Temporal pattern and prognostic value of serum NfL, GFAP and β-synuclein in acute ischemic stroke: a prospective single-center cohort study

Thesis

to obtain the academic degree of

Doctor medicinae (Dr. med.)

submitted

to the Faculty of Medicine of

Martin Luther University Halle-Wittenberg

by Yashar Bahramsari

Supervisor: Prof. Dr. med. Markus Otto

Reviewers:

1. Prof. Dr. Faramarz Dehghani, Halle (Saale)
2. Prof. Dr. Thomas Skripuletz, Hannover

Date of the defense: 10.02.2025

Abstract

Following acute ischemic stroke (AIS), clinical outcomes display extreme heterogeneity, ranging from complete recovery to severe disability and death. To date, no single serum fluid biomarker has been routinely used for the prognostic assessment of patients with AIS. We aimed to analyze the prognostic role and temporal pattern of serum levels of neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), and β-synuclein in patients with AIS. We included blood samples collected from patients with AIS (n=51) at fixed time points - within 24h (day 1), 48h (day 2), 72h (day 3) and 120h (day 5) from onset of symptoms. In the case of intravenous thrombolysis and/or mechanical thrombectomy, blood was additionally sampled before therapy and referred to as 'day 0'. We measured serum NfL and GFAP concentrations using ELLA and Simoa platforms, respectively. Serum β-synuclein was analyzed using an in-house established digital ELISA assay. We assessed the mRS at the 90-day follow-up and collected clinical and radiological data. Serum NfL and GFAP concentration were significantly higher ($p<0.05$) on each measured time point and β-synuclein on 2nd to 5th day in patients with mRS 3-6 (n=25) compared to those with mRS 0-2 (n=26) at 90-day follow-up. Serum NfL and β-synuclein reached their peak concentration in all AIS patients on 5th day with a median of 223.0 pg/ml (IQR 98.8–385.3) and a median of 19.78 pg/ml (IQR 6.66-63.3), respectively. GFAP reached peak concentrations on 3rd day with a median of 2.66 ng/ml (IQR 1.14-20.58). Serum GFAP on 3rd day reveals the highest diagnostic accuracy in the distinction between mRS 0-2 and mRS 3-6 at 90-day follow-up. In the discrimination between survivors (n=40) and non-survivors (n=11), serum NfL on 5th day showed the highest diagnostic accuracy. Serum NfL, GFAP and β-synuclein moderate to strongly correlated with each other and with NIHSS, mRS and ASPECTS. Serum NfL, GFAP, and β-synuclein levels can predict functional outcome (mRS) on 90-day follow-up in AIS patients. Serum GFAP showed the highest diagnostic accuracy in distinguishing mRS at 90-day follow-up, and NfL in distinguishing survivors from non-survivors. Blood biomarkers demonstrated distinct individual temporal patterns and were correlated with each other, as well as with clinical and radiological data. Further studies with larger sample sizes should be conducted.

Bahramsari, Yashar: Temporal pattern and prognostic value of serum NfL, GFAP and β-synuclein in acute ischemic stroke: a prospective single-center cohort study, Halle (Saale), Univ., Med. Fac., Diss., 61 Pages, 2024

Referat

Die Prognose nach einem akut ischämischen Schlaganfall (AIS) weist eine extreme Heterogenität auf, die von vollständiger Genesung bis hin zu schwerer Behinderung und dem Tod reicht. Bisher wird im klinischen Alltag kein Biomarker im Serum zum Management beim Schlaganfall verwendet. Unser Ziel ist es daher die prognostische Rolle und zeitliche Dynamik der leichten Kette des Neurofilaments (NfL), des sauren Gliafaserproteins (GFAP) und β-Synuclein beim AIS im Serum zu untersuchen. Wir haben Blutentnahmen von Patienten mit einem AIS (n=51) zu festen Zeitpunkten - innerhalb von 24 (Tag 1), 48 (Tag 2), 72 (Tag 3) und 120 Stunden (Tag 5) nach Symptombeginn - durchgeführt. Im Falle einer intravenösen Thrombolyse und/oder mechanischen Thrombektomie wurde Blut vor der Therapie entnommen und als „Tag 0“ bezeichnet. Die Serumkonzentration von NfL sowie GFAP wurde mittels ELLA und Simoa und das β-Synuclein mittels eines intern etablierten digitalen ELISA-Assays analysiert. Klinische sowie radiologische Daten wurden gesammelt und die Patienten nach 90 Tagen hinsichtlich der Modifizierten Rankin-Skala (mRS) kategorisiert. Die Serumkonzentrationen von NfL und GFAP waren zu jedem Messzeitpunkt sowie bei β-Synuclein vom 2. Tag an signifikant höher ($p<0.05$) bei Patienten mit mRS 3-6 (n=25) im Vergleich zu mRS 0-2 (n=26). Bei AIS-Patienten erreichte das NfL (Median 223.0 pg/ml mit IQR 98.8-385.3) sowie β-Synuclein (Median 19.78 pg/ml mit IQR 6.66-63.3) am 5. Tag und GFAP am 3. Tag (Median 2.66 ng/ml mit IQR 1.14 – 20.58) ihre maximale Konzentration im Serum. Das Serum GFAP am 3. Tag zeigt die höchste diagnostische Genauigkeit bei der Unterscheidung zwischen mRS 0-2 und 3-6 nach 90 Tagen und Serum NfL am 5.Tag zwischen Überlebenden (n=40) und Nicht-Überlebenden (n=11). Serum NfL, GFAP und β-Synuclein korrelieren mäßig bis stark miteinander und dem NIHSS, mRS und dem ASPECTS. Serum NfL, GFAP und β-Synuclein kann beim AIS den Verlauf (mRS) nach 90 Tagen prognostizieren. Serum GFAP zeigte die höchste diagnostische Genauigkeit bei der Unterscheidung zwischen mRS-Gruppen nach 90 Tagen und NfL hinsichtlich der Sterblichkeit. Die Biomarker zeigten individuelle longitudinale Dynamiken, korrelierten miteinander und klinisch-radiologischen Daten. Studien mit größerer Stichprobenzahl sollten durchgeführt werden.

Bahramsari, Yashar: Temporal pattern and prognostic value of serum NfL, GFAP and β-synuclein in acute ischemic stroke: a prospective single-center cohort study, Halle (Saale), Univ., Med. Fak., Diss., 61 Seiten, 2024

Table of contents

List of Abbreviations ..... **III**

1 Introduction **1**

1.1 Definition of stroke, transient ischemic attack and stroke mimics 1

1.2 Epidemiology and burden of stroke 1

1.3 Clinical presentation of AIS 2

1.4 Diagnosis of AIS 2

1.5 Therapy and prognosis of AIS 3

1.6 Blood biomarkers in clinical practice 4

1.6.1 Neurofilament light chain (NfL) 4

1.6.2 Glial fibrillary acidic protein (GFAP) 5

1.6.3 β-Synuclein (β-syn) 6

2 Objectives **8**

3 Materials and methods **9**

3.1 Inclusion criteria 9

3.2 Data collection and clinical variables 9

3.3 Definition of binary outcomes 11

3.4 Blood sampling 11

3.5 Biomarker measurement in serum samples 12

3.6 Statistical analysis 12

3.7 Study protocol approval and ethics 13

4 Results **14**

4.1 Demographic, clinical and radiological features of the study population 14

4.2 Correlations between serum biomarkers and clinical variables 17

4.3 Serum biomarkers in patients with AIS and TIA 19

4.4 Serum biomarkers and clinical data in relation to treatment groups 21

4.5 Serum biomarkers and clinical data in relation to functional outcome 25

4.6 Serum biomarkers and clinical data in relation to mortality 33

4.7 Serum biomarkers and clinical data in relation to 24h NIHSS score change 38

5 Discussion **41**

5.1 Association of biomarkers with diagnostic, clinical and treatment groups 41

5.2 Correlation between biomarkers and clinical data 42

5.3 Serum biomarkers and functional outcome 43

5.4 Longitudinal dynamics of serum biomarkers 44

5.5 Biomarker cut-off-values in cerebrovascular diseases 46

5.6 Strengths 47
5.7 Limitations 48
5.8 Conclusions 50
6 References 51
7 Theses 61
Appendix IV
Erklärung über frühere Promotionsversuche und Selbstständigkeit XII
Danksagung XIII

List of Abbreviations

Abbreviation	Definition
ADC	apparent diffusion coefficient
AIS	acute ischemic stroke
ALS	amyotrophic lateral sclerosis
ASPECTS	The Alberta Stroke Program CT score
$ASPECTS_0$	The Alberta Stroke Program CT score on admission
$ASPECTS_{24}$	The Alberta Stroke Program CT score after 24 hours
AUC	area under the curve
β-syn	β-synuclein
$β\text{-}syn_0$	β-synuclein on day 0
$β\text{-}syn_1$	β-synuclein on day 1
$β\text{-}syn_2$	β-synuclein on day 2
$β\text{-}syn_3$	β-synuclein on day 3
$β\text{-}syn_5$	β-synuclein on day 5
CI	confidence interval
CSF	cerebrospinal fluid
CT	Computed tomography
CTA	Computed tomography angiography
CTP	Computed tomography perfusion
CV	coefficients of variability
DWI	diffusion-weighted imaging
ER	emergency room
FLAIR	fluid attenuated inversion recovery
GFAP	glial fibrillary acidic protein
$GFAP_0$	glial fibrillary acidic protein on day 0
$GFAP_1$	glial fibrillary acidic protein on day 1
$GFAP_2$	glial fibrillary acidic protein on day 2
$GFAP_3$	glial fibrillary acidic protein on day 3
$GFAP_5$	glial fibrillary acidic protein on day 5
IQR	interquartile range
ICH	intracerebral hemorrhage

IVT	intravenous thrombolysis
IVT + MT	intravenous thrombolysis applied together with mechanical thrombectomy
MRI	magnetic resonance imaging
mRS	The modified Rankin Scale
$mRS_{discharge}$	The modified Rankin Scale at discharge
mRS_{90days}	The modified Rankin Scale at 90-day follow-up
MT	mechanical thrombectomy
NfH	neurofilament heavy chain
NfL	neurofilament light chain
NfL_0	neurofilament light chain on day 0
NfL_1	neurofilament light chain on day 1
NfL_2	neurofilament light chain on day 2
NfL_3	neurofilament light chain on day 3
NfL_5	neurofilament light chain on day 5
NIHSS	The National Institutes of Health Stroke Scale
$NIHSS_0$	The National Institutes of Health Stroke Scale at admission
$NIHSS_{24}$	The National Institutes of Health Stroke Scale 24hours after admission
$NIHSS_{48}$	The National Institutes of Health Stroke Scale 48hours after admission
$NIHSS_{72}$	The National Institutes of Health Stroke Scale 72hours after admission
$NIHSS_{change}$	The National Institutes of Health Stroke Scale change within 24hours
n.s.	not significant
OR	odds ratio
REDcap	Research Electronic Data Capture
ROC	receiver operating characteristic
SAH	subarachnoid hemorrhage
SD	standard deviation
TBI	traumatic brain injury
TIA	transient ischemic attack
TOAST	The Trial of Orga 10172 in Acute Stroke Treatment (classification of

	stroke etiology)
χ^2	chi – squared test

1 Introduction

1.1 Definition of stroke, transient ischemic attack and stroke mimics

Stroke can be defined as a sudden focal neurological impairment persisting for more than 24 hours and is caused by damage to the central nervous system through vascular injury, such as infarction or hemorrhage (1).

The majority of strokes are ischemic, resulting from reduced blood flow due to arterial occlusion or, more rarely, from venous sinus thrombosis (2). Approximately 30% of ischemic strokes are cardioembolic in origin, 20% are attributed to larger artery atherosclerosis, 25% are due to small-artery occlusion and 30-40% remain cryptogenic (3,4). The remaining strokes are hemorrhagic, either intracerebral or subarachnoid, resulting from the rupture of the cerebral arteries. Ruptured aneurysms are typically associated with subarachnoid hemorrhage (2).

A sudden transient episode of neurological impairment caused by focal brain, retinal, or spinal ischemia without acute infarction or tissue injury is referred to as transient ischemic attack (TIA). Because a TIA typically lasts only a few minutes and often less than an hour, the definition was changed from a time-based (referring to less than 24 hours) to a tissue-based (referring to lack of lesions on magnetic resonance imaging) version (5).

Stroke mimics can make up for 20-50% of cases of clinically suspected stroke. Mimics may be functional, such as somatization, depression, anxiety disorder, or psychiatric complications of neurological conditions, whereas others may be medical, either neurological (e.g., posterior reversible vasoconstrictive syndrome, seizures, migraine attacks, transient global amnesia, or brain neoplasm) or general (hypertensive crisis, electrolyte imbalances, acute liver failure, alcohol, hypoglycemia, or hyperglycemia) (6).

1.2 Epidemiology and burden of stroke

Acute ischemic strokes (AIS) account for approximately 87% of cases, intracerebral hemorrhages (ICH) for 10%, and subarachnoid hemorrhages (SAH) for approximately 3% (7).

The Global Burden of Diseases Study of 2021 identified stroke as the third leading cause of death worldwide following ischemic heart disease and COVID-19 (8). Stroke

is the third most prevalent cause of disability, after neonatal disorders and ischemic heart disease (9). In Germany, an annual increase of 5.6% in stroke cases from 2011 to 2017 (from 250,199 to 264,208 cases), adding up to a total of 2,544,850 cases, was noted by the German Federal Statistical Office (10). Comparing the numbers of German hospital admissions of AIS patients from 2019 to 2022 (227,258 to 215,479 cases), a slight decrease of 4.5% can be noted (11). The projected total lifetime costs of first-ever ischemic stroke survivors by 2025 are expected to be equal to 57.1 billion euros in Germany (12). The number of first-time stroke patients is expected to increase considering the association between age and disease onset, with approximately two-thirds of patients being over 65 years old and continuous improvement in life expectancy (13,14). Hence, determining a patient's rehabilitation potential by delivering an accurate clinical prognosis is crucial for stroke treatment decisions.

1.3 Clinical presentation of AIS

The clinical presentation of AIS shows high variability, depending on the territory of vascular injury and lesion size. Most commonly, the anterior circulation (70-80%) is affected, and less commonly, the posterior circulation (10-20%) (15). A middle cerebral artery stroke commonly presents with contralateral sensorimotor hemiparesis and a combination of other focal deficits (e.g., aphasia, dysarthria, neglect, gaze deviation) depending on the lesion site (2). Involvement of the posterior cerebral artery often shows hemi-or quadrant anopsia, whereas anterior cerebral artery participation leads to lower-extremity focused contralateral paresis (1,15,16). Vertebrobasilar strokes present with vertigo, ataxia, dysarthria, dysphagia, and severe disorders of consciousness, as well as motor hemi- or tetra paresis, such as in acute basilar artery occlusion (17). The National Institutes of Health Stroke Scale (NIHSS) offers an opportunity to objectively quantify the aforementioned symptoms, aiding in severity assessment, treatment decisions, and clinical monitoring of patients (18).

1.4 Diagnosis of AIS

The gold standard for the diagnostic evaluation of patients with suspected AIS is brain and neurovascular imaging. Currently, computed tomography (CT) of the head, including CT angiography (CTA) and perfusion (CTP), is the preferred choice owing to its rapid and widespread availability. Loss of grey-white matter differentiation, cortical hypodensity, hypoattenuation of deep nuclei and a hyperdense vessel sign are ischemic changes visible in approximately two-thirds of major stroke cases on CT, but are highly

insensitive to clinically milder and shortly or non-disabling strokes, referred to as minor stroke, as changes appear beyond its resolution (19–21).

Hence, this imaging modality offers only limited inclusion of patients eligible for therapy but cannot rule out the diagnosis with absolute certainty. A considerable gap remains between the estimated 20–25% of patients potentially eligible for IVT and the proportion of patients actually receiving therapy (22). Magnetic resonance imaging (MRI) offers superior spatial resolution for identifying brain ischemia compared with CT. The clinical suspicion of AIS is confirmed by a high signal on diffusion-weighted imaging (DWI) sequences with a low apparent diffusion coefficient (ADC) signal and hyperintensity on fluid-attenuated inversion recovery (FLAIR) sequences (23). It is considered the method of choice for providing a comprehensive diagnosis of TIA or minor strokes, especially when symptoms on presentation are subtle (19).

1.5 Therapy and prognosis of AIS

Two types of reperfusion therapies, intravenous thrombolysis (IVT) using recombinant plasminogen activator (0.9 mg/kg, maximal dose 90 mg) and mechanical thrombectomy (MT), are available for detecting tissue at risk (penumbra) on imaging (2). The interplay between the manifestation of symptoms and initiation of therapy seems to play a pivotal role in determining treatment success (24). Hence, the extension of the time window is the focus of research. The expansion of the time window from 3 hours to 4.5 hours and the efficacy of IVT for up to 6h in individual patients have been major achievements in recent years (25–28). The treatment of severe strokes within the first 6 h after onset of symptoms was revolutionized in 2015 as a result of positive randomized prospective studies on MT (29). The benefits of reperfusion therapy in a 24-h time window in selected patients with unclear symptoms have been previously described (30–33).

Despite the positive results of group studies, factors for individual outcome prediction remain unclear. Achieving successful recanalization in the early time window (<6h) does not necessarily ensure a good treatment outcome, and positive outcomes are observed even in cases of a late time window (6-24h) (29,30,32). Lesion volume, clinical symptom severity, and comorbidities were associated with functional outcomes (34). However, their ability to predict long-term functional outcomes remains limited.

Stroke survivors often experience chronic functional impairment, often assessed using the Modified Rankin Scale (mRS), which presents the degree of disability (35).

Rehabilitative measures enhance functional recovery in patients, mostly in the first six months after stroke (36). Stroke-induced functional impairment occurs because of neuronal dysfunction and tissue loss surrounding the affected stroke lesion (37). Functional recovery after stroke is a complex process involving multiple mechanisms, including the generation of new cells, functional remapping, angiogenesis, vascular remodeling, stroke-induced changes in inter-neuronal connectivity, adaptive responses of glial cells, neuroplasticity-mediated formation of new neuronal synapses, and axonal sprouting (38–40). Immediate care of stroke patients is best provided in a stroke unit, as evidence shows correlated benefits in terms of survival without disability and reduction of complications (i.e., aspiration pneumonia or hypertension-related secondary hemorrhage) for patients of all stroke subtypes, severity, and age (41).

1.6 Blood biomarkers in clinical practice

Blood-based biomarkers are commonly used in routine clinical practice alone or in combination with others to support decision-making, enhance diagnostic accuracy, and support prognostic assessment (e.g., Troponin T in myocardial infarction, B-type natriuretic peptide in heart failure, and creatinine to monitor kidney function) (42).

In neurological science, much effort has been made in recent years to establish robust and easily accessible biomarkers, particularly for neurodegenerative disorders (43,44). A significant milestone has been the successful creation of highly sensitive assays that allow for the detection of biomarker levels in blood, which is more conveniently accessible than cerebrospinal fluid (CSF) (45). To date, no single fluid biomarker has been used for diagnostic or prognostic purposes in patients with AIS (46).

1.6.1 Neurofilament light chain (NfL)

Neurofilament proteins, which are structural components of the neuronal cytoskeleton, have gained significant interest as CSF and blood biomarkers for several brain and spinal cord diseases, especially neurofilament light chain proteins (47). As part of the neuronal cytoskeleton, they primarily influence nerve conduction velocity by providing structural support for axons and regulating axonal diameter (48). As a result of neuroaxonal injury, NfL proteins are released into the extracellular space and can be measured in the CSF and peripheral blood as a proxy of neuronal degeneration (37).

Studies on blood NfL have reported elevated biomarker concentrations in neurodegenerative disorders, especially amyotrophic lateral sclerosis (ALS), prion

diseases, Alzheimer's disease, and neuroinflammatory diseases, such as multiple sclerosis (47). Moreover, NfL levels can be used to predict a worse disease course in virtually all neurological disorders, including AIS (49).

Studies on NfL in AIS are found extensively and more frequently compared to other neurofilament proteins (e.g., heavy chain, NfH), as the latter is more specific to ALS, and measurements delivered inconsistent results due to analyte aggregation in the former assay (50). Numerous studies have analyzed the association between AIS and NfL levels with regard to clinical and radiological variables at admission, as well as short-, middle-, and long – term functional outcomes (37,51).

Regarding diagnostic capabilities, De Marchis et al. compared serum NfL levels measured once within 24h from symptom onset between 111 TIA and 504 AIS patients and revealed a statistically significant difference between the two groups (52). Significantly elevated serum NfL levels were observed in patients with poor (mRS 3-6) compared than in those with good functional outcomes (mRS <3) in a study of 343 patients in China (53). Here, the concentrations of serum NfL at baseline in patients with an NIHSS score ≥ 5 were significantly higher than those in patients with an NIHSS score < 5 (53). Pedersen et al. showed an association between persistently elevated serum NfL levels at 90 days and worse functional outcomes at two and seven years in 320 post-stroke patients (54). In the same study, longitudinal measurements revealed an increase in serum NfL within the first 2 weeks of the acute phase, with a steady increase at 3 months and a decrease to control patients' levels at the 7th year. Serum NfL values were 22.9% higher in patients with NIHSS > 4 than in those with NIHSS ≤ 4 in a cohort of 211 patients with measurements 24 h after admission (55). The CIRCULAS cohort from Ludwig-Maximilians University found a correlation between infarct volume and elevated serum NfL levels, both seven days and six months after stroke onset (56). The latter phenomenon is quantitatively associated with secondary neurodegeneration. This supports the hypothesis of a biphasic release of serum NfL in the acute and late post-stroke phases, which is associated with neuroaxonal and synaptic damage and adaptive neural plasticity, respectively (51).

1.6.2 Glial fibrillary acidic protein (GFAP)

In addition to NfL, GFAP is a structural protein that is abundantly expressed in activated microglial cells and is used as a biomarker of astrocytic activation/injury (47,57). In cases of impaired blood-brain barrier permeability, GFAP diffuses into the

CSF and subsequently enters the bloodstream, where it can be detected (58). Astrogliosis, a process involving astrocyte damage due to proliferation and hypertrophy, results from stroke-induced glutamate excitotoxicity, and rapidly increases GFAP levels (59). As an emerging biomarker for glial damage, GFAP expression has been described in several neurological disorders, including dementia, autoimmune encephalitis, multiple sclerosis, traumatic brain injury, and malignant brain tumors (60).

Studies have revealed that the plasma GFAP level is a promising surrogate marker in the differentiation of intracerebral hemorrhage and ischemic stroke within 4.5 hours of symptom onset (61). Neurochemical monitoring of patients with traumatic brain injury revealed peak concentrations of GFAP in the serum at 24 hours followed by a decline in venous blood drawn at admission, 24 h, 5 days, and 10 days after onset (62).

Several studies have investigated the role of GFAP as a prognostic indicator of functional outcome in patients with AIS (63,64). A correlation between the stroke severity scale 1 month after stroke onset and GFAP was demonstrated in a study of 64 ischemic stroke patients (63). Elevated levels of GFAP were associated with a poor functional outcome (mRS 3-6) after 1 year and a risk of NIHSS score > 6 on admission in a study of 286 AIS patients presenting within 24 h of symptom onset (64). In patients undergoing endovascular thrombectomy for large-vessel occlusion, GFAP levels were linked to unfavorable outcomes (mRS 3-6) at the 90-day follow-up (65). Regarding the temporal dynamics of blood GFAP concentrations in AIS, only very few studies have been performed. Wunderlich et al. demonstrated peak GFAP serum concentration 48 hours after stroke onset in 53 patients, with serial venous blood samples taken on admission, 6,12,16,48,72,96 and 120 h after onset of symptoms (66).

1.6.3 β-Synuclein (β-syn)

A plethora of fluid biomarkers have been intensively investigated in AIS (46). Within this framework, synaptic proteins have raised interest in detecting and monitoring dysfunction or damage; however, their low abundance in peripheral blood hampers quantification with standard immunoassays (67). β-Synuclein is a protein mainly expressed in presynaptic terminals in different brain areas, especially in the temporal regions (45). As a part of the synuclein family, together with α- and γ-synuclein, β-synuclein exerts homeostatic activities in the synapse; however, its pathophysiological role in neurological disease has not been fully elucidated and the absence of β-synuclein expression in cells outside the brain enhances its potential as a peripheral blood

biomarker when compared to α-synuclein and other synaptic proteins (67–69). β-Synuclein concentrations were elevated in Creutzfeldt-Jakob disease and Alzheimer's disease as an indication of ongoing synaptic degeneration (70,71).

To date, only one study has investigated the association between β-synuclein and AIS. In a study of 30 patients with moderate to severe AIS, higher serum β-synuclein concentrations (measured on day 1 after symptom onset) were found to be significantly associated with clinical and radiological scores of stroke severity as well as with poorer functional outcomes at the 3-month follow-up (72). However, blood β-synuclein levels at other time points, as well as its changes over time in AIS, remain completely unexplored.

2 Objectives

The clinical outcomes following stroke can vary greatly, ranging from full recovery to significant disability and death.

In this pilot study, we aimed to assess the potential value of blood biomarkers (i.e., NfL, GFAP, and β-synuclein) measured at different time points to evaluate the prognosis of AIS. The objectives of this study were as follows:

1. The association between collected clinical data and diagnostic, therapeutic and clinico-radiological subgroups within the cohort population.

2. Correlation between serum levels of NfL, GFAP, and β-synuclein and demographic and clinical variables.

3. The association between serum biomarkers (NfL, GFAP, and β-synuclein) and diagnostic, therapeutic, and clinico-radiological subgroups with a focus on mRS at the 90-day follow-up.

4. Temporal pattern of NfL, GFAP and β-synuclein serum levels within the first 5-7 days from symptom onset

5. The sensitivity and specificity of the previously mentioned serum levels of NfL, GFAP, and β-synuclein as predictive tests for the determination of mortality and functional outcome at the 90-day follow-up.

3 Materials and methods

3.1 Inclusion criteria

This prospective single-center cohort study included 61 patients (AIS, n=51; TIA, n=7; ICH, n=3) recruited from the University Hospital of Halle (Saale). Samples of patients were collected from the 29th of April until the 9th of November 2023. All patients underwent neurological examinations upon admission to the emergency department (ER). We included patients presenting with persistent focal neurological deficits indicative of acute stroke. Furthermore, the onset of symptoms had to be within 24 h prior to admission and approximate time of onset must be known. We excluded patients with regressive or without focal neurological deficits on neurological examination, which are indicative of a stroke mimic or transient ischemic attack. Patients who presented outside the shifts of the researcher were excluded because adherence to quality standards of the study protocol could not be guaranteed in this case. Patients who did not receive a scientific blood sample within 24 hours were not included.

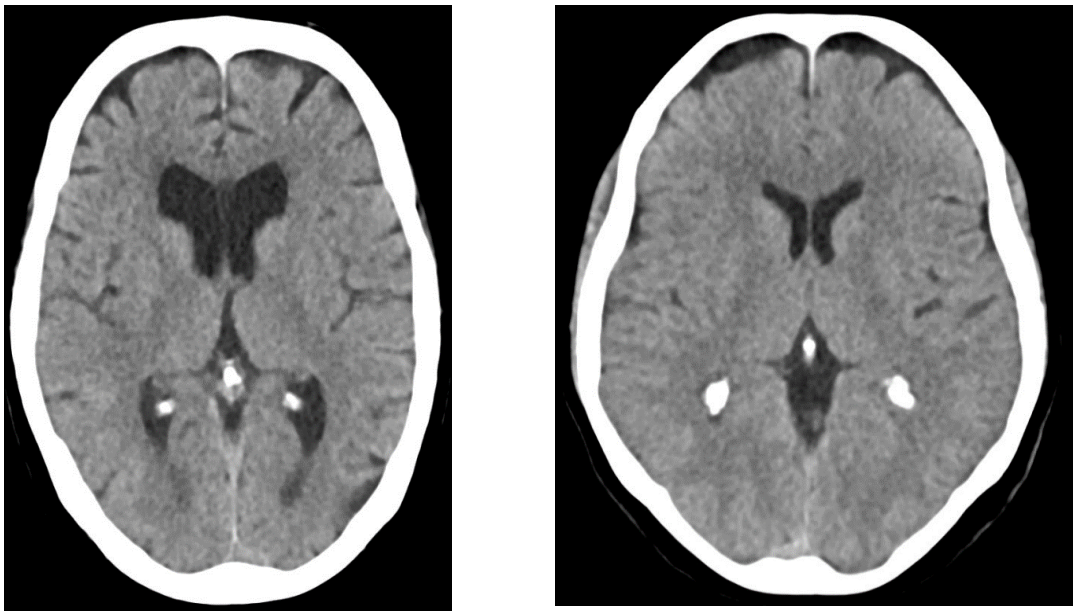
3.2 Data collection and clinical variables

A clinical database was created on a survey-based secure web application, namely Research Electronic Data Capture (REDCap), with access supplied by the University Hospital of Halle (Saale). Information from completed REDCap surveys was exported as an Excel chart for further statistical analysis. Patient information was anonymized, and each patient file was assigned a record number. Individual clinical information in ORBIS KIS (Dedalus Global Inc., Milan, Italy) was gathered only through a case number. We systematically obtained the clinical, radiological, and demographic information of the patients. Demographically, the patient's age and sex were noted. The time between blood sampling and symptom onset was noted in hours and minutes.

The National Institutes of Health Stroke Scale (NIHSS) was implemented for evaluation of stroke severity and continuous clinical guidance of focal neurological deficits. It uses a 15-item impairment scale and calculates a total score based on the following findings: consciousness, speech, language, eye movements, visual field evaluation, facial movements, strength of the upper and lower extremities, coordination, sensation, and neglect (73). A detailed scoring guide is provided in Appendix 1. A patient that is not alert, but arousable by minor stimulation with sensorimotor hemiplegia, severe aphasia, complete facial palsy, answering no questions (e.g., age, month, place), and not performing any task correctly (e.g., grip and release hand of examiner or open and close

eyes on command) would score 20 points on the NIHSS assessment. An alert and keenly responsive patient answering all questions correctly and having only complete hemianopsia would score two points on the NIHSS scale. A score of 1-4 can be categorized as minor, 5-15 as moderate, 16-20 as moderate to severe and 21-42 as severe (74). Experienced neurologists of the department assessed the NIHSS scores of the patients on admission, at 24, 48, and 72 hours, and at discharge from the hospital.

We implemented the Alberta Stroke Program CT score (ASPECTS) on admission as well as 24h after stroke onset as an imaging measure, obtained by experienced neuroradiologists. The ASPECTS measures the extent of ischemic changes using a quantitative 10-point topographic non – contrast CT scan to aid in treatment decisions in patients with middle cerebral artery stroke (75). For every involved region, one point was deducted from the initial 10 points. Examples of the study cohort are displayed in Figure 1. Appendix 2 provides a detailed scoring guide. A similar pc-ASPECTS with a 10-point scale has been established for posterior circulation stroke (76).



A. **B.**

Figure 1. ASPECTS of study patients in our cohort (from ORBIS at University Hospital Halle of patients with informed consent given). A) Patient (No. 43) with ASPECTS of 7 on admission with visible loss of grey-white matter differentiation and cortical hypodensity in the right posterior middle cerebral artery territory with extension along the operculum towards the insula. B) Patient (No. 33) with ASPECTS of 10 with intact grey-white matter differentiation and without hypodensities.

The etiology of stroke was classified according to the "Trial of Orga 10172 in Acute Stroke Treatment (TOAST)" into the following subtypes: large artery atherosclerosis, cardioembolic, small vessel occlusion, stroke of other determined etiology, and stroke of undetermined etiology (77).

We noted treatment types as IVT, MT, both or none. We grouped the diagnoses as AIS, TIA, or ICH based on MRI findings (that is, TIA was defined as the absence of an acute ischemic lesion on the MRI).

For neurological disability, we used mRS as a functional outcome at discharge and 3 months after stroke-related symptom onset with 0-2 presenting good and 3-6 poor outcome (35). A follow-up mRS score after three months was obtained using a structured telephone interview. An mRS score of 0 is interpreted as an absence of symptoms, 1 as symptoms without causing disability, 2 as slight disability, 3 as moderate disability, 4 as moderately severe disability (requiring assistance for walking and bodily needs), and 5 as severe disability (bedridden and incontinent), whereas 6 reflects death (78). A detailed scoring guide is provided in Appendix 3.

3.3 Definition of binary outcomes

The relationship of serum biomarker levels with the following binary outcomes were investigated: (I.) TIA vs. AIS; (II.) treatment groups (IVT vs. MT v.s. IVT+MT vs. no therapy) (III.) mRS 0-2 vs. mRS 3-6 at 90 days of follow – up (IV.) survivors vs. non-survivors; (V.) NIHSS score changes within 24 h: ≥ 4 vs. < 4. NIHSS change within 24 h was defined as the 24 h NIHSS subtracted from the baseline NIHSS score.

3.4 Blood sampling

We collected patients' blood samples at fixed points in time - within 24 h (day 1), 48h (day 2), 72h (day 3) and 120h (day 5) from symptom onset. If feasible, blood samples were obtained immediately upon patients' arrival at the emergency department. In the case of administration of acute therapy (IVT and/or MT), blood samples were collected before therapy and later referred to as day 0. We extracted venous blood using a 21-gauge needle, tourniquet, serum-gel tube with clotting activator (S-Monovette, 4.7 mL, Sarstedt, Germany) and lithium heparin gel tube (L-Monovette, 4.7 ml, Sarstedt, Germany) for each time point. The samples were centrifugated within two hours after collection at a speed of 3,600 × g for 10 min. We aliquoted the resulting serum and plasma samples into separate biobanking tubes with screw caps (LVL Technologies,

Germany) and arranged them into matrix boxes immediately after in a refrigerator at a temperature of -80°C.

3.5 Biomarker measurement in serum samples

We measured the NfL concentration in serum samples using a commercial microfluidic cartridge-based automated platform ELLA (BioTechne, Minneapolis, USA) to perform an enzyme-linked immunosorbent assay. For GFAP, we purchased a commercially available Simoa immunoassay run on a HD-X platform (Quanterix Inc., Lexington, USA). Because of its novelty, there are no commercially available serum kits for the purchase of β-synuclein. We determined the concentration using an in-house digital immunoassay first established by Steffen Halbgebauer et al. at the University Hospital of Ulm (79). The test protocol was run on a HD-X platform using Simoa. All measurements showed intra- and inter-assay coefficients of variability (CV) < 10 % and < 15%, respectively. For intra-assay variability, samples exceeding 20 % were re-measured. To assess comparability between runs, we measured the same sample in three replicates per plate in all runs.

3.6 Statistical analysis

We conducted statistical analysis on GraphPad Prism Version 8 (GraphPad Software Inc., Boston, USA), Microsoft Excel (2022) and R Version 4.2.2 (R Foundation, Vienna, Austria). The Shapiro – Wilk test was utilized to determine the normality of the distribution. For two – group comparisons of baseline characteristics with categorical variables, we used the chi – squared (χ^2) and Fisher equation tests. For two – group comparisons of continuous variables Mann – Whitney – U test was used with a predefined power of 80% and an α-level of 5%. We implemented the Kruskal-Wallis test with the Dunn–Bonferroni post hoc test for comparisons between ≥3 groups. Spearman's rank coefficient was used to calculate the correlations between levels of biomarkers.

Using Chan et al. interpretation of Pearson's and Spearman's correlation coefficients, relationship was evaluated very strong in rho > 0.8, strong in 0.6 to 0.8 rho, moderate between 0.3 to 0.5 rho and poor in rho < 0.3 (80).

We conducted receiver operating characteristic (ROC) analysis to assess the diagnostic specificity and sensitivity of biomarkers, considering an area under the curve (AUC) of 1 indicative of an accuracy of 100% and 0.5 of no discrimination between groups.

Youden's index was maximized to calculate the optimal threshold for the ROC curves (81). We carried out all tests two – tailed, considered p values < 0.05 as first level of statistical significance and presented odds ratios (ORs) with 95% confidence intervals (95% CI). For a better visualization of the presented figures, we transformed the biomarker concentrations logarithmically (log 10). The total number of patients in the cohort was used to present the categorical variables. Continuous variables are presented as mean with standard deviation (SD) or as median with interquartile range (IQR) depending on normal or non-normal distribution, respectively.

3.7 Study protocol approval and ethics

The procedures followed during the study adhered to the principles outlined in the Declaration of Helsinki, its recent modifications, and institutional guidelines. The study was initiated after receiving approval from the local ethics committee (registry number 2021-101). All patients provided written informed consent, either independently or through an authorized representative.

4 Results

4.1 Demographic, clinical and radiological features of the study population

We analyzed serum samples of a total of 61 patients, including 51 patients diagnosed with AIS, 7 with TIA and 3 with ICH. The mean age of the study population was 71.49 (± SD: 14.8) years and 72.20 (± SD: 15.32) years of ischemic stroke patients. Female subjects were 20 (40%) in the AIS group and 3 (43%) in the TIA group. Male subjects were 31 (60%) in the AIS group and 4 (57%) in the TIA group.

According to the TOAST classification system, 15 (29%) patients with AIS presented with LAA, cardioembolism was responsible for 18 (35%) cases, small vessel occlusion was responsible for 3 (7%) cases, and 15 (29%) patients had undetermined etiology.

Blood samples were collected after a median time of 8h 0 m (IQR 3 h–14 h 15 min) of symptom onset. A median serum creatinine concentration of 86 mg/dl (IQR 70 -102) was noted. No statistically significant differences ($p < 0.05$) were observed among the patients with AIS, TIA, and ICH in terms of age, sex, time of blood sampling, and creatinine levels.

Twenty – nine (57%) patients with AIS and 6 (86%) patients with TIA were treated with IVT. Eighteen patients (35 %) with ischemic stroke received reperfusion therapy via the MT. Both, IVT and MT (IVT+MT), was only received by 7 (14%) participants presented with an acute ischemic stroke in the emergency room (ER). Comparing the treatment options (IVT, MT, or both), no statistically significant difference was detected between patients with TIA, AIS, and ICH. Nine patients (15%) of the study population died within the hospital stay and three patients (5%) within three months of discharge, making up 12 (20%) non – survivors together, consisting of 11 (22% of subgroup) AIS and 1 (33% of subgroup) ICH patients.

At admission, ischemic stroke patients had a median ASPECTS value of 9 (IQR 9 – 10), TIA patients 10 (IQR 10 – 10) and ICH patients 7 (IQR 7 – 8). After 24 h, the median ASPECTS changed only slightly in ischemic stroke patients with values of 9 (IQR 7 – 9.5) and in ICH patients with values of 8 (IQR 7 – 8). A statistically significant difference was observed between AIS and TIA patients on the ASPECTS at admission ($p = 0.006$) and after 24 – 72 hours ($p = 0.001$).

Patients presenting to the ER had a median NIHSS score of 5 (IQR 3 – 12), TIA of 1 (IQR 1 – 2), AIS of 6 (IQR 4 – 15), and ICH of 11 (IQR 10 – 17). Patients had a median NIHSS score of 7 (IQR 3 – 21) 24 h after admission [TIA 1(IQR 0 – 1), AIS 8(IQR 4 – 23), ICH 19(IQR 17 – 38)], NIHSS score of 6 (IQR 2 – 19) after 48 h [TIA 1(IQR 0 – 1), AIS 6(IQR 3 – 22), ICH 16(IQR 15 – 32)], NIHSS score of 5 (IQR 1 – 16.25) after 72 h [TIA 0 (IQR 0 – 0), AIS 5(IQR 2 – 18), ICH 17(IQR 16 – 32)], and a median NIHSS score of 2 at discharge (IQR 1.0 – 6.25) [TIA (IQR 0 – 0), AIS 3(IQR 1 – 7), ICH 13(IQR 12 – 14)]. Patients had a median NIHSS change of 3 (IQR 1–10) within the first 24 h, including 0 in TIA, 3 in AIS (IQR 1 – 11), and 7 in ICH (IQR 0 – 28) patients. Significantly elevated NIHSS scores in AIS patients were revealed at admission (p <0.0001), after 24 h (p <0.0001), after 48 h (p <0.0001), after 72 h (p <0.0001), and at discharge (p <0.0001) compared to TIA patients. Unsurprisingly, the calculated NIHSS score change within the first 24 h also showed a statistically significant difference (p <0.004) between the groups.

Patients left the hospital with a median mRS of 2 (IQR 1 – 5) [AIS of 3 (IQR 2 – 5), ICH of 5 (IQR 4 – 6), and TIA of 0 (IQR 0 – 1)]. At functional outcome assessment after 3 months, patients had a median mRS of 3 (IQR 1 – 4.5) with AIS of 3 (IQR 1-5) and ICH of 5 (IQR 3 – 6). At discharge and after 90 days, a significant elevation (p <0.0001) was observed in AIS patients compared to TIA patients, whereas no significant differences were observed between the ICH and AIS groups.

Detailed information and respective calculated values are presented in Table 1.

Table 1. Clinical, radiological and outcome data of study population

	Total (n=61)	**TIA (n=7)**	**AIS (n=51)**	**ICH (n=3)**	***p* -value TIA vs. AIS**	***p* - value AIS vs. ICH**
Age (years)*	71.49 (±14.80)	68 (±13.89)	72.20 (±15.32)	67.67(±12.58)	0.401	0.440
Females/males	24/37	3/4	20 /31	1/2	0.853	0.839
Time from onset to blood sampling	8h (3h – 14h 15 m)	3h30m (2h – 11h)	6h (2h30m – 14h)	12h (9h30min – 14h)	0.400	0.249
Creatinine (mg/dl)	86 (70-102)	79 (73-98)	89 (70-103)	63 (39-83)	0.793	0.058
MT (yes/no)	18/43	0/7	18/33	0/3	0.058	0.208
IVT (yes/no)	35/26	6/1	29/22	0/3	0.143	0.055
IVT + MT (yes/no)	7/54	0/7	7/44	0/3	0.295	0.492
Death within hospital stay (yes/no)	9/52	0/7	8/43	1/3	0.260	0.628
Death within three months of hospital release (yes/no)	12/49	0/7	11/40	1/3	0.172	0.873
ASPECTS at admission	9 (9-10)	10 (10-10)	9 (9-10)	7 (7-8)	**0.006**	**0.006**
ASPECTS after 24h	9 (7.25 - 10)	10 (10-10)	9 (7-9.5)	8 (7-8)	**0.001**	0.294
NIHSS at admission	5 (3-12)	1(1-2)	6(4-15)	11(10-17)	**<0.0001**	0.231
NIHSS change within 24h	3 (1 – 10)	0 (0-2)	3 (1-11)	7 (0-28)	**0.004**	0.665
NIHSS after 24h	7 (3 - 21)	1 (0-1)	8 (4-23)	19 (17-38)	**<0.0001**	0.128
NIHSS after 48h	6 (2-19)	1 (0-1)	6 (3-22)	16 (15-32)	**<0.0001**	0.135
NIHSS after 72h	5 (1-16.25)	0 (0-0)	5 (2-18)	17 (16-32)	**<0.0001**	0.099
NIHSS at discharge	2 (1.0 – 6.25)	0 (0-0)	3 (1-7)	13 (12-14)	**<0.0001**	0.117
mRS at discharge	2 (1-5)	0 (0-1)	3 (2-5)	5 (4-6)	**<0.0001**	0.106
mRS after 90 days	3 (1-4.5)	0 (0-0)	3 (1-5)	5 (3-6)	**<0.0001**	0.208

4.2 Correlations between serum biomarkers and clinical variables

Correlations are presented visually in a correlation matrix (Figure 2 and 3). A comparison of serum NfL with GFAP levels showed strong correlations can be observed between NfL_0 and $GFAP_0$ (rho=0.8; p <0.001), NFL_5 and $GFAP_5$ (rho=0.7; p <0.001), and $GFAP_3$ and NfL_5 (rho=0.7; p <0.001). Moderate correlations were observed between all other time points. No association was found between Nfl_5 and $GFAP_0$. Between serum NfL and β- synuclein, strong associations were observed between Nfl_2 and $\beta\text{-syn}_3$ (rho=0.6; p <0.001), Nfl_2 and $\beta\text{-syn}_5$ (rho=0.6; p <0.001), NfL_3 and $\beta\text{-syn}_3$ (rho=0.7; p <0.001), and NfL_3 and $\beta\text{-syn}_5$ (rho=0.7; p <0.001). Moderate correlations were found at all other time points. Only $\beta\text{-syn}_0$ was not significantly correlated with NfL at any time point.

Strong correlations were found between $\beta\text{-syn}_2$ and $GFAP_2$ (rho=0.6; p <0.001), $\beta\text{-syn}_2$ and $GFAP_3$ (rho=0.7; p < 0.001), $\beta\text{-syn}_2$ and $GFAP_5$ (rho=0.6; p <0.001), $\beta\text{-syn}_3$ and $GFAP_2$ (rho=0.6; p <0.001), $\beta\text{-syn}_3$ and $GFAP_3$ (rho=0.6; p <0.001), $\beta\text{-syn}_3$ and $GFAP_5$ (rho=0.7; p <0.001), $\beta\text{-syn}_5$ and $GFAP_2$ (rho=0.6; p <0.001), $\beta\text{-syn}_5$ and $GFAP_3$ (rho=0.7; p <0.001), $\beta\text{-syn}_5$ and $GFAP_5$ (rho=0.7; p <0.001). No significant correlation was found between $\beta\text{-syn}_0$ and GFAP levels at any time point for the respective biomarkers. Moderate correlations were found at all other time points. Detailed numerical data are provided in Appendices 4 and 5, respectively.

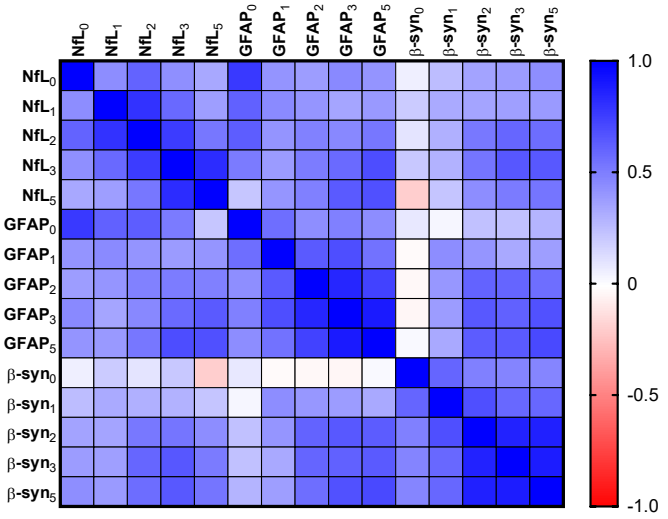


Figure 2. Correlation matrix (rho, Spearman's correlation coefficient) illustrating the relationship between serum biomarkers.

Dark blue indicates strong positive correlation and dark red indicates strong negative correlation

No strong correlation was found between serum biomarker levels in relation to NIHSS change within 24hours and discharge, as well as creatinine. Age correlated strongly with $GFAP_0$ (rho = 0.7; p = 0.001), moderately with NfL_{0-5} as well as $GFAP_1$ and $GFAP_5$ (rho=0.3-0.5; $p < 0.05$). No significant correlation was found between the β-syn_{0-5} levels and age.

The ASPECTS on admission was inversely correlated with $GFAP_2$ (rho = -0.6; p = 0.001), $GFAP_5$ (rho = -0.6; p = 0.001), and β-syn_5 (rho = -0.6; p = 0.001). The ASPECTS 24 hours after admission correlated negatively and strongly only with $GFAP_2$ (rho =-0.6; p = 0.001). A moderate inverse correlation with ASPECTS on admission and after 24 h was found in NfL_{2-5}, $GFAP_3$, and β-syn_{2-3} [rho= (- 0.3) – (-0.5); p <0.005].

The NIHSS score on admission was strongly correlated with $GFAP_3$, $GFAP_5$ and β-syn_{2-5}. NIHSS score 24h after admission was strongly correlated with most serum biomarkers among the measured time points, including NfL_{3-5}, $GFAP_{2-5}$, and β-syn_{2-5}. NIHSS score 48hours after admission strongly correlated with NfL_{2-5}, $GFAP_{3-5}$, and β-syn_{3-5}, whereas the NIHSS score after 72 hours only strongly correlated with NfL_{3-5}, $GFAP_{3-5}$, and β-syn_3.

Biomarker concentration in serum correlated strongly with mRS at discharge, as shown by NfL_3, NfL_5, $GFAP_3$, and $GFAP_5$, whereas stronger correlations were observed in mRS at 90 days follow-up, including NfL_{2-5}, $GFAP_3$, and β-syn_{3-5}.

No correlation was found between age and the NIHSS, ASPECTS, and mRS scores. On admission, the ASPECTS was only moderately and inversely correlated with $NIHSS_0$ (rho=-0.3; p<0.024) and $NIHSS_{24}$ (rho=-0.3; p<0.013). The ASPECTS after 24 h was moderately correlated with $NIHSS_0$, $NIHSS_{24}$, $NIHSS_{48}$, $NIHSS_{72}$, and mRS. The mRS showed a moderate correlation with NIHSS change within 24 h. A strong correlation was found between the mRS after 90 days and NIHSS score on admission (rho=0.6; p <0.001), 24h (rho=0.7; p <0.001), 48h (rho=0.8; p <0.001), 72h (rho=0.7; p <0.001), and discharge (rho=0.7; p <0.001).

All correlations and respective rho- and p-values are described in detail in Appendices 6 and 7.

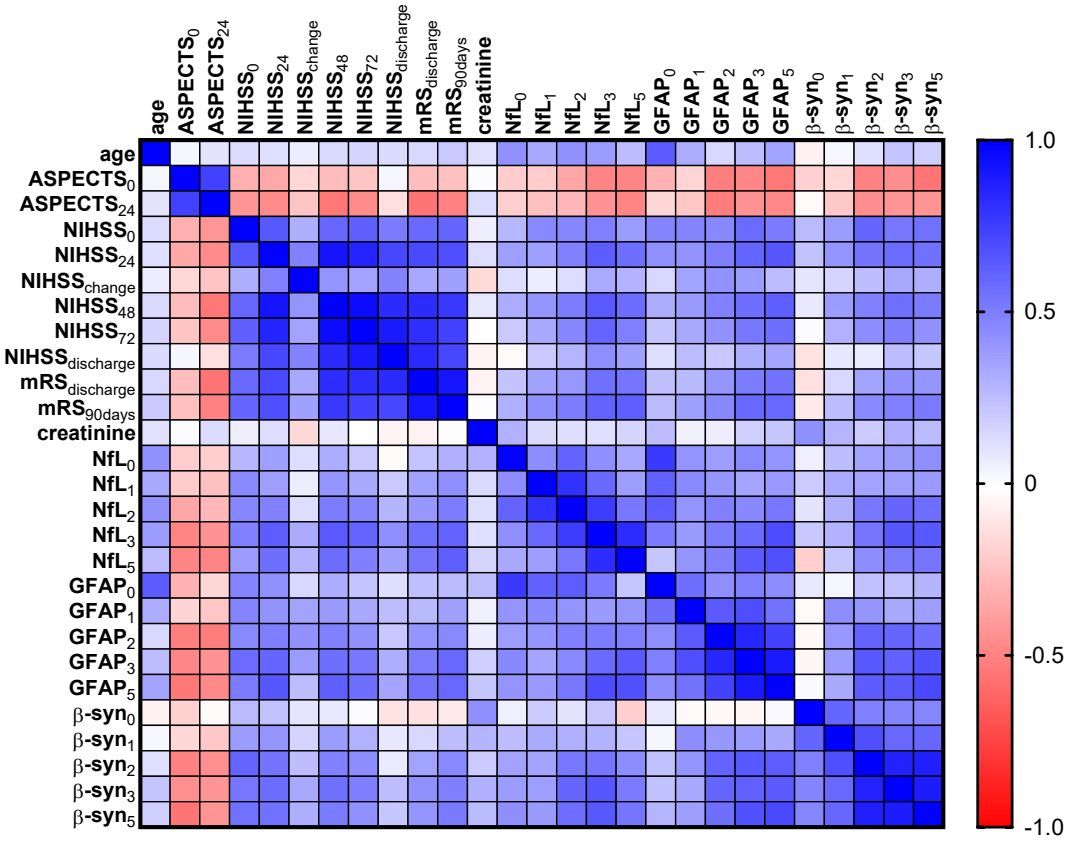


Figure 3. Correlation matrix (rho, Spearman's correlation coefficient) illustrating the relationship between serum biomarkers and demographic and clinical variables.

Dark blue indicates strong positive correlation and dark red indicates strong negative correlation

4.3 Serum biomarkers in patients with AIS and TIA

Regarding NfL concentration, significant elevation (p <0.05) was observed in AIS patients compared to TIA patients only on the 2nd day (p =0.033), and on the 3rd day (p =0.034) after symptom onset. No statistically significant difference was observed when comparing the NfL concentration before therapy, on the 1st and on 5th day. GFAP concentration was significantly elevated in patients on 1st (p =0.004), 2nd (p =0.001) 3rd (p =0.001) and 5th (p =0.005) day after symptom onset. No statistically significant difference in GFAP concentration was observed between the groups before the therapy. β-synuclein concentration showed no statistically significant difference between the diagnostic groups on any of the analyzed days or before therapy. Peak concentrations of NfL [median 223 pg/ml (IQR 98.78 – 385.3)] are reached on 5th day, GFAP on 3rd day [median 2.66 ng/ml (1.14-20.58)] and β-syn on 5th day [19.78 pg/ml (IQR 6.66-63.3)] in all AIS patients. All detailed values are provided in Table 2, and the temporal patterns of the respective serum biomarker levels are presented with a level of significance in Figure 4.

Table 2. Serum biomarker concentrations in AIS and TIA

	Total (n=61)	**All TIA (n=7)**	**All AIS (n=51)**	***p* - value TIA vs. AIS**
NfL_0 (pg/ml)	39.0 (19 - 91.2)	26.9 (6.6-127.8)	42.2 (19.7 - 86.3)	0.470
NfL_1 (pg/ml)	77.5 (26.3 - 140.5)	53.0 (11.6-98.4)	79.4 (26.6 - 153.5)	0.197
NfL_2 (pg/ml)	112.0 (59.6 - 175.3)	47.6 (13.4-110)	115.0 (62.4 - 187.5)	**0.033**
NfL_3 (pg/ml)	154.0 (52.5 - 252.5)	33.9 (15.4-172.8)	155.0 (57.8 - 265.0)	**0.034**
NfL_5 (pg/ml)	215.0 (97.9 - 350.5)	100.8 (32.2-219.8)	223.0 (98.8 - 385.3)	0.105
$GFAP_0$ (ng/ml)	0.39 (0.25 - 0.65)	0.36 (0.26 - 0.64)	0.39 (0.25 - 0.65)	0.947
$GFAP_1$ (ng/ml)	1.59 (0.49 - 5.39)	0.37 (0.21 - 0.54)	1.72 (0.67 - 4.50)	**0.004**
$GFAP_2$ (ng/ml)	2.31 (0.64 - 8.47)	0.32 (0.29 - 0.62)	2.48 (1.02 - 8.81)	**0.001**
$GFAP_3$ (ng/ml)	2.58 (0.86 - 20.32)	0.41 (0.23 - 0.71)	2.66 (1.14 - 20.58)	**0.001**
$GFAP_5$ (ng/ml)	2.10 (0.83 - 12.02)	0.26 (0.16 - 0.49)	2.10 (0.89 - 11.99)	**0.005**
β-syn_0 (pg/ml)	5.46 (2.46 - 12.17)	4.44 (1.44 - 15.15)	5.46 (2.81 – 14.54)	0.581
β-syn_1 (pg/ml)	8.83 (2.78 - 23.24)	5.55 (2.07 - 25.09)	8.97 (2.82 – 23.03)	0.576
β-syn_2 (pg/ml)	12.05 (4.56 - 27.88)	4.52 (2.69 - 27.88)	12.21 (5.11 - 35.89)	0.222
β-syn_3 (pg/ml)	12.83 (4.76 - 34.28)	5.08 (1.79 - 27.88)	16.54 (6.20 - 37.93)	0.127
β-syn_5 (pg/ml)	16.16 (6.13 - 56.69)	5.44 (3.17 - 51.02)	19.78 (6.66-63.3)	0.203

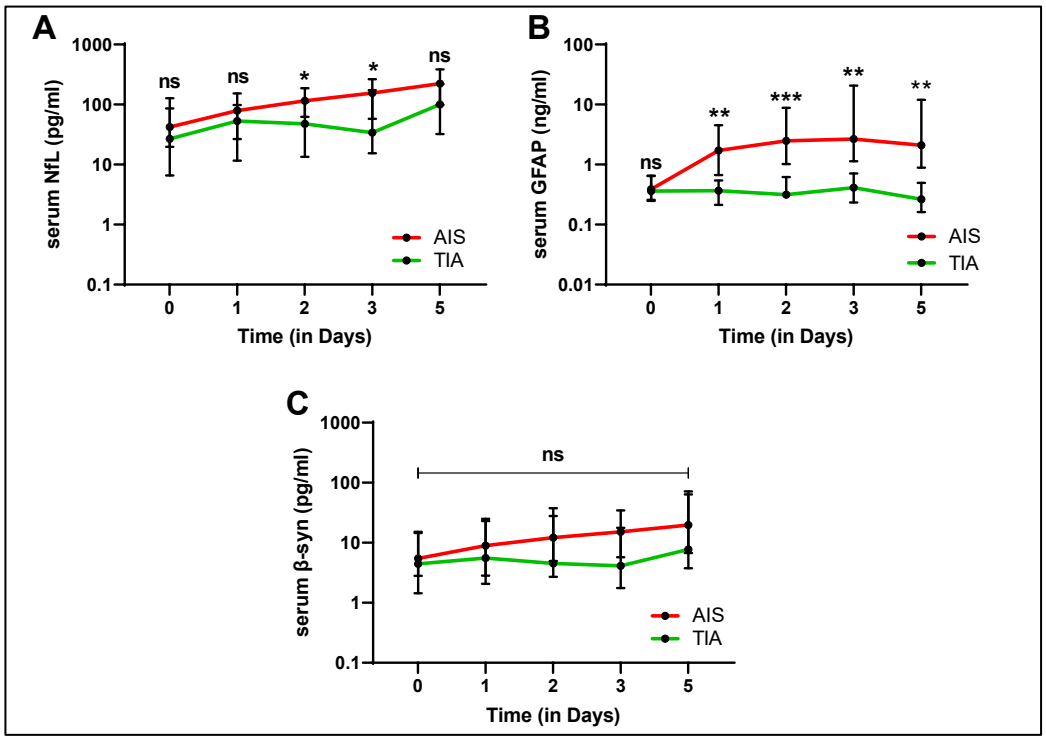


Figure 4. Comparison of serum biomarker concentrations and temporal patterns in AIS vs. TIA

(A) serum NfL (pg/ml), (B) serum GFAP (ng/ml), and (C) serum β-syn (pg/ml). ^{ns}not significant

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.4 Serum biomarkers and clinical data in relation to treatment groups

The treatment of acute AIS patients was divided into the following groups: IVT (n=22), MT (n=11), IVT + MT (n=7), and patients without treatment (n=11). No statistically significant differences were revealed regarding age, sex, creatinine levels, mortality, NIHSS change within 24h, NIHSS 48h after symptom onset and mRS. Time from onset of symptoms to blood sampling showed statistically considerable difference (p = 0.0001) between treatment groups [IVT vs. MT (p = 0.037), IVT vs. No therapy (p = 0.0001)].

In patients treated with IVT compared to those treated with MT, we found substantially lower ASPECTs at admission and after 24-72h (p = 0.001 for both). In other comparisons between the groups, only IVT compared to IVT together with MT showed a significantly lower ASPECTS on the Dunn-Bonferroni post hoc test (p = 0.016). NIHSS score at admission (p = 0.009), NIHSS 24h (p = 0.010) and 72h (p = 0.037) after symptom onset were significantly lower in IVT patients than in MT patients. The detailed information is presented in Table 3.

No differences in serum NfL concentrations were detected between the treatment groups. On the 3rd (p = 0.031) and 5th days (p = 0.025) of hospital stay, GFAP concentrations were significantly lower in IVT than in MT patients. No differences were observed on other days in terms of GFAP concentration. β-synuclein showed significantly higher concentration on 2nd, 3rd and 5th day in IVT+MT compared to no treatment (p = 0.037, 0.043, p = 0.044, respectively) and compared to IVT alone on 2nd day (p = 0.036) and 5th day (p = 0.04). On 3rd day, β-synuclein levels were significantly higher in patients with MT than in those without treatment (p = 0.047).

Peak concentrations of NfL were reached on the 5th day in all treatment groups [IVT 164 pg/ml (IQR 53.6-282.8); MT 292 pg/ml (IQR 129.3-1161); IVT+MT 305 pg/ml (IQR 233-864); no therapy 255 pg/ml (IQR122.5-337.5)]. Patients treated with MT or without treatment reached peak GFAP concentrations on the 3rd day [MT 21.89 ng/ml (IQR 4.09-50.35); no therapy 2.26 ng/ml (IQR 0.97-3.76)], whereas patients treated with IVT reached peak concentration on the 2nd day [2.28 ng/ml (IQR 0.29-3.94)] and patients treated with IVT + MT on the 5th day [4.32 ng/ml (IQR 2.1-11.97)]. Peak concentration of β-synuclein was reached on the 1st day in IVT patients [8.70 pg/ml (IQR 2.80-23,57)], on the 5th day in MT patients [47.77 (IQR 20.48-183.3)], on the 2nd day in IVT+MT patients [65.53 (IQR 13.66-115.2)] and on the 3rd day in patients

receiving no therapy [9.62 (IQR 3.81-12.93)]. Detailed information regarding the serum biomarker concentrations is displayed in Table 4. The respective serum biomarker concentrations in relation to the treatment options with the level of significance are presented in Figure 5.

Table 3. Clinical, radiological and outcome data of treatment groups

	AIS				***p* - value**
	IVT (n=22)	**MT (n=11)**	**IVT + MT (n=7)**	**No therapy (n=11)**	***p* - value Kruskal-Wallis**
Age (years)*	70.86 (± 18.15)	70.18 (± 17.26)	76 (±8.62)	74.45 (±10.71)	0.968
Females/males	8/14	5/6	4/3	3/8	0.603
Time from onset to blood sampling	2h30min (1h30min – 3h38min)	8h (5h-16h)	8h (3h-17h)	15h 30min (9h-20h)	**0.0001**
Creatinine (mg/dl)	82 (70-122.8)	91 (68-102)	86 (72-93)	90 (64-104)	0.953
Death within hospital stay (yes/no)	1/21	3/8	2/5	2/9	0.261
Death within three months of hospital release (yes/no)	3/19	4/7	2/5	3/8	0.496
ASPECTS at admission	10 (9 - 10)	8 (8 - 9)	8 (8 - 9)	9 (9 - 10)	**0.0004**
ASPECTS after 24h	9 (9 - 10)	7 (5.25 - 8.25)	8 (7 - 9)	9 (5 - 9)	**0.001**
NIHSS at admission	4.5 (3 - 7,5)	15 (5 - 38)	10 (5 - 24)	7(4 - 11)	**0.009**
NIHSS score change within 24h	3 (1 - 6.25)	8 (2 - 22)	3 (0 - 14)	3 (1-7)	0.315
NIHSS after 24h	5 (3.75 - 9.25)	33 (9 - 38)	14 (5 - 38)	4 (2 - 22)	**0.010**
NIHSS after 48h	5.5 (2.75 - 9.5)	19 (8 - 38)	5 (2 - 31)	5 (2 - 22)	0.074
NIHSS after 72h	4.5 (1 - 6.5)	18 (6 - 38)	5 (1 - 26)	4.5 (1.75 - 10.25)	0.037
NIHSS at discharge	3 (1 - 4)	11 (2.25 - 15.75)	1 (1 - 9.25)	4 (0.5 - 6.5)	0.149
mRS at discharge	3 (1 - 4)	4 (3 - 6)	3 (1 - 6)	3 (2 - 5)	0.108
mRS after 90 days	2 (1 - 3.25)	5 (3 - 6)	2 (1 - 6)	2 (1 - 6)	0.136

Table 4. Serum biomarker concentrations in treatment groups

	AIS				***p* -value**
	IVT (n=22)	**MT (n=11)**	**IVT+ MT (n=7)**	**No therapy (n=11)**	***p* - value Kruskal-Wallis**
NfL_0 (pg/ml)	45 (20.6-81.5)	24.6 (11.36-83.65)	21.9 (18.6-115)	/	0.809
NfL_1 (pg/ml)	61 (22.5-165)	57.1 (9.71-136)	103 (75.9-189)	94.7 (58.8 -354)	0.463
NfL_2 (pg/ml)	90 (46.65-161)	133 (85.7-194)	152.1 (92.2-338)	114.5 (66.6-226.8)	0.449
NfL_3 (pg/ml)	85.8 (38.2-211)	226 (131-462)	184 (145 - 317)	129 (66.50-236)	0.069
NfL_5 (pg/ml)	164(53.6-282.8)	292 (129.3-1161)	305 (233-864)	255 (122.5-337.5)	0.184
$GFAP_0$ (ng/ml)	0.36 (0.19-0.66)	0.35 (0.30-1.31)	0.49 (0.25-0.87)	/	0.784
$GFAP_1$ (ng/ml)	1.63 (0.26-4.02)	2.79 (1.27-11.10)	1.68 (0.78-30.50)	1.17 (0.56-2.86)	0.541
$GFAP_2$ (ng/ml)	2.28 (0.29-3.94)	9.50 (3.77-18.55)	3.31 (0.59-33.95)	1.62 (1.03-7.98)	0.091
$GFAP_3$ (ng/ml)	1.87 (0.28-8.02)	21.89 (4.09-50.35)	3.20 (2.45-22.41)	2.26 (0.97-3.76)	**0.031**
$GFAP_5$ (ng/ml)	1.20 (0.29-3.48)	13.46 (1.75-184.6)	4.32 (2.1-11.97)	1.56 (0.98-3.14)	**0.025**
$\beta\text{-}syn_0$ (pg/ml)	4.77 (2.5-8.38)	15.72 (2.39-20.4)	7.35 (4.12-51.25)	/	0.427
$\beta\text{-}syn_1$ (pg/ml)	8.70 (2.80-23.57)	14.19 (5.48 -25.15)	15.31 (7.79-39.33)	4.37 (1.97-20.63)	0.399
$\beta\text{-}syn_2$ (pg/ml)	6.98 (4.44-23.78)	17.73 (11.74-75.28)	65.53 (13.66-115.2)	8.11 (2.25-12.67)	**0.008**
$\beta\text{-}syn_3$ (pg/ml)	7.76 (3.82-28.31)	29.68 (17.29-103.5)	34.79 (23.95-99.03)	9.62 (3.81 -12.93)	**0.003**
$\beta\text{-}syn_5$ (pg/ml)	8.45 (4.82-34.35)	47.77 (20.48-183.3)	63.55 (34.56 -81.41)	9.17 (4.95 - 20.01)	**0.003**

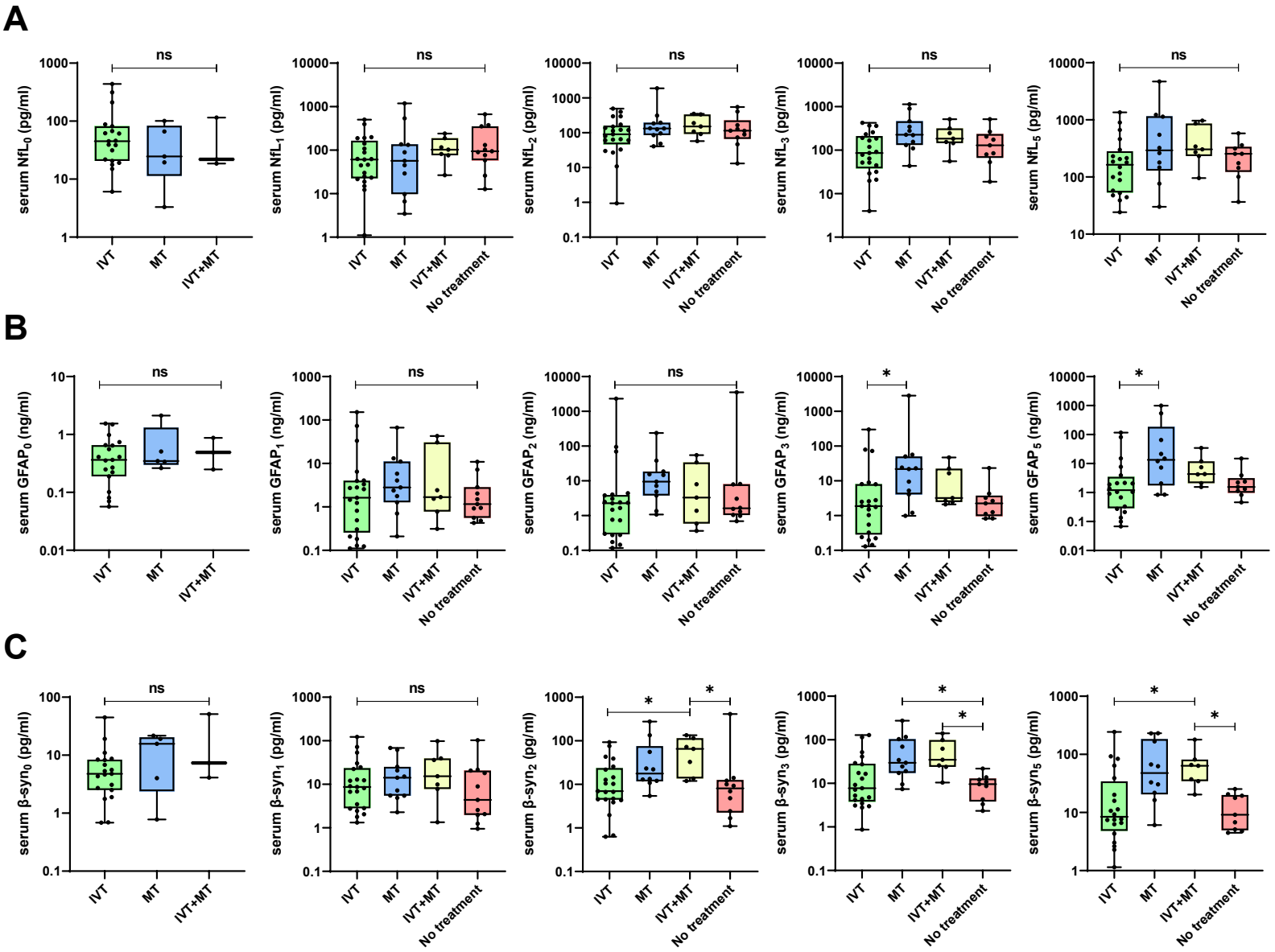


Figure 5. Comparison of serum biomarker concentrations in the treatment groups

(A) serum NfL (pg/ml), (B) serum GFAP (ng/ml), (C) serum β-syn (pg/ml). ^{ns}not significant $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

4.5 Serum biomarkers and clinical data in relation to functional outcome

Evaluating the functional outcome at the 90-day follow-up, 25 patients experienced good outcomes (mRS 0-2) and 26 patients had poor functional outcomes (mRS 3-6). We observed no statistically significant differences between the groups in terms of sex, treatment choice (IVT, MT, IVT+MT, no therapy), time from onset to blood sampling, ASPECTS at admission and after 24 hours. The age of patients with mRS 3-6 was significantly higher than that of patients with mRS 0-2 patients (76.81 ± SD 11.76 vs. 67.40 ± SD 17.26). We detected statistically significant differences in the NIHSS scores at every assessment period between the two groups (p <0.001). As expected, a significant difference between death within hospital stay (31% of mRS 3-6, 0% of mRS 0-2) and within three months of hospital release (42.3% of mRS 3-6, 0% of mRS 0-2) was observed between the groups. Detailed information is presented in Table 5.

Table 5. Clinical, radiological and outcome data of mRS groups at 90-day follow-up

	mRS 0-2 (n= 25)	**mRS 3-6 (n=26)**	***p* value mRS 0-2 vs 3-6**
Age (years)*	67.40 (±17.26)	76.81 (±11.76)	0.034
Females/males	9/16	11/15	0.645
Time from onset to blood sampling	8h0min (1h45min-14h)	5h 30min (2h 30m – 15h 38min)	0.603
Creatinine (mg/dl)	87 (70.50-103.5)	90.50 (68.75-102.5)	0.937
MT (yes/no)	6/19	12/14	0.098
IVT (yes/no)	17/8	12/14	0.115
IVT + MT (yes/no)	4/201	3/23	0.644
Death within hospital stay (yes/no)	0/25	8/18	**0.003**
Death within three months of hospital release (yes/no)	0/25	11/15	**0.0002**
ASPECTS at admission	9 (9-10)	9 (8-10)	0.325
ASPECTS after 24h	9 (8.25-9.75)	8 (5.5-9.5)	**0.061**
NIHSS at admission	4 (3-6)	14.50 (5.75-21.75)	**<0.0001**
NIHSS score change within 24h	3 (1-4)	7 (1-17)	**0.009**
24h NIHSS	4 (1.5-9)	22.5 (5-38)	**<0.0001**
48h NIHSS	3 (1-6.5)	20.5 (5.75-31.75)	**<0.0001**
72h NIHSS	2 (1-5)	18 (4.5-30.50)	**<0.0001**
NIHSS at discharge	1 (1-3)	8 (4-15)	**<0.0001**
mRS at discharge	2 (1-3)	4.5 (3.75-6)	**<0.0001**
mRS after 90 days	1 (1-2)	5 (3-6)	**<0.0001**

NfL concentration in serum was significantly elevated in mRS 3-6 before therapy (p = 0.011), on 1st (p = 0.019), 2nd (p = 0.003), 3rd (p = 0.001) and 5th day (p = 0.003) compared to the mRS 0-2 group at the 90-day follow-up. The peak concentration of NfL was reached on the 5th day in both the groups. Serum GFAP concentration in the mRS 3-6 group was significantly higher before therapy (p = 0.046), on 1st (p = 0.022), 2nd (p = 0.018), 3rd (p = 0.0002), and 5th day (p = 0.001) compared to the mRS 0-2 group. Peak GFAP concentration levels were reached on 2nd day in patients with mRS 0-2 and on 3rd day in patients with mRS 3-6. β-synuclein levels showed statistically significant difference in concentration levels on the 2nd (p = 0.008), 3rd (p = 0.006) and 5th day (p = 0.001) of symptom onset. No statistically significant difference was observed before therapy and on 1st day. β-synuclein reaches peak levels on 2nd day in mRS 0-2 group and on 5th day in mRS 3-6 group. Detailed information is presented in Table 6. The respective biomarker concentrations with the level of significance and temporal pattern in the mRS groups are presented in Figure 6-8.

Table 6. Serum biomarker concentrations in mRS groups at 90-day follow-up

	AIS		***p* - value**
	mRS 0-2 (n= 25)	**mRS 3-6 (n=26)**	***p* - value mRS 0-2 vs 3-6**
NfL_0 (pg/ml)	21.90 (18.60 - 45.00)	66.30 (38.65 - 262.0)	**0.011**
NfL_1 (pg/ml)	46.20 (22.50 - 105.5)	103.0 (59.05 - 365.0)	**0.019**
NfL_2 (pg/ml)	92.20 (52.95 - 125.0)	151.1 (86.78 - 346.3)	**0.003**
NfL_3 (pg/ml)	85.80 (44.15 -170.5)	226.0 (131.0 - 426.0)	**0.001**
NfL_5 (pg/ml)	159.0 (79.38 - 249.5)	331.5 (171.5 - 872.8)	**0.003**
$GFAP_0$ (ng/ml)	0.35 (0.10 - 0.49)	0.56 (0.30 - 0.81)	**0.046**
$GFAP_1$ (ng/ml)	1.23 (0.37 - 3.02)	2.80 (1.10 - 11.00)	**0.022**
$GFAP_2$ (ng/ml)	1.56 (0.66 - 4.47)	4.11 (1.46 - 30.10)	**0.018**
$GFAP_3$ (ng/ml)	1.50 (0.69 - 2.93)	8.96 (2.65 - 24.06)	**0.0002**
$GFAP_5$ (ng/ml)	1.34 (0.52 - 2.32)	7.50 (1.94 - 24.67)	**0.001**
β-syn_0 (pg/ml)	5.33 (3.09 - 8.705)	7.35 (2.19 - 17.43)	0.964
β-syn_1 (pg/ml)	8.70 (2.24 - 21.16)	14.19 (4.19 - 32.24)	0.209
β-syn_2 (pg/ml)	9.29 (4.44 - 14.27)	22 (10.21 - 70.39)	**0.008**
β-syn_3 (pg/ml)	9.20 (4.332-20.65)	26.69 (11.31 - 111.6)	**0.006**
β-syn_5 (pg/ml)	7.51 (4.81 - 26.80)	30.45 (16.39 - 92.38)	**0.001**

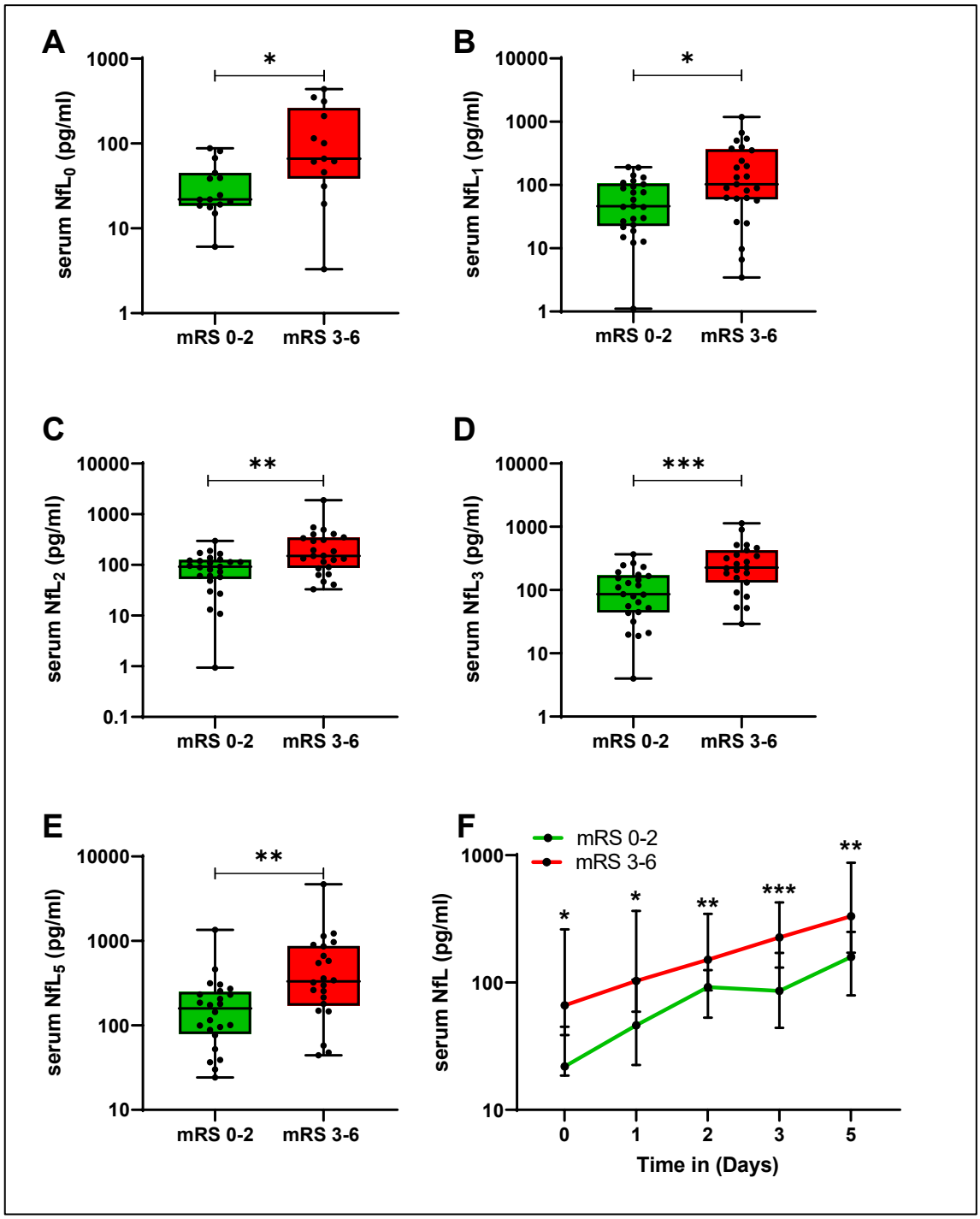


Figure 6. Comparison of serum NfL concentrations in mRS groups at the 90-day follow-up (A) serum NfL_0 (pg/ml), (B) serum NfL_1 (pg/ml), (C) serum NfL_2 (pg/ml), (D) serum NfL_3 (pg/ml), (E) serum NfL_5 (pg/ml), (F) Temporal pattern of serum NfL. ^{ns}not significant $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

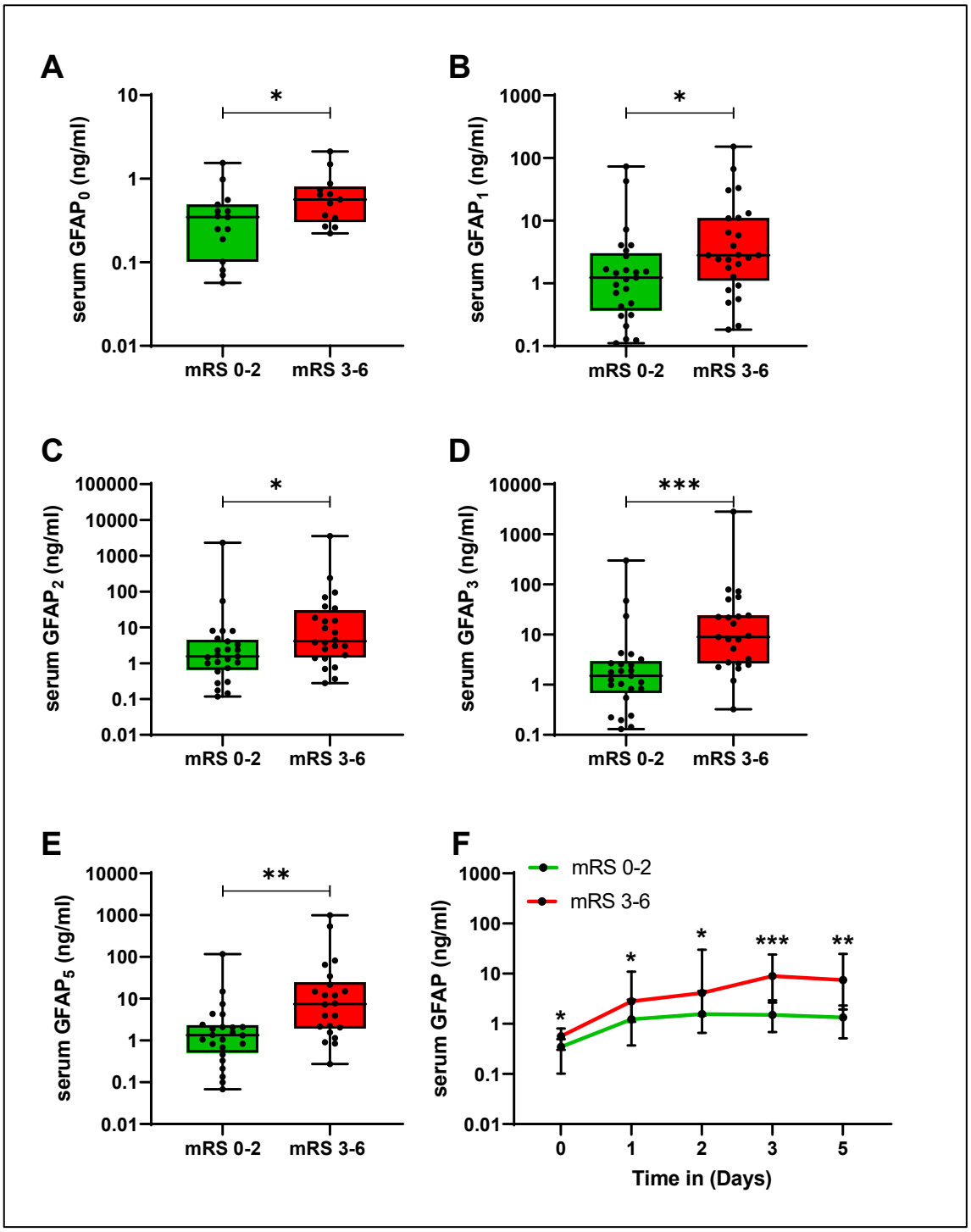


Figure 7. Comparison of serum GFAP concentrations in mRS groups at the 90-day follow-up (A) serum $GFAP_0$ (ng/ml), (B) serum $GFAP_1$ (ng/ml), (C) serum $GFAP_2$ (ng/ml), (D) serum $GFAP_3$ (ng/ml), (E) serum $GFAP_5$ (ng/ml), (F) Temporal pattern of serum GFAP . ^{ns}not significant $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

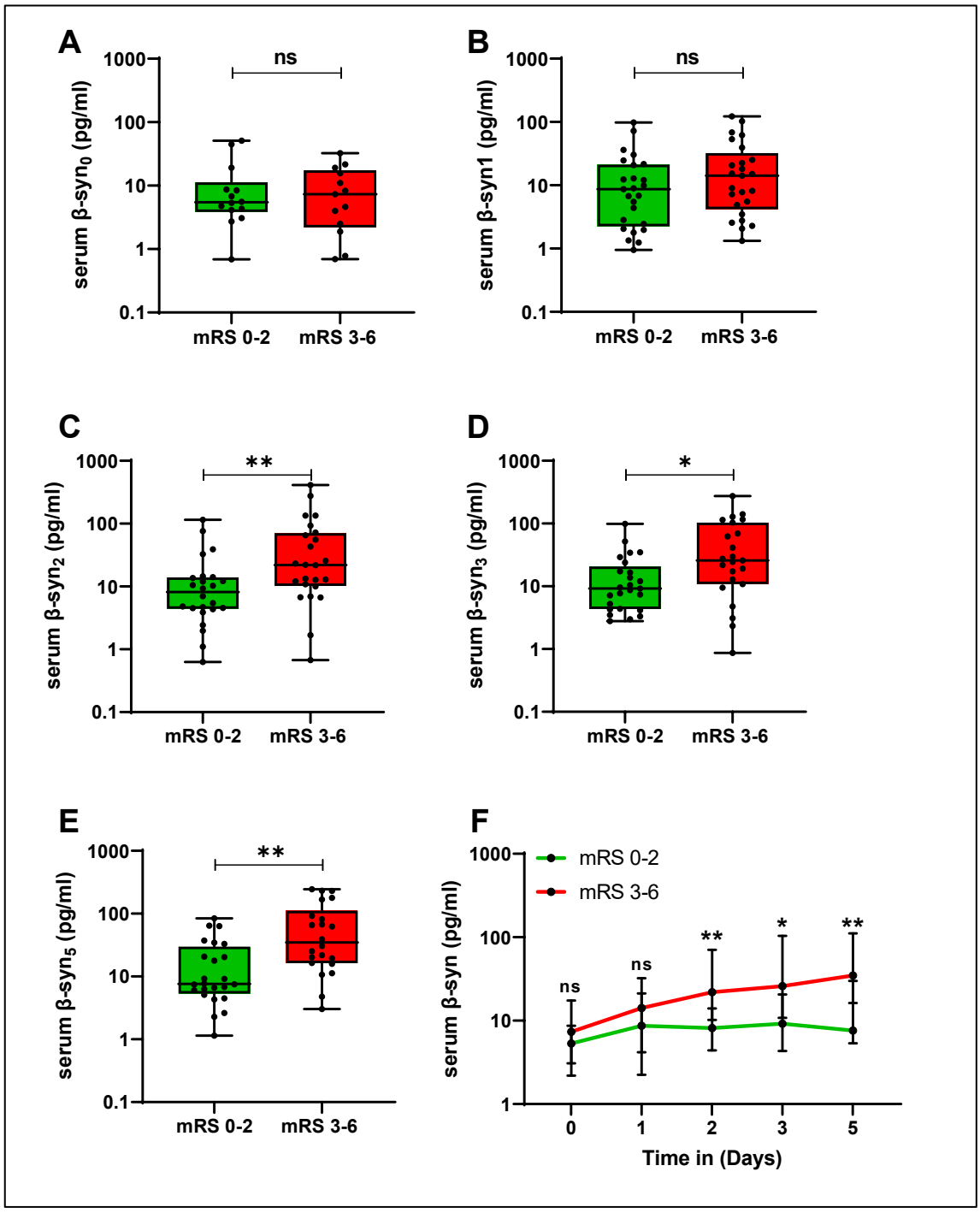


Figure 8. Comparison of serum β-synuclein concentrations in mRS groups at the 90-day follow-up

(A) serum β-syn$_0$ (pg/ml), (B) serum β-syn $_1$ (pg/ml), (C) serum β-syn $_2$ (pg/ml), (D) serum β-syn $_3$ (pg/ml), (E) serum β-syn $_5$ (pg/ml), (F) Temporal pattern of serum β-synuclein. ^{ns}not significant *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The ROC curve data for all blood biomarkers in the differential diagnosis between patients with mRS 0-2 and mRS 3-6 at the 90-day follow-up are reported in Table 7 and are graphically presented in Figure 9.

We compared Nfl, GFAP, and β-synuclein at each assessed time point to distinguish between the functional outcome groups.

NfL provided higher diagnostic accuracy among the biomarkers before treatment [AUC 0.779 (0.59-0.96 CI 95%)], on 1st [AUC 0.692 (0.54 to 0.84 CI 95%)] and 2nd [AUC 0.743 (0.60-0.88 CI 95%)] day. GFAP revealed the highest accuracy on 3rd day [AUC 0.807 (0.68 - 0.93 CI 95%)]. On 5th day, the highest accuracy was noted for GFAP [AUC 0.775 (0.64-0.91 CI 95%)] and β-synuclein [AUC 0.775 (0.64-0.91 CI 95%)].

In the overall discrimination between good (mRS 0-2) and bad (mRS 3-6) functional outcomes after 90 days, the ROC curve analysis demonstrated the highest accuracy among all measured biomarkers and time points for serum GFAP on 3rd day.

At a cut-off of 2.005 ng/ml, calculated by means of the maximized Youden's index, a sensitivity of 64 % and a specificity of 91.3% were achieved.

Table 7. The ROC curve data of all serum biomarkers in the differential diagnosis between patients with mRS 0-2 and mRS 3-6 at 90-day follow-up

mRS 0-2 vs. 3-6	AUC	Std. Error	95% CI	*p* -value	Cut-off	Sensitivity%	95% CI	Specificity%	95% CI	Youden	Likelihood ratio
NfL_0	0.779	0.094	0.59 to 0.96	0.012	>45.45	76.92	49.74% to 91.82%	80.0	54.81% to 92.95%	156.92	3.846
NfL_1	0.692	0.076	0.54 to 0.84	0.020	>59.90	76.0	56.57% to 88.50%	56.0	37.07% to 73.33%	132.0	1.727
NfL_2	0.743	0.071	0.60 to 0.88	0.004	<130.5	80.0	60.87% to 91.14%	62.5	42.71% to 78.84%	142.5	2.133
NfL_3	0.780	0.067	0.65 to 0.91	0.001	<179.5	80.0	60.87% to 91.14%	69.57	49.13% to 84.40%	149.57	2.629
NfL_5	0.754	0.074	0.61 to 0.90	0.003	<258.5	79.17	59.53% to 90.76%	63.64	42.95% to 80.27%	142.81	2.177
$GFAP_0$	0.723	0.098	0.53 to 0.91	0.045	<0.5000	80.0	54.81% to 92.95%	61.54	35.52% to 82.29%	141.54	2.08
$GFAP_1$	0.688	0.076	0.54 to 0.84	0.023	<1.724	72.0	52.42% to 85.72%	72.0	52.42% to 85.72%	144.0	2.571
$GFAP_2$	0.697	0.076	0.55 to 0.85	0.018	<2.446	68.0	48.41% to 82.79%	70.83	50.83% to 85.09%	138.83	2.331
$GFAP_3$	0.807	0.065	0.68 to 0.93	<0.001	<2.005	64.0	44.52% to 79.75%	91.3	73.20% to 98.45%	155.3	7.36
$GFAP_5$	0.775	0.069	0.64 to 0.91	0.001	<2.123	75.0	55.10% to 88.00%	72.73	51.85% to 86.85%	147.73	2.75
$\beta - syn_0$	0.517	0.117	0.29 to 0.75	0.884	<2.607	30.77	12.68% to 57.63%	92.86	68.53% to 99.63%	123.63	4.308
$\beta - syn_1$	0.605	0.080	0.45 to 0.76	0.204	>13.52	52.0	33.50% to 69.97%	72.0	52.42% to 85.72%	124.0	1.857
$\beta - syn_2$	0.726	0.074	0.58 to 0.87	0.007	>18.16	54.17	35.07% to 72.11%	83.33	64.15% to 93.32%	137.5	3.25
$\beta - syn_3$	0.715	0.078	0.56 to 0.87	0.011	>18.22	65.22	44.89% to 81.19%	76	56.57% to 88.50%	141.22	2.717
$\beta - syn_5$	0.775	0.069	0.64 to 0.91	0.001	>9.966	90.91	72.19% to 98.38%	62.5	42.71% to 78.84%	153.41	2.424

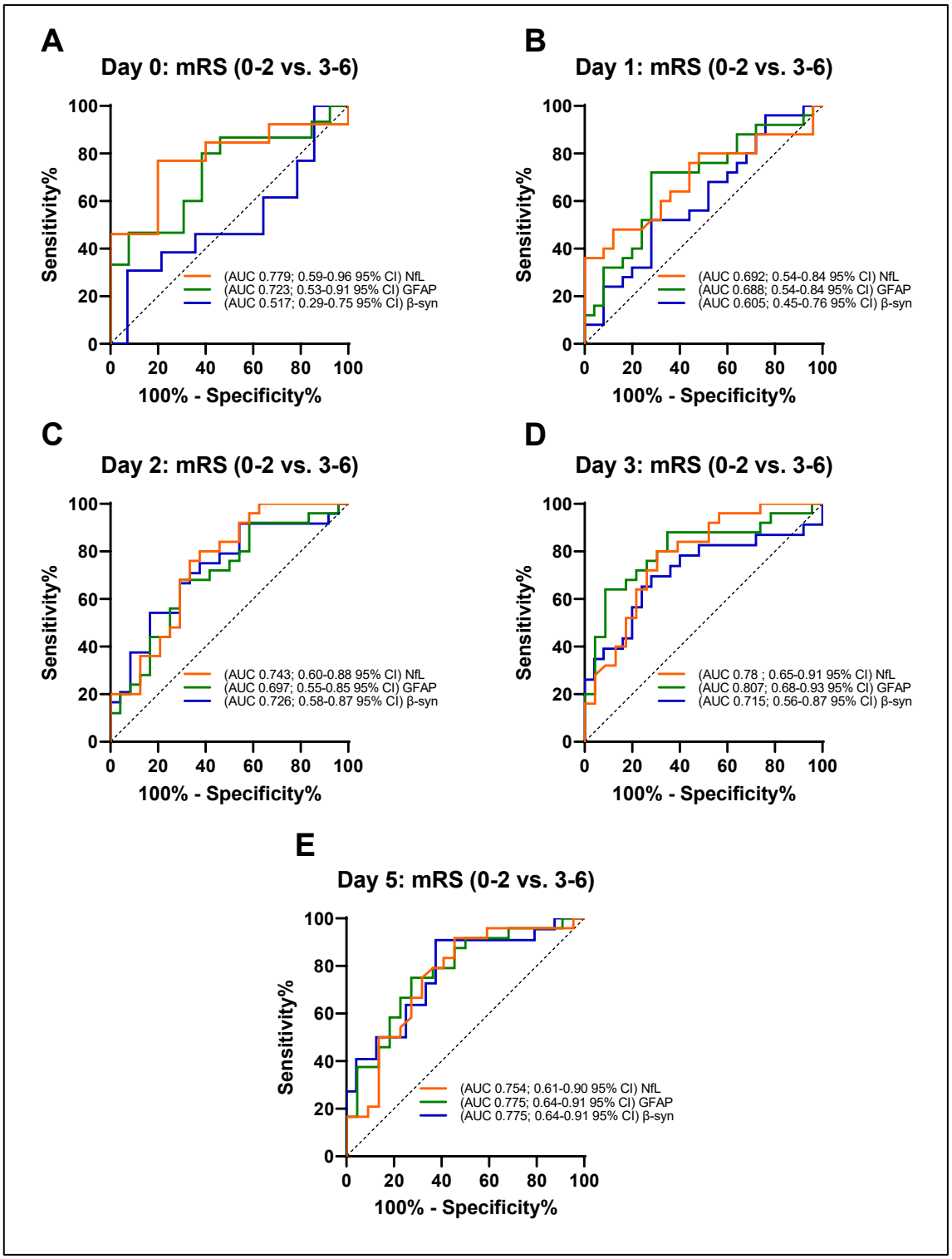


Figure 9. Receiver operating characteristic analysis. ROC curves relative to serum NFL, GFAP and β-synuclein for discrimination of mRS 0-2 vs. 3-6 at 90-day follow-up

(A) Day 0, (B) Day 1, (C) Day 2, (D) Day 3, (E) Day 5

4.6 Serum biomarkers and clinical data in relation to mortality

In the study population, among AIS patients, 40 participants survived and 11 died (27.5%), including eight during the hospital stay and three within 3 months of discharge. In terms of age, sex, chosen treatment, creatinine concentration, and time from onset to blood sampling, there were no statistically significant differences between survivors and non-survivors. Non – survivors showed significantly elevated NIHSS scores at admission (p = 0.009), 24 h (p = 0.0002), 48 h (p <0.0001), and 72 h (p = 0.0002) after symptom onset compared with survivors. No significant difference was observed in the NIHSS score within the first 24 h. The ASPECTS was significantly discernible between both groups on admission and after 24hours. Unsurprisingly, a statistically significant difference in the mRS scores was observed (p <0.0001). The detailed information is presented in Table 8.

Table 8. Clinical, radiological, and outcome data of survivors versus non-survivors.

AIS patients	**Non-survivors (n=11)**	**survivors (n=40)**	***p* - value**
Age (years)*	77.64 (±6,83)	70.70 (±16.68)	0.344
Females/males	7/4	13/27	0.061
Time from onset to blood sampling	8h (4h-18h)	5h30min (2h-13h45min)	0.248
Creatinine (mg/dl)	91 (70-104)	88 (70-102.8)	0.680
MT (yes/no)	6/5	12/28	0.131
IVT (yes/no)	4/7	25/15	0.121
IVT + MT (yes/no)	2/9	5/35	0.628
Death within hospital stay (yes/no)	8/3	0/40	**<0.0001**
Death within three months of hospital release (yes/no)	3/8	0/40	**<0.0001**
ASPECTS at admission	8 (6-9)	9 (9-10)	**0.009**
ASPECTS after 24h	5 (3-8)	9 (8-10)	**<0.0001**
NIHSS at admission	15 (6-38)	5 (4-9.75)	**0.009**
NIHSS score change within 24h	6 (0 – 17)	3 (1-9.5)	0.558
NIHSS after 24h	38 (22-38)	5.5 (3.25-13)	**0.0002**
NIHSS after 48h	31 (22-38)	5 (2-9.75)	**<0.0001**
NIHSS after 72h	30 (13.75-38)	4.5 (1.25-7.5)	**0.0002**
NIHSS at discharge	8 (1-15)	3 (1-6)	0.381
mRS at discharge	6 (5-6)	3 (1-4)	**<0.0001**
mRS after 90 days	6 (6-6)	2 (1-3)	**<0.0001**

NfL showed significantly lower levels in serum in survivors than in non-survivors on every measured day and before therapy, reaching peak concentration on the 5th day of the measured episode [non-survivors 721.5 pg/ml (IQR 378.5 – 953) vs. survivors 179 pg/ml (IQR 85.53 – 301.3)]. Comparing both groups, GFAP was significantly elevated in non-survivors only on 2nd (p = 0.011), 3rd (p = 0.008) and 5th day (p = 0.002), whereas no significant difference was detected before therapy and on the first day. The peak concentration in both groups was reached on the 3rd day [Non – survivors 19.26 ng/ml (IQR 8.34- 48.04) vs. survivors 2.47 ng/ml (IQR 1–7.38)]. Regarding β-synuclein concentration, a significant difference between survivors and non-survivors was observed on every measured day, with peak concentration on the 3rd day in non – survivors [69.57 pg/ml (IQR 14.04 – 134.5)] and on the 5th day in survivors [13.61 pg/ml (IQR 6.127 – 36.51)]. No statistically significant difference was found in serum samples before therapy (p = 0.771). Further information is provided in Table 9. The respective biomarker levels in the serum with a level of significance are shown in Figure 10.

Table 9. Serum biomarker concentrations in survivors vs. non-survivors

	Only AIS		***p* - value**
	Non-survivors (n=11)	**Survivors (n=40)**	***p* - value survivors vs. non-survivors**
NfL_0 (pg/ml)	115 (66.2 - 393.5)	38.50 (19.10 - 66.3)	**0.010**
NfL_1 (pg/ml)	297 (75.70 - 545.8)	62.7 (25.13 - 114)	**0.009**
NfL_2 (pg/ml)	349 (138.6 - 521.5)	102.5 (58.28 - 147)	**0.008**
NfL_3 (pg/ml)	352.5 (222.3 - 515.3)	124 (52.25 - 224.5)	**0.007**
NfL_5 (pg/ml)	721.5 (378.5 - 953)	179 (85.53 - 301.3)	**0.0004**
$GFAP_0$ (ng/ml)	0.88 (0.39 - 1.80)	0.36 (0.25 - 0.56)	0.062
$GFAP_1$ (ng/ml)	4.65 (1.76 - 11.62)	1.52 (0.51 - 3.81)	0.097
$GFAP_2$ (ng/ml)	15.45 (3.72 - 136.1)	1.98 (0.82 - 6.59)	**0.011**
$GFAP_3$ (ng/ml)	19.26 (8.34 - 48.04)	2.47 (1 - 7.38)	**0.008**
$GFAP_5$ (ng/ml)	4.78 (13.40 - 415.4)	1.73 (0.83 - 5.11)	**0.002**
$β\text{-}syn_0$ (pg/ml)	7.35 (2.35 - 25.86)	5.33 (2.71 - 10.99)	0.771
$β\text{-}syn_1$ (pg/ml)	22.89 (7.22 - 57.26)	8.44 (2.47 - 19.40)	**0.025**
$β\text{-}syn_2$ (pg/ml)	55.65 (17.73 - 205.3)	10.65 (4.59 - 21.58)	**0.002**
$β\text{-}syn_3$ (pg/ml)	69.57 (14.04 - 134.5)	12.45 (4.47 - 29.54)	**0.009**
$β\text{-}syn_5$ (pg/ml)	64.29 (20.02 - 217.1)	13.61 (6.13 - 36.51)	**0.009**

The ROC curve data for all blood biomarkers in the differential diagnosis between survivors and non-survivors at 90 days follow-up are presented in Table 10. The ROC curve for all serum biomarkers and days is graphically displayed in Figure 9.

We compared Nfl, GFAP, and β-synuclein levels at each assessed time point for accuracy in distinguishing between survivors and non-survivors after 90 days. NfL provided higher diagnostic accuracy among the biomarkers before treatment [AUC 0.861 (0.67-1.0 CI 95%)], on 1st [AUC 0.765 (0.57-0.96 CI 95%)], 2nd [AUC 0.847 (0.71-0.99 CI 95%)], 3rd [0.866 (AUC 0.057 CI 95%)], and 5th [AUC 0.875 0.77 to 0.98 CI 95%)] day. In the discrimination between survivors and non-survivors, ROC curve analysis demonstrated the highest accuracy among all measured biomarkers for serum NfL on 5th day.

At a cut-off of 258.5 pg/ml, calculated by means of the maximized Youden's index, a sensitivity of 71.5 % and a specificity of 100% were achieved.

Table 10. The ROC curve data of all serum biomarkers in the differential diagnosis between survivors vs. non survivors at 90-day follow-up

90-day mortality	AUC	Std. Error	95% CI	*p* - value	Cut-off	Sens.%	95% CI	Spec.%	95% CI	Youden	Likelihood ratio
NfL$_0$	0.861	0.097	0.67 to 1.0	0.013	94.45	91.3	73.20% to 98.45%	80.0	37.55% to 98.97%	171.3	4.565
NfL$_1$	0.765	0.102	0.57 to 0.96	0.010	219.5	95.0	83.50% to 99.11%	60.0	31.27% to 83.18%	155.0	2.375
NfL$_2$	0.847	0.073	0.71 to 0.99	0.001	343.5	97.5	87.12% to 99.87%	55.56	26.67% to 81.12%	153.06	2.194
NfL$_3$	0.866	0.057	0.75 to 0.98	0.001	179.5	67.5	52.02% to 79.92%	100	67.56% to 100.0%	167.5	-
NfL$_5$	0.875	0.052	0.77 to 0.98	0.001	258.5	71.5	55.24% to 83.00%	100	67.56% to 100.0%	171.5	-
GFAP$_0$	0.772	0.166	0.45 to 1.0	0.088	0.8077	91.3	73.20% to 98.45%	75.0	30.06% to 98.72%	166.3	3.652
GFAP$_1$	0.650	0.098	0.46 to 0.84	0.163	1.902	60.0	44.60% to 73.65%	77.78	45.26% to 96.05%	137.78	2.7
GFAP$_2$	0.770	0.092	0.59 to 0.95	0.012	4.225	72.5	57.17% to 83.89%	77.78	45.26% to 96.05%	150.28	3.263
GFAP$_3$	0.794	0.071	0.65 to 0.93	0.009	6.600	75.0	59.81% to 85.81%	87.5	52.91% to 99.36%	162.5	6.0
GFAP$_5$	0.839	0.061	0.72 to 0.96	0.003	2.185	65.79	49.89% to 78.79%	100.0	67.56% to 100.0%	165.79	-
β - syn$_0$	0.548	0.160	0.23 to 0.86	0.742	19.11	86.96	67.87% to 95.46%	40.0	7.107% to 76.93%	126.96	1.449
β - syn$_1$	0.730	0.083	0.57 to 0.89	0.026	<16.72	75.0	59.81% to 85.81%	70.0	39.68% to 89.22%	145.0	2.5
β - syn$_2$	0.828	0.074	0.68 to 0.97	0.002	< 22.00	77.5	62.50% to 87.68%	77.78	45.26% to 96.05%	155.28	3.488
β - syn$_3$	0.778	0.084	0.61 to 0.94	0.010	< 65.94	90.0	76.95% to 96.04%	55.56	26.67% to 81.12%	145.56	2.025
β - syn$_5$	0.788	0.078	0.63 to 0.94	0.010	<16.16	52.5	37.50% to 67.06%	100.0	67.56% to 100.0%	152.5	-

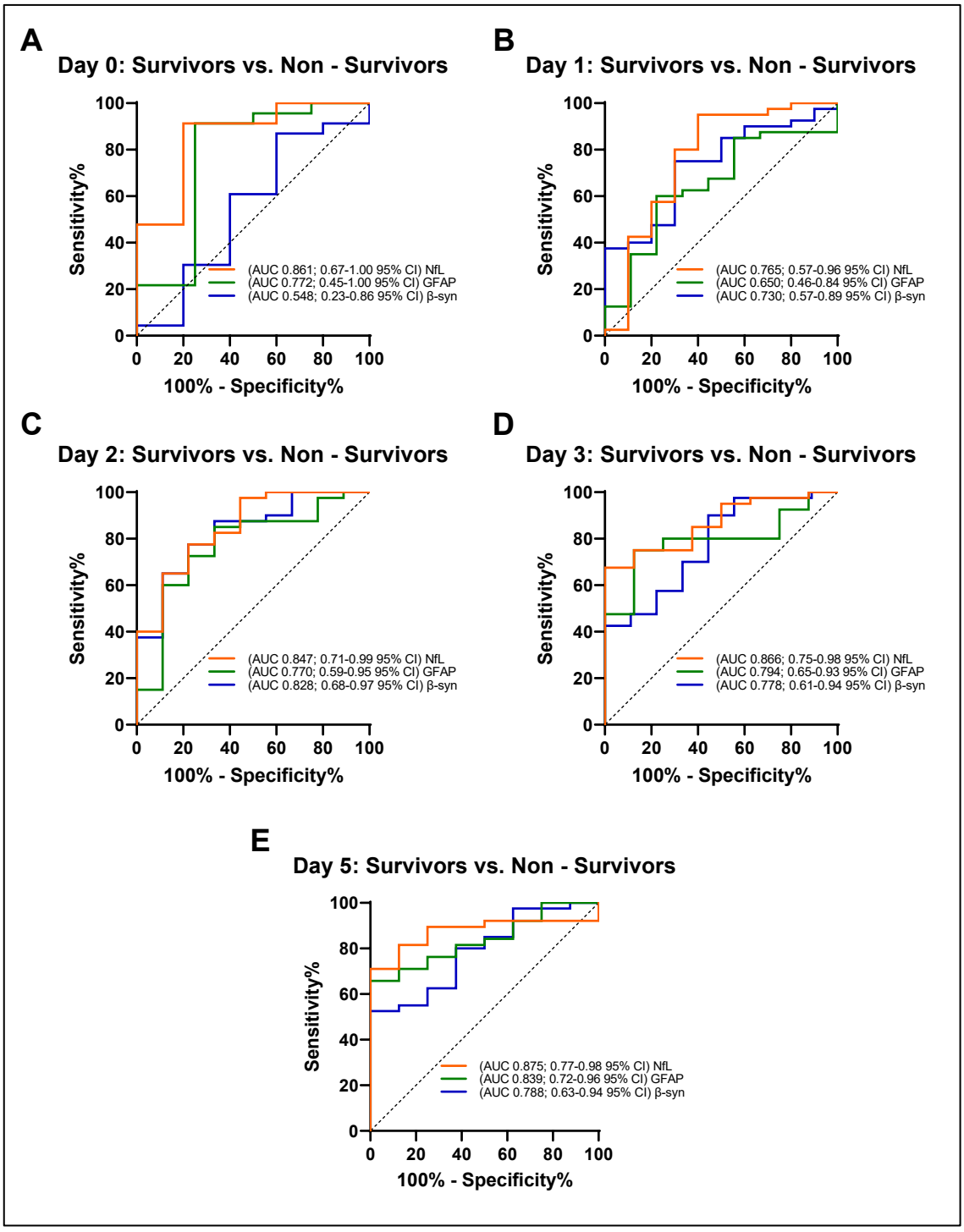


Figure 10. Receiver operating characteristic analysis. ROC curves relative to serum NfL, GFAP and β-synuclein for discrimination of survivors vs. non-survivors at 90-day follow-up

(A) Day 0, (B) Day 1, (C) Day 2, (D) Day 3, (E) Day

4.7 Serum biomarkers and clinical data in relation to 24h NIHSS score change

A NIHSS score change of ≥ 4 in the first 24 h was observed in 25 patients and a score change of <4 in 26 patients. No statistically significant differences were observed between the groups in terms of sex, age, time from onset to blood sampling, ASPECTs (admission and after 24h), creatinine levels, treatment, and death rate. Statistically significant discernability in NIHSS score at admission [change ≥ 4 = 10 (IQR 5-18) and change < 4 = 5 (IQR 2.75-7.25)], 24 (p = 0.001), 48 (p = 0.002), and 72 hours after admission (p = 0.008) and at discharge admission [change ≥ 4 = 4 (IQR 2-14.5) and change < 4 = 1.5 (IQR 1-3.25)] was observed between patients with NIHSS score change of < 4 in comparison to ≥ 4. Detailed information is presented in Table 11.

Table 11. Clinical, radiological, and outcome data of 24h NIHSS score change < 4 vs. ≥ 4

Only AIS	**NIHSS score change within 24h ≥4 (n=25)**	**NIHSS score change within 24h <4 (n=26)**	***p* - value NIHSS score change <4 vs. ≥4**
Age (years)*	71.56 (± 16.52)	72.81 (±14.37)	0.959
Females/males	8/17	12/14	0.301
Time from onset to blood sampling	6h (2h-15h45min)	6h30min (2h52min - 13h15min)	0.944
Creatinine (mg/dl)	86 (67.5 - 96.5)	91 (74.75 - 106.5)	0.106
MT (yes/no)	11/14	7/19	0.202
IVT (yes/no)	12/13	17/9	0.210
IVT+MT (yes/no)	3/22	4/22	0.725
Death within hospital stay (yes/no)	4/21	4/22	0.952
Death within three months of hospital release (yes/no)	2/23	1/25	0.529
ASPECTS at admission	9 (8 - 10)	9 (9 - 10)	0.390
ASPECTS after 24h	8.5 (7 - 9)	9 (8 - 10)	0.123
NIHSS at admission	10 (5 - 18)	5 (2.75 - 7.25)	**0.022**
NIHSS score change within 24h	11 (5 - 17)	1 (0 - 3)	**<0.0001**
NIHSS after 24h	18 (8 - 32)	4 (3 - 7.5)	**0.001**
NIHSS after 48h	13 (6 - 26)	4 (2 - 6.5)	**0.002**
NIHSS after 72h	11 (5 - 22)	3 (1 - 6)	**0.008**
NIHSS at discharge	4 (2 - 14.5)	1.5 (1 - 3.25)	**0.001**
mRS at discharge	4 (3 - 5)	2.5 (1 - 3)	**0.006**
mRS after 90 days	4 (2 - 5.5)	1 (1 - 3)	**0.006**

In patients experiencing NIHSS score change < 4 in the first 24 hours, significantly lower NfL concentrations were observed on the 3rd [change < 4 = 110 pg/ml (IQR 48.3-215); change ≥ 4 =191 pg/ml (IQR 91.70-361)] and 5th day [change < 4 = 176.5 pg/ml (IQR 48.75-293); change ≥ 4 =285 pg/ml (IQR 148.5-601)] in comparison to patients with a change ≥ 4. The NfL concentration peaked in both groups on the 5th day. No significant differences (p < 0.05) were observed in the NfL concentration before therapy, 1st and 2nd day. Looking at GFAP concentration, we observed statistically significant discernability on 1st (p = 0.014), 2nd (p = 0.003) and 3rd day (p = 0.018), but not on 5th day and before therapy. Peak concentrations were measured on the 2nd day in patients with a change ≥ 4 in 24 hours [4.47 ng/ml (IQR 2.38-29.16)] and on the 5th day in those with a change < 4 [1.83 ng/ml (IQR 0.55-6.67)]. We observed no statistically significant differences in β-synuclein measurements at any time point between groups. Peak concentrations in both groups were reached on 5th day [change < 4 = 10.70 pg/ml (IQR 5.873-49.06); change ≥ 4 = 21.85 pg/ml (IQR 9.231-67.19)]. Detailed information is presented in Table 12. The respective biomarker levels of NfL, GFAP, and β-syn with levels of statistical significance as temporal patterns are displayed in Figure 11.

Table 12. Serum biomarker concentrations in 24h NIHSS score change < 4 vs. ≥ 4

	Only AIS		**p-value**
	NIHSS score change within 24h ≥ 4 (n=25)	**NIHSS score change within 24h < 4 (n=26)**	**p-value NIHSS score change < 4 vs. ≥ 4**
NfL_0 (pg/ml)	53.55 (16.10 - 67.13)	34.95 (20.90 - 97.73)	0.837
NfL_1 (pg/ml)	89.3 (45.65 - 189.5)	75.90 (24.10 - 120)	0.399
NfL_2 (pg/ml)	129 (63.95 - 297.8)	114 (58.95 - 151.1)	0.333
NfL_3 (pg/ml)	191 (91.70 - 361)	110 (48.3 - 215)	**0.025**
NfL_5 (pg/ml)	285 (148.5 - 601)	176.5 (48.75 - 293)	**0.031**
$GFAP_0$ (ng/ml)	0.53 (0.26 - 0.65)	0.36 (0.19 - 0.68)	0.507
$GFAP_1$ (ng/ml)	2.82 (1.45 - 9.06)	1.17 (0.46 - 2.62)	**0.014**
$GFAP_2$ (ng/ml)	4.47 (2.38 - 29.16)	1.31 (0.48 - 3.85)	**0.003**
$GFAP_3$ (ng/ml)	4.28 (2.26 - 22.96)	1.50 (0.69 - 12.29)	**0.018**
$GFAP_5$ (ng/ml)	2.17 (1.28 - 14.42)	1.83 (0.55 - 6.67)	0.171
$β\text{-}syn_0$ (pg/ml)	6.34 (2.65 - 14.54)	5.17 (3.04 - 16.49)	0.909
$β\text{-}syn_1$ (pg/ml)	12.37 (3.82 - 23.57)	7.79 (2.64 - 25.94)	0.564
$β\text{-}syn_2$ (pg/ml)	13.48 (7.77 - 48.16)	10.02 (4.22 - 35.89)	0.185
$β\text{-}syn_3$ (pg/ml)	21.52 (9.23 - 59.67)	9.62 (4.32 - 31.69)	0.095
$β\text{-}syn_5$ (pg/ml)	21.85 (9.23 - 67.19)	10.70 (5.87 - 49.06)	0.259

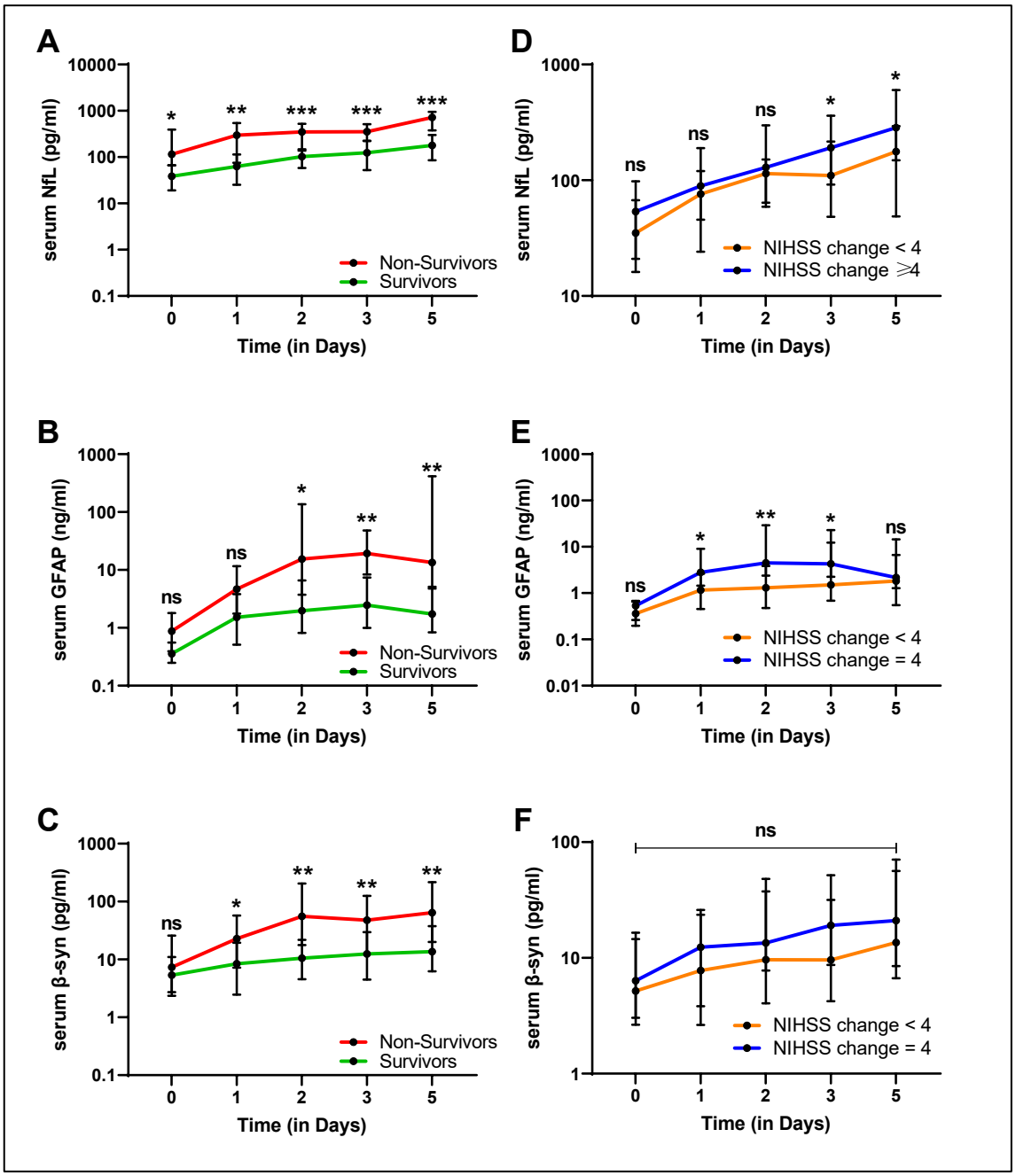


Figure 11. Comparison of serum biomarker concentrations in survivors vs. non-survivors (A-C) and 24h NIHSS score change < 4 vs. ≥ 4 (D-F)

(A,D) Temporal pattern of serum NfL (pg/ml); (B,E) Temporal pattern of serum GFAP (ng/ml), (C,F) Temporal pattern of serum β-synuclein (pg/ml) ^{ns}not significant *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

5 Discussion

In this single-center pilot study, we described for the first time the longitudinal dynamics of a panel of serum biomarkers (NfL, GFAP, and β-synuclein) in a well-characterized cohort of patients with AIS and assessed their potential prognostic value.

5.1 Association of biomarkers with diagnostic, clinical and treatment groups

First, we found significantly increased levels of NfL and GFAP, but not of β-syn, in patients with AIS compared to those in patients with TIA. On the one hand, our findings corroborate reports from several previous studies on serum NfL, which was described to be elevated during the acute phase of AIS (82–84). On the other hand, this study deepens our knowledge on GFAP in AIS. Indeed, GFAP serum levels were found to be unchanged in a previous study comparing patients with AIS and stroke mimics (85). Instead, studies have demonstrated substantially higher GFAP levels in patients with ICH than in AIS, with up to 16-fold elevation (61,86). Indeed, in the hyperacute phase of ICH, the increase in GFAP level in serum is supposed to be due to the rapid blood-brain-barrier disruption and subsequent diffusion of CSF/brain proteins into peripheral blood occurring quicker than in AIS (60). In our study, the small sample size of patients with ICH recruited (n=3) hampered the analyses of GFAP in ICH. Moreover, we provided the first data on the temporal trajectories of a novel marker of synaptic damage (β-synuclein) after AIS, which has only been explored in a small exploratory cohort to date (n=30) (72).

In addition, we explored the associations between treatment options and biomarkers and found an overall increase in GFAP and β-syn levels in patients undergoing MT (both in combination with IVT and alone) compared to patients who did not receive MT. However, previous reports have demonstrated a biochemical positive effect of acute treatment for AIS (i.e., reduced biomarker levels after treatment) (72,87,88). This finding could be attributed to the different clinical and radiological characteristics of the subgroups. Indeed, in our cohort, patients who underwent MT had significantly reduced ASPECTS and higher NIHSS scores at baseline, which correlated with higher marker concentrations. As an alternative explanation, histological studies on thrombus composition have highlighted the mechanisms through which IVT may help dissolve smaller thrombi, in addition to the single thrombi removed with MT (89). Precisely, IVT-induced thinning of superficial fibrin layers may create a more porous structural

composition of the clot (89–91). Furthermore, an increased sensitivity of red blood cell-rich thrombi towards IVT compared to fibrin-rich thrombi has been demonstrated (91–93).

Patients experiencing NIHSS score change < 4 in the first 24 hours showed significantly lower concentrations of serum NfL_3, NfL_5 and $GFAP_{1-3}$ compared to patients with a NIHSS change ≥ 4. Serum β-synuclein showed no significant discrimination between the two compared groups. Barba et al. demonstrated higher levels of serum β-synuclein and NfL in AIS patients with a 24-h NIHSS change ≥ 4 compared to those with a change < 4 (72). In accordance with our results, we hypothesized that a rapid clinical change increases blood biomarker levels in patients with AIS.

5.2 Correlation between biomarkers and clinical data

On another issue, serum levels of NfL, GFAP and β-syn were moderately to strongly correlated with each other at multiple time points. This finding is consistent with previous reports of traumatic brain injury (TBI) (62). Here, plasma levels of GFAP and β-syn were significantly associated with each other, while no association between β-syn and NfL was demonstrated. Moreover, our results support previous findings on AIS using the same biomarkers measured on day 1 after AIS (72). Hence, these multiple correlations between markers reflecting different pathophysiological mechanisms may indicate that glial activation, neuroaxonal damage, and synaptic damage co-occur after AIS and contribute to the clinical outcome.

Furthermore, increasing age showed a significant positive correlation with serum GFAP and NfL concentrations but not with β-syn. The association between age and NfL levels in the blood has been described in studies with a yearly 2.2% increase in healthy controls between the ages of 18 and 70 years, as well as for GFAP in patients following TBI (65,94–97). However, whether this may apply also in AIS is not univocally elucidated, given that plasma NfL levels were not associated with age in a previous study of 60 AIS patients undergoing MT (87).

Of great interest, serum biomarker levels were well correlated with clinical (i.e., NIHSS) and radiological (i.e., ASPECTS) scores of disease severity. No correlations were found between NIHSS changes within 24 h and creatinine in relation to serum biomarker levels. On the one hand, the observed correlation between measured serum markers and ASPECTS suggests a direct connection between extent of ischemic injury

and biomarker levels. The strongest correlations were found from 2nd day onwards, consistent with previous studies associating blood biomarker levels and infarct volume after 48h (52,72,98). On the other hand, higher biomarker concentrations may indicate an overall greater structural damage burden which underlie a more disabling disease (i.e., higher NIHSS scores). In fact, we also observed that patients with rapid clinical improvement (as determined by the NIHSS score change within 24 hours) had lower NfL and GFAP concentrations in serum. This suggests a close relationship between the biochemical and clinical progression of the disease. Coherently, levels of serum GFAP and NfL were strongly associated with the severity of neurological deficits assessed by NIHSS score (52,55,59,83). Further, in a study of 286 AIS patients, elevated serum GFAP levels measured at baseline were significantly associated with an increased risk of an NIHSS score > 6 (64). The analysis of 211 AIS patients showed serum NfL values to be 22.9% higher in cases of NIHSS > 4 than in cases of NIHSS ≤ 4 (55). These findings are even more interesting because of the lack of a strict association between the NIHSS score and ASPECTS (99). Hence, such biomarkers may have distinct value in the clinical and radiological evaluation of AIS. Sellner et al. observed no correlation between serum neurofilament levels of NIHSS in 16 AIS patients (100). This might potentially be caused by limited sample size (n=18) and the utilization of NfH instead of NfL. Correlating clinical data, a moderate-to-strong relationship between NIHSS score, NIHSS change, ASPECTS, and mRS was demonstrated. No correlation was found looking at age in relation to NIHSS, ASPECTS and mRS.

5.3 Serum biomarkers and functional outcome

As a main result of our study, NfL, GFAP, and β-syn serum concentrations assessed at virtually all time points were significantly higher in patients with bad (i.e., mRS 3-6) vs. good (i.e., mRS 0-2) clinical outcomes at the 3-month follow-up. Of note, in the overall discrimination between these two groups, the highest accuracy was demonstrated for serum GFAP on day 3 with a sensitivity of 64% and a specificity of 91.3% with an optimal cut-off value of 2.005 ng/ml.

However, the relationship between blood biomarkers and functional outcomes can be of ambivalent interpretation. Evidence from the literature supports our findings of significant associations between NfL and GFAP and middle- and long-term functional outcomes (55,63,65,66,72,87,88,101).

However, other authors could not replicate these results in a cohort of 408 patients with AIS after adjusting for age, NIHSS score, and infarct size (52). Here, the stroke severity at baseline [median NIHSS at admission: 5 (IQR 2-10)] was similar to that of our cohort [median NIHSS at admission: 6 (IQR 4-15)]. Thus, differences may be due to other factors, such as stroke etiologies and/or localization (i.e., anterior or posterior circulation), different methods for biomarker quantification as well as coexisting disorders, such as heart or renal diseases, diabetes mellitus and others that are known influencing factors of GFAP and NfL blood concentrations (56,102). For example, some studies were conducted using electrochemiluminescence-based immunoassays, which were proven to be less sensitive than the Simoa or Ella systems (103,104). Hence, especially in minor strokes, where the burden of neuronal damage is expected to be lower, it might be challenging to differentiate between stroke- and comorbidity-related elevation in serum biomarkers.

In terms of overall mortality at 90 days, serum levels of $NfL_{0\text{-}5}$, $GFAP_{2\text{-}5}$ and $\beta\text{-syn}_{0\text{-}5}$ were significantly lower in stroke survivors compared to non-survivors. In the discrimination between survivors and non-survivors, serum NfL_5 demonstrated the highest accuracy among all measured biomarkers and time points, with a sensitivity of 71.5% and a specificity of 100% at a cut-off of 258.5 pg/ml. The association between mortality and biomarker levels aligns well with previous studies, particularly regarding NfL (72,105). The assessment of such biomarkers during the acute phase after AIS may aid clinicians in identifying patients at a higher risk of all-cause mortality at follow-up.

5.4 Longitudinal dynamics of serum biomarkers

Of great relevance, we observed in patients with AIS peak serum levels of NfL and of β-syn at day 5 and of GFAP at day 3. After separating AIS patients according to mRS at the 90-day follow-up, serum levels of NfL still peaked on the 5th day in both groups and GFAP and β-syn in the poor outcome group (mRS 3-6) on 3rd and 5th day, respectively. In the good functional outcome group (mRS 0-2), serum GFAP and β-syn reached peak concentrations earlier on the 2nd day. Serum NfL levels peaked on the 5th day in all treatment groups. Peak serum GFAP concentration was reached on the 3rd day in patients treated with MT or in those without therapy, on the 2nd day in patients treated with IVT only, and on the 5th day in those treated with IVT+MT. The peak concentration of β-syn in serum was reached on the 1st day in IVT patients, on the 2nd

day in IVT+MT patients, on the 3rd day in patients receiving no treatment, and on the 5th day in MT patients.

In a study of AIS patients with serum NfL measurements on 1st, 2nd, 3rd and 7th day after admission to hospital admission, peak concentrations were measured on the 7th day (56). Pedersen et al. demonstrated an increase in serum NfL in 320 AIS patients within the first 2 weeks, with a peak at 3 months, followed by a decrease to control patient's level at the 7th year (54). Neurofilaments can be abundantly found in myelinated axons that are prone to stroke-induced Wallerian degeneration, representing anterograde axonal and myelin sheath deterioration (106,107). Furthermore, a study demonstrated a correlation between a MRI – based quantitative measure of secondary neurodegeneration and serum NfL obtained 6 months after stroke (56). Within this frame, the constant increase in blood NfL during the subacute and chronic phases post-event may indicate secondary neurodegenerative processes, which frequently occur after AIS and affect functional recovery and cognitive decline (108).

The observed longitudinal dynamics of GFAP, however, suggest that the post-ischemic inflammatory response can be detected early in the disease course. In previous studies, the peak concentration of serum GFAP was between 48h and 72 h after AIS onset, similar to the findings of our study (66,109). The brain cells and the blood-brain barrier can maintain their structural integrity for an extended period, resulting in brain cell death from necrosis and lysis occurring 6-12 hours after the onset of blood vessel blockage in ischemic stroke (59). GFAP is a marker of astrocyte activation and injury (57,59). Excessive activation of reactive astrocytes induces the production of proinflammatory and cytotoxic cytokines, posing a threat to neurons and oligodendrocytes in the injured brain (110). The exact contribution of astrogliosis in the development of cerebral ischemic lesions is not yet fully understood, but the disruption of astrocytes is associated with a reduced immune response of the body, exacerbating the inflammatory cascade and ultimately leading to an expansion in infarct volume (111,112).

Finally, this is the first study to longitudinally analyze a biomarker of synaptic damage/dysfunction in the serum (β-syn) of AIS patients. Preliminary data were provided for TBI on plasma β-syn quantified in serial samples on hospital admission and after 24h, 5 and 10 days (62). Here, peak plasma concentrations of β-syn were reached 24h after admission [median: 34.1 pg/ml (IQR 16.6-119)] with steady decrease

afterwards (62). Considering also the correlation with the severity of TBI, high blood β-syn levels have been suggested to indicate early synaptic disruption occurring as a consequence of head trauma. In comparison to TBI, we found that the peak concentration of serum β-syn was delayed from day 2 to day 5 only in patients with an mRS 3-6. This suggests that long-lasting synaptic damage after severe AIS may be one of the main pathophysiological mechanisms leading to worse clinical outcomes. Such biomarkers may be used to monitor experimental therapies targeting synaptic integrity and/or other studies assessing synaptic plasticity after AIS (113).

5.5 Biomarker cut-off-values in cerebrovascular diseases

As one of the main factors limiting the use of blood biomarkers in the management of patients with cerebrovascular diseases, reliable cut-off values for NfL and GFAP have not yet been validated. Using a serum GFAP cut-off of 0.34 ng/ml, Ren et al. yielded an AUC of 0.86 with a sensitivity of 61% and a specificity of 96% for distinguishing between ischemic and hemorrhagic stroke within 4.5 hours of symptom onset (114). At a cut-off point of 0.15 µg/L, serum GFAP predicted unfavorable outcomes after 1 year (measured by Fisher grade on CT and the World Federation of Neurological Surgeons subarachnoid hemorrhage scale) with a sensitivity of 92% and specificity of 40% in patients with aneurysmal SAH (115). In a study of 64 patients with AIS, serum GFAP concentration at a cut-off value of 0.112 ng/ml within 72 hours predicted more severe stroke (NIHSS of 16-42) until day 7 (63). For the discrimination between good and bad functional outcomes at the 90-day follow-up by mRS, our calculated serum GFAP cut-off of 2.005 ng/ml with a sensitivity of 64% and a specificity of 91.3% noticeably exceeded the absolute concentration values of the mentioned study. One reason could be the methodological difference in biomarker measurement (e.g., assays, protocols) and second, the difference in choice of outcome measure (e.g. mRS of 0-2 or 0-1 as good outcome). AIS patients with a serum NfL concentration above 33 pg/ml measured 24 h after stroke experienced a significantly higher risk of recurrent stroke and death during a median follow-up of 41.8 months, as revealed by a study of Uphaus et al. (55). At a cut-off point of 49.35pg/ml serum NfL was shows to distinguish between AIS and TIA with a sensitivity of 73% and specificity of 80% with blood sampled approximately 63.8 ± 50.1 hours after hospital admission (83). This was confirmed by the serum NfL values measured in our AIS patients.

In the study of Vollmuth et al. a median serum NfL of 96 pg/ml (IQR 51-228) and $GFAP_1$ of 5.7 ng/ml (IQR 1.5-22) was measured in patients with a median NIHSS score of 13 (116). Barba et al. demonstrated a median serum NfL_1 of 50.4 pg/ml (IQR 34.0–113.1), $GFAP_1$ of 6.3 ng/ml (IQR 1.0-20.5) and β-syn_1 of 20.1 pg/ml (IQR 5.8–43.8) in AIS patients with a median NIHSS score of 14 (72).

Comparing the median values of serum NfL and GFAP with these studies using the same assays for measurement, our results align well (72,116). Instead, no other cut-off values for blood β-syn have been published to date in the literature. Serum NfL_1 in our study was a median of 79.4 pg/ml (IQR 26.6-153.5), $GFAP_1$ of 2.48 ng/ml (IQR 1.02-8.81) and β-syn_1 of 8.97 pg/ml (IQR 2.82-23.03). Hence, more harmonious and standardized methods for biomarker quantification and reference materials for inter-laboratory validation are required to make biomarker cut-offs usable for routine clinical purposes in AIS.

5.6 Strengths

The main strengths of this study were the deep clinical and biochemical characterization of the study population, as well as the collection of longitudinal samples during the first week after AIS.

The temporal pattern resulting from repeated measurements provided more detailed information about the association between serum biomarker levels and clinical progression as well as outcome measures. For example, the NIHSS score 24h after admission was more strongly correlated with serum biomarkers measured from the 2nd day onwards. Furthermore, the prognostic value of biomarkers for identifying patients with poor outcomes at follow-up (both overall mortality and mRS score) was highest when the biomarkers were quantified on days 3 or 5. Studies with a single measurement miss ideal serum levels for an association or correlation with clinical and prognostic data. This is of particular relevance, considering the rapid trajectories of such biomarkers after acute brain injury (62). The temporal pattern of biomarkers in AIS patients has been described extensively in serum NfL but varies greatly in papers about serum GFAP and has not been analyzed in β-syn before. Here, we provided further comparable results for first and novel results for the latter one.

Finally, we measured two of the three biomarkers with robust and reproducible methods, that is, commercially available immunoassays on the Ella platform (NfL) and

with Simoa technology. However, no commercial assays are currently available for the quantification of β-syn. Here, we used an in-house established assay that was previously published (71,72,116). Additionally, the systematic collection of clinical data using the REDcap web application offers an easy tool for including more participants in this project in the future.

Potential blood biomarkers have been described in previous years in stroke research (for example, NSE, S-100B), but many failed to show correlation with functional outcome (117,118). The choice of NfL, GFAP and β-syn is a strength of the study because they represent biomarkers that are of high interest in ongoing research. We took advantage of a panel of biomarkers to assess different pathophysiological mechanisms occurring after AIS, thus being able to monitor the burden of neuroaxonal, glial, and synaptic injury simultaneously. To our knowledge, this is the first study to assess this aspect in AIS.

5.7 Limitations

As the main limitation of this pilot study, we acknowledge the small sample size of our cohort (n=51), which hampers the generalizability of our results. However, most studies on serum biomarkers in AIS have included < 500 patients (54–56,83,84). Most of previous studies on temporal pattern of biomarkers in AIS are even performed with n <100 patients (54,66,109). In order to confirm our findings, it is essential to conduct further research with larger patient cohorts. The validation of presented results can only be achieved by internal and external replication in independent multicentric cohorts.

Another limitation is the lack of knowledge about the basal biomarker levels of the included patients prior to inclusion. Naturally, stroke onset cannot be precisely predicted. Hence, data on comorbidities that could possibly influence biomarker concentrations were only partly available. Indeed, cardiovascular (e.g. heart failure), renal and neurological comorbidities are known influencing factors for NfL and GFAP blood concentrations and may lead to biased interpretation of our results (60,102,119).

Third, quantitative neuroimaging parameters, such as the infarct size volume, were not available for the present study population. Indeed, given the assumption that biomarker levels may reflect the ongoing neuronal (e.g. NfL) or synaptic (e.g. β-synuclein) damage, future studies should better test associations with the cortical and subcortical lesion burden. Indeed, previous studies have reported significant correlations between infarct volume (assessed using MRI) and serum NfL levels measured after day 3 (56).

The decision to choose the ASPECTS over infarct volume was based on practical limitations. However, in a larger cohort of 1046 patients, the ASPECTS and infarct volume measured by CT perfusion imaging showed a strong correlation in large-vessel occlusion with a median ASPECTS of 9 (120). In other studies, especially MRI-based calculation of infarct volume (using DWI sequence), difference maps of lesions between baseline and follow-up (using FLAIR sequence) and quantification of microstructural damage within white matter tracts as measure of secondary neurodegeneration (using mean diffusivity maps) were performed (23,56,121).

Fourth, when assessing the relationship between biomarkers and mortality, data on the cause of death were not available. In previous reports from our group, serum NfL and β-syn were differentially associated with overall mortality and death only due to severe neurological complications (i.e. hemorrhagic transformation or malignant infarction), respectively (72). However, systemic complications, such as aspiration pneumonia, heart failure, and acute kidney injury, are among the most frequent complications and may contribute significantly to biomarker concentrations. Hence, future studies should better assess this aspect (122).

Fifth, we only focused on biomarker trajectories during the acute phase of AIS and did not assess biomarker concentrations at the follow-up. Indeed, NfL levels were demonstrated to increase steadily up to 3 weeks until 3 months after the acute event (56,98). For serum GFAP, the measured time points were sufficient to detect peak concentration, but for serum NfL, our data could only insufficiently identify a post-stroke peak. Hence, further studies on blood biomarkers in the post-acute and chronic phases of AIS are needed to better evaluate their prognostic role. For β-syn, no studies on the longitudinal release pattern of AIS have been conducted. Instead of solely assessing mRS at 90 days post-stroke by telephone interview, patients could have been invited to the hospital for a follow-up blood sampling and a neurological examination by experienced clinicians.

A general limitation not only to our but also to most biomarker studies is a lack of formally standardized reference material (e.g., plasma, serum) and the diversity of assays used to measure concentration. Hence, comparisons between studies are only indicative, and no reliable cut-off values can be calculated. For example, recent studies use commercially available kits from Simoa for GFAP measurement, but previous studies used individual in-house protocols.

A potential limitation of the study is the involvement of patients with mainly moderate and not severe stroke severity according to the NIHSS score [median NIHSS 6 (IQR 4-15)]. This unintended bias in patient selection might have emerged from the more convenient inclusion of patients who could provide informed consent and the anticipated survival for a follow-up assessment at the 90-day follow-up. An alternative explanation could be that patients who presented at the ER at the time of patient inclusion coincidentally presented with moderate NIHSS scores.

5.8 Conclusions

Serum NfL, GFAP and β-synuclein concentration measured within the first 5 days of symptom-onset in AIS showed significant prognostic capabilities for functional outcome (mRS) at 90-day follow-up. Serum biomarker levels were significantly associated with the clinical and radiological scores of disease severity. The diagnostic accuracy for discrimination of mRS at the 90-day follow-up was highest for GFAP on day 3 and for overall mortality in NfL on day 5. Biomarkers correlated with each other and revealed individual temporal patterns. Such biomarkers could be implemented in the routine assessment of patients with AIS for tailored decision making at an individual level. Further studies with larger sample sizes are required to validate our findings.

6 References

1. Murphy SJ, Werring DJ. Stroke: causes and clinical features. Med Abingdon Engl UK Ed. 2020 Sep;48(9):561–6.

2. Campbell BCV, Khatri P. Stroke. The Lancet. 2020 Jul 11;396(10244):129–42.

3. Kolominsky-Rabas PL, Weber M, Gefeller O, Neundoerfer B, Heuschmann PU. Epidemiology of Ischemic Stroke Subtypes According to TOAST Criteria. Stroke. 2001 Dec;32(12):2735–40.

4. Yaghi S, Bernstein RA, Passman R, Okin PM, Furie KL. Cryptogenic Stroke: Research and Practice. Circ Res. 2017 Feb 3;120(3):527–40.

5. Amarenco Pierre. Transient Ischemic Attack. N Engl J Med. 2020 May 14;382(20):1933–41.

6. H Buck B, Akhtar N, Alrohimi A, Khan K, Shuaib A. Stroke mimics: incidence, aetiology, clinical features and treatment. Ann Med. 2021 Dec;53(1):420–36.

7. Virani SS, Alonso A, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP, et al. Heart Disease and Stroke Statistics-2020 Update: A Report From the American Heart Association. Circulation. 2020 Mar 3;141(9):e139–596.

8. GBD 2021 Causes of Death Collaborators. Global burden of 288 causes of death and life expectancy decomposition in 204 countries and territories and 811 subnational locations, 1990-2021: a systematic analysis for the Global Burden of Disease Study 2021. Lancet Lond Engl. 2024 May 18;403(10440):2100–32.

9. GBD 2019 Stroke Collaborators. Global, regional, and national burden of stroke and its risk factors, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet Neurol. 2021 Oct;20(10):795–820.

10. Eyding J, Bartig D, Weber R, Katsanos AH, Weimar C, Hacke W, et al. Inpatient TIA and stroke care in adult patients in Germany - retrospective analysis of nationwide administrative data sets of 2011 to 2017. Neurol Res Pract. 2019;1:39.

11. Ungerer MN, Bartig D, Richter D, Krogias C, Hacke W, Gumbinger C. The evolution of acute stroke care in Germany from 2019 to 2021: analysis of nation-wide administrative datasets. Neurol Res Pract. 2024 Jan 11;6(1):4.

12. Kolominsky-Rabas PL, Heuschmann PU, Marschall D, Emmert M, Baltzer N, Neundörfer B, et al. Lifetime cost of ischemic stroke in Germany: results and national projections from a population-based stroke registry: the Erlangen Stroke Project. Stroke. 2006 May;37(5):1179–83.

13. Donkor ES. Stroke in the 21st Century: A Snapshot of the Burden, Epidemiology, and Quality of Life. Stroke Res Treat. 2018;2018:3238165.

14. Grefkes C, Fink GR. Recovery from stroke: current concepts and future perspectives. Neurol Res Pract. 2020;2:17.

15. Nouh A, Remke J, Ruland S. Ischemic posterior circulation stroke: a review of anatomy, clinical presentations, diagnosis, and current management. Front Neurol. 2014;5:30.

16. Maulaz AB, Bezerra DC, Bogousslavsky J. Posterior cerebral artery infarction from middle cerebral artery infarction. Arch Neurol. 2005 Jun;62(6):938–41.

17. Salerno A, Strambo D, Nannoni S, Dunet V, Michel P. Patterns of ischemic posterior circulation strokes: A clinical, anatomical, and radiological review. Int J Stroke. 2022 Aug 1;17(7):714–22.

18. Farooque U, Lohano AK, Kumar A, Karimi S, Yasmin F, Bollampally VC, et al. Validity of National Institutes of Health Stroke Scale for Severity of Stroke to Predict Mortality Among Patients Presenting With Symptoms of Stroke. Cureus. 2020 Sep 5;12(9):e10255.

19. Barber PA, Hill MD, Eliasziw M, Demchuk AM, Pexman JHW, Hudon ME, et al. Imaging of the brain in acute ischaemic stroke: comparison of computed tomography and magnetic resonance diffusion-weighted imaging. J Neurol Neurosurg Psychiatry. 2005 Nov;76(11):1528–33.

20. van Poppel LM, Majoie CBLM, Marquering HA, Emmer BJ. Associations between early ischemic signs on non-contrast CT and time since acute ischemic stroke onset: A scoping review. Eur J Radiol. 2022 Oct;155:110455.

21. Fischer U, Baumgartner A, Arnold M, Nedeltchev K, Gralla J, Marco De Marchis G, et al. What Is a Minor Stroke? Stroke. 2010 Apr;41(4):661–6.

22. Boode B, Welzen V, Franke C, van Oostenbrugge R. Estimating the Number of Stroke Patients Eligible for Thrombolytic Treatment if Delay Could Be Avoided. Cerebrovasc Dis. 2006 Dec 29;23(4):294–8.

23. Allen LM, Hasso AN, Handwerker J, Farid H. Sequence-specific MR imaging findings that are useful in dating ischemic stroke. Radiogr Rev Publ Radiol Soc N Am Inc. 2012;32(5):1285–97; discussion 1297-1299.

24. Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, et al. Guidelines for the Early Management of Patients With Acute Ischemic Stroke: 2019 Update to the 2018 Guidelines for the Early Management of Acute Ischemic Stroke: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. Stroke. 2019 Dec;50(12):e344–418.

25. Emberson J, Lees KR, Lyden P, Blackwell L, Albers G, Bluhmki E, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. Lancet Lond Engl. 2014 Nov 29;384(9958):1929–35.

26. Hacke W, Kaste M, Bluhmki E, Brozman M, Dávalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. N Engl J Med. 2008 Sep 25;359(13):1317–29.

27. IST-3 collaborative group, Sandercock P, Wardlaw JM, Lindley RI, Dennis M, Cohen G, et al. The benefits and harms of intravenous thrombolysis with recombinant tissue plasminogen activator within 6 h of acute ischaemic stroke (the third international stroke trial [IST-3]): a randomised controlled trial. Lancet Lond Engl. 2012 Jun 23;379(9834):2352–63.

28. Wardlaw JM, Murray V, Berge E, del Zoppo G, Sandercock P, Lindley RL, et al. Recombinant tissue plasminogen activator for acute ischaemic stroke: an updated systematic review and meta-analysis. Lancet Lond Engl. 2012 Jun 23;379(9834):2364–72.

29. Goyal M, Menon BK, van Zwam WH, Dippel DWJ, Mitchell PJ, Demchuk AM, et al. Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomised trials. Lancet Lond Engl. 2016 Apr 23;387(10029):1723–31.

30. Albers GW, Marks MP, Kemp S, Christensen S, Tsai JP, Ortega-Gutierrez S, et al. Thrombectomy for Stroke at 6 to 16 Hours with Selection by Perfusion Imaging. N Engl J Med. 2018 Feb 22;378(8):708–18.

31. Campbell BCV, Ma H, Ringleb PA, Parsons MW, Churilov L, Bendszus M, et al. Extending thrombolysis to 4·5-9 h and wake-up stroke using perfusion imaging: a systematic review and meta-analysis of individual patient data. Lancet Lond Engl. 2019 Jul 13;394(10193):139–47.

32. Nogueira RG, Jadhav AP, Haussen DC, Bonafe A, Budzik RF, Bhuva P, et al. Thrombectomy 6 to 24 Hours after Stroke with a Mismatch between Deficit and Infarct. N Engl J Med. 2018 Jan 4;378(1):11–21.

33. Thomalla G, Simonsen CZ, Boutitie F, Andersen G, Berthezene Y, Cheng B, et al. MRI-Guided Thrombolysis for Stroke with Unknown Time of Onset. N Engl J Med. 2018 Aug 16;379(7):611–22.

34. Vogt G, Laage R, Shuaib A, Schneider A, VISTA Collaboration. Initial lesion volume is an independent predictor of clinical stroke outcome at day 90: an analysis of the Virtual International Stroke Trials Archive (VISTA) database. Stroke. 2012 May;43(5):1266–72.

35. Banks JL, Marotta CA. Outcomes validity and reliability of the modified Rankin scale: implications for stroke clinical trials: a literature review and synthesis. Stroke. 2007 Mar;38(3):1091–6.

36. Hankey GJ, Spiesser J, Hakimi Z, Bego G, Carita P, Gabriel S. Rate, degree, and predictors of recovery from disability following ischemic stroke. Neurology. 2007 May 8;68(19):1583–7.

37. Pekny M, Wilhelmsson U, Stokowska A, Tatlisumak T, Jood K, Pekna M. Neurofilament Light Chain (NfL) in Blood-A Biomarker Predicting Unfavourable Outcome in the Acute Phase and Improvement in the Late Phase after Stroke. Cells. 2021 Jun 18;10(6):1537.

38. Gauthier LV, Taub E, Mark VW, Barghi A, Uswatte G. Atrophy of spared gray matter tissue predicts poorer motor recovery and rehabilitation response in chronic stroke. Stroke. 2012 Feb;43(2):453–7.

39. Kuchcinski G, Munsch F, Lopes R, Bigourdan A, Su J, Sagnier S, et al. Thalamic alterations remote to infarct appear as focal iron accumulation and impact clinical outcome. Brain J Neurol. 2017 Jul 1;140(7):1932–46.

40. Duering M, Righart R, Wollenweber FA, Zietemann V, Gesierich B, Dichgans M. Acute infarcts cause focal thinning in remote cortex via degeneration of connecting fiber tracts. Neurology. 2015 Apr 21;84(16):1685–92.

41. Langhorne P, Ramachandra S, Stroke Unit Trialists' Collaboration. Organized Inpatient (Stroke Unit) Care for Stroke: Network Meta-Analysis. Stroke. 2020 Dec;51(12):e349–50.

42. Kamtchum-Tatuene J, Jickling GC. Blood Biomarkers for Stroke Diagnosis and Management. Neuromolecular Med. 2019 Dec;21(4):344–68.

43. Cullen NC, Leuzy A, Janelidze S, Palmqvist S, Svenningsson AL, Stomrud E, et al. Plasma biomarkers of Alzheimer's disease improve prediction of cognitive decline in cognitively unimpaired elderly populations. Nat Commun. 2021 Jun 11;12(1):3555.

44. Jack CR, Bennett DA, Blennow K, Carrillo MC, Dunn B, Haeberlein SB, et al. NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. Alzheimers Dement J Alzheimers Assoc. 2018 Apr;14(4):535–62.

45. Oeckl P, Anderl-Straub S, Danek A, Diehl-Schmid J, Fassbender K, Fliessbach K, et al. Relationship of serum beta-synuclein with blood biomarkers and brain atrophy. Alzheimers Dement. 2023;19(4):1358–71.

46. Montellano FA, Ungethüm K, Ramiro L, Nacu A, Hellwig S, Fluri F, et al. Role of Blood-Based Biomarkers in Ischemic Stroke Prognosis: A Systematic Review. Stroke. 2021 Jan;52(2):543–51.

47. Neurofilaments as biomarkers in neurological disorders - towards clinical application - PubMed [Internet]. [cited 2024 May 13]. Available from: https://pubmed.ncbi.nlm.nih.gov/38609644/

48. Yuan A, Rao MV, Veeranna null, Nixon RA. Neurofilaments and Neurofilament Proteins in Health and Disease. Cold Spring Harb Perspect Biol. 2017 Apr 3;9(4):a018309.

49. Lee Y, Lee BH, Yip W, Chou P, Yip BS. Neurofilament Proteins as Prognostic Biomarkers in Neurological Disorders. Curr Pharm Des. 2020;25(43):4560–9.

50. Lu CH, Petzold A, Topping J, Allen K, Macdonald-Wallis C, Clarke J, et al. Plasma neurofilament heavy chain levels and disease progression in amyotrophic lateral sclerosis: insights from a longitudinal study. J Neurol Neurosurg Psychiatry. 2015 May;86(5):565–73.

51. Sanchez JD, Martirosian RA, Mun KT, Chong DS, Llorente IL, Uphaus T, et al. Temporal Patterning of Neurofilament Light as a Blood-Based Biomarker for Stroke: A Systematic Review and Meta-Analysis. Front Neurol. 2022;13:841898.

52. De Marchis GM, Katan M, Barro C, Fladt J, Traenka C, Seiffge DJ, et al. Serum neurofilament light chain in patients with acute cerebrovascular events. Eur J Neurol. 2018 Mar;25(3):562–8.

53. Wang P, Fan J, Yuan L, Nan Y, Nan S. Serum Neurofilament Light Predicts Severity and Prognosis in Patients with Ischemic Stroke. Neurotox Res. 2020 Apr;37(4):987–95.

54. Pedersen A, Stanne TM, Nilsson S, Klasson S, Rosengren L, Holmegaard L, et al. Circulating neurofilament light in ischemic stroke: temporal profile and outcome prediction. J Neurol. 2019 Nov;266(11):2796–806.

55. Uphaus T, Bittner S, Gröschel S, Steffen F, Muthuraman M, Wasser K, et al. NfL (Neurofilament Light Chain) Levels as a Predictive Marker for Long-Term Outcome After Ischemic Stroke. Stroke. 2019 Nov;50(11):3077–84.

56. Tiedt S, Duering M, Barro C, Kaya AG, Boeck J, Bode FJ, et al. Serum neurofilament light: A biomarker of neuroaxonal injury after ischemic stroke. Neurology. 2018 Oct 2;91(14):e1338–47.

57. Barthel PC, Staabs F, Li LY, Buthut M, Otto C, Ruprecht K, et al. Immunoreactivity to astrocytes in different forms of dementia: High prevalence of autoantibodies to GFAP. Brain Behav Immun - Health. 2023 Mar 2;29:100609.

58. Kim H, Lee EJ, Lim YM, Kim KK. Glial Fibrillary Acidic Protein in Blood as a Disease Biomarker of Neuromyelitis Optica Spectrum Disorders. Front Neurol. 2022;13:865730.

59. Amalia L. Glial Fibrillary Acidic Protein (GFAP): Neuroinflammation Biomarker in Acute Ischemic Stroke. J Inflamm Res. 2021;14:7501–6.

60. Abdelhak A, Foschi M, Abu-Rumeileh S, Yue JK, D'Anna L, Huss A, et al. Blood GFAP as an emerging biomarker in brain and spinal cord disorders. Nat Rev Neurol. 2022 Mar;18(3):158–72.

61. Foerch C, Niessner M, Back T, Bauerle M, De Marchis GM, Ferbert A, et al. Diagnostic accuracy of plasma glial fibrillary acidic protein for differentiating intracerebral hemorrhage and cerebral ischemia in patients with symptoms of acute stroke. Clin Chem. 2012 Jan;58(1):237–45.

62. Halbgebauer R, Halbgebauer S, Oeckl P, Steinacker P, Weihe E, Schafer MKH, et al. Neurochemical Monitoring of Traumatic Brain Injury by the Combined Analysis of Plasma Beta-Synuclein, NfL, and GFAP in Polytraumatized Patients. Int J Mol Sci. 2022 Aug 25;23(17):9639.

63. Puspitasari V, Gunawan PY, Wiradarma HD, Hartoyo V. Glial Fibrillary Acidic Protein Serum Level as a Predictor of Clinical Outcome in Ischemic Stroke. Open Access Maced J Med Sci. 2019 May 15;7(9):1471–4.

64. Liu G, Geng J. Glial fibrillary acidic protein as a prognostic marker of acute ischemic stroke. Hum Exp Toxicol. 2018 Oct;37(10):1048–53.

65. Pujol-Calderón F, Zetterberg H, Portelius E, Löwhagen Hendén P, Rentzos A, Karlsson JE, et al. Prediction of Outcome After Endovascular Embolectomy in Anterior Circulation Stroke Using Biomarkers. Transl Stroke Res. 2022;13(1):65–76.

66. Wunderlich MT, Wallesch CW, Goertler M. Release of glial fibrillary acidic protein is related to the neurovascular status in acute ischemic stroke. Eur J Neurol. 2006 Oct;13(10):1118–23.

67. Barba L, Paolini Paoletti F, Bellomo G, Gaetani L, Halbgebauer S, Oeckl P, et al. Alpha and Beta Synucleins: From Pathophysiology to Clinical Application as Biomarkers. Mov Disord. 2022 Apr;37(4):669–83.

68. Carnazza KE, Komer LE, Xie YX, Pineda A, Briano JA, Gao V, et al. Synaptic vesicle binding of α-synuclein is modulated by β- and γ-synucleins. Cell Rep [Internet]. 2022 Apr 12 [cited 2024 Apr 25];39(2). Available from: https://www.cell.com/cell-reports/abstract/S2211-1247(22)00427-2

69. Hayashi J, Carver JA. β-Synuclein: An Enigmatic Protein with Diverse Functionality. Biomolecules. 2022 Jan 16;12(1):142.

70. Oeckl P, Halbgebauer S, Anderl-Straub S, von Arnim CAF, Diehl-Schmid J, Froelich L, et al. Targeted Mass Spectrometry Suggests Beta-Synuclein as Synaptic Blood Marker in Alzheimer's Disease. J Proteome Res. 2020 Mar 6;19(3):1310–8.

71. Halbgebauer S, Oeckl P, Steinacker P, Yilmazer-Hanke D, Anderl-Straub S, von Arnim C, et al. Beta-synuclein in cerebrospinal fluid as an early diagnostic marker of Alzheimer's disease. J Neurol Neurosurg Psychiatry. 2021 Apr;92(4):349–56.

72. Barba L, Vollmuth C, Abu-Rumeileh S, Halbgebauer S, Oeckl P, Steinacker P, et al. Serum β-synuclein, neurofilament light chain and glial fibrillary acidic protein as prognostic biomarkers in moderate-to-severe acute ischemic stroke. Sci Rep. 2023 Nov 28;13(1):20941.

73. Kwah LK, Diong J. National Institutes of Health Stroke Scale (NIHSS). J Physiother. 2014 Mar;60(1):61.

74. Kogan E, Twyman K, Heap J, Milentijevic D, Lin JH, Alberts M. Assessing stroke severity using electronic health record data: a machine learning approach. BMC Med Inform Decis Mak. 2020 Jan 8;20:8.

75. Pop NO, Tit DM, Diaconu CC, Munteanu MA, Babes EE, Stoicescu M, et al. The Alberta Stroke Program Early CT score (ASPECTS): A predictor of mortality in acute ischemic stroke. Exp Ther Med. 2021 Dec;22(6):1371.

76. Puetz V, Sylaja PN, Coutts SB, Hill MD, Dzialowski I, Mueller P, et al. Extent of hypoattenuation on CT angiography source images predicts functional outcome in patients with basilar artery occlusion. Stroke. 2008 Sep;39(9):2485–90.

77. Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a

multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke. 1993 Jan;24(1):35–41.

78. van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. Stroke. 1988 May;19(5):604–7.

79. Halbgebauer S, Abu-Rumeileh S, Oeckl P, Steinacker P, Roselli F, Wiesner D, et al. Blood β-Synuclein and Neurofilament Light Chain During the Course of Prion Disease. Neurology. 2022 Apr 5;98(14):e1434–45.

80. Chan YH. Biostatistics 104: correlational analysis. Singapore Med J. 2003 Dec;44(12):614–9.

81. Youden WJ. Index for rating diagnostic tests. Cancer. 1950 Jan;3(1):32–5.

82. Hjalmarsson C, Bjerke M, Andersson B, Blennow K, Zetterberg H, Aberg ND, et al. Neuronal and glia-related biomarkers in cerebrospinal fluid of patients with acute ischemic stroke. J Cent Nerv Syst Dis. 2014;6:51–8.

83. Onatsu J, Vanninen R, Jäkälä P, Mustonen P, Pulkki K, Korhonen M, et al. Serum Neurofilament Light Chain Concentration Correlates with Infarct Volume but Not Prognosis in Acute Ischemic Stroke. J Stroke Cerebrovasc Dis Off J Natl Stroke Assoc. 2019 Aug;28(8):2242–9.

84. Pujol-Calderón F, Portelius E, Zetterberg H, Blennow K, Rosengren LE, Höglund K. Neurofilament changes in serum and cerebrospinal fluid after acute ischemic stroke. Neurosci Lett. 2019 Apr 17;698:58–63.

85. Kalra LP, Khatter H, Ramanathan S, Sapehia S, Devi K, Kaliyaperumal A, et al. Serum GFAP for stroke diagnosis in regions with limited access to brain imaging (BE FAST India). Eur Stroke J. 2021 Jun;6(2):176–84.

86. Luger S, Witsch J, Dietz A, Hamann GF, Minnerup J, Schneider H, et al. Glial Fibrillary Acidic Protein Serum Levels Distinguish between Intracerebral Hemorrhage and Cerebral Ischemia in the Early Phase of Stroke. Clin Chem. 2017 Jan;63(1):377–85.

87. Chen CH, Chu HJ, Hwang YT, Lin YH, Lee CW, Tang SC, et al. Plasma neurofilament light chain level predicts outcomes in stroke patients receiving endovascular thrombectomy. J Neuroinflammation. 2021 Sep 12;18(1):195.

88. Correia M, Silva I, Gabriel D, Simrén J, Carneiro A, Ribeiro S, et al. Early plasma biomarker dynamic profiles are associated with acute ischemic stroke outcomes. Eur J Neurol. 2022 Jun;29(6):1630–42.

89. Jolugbo P, Ariëns R. Thrombus composition and efficacy of thrombolysis and thrombectomy in acute ischaemic stroke. Stroke. 2021 Mar 1;52(3):1131–42.

90. Stanford SN, Sabra A, D'Silva L, Lawrence M, Morris RHK, Storton S, et al. The changes in clot microstructure in patients with ischaemic stroke and the effects of therapeutic intervention: a prospective observational study. BMC Neurol. 2015 Mar 15;15:35.

91. Fibrin Clot Architecture in Acute Ischemic Stroke Treated With Mechanical Thrombectomy With Stent-Retrievers - Cohort Study - PubMed [Internet]. [cited 2024 May 21]. Available from: https://pubmed.ncbi.nlm.nih.gov/29176266/

92. Kim YD, Nam HS, Kim SH, Kim EY, Song D, Kwon I, et al. Time-Dependent Thrombus Resolution After Tissue-Type Plasminogen Activator in Patients With Stroke and Mice. Stroke. 2015 Jul;46(7):1877–82.

93. Choi MH, Park GH, Lee JS, Lee SE, Lee SJ, Kim JH, et al. Erythrocyte Fraction Within Retrieved Thrombi Contributes to Thrombolytic Response in Acute Ischemic Stroke. Stroke. 2018 Mar;49(3):652–9.

94. Gao Y, Su D, Xue Z, Ji L, Wang S. Association Between Serum Neurofilament Light Chain and Cognitive Performance Among Older Adults in the United States: A Cross-Sectional Study. Neurol Ther. 2023 Dec;12(6):2147–60.

95. Disanto G, Barro C, Benkert P, Naegelin Y, Schädelin S, Giardiello A, et al. Serum Neurofilament light: A biomarker of neuronal damage in multiple sclerosis. Ann Neurol. 2017 Jun;81(6):857–70.

96. Huebschmann NA, Luoto TM, Karr JE, Berghem K, Blennow K, Zetterberg H, et al. Comparing Glial Fibrillary Acidic Protein (GFAP) in Serum and Plasma Following Mild Traumatic Brain Injury in Older Adults. Front Neurol. 2020;11:1054.

97. Ward MD, Weber A, Merrill VD, Welch RD, Bazarian JJ, Christenson RH. Predictive Performance of Traumatic Brain Injury Biomarkers in High-Risk Elderly Patients. J Appl Lab Med. 2020 Jan 1;5(1):91–100.

98. Gendron TF, Badi MK, Heckman MG, Jansen-West KR, Vilanilam GK, Johnson PW, et al. Plasma neurofilament light predicts mortality in patients with stroke. Sci Transl Med. 2020 Nov 11;12(569):eaay1913.

99. Deng PP, Wu N, Chen XJ, Chen FL, Xu HS, Bao GS. NIHSS-the Alberta Stroke Program Early CT Score mismatch in guiding thrombolysis in patients with acute ischemic stroke. J Neurol. 2022 Mar;269(3):1515–21.

100. Sellner J, Patel A, Dassan P, Brown MM, Petzold A. Hyperacute detection of neurofilament heavy chain in serum following stroke: a transient sign. Neurochem Res. 2011 Dec;36(12):2287–91.

101. Wu J, Wu D, Liang Y, Zhang Z, Zhuang L, Wang Z. Plasma neurofilament light chain: A biomarker predicting severity in patients with acute ischemic stroke. Medicine (Baltimore). 2022 Jul 1;101(26):e29692.

102. Traub J, Otto M, Sell R, Göpfert D, Homola G, Steinacker P, et al. Serum phosphorylated tau protein 181 and neurofilament light chain in cognitively impaired heart failure patients. Alzheimers Res Ther. 2022 Oct 10;14(1):149.

103. Traenka C, Disanto G, Seiffge DJ, Gensicke H, Hert L, Grond-Ginsbach C, et al. Serum Neurofilament Light Chain Levels Are Associated with Clinical Characteristics and Outcome in Patients with Cervical Artery Dissection. Cerebrovasc Dis Basel Switz. 2015;40(5–6):222–7.

104. Kuhle J, Barro C, Andreasson U, Derfuss T, Lindberg R, Sandelius Å, et al. Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. Clin Chem Lab Med. 2016 Oct 1;54(10):1655–61.

105. Rübsamen N, Maceski A, Leppert D, Benkert P, Kuhle J, Wiendl H, et al. Serum neurofilament light and tau as prognostic markers for all-cause mortality in the elderly general population-an analysis from the MEMO study. BMC Med. 2021 Feb 15;19(1):38.

106. Thomalla G, Glauche V, Koch MA, Beaulieu C, Weiller C, Röther J. Diffusion tensor imaging detects early Wallerian degeneration of the pyramidal tract after ischemic stroke. NeuroImage. 2004 Aug;22(4):1767–74.

107. Lee EJ, Lim YM. Wallerian Degeneration in the Spinal Cord after Stroke. Neurol India. 2022;70(5):2300–1.

108. Datta A, Sarmah D, Kalia K, Borah A, Wang X, Dave KR, et al. Advances in Studies on Stroke-Induced Secondary Neurodegeneration (SND) and Its Treatment. Curr Top Med Chem. 2020;20(13):1154–68.

109. Dvorak F, Haberer I, Sitzer M, Foerch C. Characterisation of the diagnostic window of serum glial fibrillary acidic protein for the differentiation of intracerebral haemorrhage and ischaemic stroke. Cerebrovasc Dis Basel Switz. 2009;27(1):37–41.

110. Chen Y, Swanson RA. Astrocytes and brain injury. J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab. 2003 Feb;23(2):137–49.

111. Li L, Lundkvist A, Andersson D, Wilhelmsson U, Nagai N, Pardo AC, et al. Protective role of reactive astrocytes in brain ischemia. J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab. 2008 Mar;28(3):468–81.

112. Glial fibrillary acidic protein is a body fluid biomarker for glial pathology in human disease - PubMed [Internet]. [cited 2024 May 15]. Available from: https://pubmed.ncbi.nlm.nih.gov/25543069/

113. Shi X, Luo L, Wang J, Shen H, Li Y, Mamtilahun M, et al. Stroke subtype-dependent synapse elimination by reactive gliosis in mice. Nat Commun. 2021 Nov 26;12(1):6943.

114. Ren C, Kobeissy F, Alawieh A, Li N, Li N, Zibara K, et al. Assessment of Serum UCH-L1 and GFAP in Acute Stroke Patients. Sci Rep. 2016 Apr 14;6:24588.

115. Nylén K, Csajbok LZ, Ost M, Rashid A, Blennow K, Nellgård B, et al. Serum glial fibrillary acidic protein is related to focal brain injury and outcome after aneurysmal subarachnoid hemorrhage. Stroke. 2007 May;38(5):1489–94.

116. Vollmuth C, Fiessler C, Montellano FA, Kollikowski AM, Essig F, Oeckl P, et al. Incremental value of serum neurofilament light chain and glial fibrillary acidic protein as blood-based biomarkers for predicting functional outcome in severe acute ischemic stroke. Eur Stroke J. 2024 Feb 24;23969873241234436.

117. Selçuk Ö, Yayla V, Çabalar M, Güzel V, Uysal S, Gedikbaşi A. The Relationship of Serum S100B Levels with Infarction Size and Clinical Outcome in Acute Ischemic Stroke Patients. Noro Psikiyatri Arsivi. 2014 Dec;51(4):395–400.

118. Anand N, Stead LG. Neuron-specific enolase as a marker for acute ischemic stroke: a systematic review. Cerebrovasc Dis Basel Switz. 2005;20(4):213–9.

119. Giacomucci G, Mazzeo S, Bagnoli S, Ingannato A, Leccese D, Berti V, et al. Plasma neurofilament light chain as a biomarker of Alzheimer's disease in Subjective Cognitive Decline and Mild Cognitive Impairment. J Neurol. 2022;269(8):4270–80.

120. Nannoni S, Ricciardi F, Strambo D, Sirimarco G, Wintermark M, Dunet V, et al. Correlation between ASPECTS and Core Volume on CT Perfusion: Impact of Time since Stroke Onset and Presence of Large-Vessel Occlusion. AJNR Am J Neuroradiol. 2021 Mar;42(3):422–8.

121. Singh P, Yan J, Hull R, Read S, O'Sullivan J, Henderson RD, et al. Levels of phosphorylated axonal neurofilament subunit H (pNfH) are increased in acute ischemic stroke. J Neurol Sci. 2011 May 15;304(1–2):117–21.

122. de Jonge JC, van de Beek D, Lyden P, Brady MC, Bath PM, van der Worp HB. Temporal Profile of Pneumonia After Stroke. Stroke. 2022 Jan;53(1):53–60.

123. Meyer BC, Lyden PD. The modified National Institutes of Health Stroke Scale: its time has come. Int J Stroke Off J Int Stroke Soc. 2009 Aug;4(4):267–73.

124. Runde D. Calculated decisions: modified Rankin Scale for neurologic disability. Emerg Med Pract. 2021 Jun 15;23(Suppl 6):CD1–3.

125. Barber PA, Demchuk AM, Zhang J, Buchan AM. Validity and reliability of a quantitative computed tomography score in predicting outcome of hyperacute stroke before thrombolytic therapy. ASPECTS Study Group. Alberta Stroke Programme Early CT Score. Lancet Lond Engl. 2000 May 13;355(9216):1670–4.

7 Theses

1. Serum NfL, GFAP, and β-syn levels can predict functional outcome (mRS) on 90-day follow-up in AIS as concentrations are significantly higher in patients with mRS 3-6 compared to those with mRS 0-2 at 90-day follow-up.

2. Serum biomarkers demonstrate an individual temporal pattern with peak serum concentrations of NfL [median 223 pg/ml (IQR 98.78 – 385.3)] as well as β-syn reached on the 5th day [median 19.78 pg/ml (IQR 6.66-63.3)] and GFAP on the 3rd day [median 2.66 ng/ml (1.14-20.58)].

3. In the discrimination between good (mRS 0-2) and bad (mRS 3-6) functional outcome at 90-day follow-up after AIS, serum GFAP on the 3rd day demonstrates highest diagnostic accuracy among all measured biomarkers with a sensitivity of 64% and a specificity of 91.3% at a cut-off of 2.005 ng/ml.

4. In the discrimination between survivors and non-survivors following AIS, serum NfL on the 5th day showed the highest diagnostic accuracy among all measured biomarkers at a cut-off of 258.5 pg/ml with a sensitivity of 71.5 % and a specificity of 100%.

5. Serum NfL, GFAP and β-synuclein correlate with each other and with clinical as well as radiological scores (namely ASPECTS, NIHSS and mRS).

Appendix

Appendix 1. The National Institutes of Health Stroke Scale (73,123,124)

Item	Item Name	Scoring Guide
1a.	Level of consciousness (LOC)	0 = alert; keenly responsive 1 = not alert; arouses to minor stimulation 2 = not alert; arouses to repeated stimulation or to pain 3 = postures or unresponsive
1b.	Level of consciousness questions (month and age)	0 = answers 2 questions correctly 1 = answers 1 question correctly 2 = answers 0 question correctly
1c.	Level of consciousness commands (blink eyes and squeeze hands)	0 = performs 2 tasks correctly 1 = performs 1 task correctly 2 = performs 0 task correctly
2.	Gaze	0 = normal 1 = partial gaze palsy; can be overcome or corrects with oculocephalic reflex 2 = forced gaze paresis cannot be overcome
3.	Visual fields	0 = no loss of vision 1 = hemianopia; partial 2 = hemianopia; complete 3 = hemianopia; bilateral
4.	Facial palsy	0 = normal symmetry 1 = minor paralysis; flat nasolabial fold, asymmetry when smiling 2 = partial paralysis; total or near-total paralysis of lower face 3 = complete paralysis of one or both sides; absence of facial movement in the upper and lower face
5a.	Motor: left arm	0 = no drift; limb holds for full 10 seconds 1 = drift; limb holds, but drifts down before full 10 seconds 2 = some effort against gravity; drifts down to bed immediately, but has some effort against gravity 3 = no effort against gravity, limb falls to bed immediately

		4 = no movement
5b.	Motor: right arm	0 = no drift; limb holds for full 10 seconds 1 = drift; limb holds, but drifts down before full 10 seconds 2 = some effort against gravity; drifts down to bed immediately, but has some effort against gravity 3 = no effort against gravity 4 = no movement
6a.	Motor: left leg	0 = no drift; leg holds up for full 5 seconds 1 = drift; leg falls by the end of the 5-second period 2 = some effort against gravity; leg falls to bed 3 = no effort against gravity; leg falls to bed immediately. 4 = no movement
6b.	Motor: right leg	0 = no drift; leg holds up for full 5 seconds 1 = drift; leg falls by the end of the 5-second period 2 = some effort against gravity; leg falls to bed 3 = no effort against gravity; leg falls to bed immediately. 4 = no movement
7.	Limb ataxia	0 = no ataxia 1 = ataxia in one limb 2 = ataxia in two limbs
8.	Sensation	0 = normal; no sensory loss 1 = mild-to-moderate sensory loss; less sharp/more dull 2 = severe to total sensory loss; patient is not aware of being touched in the face, arm, and leg
9.	Language	0 = normal; no aphasia 1 = mild-moderate aphasia; some obvious changes; without significant disability 2 = severe aphasia; cannot identify materials, fragmentary expression, inference needed
10.	Dysarthria	0 = normal 1 = mild to moderate; understood, but slurring 2 = severe or anarthric; unintelligible slurring or out of proportion to dysphasia
11.	Extinction/inattention	0 = no abnormality 1 = visual, tactile, auditory, spatial, or personal inattention or extinction to bilateral simultaneous stimulation in one of the sensory modalities

		2 = profound hemi-inattention or extinction to more than one modality; does not recognize own hand or orients to only one side of space

Appendix 2. Alberta Stroke Program Early CT Score (75,125)

Points (10 = no lesion)	Description
- 1	Caudate
- 1	Putamen
- 1	internal capsule
- 1	insular cortex
- 1	M1; anterior middle cerebral artery cortex, corresponding to the frontal operculum
- 1	M2; middle cerebral artery cortex lateral to insular ribbon, corresponding to the anterior temporal lobe
- 1	M3; posterior middle cerebral artery cortex, corresponding to the posterior temporal lobe
- 1	M4; anterior middle cerebral artery territory immediately superior to M1
- 1	M5; lateral middle cerebral artery territory immediately superior to M2
- 1	M6; posterior middle cerebral artery territory immediately superior to M3

Appendix 3. The modified Rankin Scale (35,124)

Points	Description
0	no symptoms
1	no significant disability; able to carry out all activities, despite some symptoms
2	slight disability; able to look after own affairs without assistance, but unable to carry out all previous activities
3	moderate disability; requires some help, but able to walk unassisted
4	moderate severe disability; unable to attend to own bodily needs without assistance or unable to walk unassisted
5	severe disability; requires constant nursing care and attention, bedridden, incontinent
6	Dead

Appendix 4. Spearman correlation of serum biomarkers with each other (*p*-value)

p-value	NfL_0	NfL_1	NfL_2	NfL_3	NfL_5	$GFAP_0$	$GFAP_1$	$GFAP_2$	$GFAP_3$	$GFAP_5$	$\beta\text{-syn}_0$	$\beta\text{-syn}_1$	$\beta\text{-syn}_2$	$\beta\text{-syn}_3$	$\beta\text{-syn}_5$
NfL_0	1	<0.001	<0.001	0.022	0.207	<0.001	0.007	0.048	0.02	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NfL_1	<0.001	1	<0.001	<0.001	0.01	<0.001	<0.001	0.003	0.014	0.006	n.s.	0.018	0.013	0.008	0.006
NfL_2	<0.001	<0.001	1	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	n.s.	0.03	<0.001	<0.001	<0.001
NfL_3	0.022	<0.001	<0.001	1	<0.001	0.007	0.006	<0.001	<0.001	<0.001	n.s.	0.039	<0.001	<0.001	<0.001
NfL_5	n.s.	0.01	<0.001	<0.001	1	n.s.	0.004	<0.001	<0.001	<0.001	n.s.	n.s.	0.002	<0.001	<0.001
$GFAP_0$	<0.001	<0.001	<0.001	0.007	n.s.	1	0.002	0.025	0.01	0.029	n.s.	n.s.	n.s.	n.s.	n.s.
$GFAP_1$	0.007	<0.001	0.009	0.006	0.004	0.002	1	<0.001	<0.001	<0.001	n.s.	0.001	0.003	0.018	0.008
$GFAP_2$	0.048	0.003	<0.001	<0.001	<0.001	0.025	<0.001	1	<0.001	<0.001	n.s.	0.004	<0.001	<0.001	<0.001
$GFAP_3$	0.02	0.014	<0.001	<0.001	<0.001	0.01	<0.001	<0.001	1	<0.001	n.s.	0.007	<0.001	<0.001	<0.001
$GFAP_5$	n.s.	0.006	<0.001	<0.001	<0.001	0.029	<0.001	<0.001	<0.001	1	n.s.	0.023	<0.001	<0.001	<0.001
$\beta\text{-syn}_0$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1	<0.001	0.01	0.013	0.014
$\beta\text{-syn}_1$	n.s.	0.018	0.03	0.039	n.s.	n.s.	0.001	0.004	0.007	0.023	<0.001	1	<0.001	<0.001	<0.001
$\beta\text{-syn}_2$	n.s.	0.013	<0.001	<0.001	0.002	n.s.	0.003	<0.001	<0.001	<0.001	0.01	<0.001	1	<0.001	<0.001
$\beta\text{-syn}_3$	n.s.	0.008	<0.001	<0.001	<0.001	n.s.	0.018	<0.001	<0.001	<0.001	0.013	<0.001	<0.001	1	<0.001
$\beta\text{-syn}_5$	n.s.	0.006	<0.001	<0.001	<0.001	n.s.	0.008	<0.001	<0.001	<0.001	0.014	<0.001	<0.001	<0.001	1

Appendix 5. Spearman correlation of serum biomarkers with each other (*rho*, Spearman correlation coefficient)

rho	NfL_0	NfL_1	NfL_2	NfL_3	NfL_5	$GFAP_0$	$GFAP_1$	$GFAP_2$	$GFAP_3$	$GFAP_5$	$\beta\text{-syn}_0$	$\beta\text{-syn}_1$	$\beta\text{-syn}_2$	$\beta\text{-syn}_3$	$\beta\text{-syn}_5$
NfL_0	1	0.8	0.8	0.4	0.3	0.8	0.5	0.4	0.5	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NfL_1	0.8	1	0.8	0.6	0.4	0.6	0.5	0.4	0.4	0.4	n.s.	0.3	0.4	0.4	0.4
NfL_2	0.8	0.8	1	0.8	0.5	0.6	0.4	0.5	0.5	0.5	n.s.	0.3	0.5	0.6	0.6
NfL_3	0.4	0.6	0.8	1	0.8	0.5	0.4	0.5	0.6	0.7	n.s.	0.3	0.5	0.7	0.7
NfL_5	n.s.	0.4	0.5	0.8	1	n.s.	0.4	0.5	0.7	0.7	n.s.	n.s.	0.4	0.5	0.5
$GFAP_0$	0.8	0.6	0.6	0.5	n.s.	1	0.6	0.4	0.5	0.5	n.s.	n.s.	n.s.	n.s.	n.s.
$GFAP_1$	0.5	0.5	0.4	0.4	0.4	0.6	1	0.7	0.7	0.6	n.s.	0.5	0.4	0.3	0.4
$GFAP_2$	0.4	0.4	0.5	0.5	0.5	0.4	0.7	1	0.9	0.7	n.s.	0.4	0.6	0.6	0.6
$GFAP_3$	0.5	0.4	0.5	0.6	0.7	0.5	0.7	0.9	1	0.9	n.s.	0.4	0.7	0.6	0.7
$GFAP_5$	n.s.	0.4	0.5	0.7	0.7	0.5	0.6	0.7	0.9	1	n.s.	0.3	0.6	0.7	0.7
$\beta\text{-syn}_0$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1	0.6	0.5	0.5	0.5
$\beta\text{-syn}_1$	n.s.	0.3	0.3	0.3	n.s.	n.s.	0.5	0.4	0.4	0.3	0.6	1	0.7	0.6	0.6
$\beta\text{-syn}_2$	n.s.	0.4	0.5	0.5	0.4	n.s.	0.4	0.6	0.7	0.6	0.5	0.7	1	0.9	0.9
$\beta\text{-syn}_3$	n.s.	0.4	0.6	0.7	0.5	n.s.	0.3	0.6	0.6	0.7	0.5	0.6	0.9	1	0.9
$\beta\text{-syn}_5$	n.s.	0.4	0.6	0.7	0.5	n.s.	0.4	0.6	0.7	0.7	0.5	0.6	0.9	0.9	1

Appendix 6. Spearman correlation of serum biomarkers with clinical and radiological variables (*p*-value)

***p*-value**	**age**	**$ASPECTS_0$**	**$ASPECTS_{24}$**	**$NIHSS_0$**	**$NIHSS_{24}$**	**$NIHSS_{change}$**	**$NIHSS_{48}$**	**$NIHSS_{72}$**	**$NIHSS_{discharge}$**	**$mRS_{discharge}$**	**mRS_{90days}**	**creatinine**
age		0.81	0.504	0.349	0.384	0.64	0.32	0.256	0.377	0.28	0.142	0.42
$ASPECTS_0$	0.81		0.001	0.024	0.014	0.263	0.06	0.104	0.837	0.064	0.078	0.939
$ASPECTS_{24}$	0.504	0.001		0.005	0.002	0.122	0.001	0.002	0.447	0.001	0.001	0.379
$NIHSS_0$	0.349	0.024	0.005		0.001	0.026	0.001	0.001	0.001	0.001	0.001	0.646
$NIHSS_{24}$	0.384	0.014	0.002	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.359
$NIHSS_{change}$	0.64	0.263	0.122	0.026	0.001		0.003	0.011	0.001	0.015	0.008	0.273
$NIHSS_{48}$	0.32	0.06	0.001	0.001	0.001	0.003		0.001	0.001	0.001	0.001	0.512
$NIHSS_{72}$	0.256	0.104	0.002	0.001	0.001	0.011	0.001		0.001	0.001	0.001	0.992
$NIHSS_{discharge}$	0.377	0.837	0.447	0.001	0.001	0.001	0.001	0.001		0.001	0.001	0.747
$mRS_{discharge}$	0.28	0.064	0.001	0.001	0.001	0.015	0.001	0.001	0.001		0.001	0.718
mRS_{90days}	0.142	0.078	0.001	0.001	0.001	0.008	0.001	0.001	0.001	0.001		0.958
creatinine	0.42	0.939	0.379	0.646	0.359	0.273	0.512	0.992	0.747	0.718	0.958	
NfL_0	0.004	0.201	0.264	0.068	0.014	0.4	0.032	0.183	0.908	0.131	0.045	0.05
NfL_1	0.017	0.156	0.104	0.001	0.008	0.637	0.003	0.017	0.178	0.009	0.002	0.325
NfL_2	0.002	0.015	0.065	0.001	0.001	0.365	0.001	0.001	0.061	0.004	0.001	0.388
NfL_3	0.007	0.001	0.005	0.001	0.001	0.021	0.001	0.001	0.003	0.001	0.001	0.409
NfL_5	0.079	0.001	0.002	0.009	0.001	0.046	0.001	0.001	0.018	0.001	0.001	0.271
$GFAP_0$	0.001	0.117	0.414	0.01	0.022	0.434	0.098	0.248	0.555	0.205	0.176	0.178
$GFAP_1$	0.023	0.237	0.14	0.001	0.003	0.01	0.006	0.017	0.091	0.057	0.008	0.688

$GFAP_2$	0.283	0.001	0.001	0.001	0.001	0.003	0.001	0.003	0.173	0.004	0.001	0.649
$GFAP_3$	0.072	0.001	0.005	0.001	0.001	0.007	0.001	0.001	0.04	0.001	0.001	0.193
$GFAP_5$	0.013	0.001	0.003	0.001	0.001	0.081	0.001	0.001	0.025	0.001	0.001	0.14
β-syn_0	0.771	0.349	0.912	0.165	0.226	0.612	0.654	0.964	0.603	0.549	0.666	0.02
β-syn_1	0.831	0.276	0.157	0.006	0.003	0.245	0.006	0.035	0.565	0.278	0.068	0.04
β-syn_2	0.392	0.001	0.003	0.001	0.001	0.076	0.001	0.002	0.627	0.011	0.001	0.161
β-syn_3	0.114	0.002	0.005	0.001	0.001	0.018	0.001	0.001	0.098	0.002	0.001	0.032
β-syn_5	0.201	0.001	0.006	0.001	0.001	0.03	0.001	0.003	0.158	0.004	0.001	0.071

Appendix 7. Spearman correlation of serum biomarkers with clinical and radiological variables (*rho*, Spearman correlation coefficient)

rho	**age**	**$ASPECTS_0$**	**$ASPECTS_{24}$**	**$NIHSS_0$**	**$NIHSS_{24}$**	**$NIHSS_{change}$**	**$NIHSS_{48}$**	**$NIHSS_{72}$**	**$NIHSS_{discharge}$**	**$mRS_{discharge}$**	**mRS_{90days}**	**creatinine**
age	1	0.1	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.3	0.2
$ASPECTS_0$	0.1	1	0.8	-0.4	-0.4	-0.2	-0.3	-0.3	0.1	-0.3	-0.3	0.1
$ASPECTS_{24}$	0.2	0.8	1	-0.5	-0.5	-0.3	-0.6	-0.5	-0.2	-0.6	-0.5	0.2
$NIHSS_0$	0.2	-0.4	-0.5	1	0.7	0.4	0.6	0.7	0.6	0.6	0.7	0.1
$NIHSS_{24}$	0.2	-0.4	-0.5	0.7	1	0.5	1	0.9	0.8	0.8	0.7	0.2
$NIHSS_{change}$	0.1	-0.2	-0.3	0.4	0.5	1	0.5	0.4	0.5	0.4	0.4	-0.2
$NIHSS_{48}$	0.2	-0.3	-0.6	0.6	1	0.5	1	1	0.9	0.9	0.8	0.1
$NIHSS_{72}$	0.2	-0.3	-0.5	0.7	0.9	0.4	1	1	0.9	0.9	0.8	0.1
$NIHSS_{discharge}$	0.2	0.1	-0.2	0.6	0.8	0.5	0.9	0.9	1	0.9	0.8	-0.1
$mRS_{discharge}$	0.2	-0.3	-0.6	0.6	0.8	0.4	0.9	0.9	0.9	1	1	-0.1

mRS_{90days}	0.3	-0.3	-0.5	0.7	0.7	0.4	0.8	0.8	0.8	1	1	0.1
creatinine	0.2	0.1	0.2	0.1	0.2	-0.2	0.1	0.1	-0.1	-0.1	0.1	1
NfL_0	0.5	-0.2	-0.2	0.3	0.4	0.2	0.4	0.3	-0.1	0.3	0.4	0.4
NfL_1	0.4	-0.3	-0.3	0.5	0.4	0.1	0.5	0.4	0.3	0.4	0.5	0.2
NfL_2	0.5	-0.4	-0.3	0.5	0.5	0.2	0.6	0.5	0.3	0.5	0.6	0.2
NfL_3	0.4	-0.5	-0.5	0.5	0.7	0.4	0.7	0.7	0.5	0.6	0.7	0.2
NfL_5	0.3	-0.5	-0.5	0.4	0.6	0.3	0.6	0.6	0.4	0.6	0.7	0.2
$GFAP_0$	0.7	-0.4	-0.2	0.5	0.5	0.2	0.4	0.3	0.2	0.3	0.3	0.3
$GFAP_1$	0.4	-0.2	-0.3	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.4	0.1
$GFAP_2$	0.2	-0.6	-0.6	0.5	0.6	0.5	0.5	0.5	0.3	0.5	0.5	0.1
$GFAP_3$	0.3	-0.5	-0.5	0.6	0.7	0.4	0.6	0.6	0.4	0.6	0.6	0.2
$GFAP_5$	0.4	-0.6	-0.5	0.6	0.7	0.3	0.7	0.6	0.4	0.6	0.6	0.3
$\beta\text{-}syn_0$	-0.1	-0.2	-0.1	0.3	0.3	0.2	0.1	0.1	-0.2	-0.2	-0.1	0.5
$\beta\text{-}syn_1$	0.1	-0.2	-0.3	0.4	0.5	0.2	0.4	0.4	0.1	0.2	0.3	0.3
$\beta\text{-}syn_2$	0.2	-0.5	-0.5	0.7	0.6	0.3	0.5	0.5	0.1	0.4	0.5	0.3
$\beta\text{-}syn_3$	0.3	-0.5	-0.5	0.6	0.6	0.4	0.6	0.6	0.3	0.5	0.6	0.4
$\beta\text{-}syn_5$	0.2	-0.6	-0.5	0.6	0.6	0.4	0.6	0.5	0.3	0.5	0.6	0.3

Erklärung über frühere Promotionsversuche und Selbstständigkeit

Ich erkläre, dass ich mich an keiner anderen Hochschule einem Promotionsverfahren unterzogen bzw. eine Promotion begonnen habe.

Ich erkläre die Angaben wahrheitsgemäß gemacht und die wissenschaftliche Arbeit an keiner anderen wissenschaftlichen Einrichtung zur Erlangung eines akademischen Grades eingereicht zu haben.

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst habe. Alle Regeln der guten wissenschaftlichen Praxis wurden eingehalten; es wurden keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht.

Halle (Saale), den 30.05.2024

_____

Yashar Bahramsari

Danksagung

Ich bedanke mich zutiefst bei Prof. Dr. med. Markus Otto für seine herausragende Betreuung während meiner Promotion, die sich durch strukturierte wissenschaftliche Anleitung und eine engagierte Vermittlung seiner wertvollen Expertise auszeichnete. Beim Übergang vom Studium in das Berufsleben hat er mich persönlich sehr gefördert und entscheidend geprägt.

Mein herzlicher Dank gilt Dr. med. Lorenzo Barba für seine großzügige Hilfestellung während meiner Promotion. Seine fachkundigen Ratschläge, warme Menschlichkeit und Freundschaft haben meine Arbeit sowie mein Leben maßgeblich bereichert. Die Verwirklichung dieses Traums wäre ohne ihn nicht möglich gewesen.

Zudem möchte ich mich bei dem gesamten Pflegepersonal auf Stroke-Unit und neurologischer Intensivstation für die unentwegte Unterstützung bei der Entnahme von Blutproben und logistischen Umsetzung des Projekts bedanken.

Meinen geschätzten Eltern, Dr. Mojgan Kowsari und Dr. Rahim Bahramsari, sowie meiner Partnerin Shabnam Rafieipour, möchte ich meine Dankbarkeit für ihre unermüdliche Unterstützung zum Ausdruck bringen. Ihr unerschütterlicher Glaube an mich, Ermutigungen und bedingungslose Liebe haben mich inspiriert und maßgeblich zum Erfolg meiner Arbeit beigetragen.