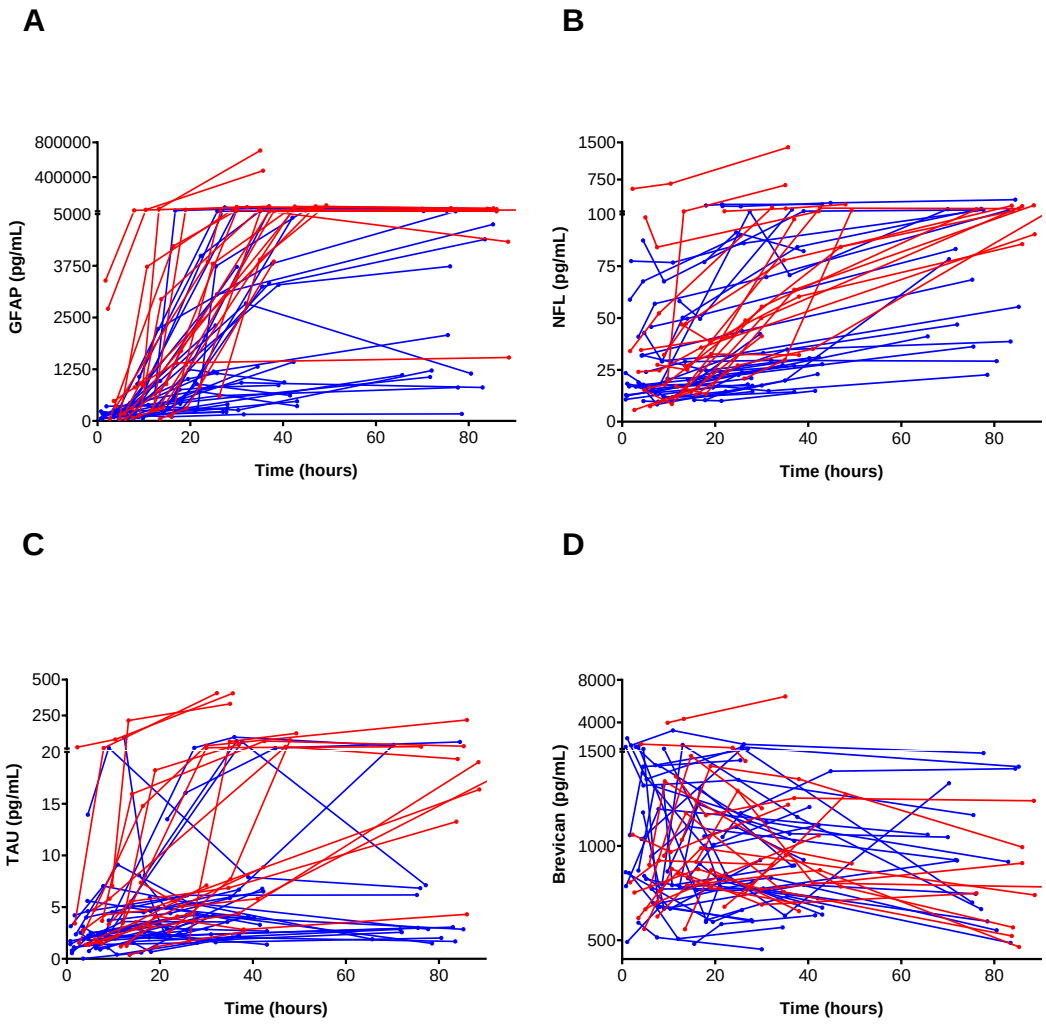
	Clinical outcome					Neuroradiological outcome				
	Bad (n=19)		Good (n=35)		Adjusted	Bad (n=19)		Good (n=35)		Adjusted
ln(Biomarker)	Mean	SD	Mean	SD	P value	Mean	SD	Mean	SD	P value
GFAP (pg/mL)										
At admission (T_0)	5.699	(1.253)	5.169	(0.760)	0.097	5.496	(1.286)	5.279	(0.787)	0.293
After treatment (T_1)	8.229	(1.614)	6.141	(1.127)	**<0.001**	7.967	(1.523)	6.287	(1.410)	**<0.001**
At 24h (T_2)	9.979	(1.586)	7.606	(1.670)	**<0.001**	10.168	(1.569)	7.497	(1.502)	**<0.001**
At 72h (T_3)	9.323	(1.107)	8.006	(1.587)	**<0.001**	8.580	(1.173)	7.944	(1.420)	**<0.001**
NfL (pg/mL)										
At admission (T_0)	3.363	(1.120)	3.240	(0.747)	0.718	3.315	(1.038)	3.266	(0.810)	0.677
After treatment (T_1)	3.759	(1.063)	3.248	(0.798)	0.078	3.631	(1.007)	3.320	(0.872)	0.159
At 24h (T_2)	4.493	(1.111)	3.682	(0.778)	**0.004**	4.567	(0.999)	3.639	(0.813)	**<0.001**
At 72h (T_3)	4.868	(0.651)	4.335	(0.679)	**0.001**	4.981	(0.368)	4.305	(0.742)	**<0.001**
t-Tau (pg/mL)										
At admission (T_0)	1.282	(1.227)	0.697	(1.114)	0.114	0.941	(0.920)	0.882	(1.309)	0.672
After treatment (T_1)	2.112	(1.366)	1.155	(0.973)	**0.006**	2.141	(1.397)	1.139	(0.932)	**0.001**
At 24h (T_2)	3.083	(1.763)	1.642	(1.071)	**<0.001**	3.153	(1.719)	1.601	(1.053)	**<0.001**
At 72h (T_3)	3.038	(1.376)	1.582	(1.017)	**<0.001**	3.123	(1.048)	1.626	(1.226)	**<0.001**
Brevican (pg/mL)										
At admission (T_0)	6.883	(0.432)	6.867	(0.411)	0.743	6.808	(0.473)	6.908	(0.382)	0.337
After treatment (T_1)	7.011	(0.406)	6.842	(0.400)	0.121	7.001	(0.412)	6.845	(0.398)	0.242
At 24h (T_2)	6.954	(0.509)	6.818	(0.358)	0.219	6.957	(0.504)	6.817	(0.361)	0.324
At 72h (T_3)	6.763	(0.394)	6.725	(0.333)	0.155	6.607	(0.318)	6.815	(0.357)	0.925

SD, Standard deviation; Adjusted P values for marginal contrasts in LMM at predefined time points

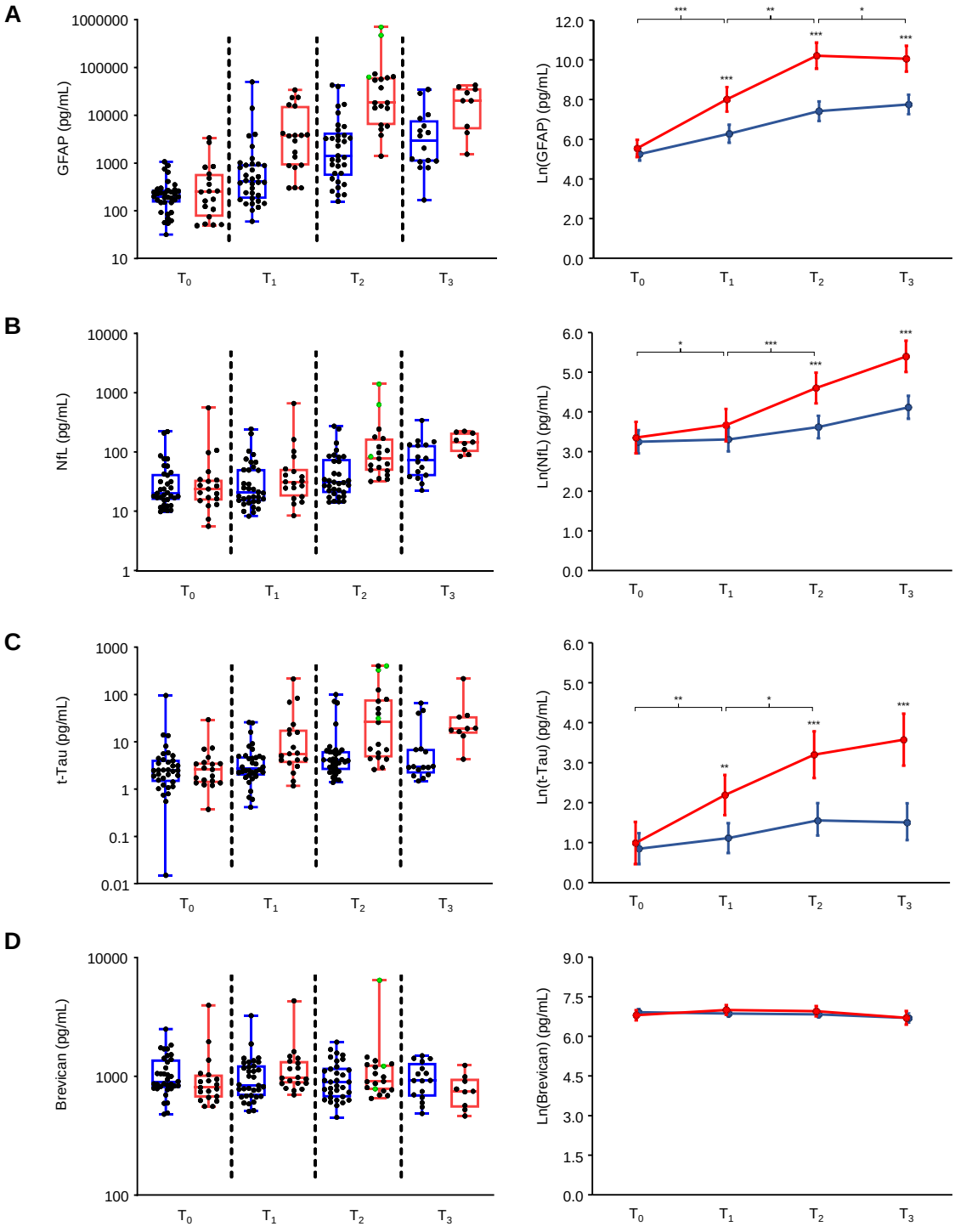
Supplemental Table 4. Rate of change between T0 and T1 according to clinical and neuroradiological outcome according to clinical and neuroradiological outcome

	Clinical outcome					Neuroradiological outcome				
	Bad (n=19)		Good (n=35)			Bad (n=19)		Good (n=35)		
Biomarker	Median	IQR	Median	IQR	P	Median	IQR	Median	IQR	P
GFAP (pg/mL/h)	305	(95 to 2598)	34	(9 to 135)	**<0.001**	305	(95 to 1135)	36	(9 to 135)	**<0.001**
NfL (pg/mL/h)	0.78	(0.07 to 3.18)	0.09	(-0.57 to 0.71)	0.056	0.79	(-0.39 to 2.95)	0.09	(-0.57 to 0.65)	0.088
t-Tau (pg/mL/h)	0.39	(0.14 to 1.21)	0.09	(-0.07 to 0.26)	**0.012**	0.39	(0.14 to 2.11)	0.05	(-0.10 to 0.26)	**0.002**
Brevican (pg/mL/h)	0.03	(-0.01 to 0.06)	-0.01	(-0.04 to 0.03)	0.072	0.03	(0.01 to 0.06)	-0.02	(-0.06 to 0.04)	**0.020**

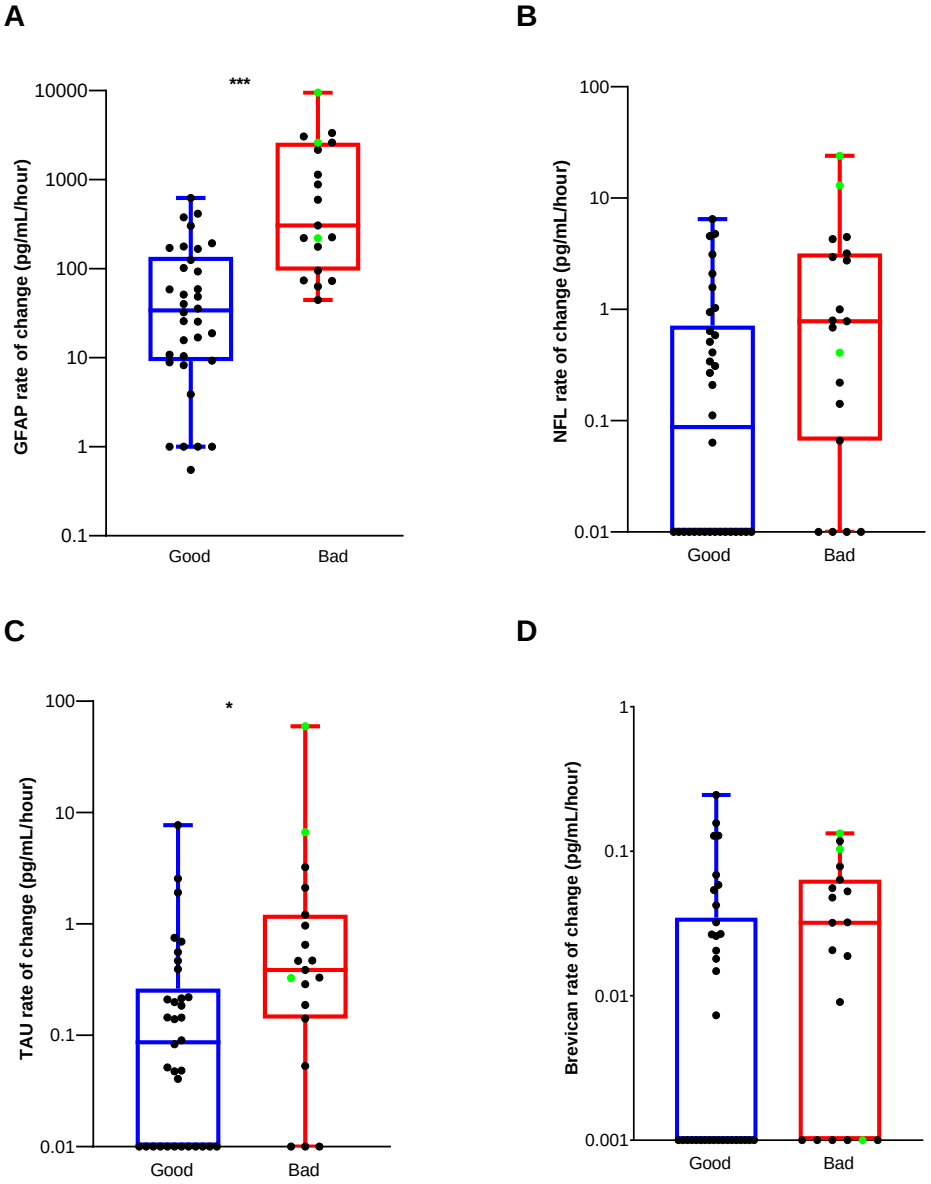
IQR, Interqurtile range; Mann-Whitney test was used to compare outcomes.



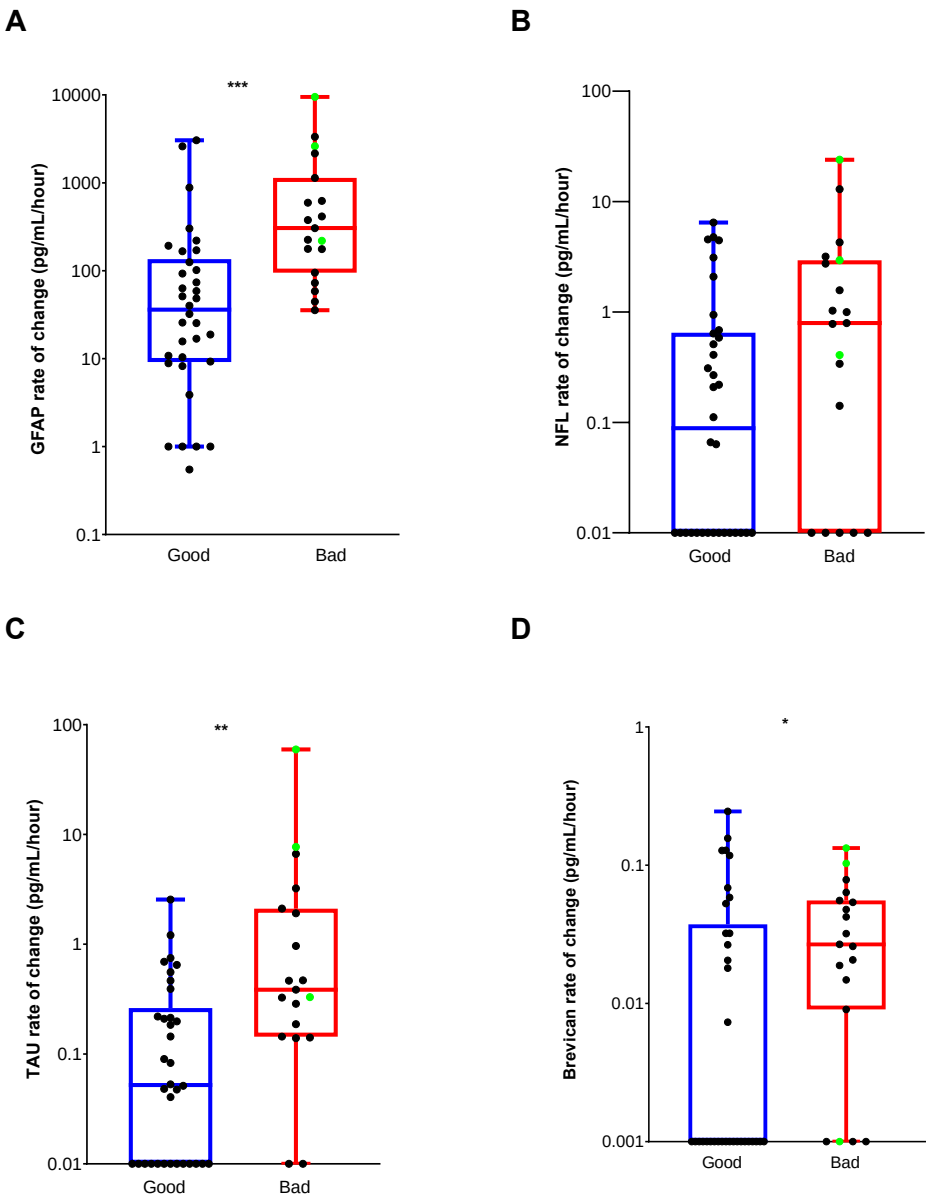
Supplemental Figure 1. Longitudinal biomarker panel profile in the acute phase of stroke patient care. A to **D** spaghetti plot showing longitudinal plasma GFAP(**A**), NfL(**B**), t-Tau(**C**) and brevican (**D**) from individual AIS patients with good neuroradiological outcome (blue, n=35) and bad neuroradiological outcome (red, n=19). In the *x axis* time "0" is time of symptom onset or wake-up time. Y axis biomarker is in logarithmic scale and units were selected to fit all sample values.



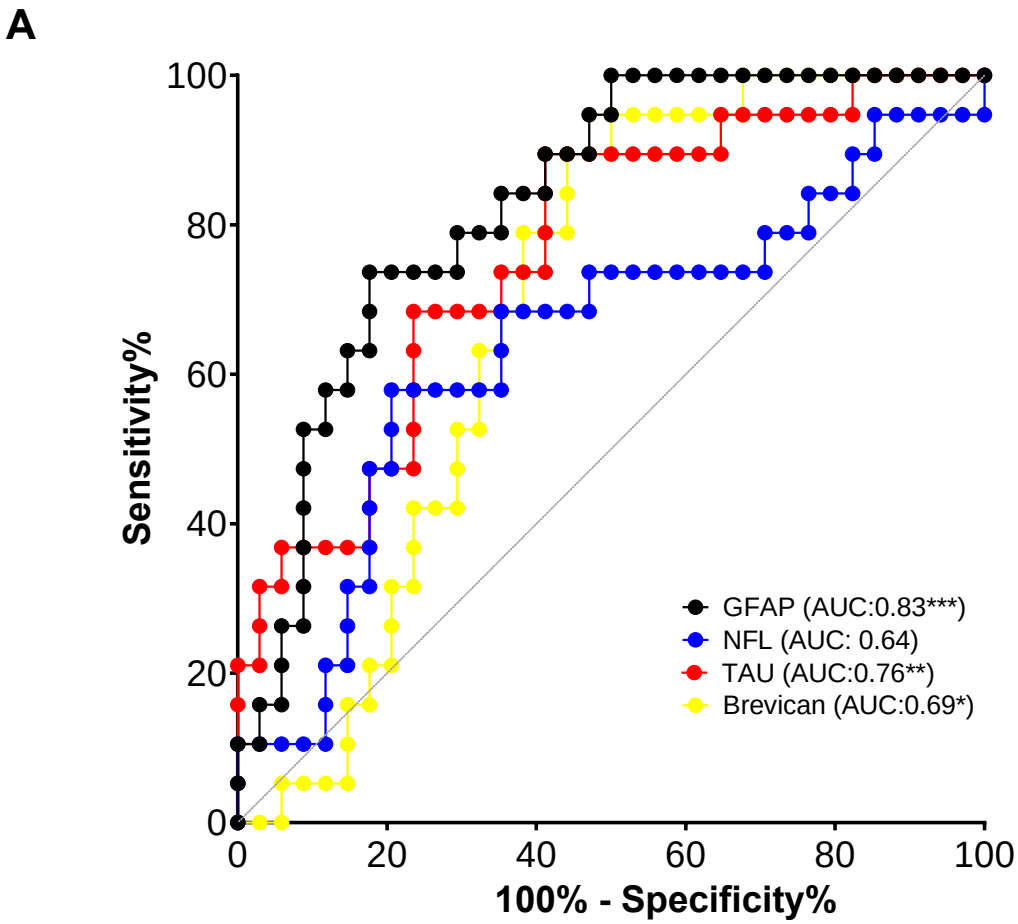
Supplemental Figure 2. Biomarker levels in AIS patients with good and bad neuroradiological outcome with time. Left panel Boxplots represent the median and max and minimal values of each biomarker at predetermined time points (T_0 admission; T_1 after treatment; T_2 at 24 hours; T_3 at 72hours) GFAP(**A**), NfL(**B**), t-Tau(**C**) and brevican (**D**). AIS patients with good clinical outcome (n=35) are represented in blue and bad clinical outcome (n=19) in red. Three patients with a symptomatic intracranial hemorrhage at 24 hours are highlighted in green. Y axis in logarithm scale. **Right panel** Estimated means and 95% confidence levels based on the LMM model (**A**) GFAP (**B**) NfL (**C**) t-Tau and (**D**) brevican. **P*<0.05, ***P*<0.01, ****P*<0.001.



Supplemental Figure 3. Biomarker rate of change in patients with good and bad clinical outcome. Boxplots represent the median and max and minimal values of each biomarker rate of change between admission (T_0) and treatment (T_1): (**A**) GFAP (**B**) NfL (**C**) t-Tau and (**D**) brevican. AIS patients with good clinical outcome (n=35) are represented in blue and bad clinical outcome (n=19) in red. 3 patients that had a symptomatic intracranial hemorrhage at 24 hours are highlighted in green. In the *Y axis* biomarker rate of change is expressed in in pg/mL/hour. We used a logarithm scale (log_{10}) to improve comparison between groups, negative values are at the base of each box-plot. Mann-Whitney test was used for group comparisons. **P*<0.05, ***P*<0.01, ****P*<0.001.



Supplemental Figure 4. Biomarker rate of change in patients with good and bad neurorradiogical outcome. Boxplots represent the median and max and minimal values of each biomarker rate of change between admission (T_0) and treatment (T_1): (**A**) GFAP (**B**) NfL (**C**) t-Tau and (**D**) brevican. AIS patients with good clinical outcome (n=35) are represented in blue and bad clinical outcome (n=19) in red. 3 patients that had a symptomatic intracranial hemorrhage at 24 hours are highlighted in green. In the *Y axis* biomarker rate of change is expressed in in pg/mL/hour. We used a logarithm scale (log_{10}) to improve comparison between groups, negative values are at the base of each box-plot. Mann-Whitney test was used for group comparisons. **P*<0.05, ***P*<0.01, ****P*<0.001.



Supplemental Figure 5. Biomarker panel performance in early neuroradiologic outcome prediction Receiver-operator characteristics (ROC) curve to differentiate AIS patients with good (n=35) and bad (n=19) neuroradiologic outcome based on individual biomarkers rate of change (pg/mL/h). Based on observed values, GFAP (black) has good, t-Tau (red) has fair and brevican (yellow) has poor discriminating capacity between good and bad outcome. Logistic regression derived ROC confirmed this observation in GFAP and t-Tau rates of change. GFAP AUC = 0.81** [95% CI 0.70-0.92], with an optimal cutoff point of 176.4 pg/mL/h. The observed t-Tau rate of change performance was also confirmed after logistic regression modeling, AUC = 0.75* [95% CI 0.62-0.88], with an optimal cutoff point of 6.6pg/mL/h. Both NfL and brevican did not differentiate between neuroradiologic outcomes. *P<0.05, **P<0.01, ***P<0.001.

Appendix Authors

Author	Design or conceptualization of the study	major role in the acquisition of data	analysis or interpretation of the data	drafting or revising the manuscript for intellectual content
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Hugo Dória, MD		x		x
Ricardo Varela, MD		x		x
Ana Aires, MD		x		x
Rui Antunes, MD		x		x
Rui Felgueiras, MD		x		x
Pedro Castro, MD-PhD		x		x
Kaj Blennow, MD-PhD				x
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Henrik Zetterberg, MD-PhD	x		x	x
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