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ORIGINAL ARTICLE

Prognostic serum biomarkers of synaptic, neuronal and glial injury in patients with acute ischemic stroke of the anterior circulation

Lorenzo Barba[1] | Christoph Vollmuth[2] | Steffen Halbgebauer[3,4] | Kathrin Ungethüm[5,6] | Christian Hametner[2] | Fabian Essig[2] | Alexander M. Kollikowski[7] | Mirko Pham[7] | Michael K. Schuhmann[2] | Peter U. Heuschmann[5,6,8] | Patrick Oeckl[3,4] | Petra Steinacker[1] | Michele Romoli[9] | Lucio D'Anna[10,11] | Samir Abu-Rumeileh[1] | Karl Georg Haeusler[3] | Guido Stoll[12] | Hermann Neugebauer[2] | Markus Otto[1]

[1]Department of Neurology, Martin-Luther-University of Halle-Wittenberg, Halle (Saale), Germany

[2]Department of Neurology, University Hospital Würzburg (UKH), Würzburg, Germany

[3]Department of Neurology, University of Ulm, Ulm, Germany

[4]German Center for Neurodegenerative Diseases (DZNE e.V.), Ulm, Germany

[5]Institute for Clinical Epidemiology and Biometry, University of Würzburg, Wurzburg, Germany

[6]Institute for Medical Data Science, University Hospital Würzburg, Wurzburg, Germany

[7]Department of Neuroradiology, University Hospital Würzburg, Wurzburg, Germany

[8]Clinical Trial Centre, University Hospital Würzburg, Wurzburg, Germany

[9]Neurology and Stroke Unit, "Bufalini" Hospital, Cesena, Italy

[10]Department of Stroke and Neuroscience, Charing Cross Hospital, Imperial College London NHS Healthcare Trust, London, UK

[11]Department of Brain Sciences, Imperial College London, London, UK

[12]Institute of Experimental Biomedicine I, University Hospital Würzburg, Wurzburg, Germany

Correspondence
Markus Otto, Department of Neurology, Martin-Luther-University Halle-Wittenberg, Ernst-Grube Strasse 40, 06120 Halle (Saale), Germany.
Email: markus.otto@uk-halle.de

Hermann Neugebauer, Department of Neurology, University of Würzburg, Josef-Schneider-Straße 11, 97080 Wurzburg, Germany.
Email: hermann.neugebauer@icloud.com

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Abstract

Background: We aimed to investigate the prognostic role of β-synuclein in comparison to that of neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) for predicting functional outcome after acute ischemic stroke (AIS).

Methods: We measured serum concentrations of β-synuclein, NfL and GFAP 24 h after hospital admission in 213 consecutive patients with moderate-to-severe AIS. We investigated the association between serum biomarkers and radiological/clinical characteristics, 3-months mortality and functional outcome on the modified Rankin Scale (mRS).

Results: In 213 patients with AIS [mean age: 76.1 (±12.5) years, 53.1% males, median NIHSS score on admission: 13 (IQR: 9–17)], higher levels of β-synuclein, NfL and GFAP were associated with higher NIHSS scores and with lower Alberta Stroke Program CT Score (ASPECTS) points on admission. Serum β-synuclein levels was significantly correlated with NfL (rho = 0.715, $p < 0.001$) and GFAP concentrations (rho = 0.684, $p < 0.001$).

Lorenzo Barba, Christoph Vollmuth, Hermann Neugebauer and Markus Otto, contributed equally.

The inclusion of serum β-synuclein significantly improved the accuracy of prediction models without biomarkers for overall mortality (AUC: 0.836 vs. 0.752, $p < 0.001$) and mRS 3–6 vs. 0–2 (AUC: 0.812 vs. 0.624, $p < 0.001$). Combination models with NfL and/or GFAP showed a similar accuracy.

Conclusions: Serum β-synuclein may be used to assess synaptic damage/dysfunction and to predict 3-months clinical outcomes in patients with AIS.

KEYWORDS
beta-synuclein, biomarkers, GFAP, NfL, stroke

INTRODUCTION

The clinical outcome after an acute ischemic stroke (AIS) is extremely variable, even after successful revascularization therapy with intravenous thrombolysis (IVT) and/or mechanical thrombectomy (MT). In routine clinical practice, the prognostic evaluation of patients with AIS is based on multiple clinical and diagnostic parameters, such as the disease severity at hospital admission, the size of ischemic stroke lesion, the localization of vessel occlusion and the burden of comorbidities. Blood-based biomarkers that specifically reflect the extent of the ischemic brain damage could assist stroke clinicians especially in the prognostic evaluation of patients, although they have not been implemented yet in clinical routine [1].

Neurofilament light chain (NfL) and glial fibrillary acidic proteins (GFAP) are well-recognized blood-based biomarkers indicative of neuroaxonal and astroglial injury in several neurological disorders [2, 3]. Blood levels of NfL and GFAP were found to be increased in patients with AIS compared to healthy controls [2, 3]. In a previous study, we showed that serum NfL and GFAP level may improve the prognostic assessment in AIS patients in addition to clinical evaluation, as higher biomarker levels were associated with disease severity as well as with worse functional outcome at 3-months follow-up [4]. However, reliable cutoff values for NfL and GFAP are still lacking, given the variable timing of blood sampling across studies and the multiple factors impacting on their serum levels [2, 3]. Notably, other aspects of ischemic brain injury, such as synaptic derangement, have not been assessed using fluid biomarkers in AIS. Beta-synuclein (β-synuclein) is a novel candidate biomarker for synaptic damage/dysfunction that has shown promising results in neurodegenerative disorders (e.g. Alzheimer's and prion disease) and traumatic brain injury [5–10]. In a pilot study, we explored the value of serum β-synuclein in a small cohort of patients with AIS and demonstrated an association between higher biomarker levels and stroke severity as well as poorer clinical outcome [11].

In this study, we aimed to assess the role of serum β-synuclein-as a biomarker of synaptic damage-in a well-characterized cohort of patients with AIS of the anterior circulation. We investigated its association with clinical and radiological variables collected on hospital admission, stroke etiology and acute stroke treatment. In addition, we assessed the prognostic role of β-synuclein for mortality and functional outcome at 3 months in comparison to NfL and GFAP [4]. Moreover, we expanded our previous findings by testing preliminary biomarker cutoffs and combinations of biomarkers for prognostic purposes.

METHODS

Study population

Patients included in the present study were enrolled into a single-center prospective observational cohort at the University of Würzburg (Würzburg, Germany) between 06/2020 and 08/2021. Details on the study protocol and population have been published previously [4, 11, 12]. For the aim of this study, we included patients fulfilling the following inclusion criteria: (1) age ≥18 years; (2) AIS of the anterior circulation (i.e., internal carotid artery, ICA; middle cerebral artery, MCA; anterior cerebral artery, ACA); (3) NIHSS at admission ≥6 and/or acute treatment with MT [4, 11, 12]. We collected clinical, biochemical and radiological data of patients on hospital admission and at follow-up, namely NIHSS scores on hospital admission, 24 h, 48 h, 72 h and at discharge; AIS etiology according to the trial of ORG 10172 in acute stroke treatment (TOAST) classification system [13]; Alberta Stroke Program CT Score (ASPECTS) on hospital admission; data on hyperacute treatment (i.e., intravenous thrombolysis, IVT, and/or mechanical thrombectomy, MT); modified Thombolysis in Cerebral Ischemia (mTICI) score after MT; mRS on admission (pre-event mRS) and at 3 months assessed by a blinded rater by structured telephone interview.

Blood sample collection and biomarker analysis

Serum samples were collected at scheduled timepoints (10 am) the day after hospital admission [median time from clinical onset to blood sampling: 31 (interquartile range, IQR: 21–59) hours] and processed according to standard protocols. We quantified serum NfL with commercially available kits for the Ella microfluidic system (BioTechne), serum GFAP with digital Simoa immunoassay kits run on a HD-X platform (Quanterix Inc) and serum β-synuclein with a previously described in-house established digital Simoa immunoassay [7, 8]. As internal controls, we run the same sample in all plates and calculated the intra- and inter-assay coefficients of variability to be <15% and <20%, respectively.

Definition of variables of interest

To evaluate the prognostic value of serum markers for AIS, we explored their associations with the following binary outcomes: (1) 3-months functional outcome defined as good (mRS 0–2 or mRS unchanged compared to pre-event mRS) vs. poor (mRS 3–6); (2) all-cause mortality within 3 months. We also tested associations between serum biomarker levels and the temporal changes of the NIHSS score, i.e., the NIHSS change within 24 h (admission NIHSS–NIHSS at 24 h). Dependently on the NIHSS change within 24 h, early neurological improvement (ENI) was defined as NIHSS score improvement within 24 h ≥4 [14]. In addition, we compared biomarker levels dependently on the ASPECTS by using a previously described cutoff of 8 arbitrary units (AU) because of its association with the 3-month functional outcome [15].

Statistical analysis

Statistical analyses were performed with R studio v4.2.2 (R foundation) and GraphPad v8 (GraphPad Software). For comparisons of continuous variables, we used the Mann–Whitney *U* test and the Kruskal-Wallis test (followed by Dunn-Bonferroni's post-hoc test). The Chi-squared test was adopted for comparisons of categorial variables. Correlation between continuous variables were computed with Spearman's correlations. Generalized linear regression was modeled (GLMs) to test associations between variables after accounting for covariables of interest (see below) [16]. For group differences, we calculated the standardized effect size with the Cohen's d. To test the accuracy of biomarkers for binary outcomes, we performed receiver operating characteristic (ROC) analysis and calculated the best cut-off values by maximizing the Youden index. Then, we tested the associations of serum biomarkers (both as continuous and as discrete variable) in logistic regression models after accounting for covariables (if not other specified: age, sex, GFR, time in hours from clinical onset to blood sampling, NIHSS on admission, acute therapy with IVT and/or MT). Then, we calculated the area under the curve (AUC) values from the ROC analysis derived from the GLMs. We compared the ROC values of different models with the DeLong test. Statistical significance was set at *p*-values <0.05.

RESULTS

Descrition of the study population

We included 213 patients with moderate-to-severe AIS [mean age: 76.1 (±12.5) years, 113 (53.1%) male and 100 (46.9%) female participants] with blood samples collected 1 day after hospital admission [median time from onset to blood sampling: 31 (IQR: 21–59) hours]. Demographic and clinical data are summarized in Table 1 and were previously described [4]. On hospital admission, median NIHSS score was 13 (IQR: 9–17) points and the ASPECTS was <8 in 103 (48.4%) patients. One-hundred eighty-six patients underwent reperfusion therapy (87.3%) (IVT in 28, 13.1%; MT in 101; 47.4%; IVT + MT in 57, 26.8%). Median serum biomarker concentrations in AIS were: β-synuclein 27.2 pg/mL (IQR: 8.7–85.3 pg/mL), NfL 95.8 pg/mL (IQR: 51.1–223.0 pg/mL) and GFAP 5.7 ng/mL (IQR: 1.6–21.8 ng/mL).

Serum biomarkers and functional outcomes at 3 months

In our cohort, 158 patients (74.2%) had poor functional outcome (mRS 3–6) at 3 months after stroke (Table 1). Serum biomarker concentrations measured 1 day after hospital admission correlated with 3-months mRS scores in AIS patients (NfL: rho = 0.430, $p < 0.001$; GFAP: rho = 0.497, $p < 0.001$; β-synuclein: rho = 0.510, $p < 0.001$) (Figure 1a, Table S1–S7). Patients with poor (mRS 3–6) vs. good (mRS 0–2 or unchanged to pre-event mRS) functional outcome at 3 months showed significantly increased serum levels of β-synuclein [median: 418.0 vs. 7.6 pg/mL, Cohen's d: 0.79], NfL [median: 137.5 vs. 49.7 pg/mL, Cohen's d: 0.73] and GFAP [median: 9.9 vs. 0.8 ng/mL, Cohen's d: 0.40] ($p < 0.001$ for all comparisons) (Figure 1b, Table 1). ROC analysis revealed a good accuracy (AUC ≥0.80) of serum biomarkers for discriminating 3-months functional outcomes. Best cutoff values were 73.3 pg/mL for NfL, 3.0 pg/mL for GFAP and 19.0 pg/mL for β-synuclein (Figure 1c, Table 2). Regression analysis showed that multivariable models including any serum biomarker were significantly more accurate in predicting 3-months functional outcomes than models without a biomarker, especially when considering cutoff values (Table S2). The best discrimination performance was obtained including GFAP (with cutoff 3.0 ng/mL), either alone (AUC: 0.849) or in combination with NfL (with cutoff 73.3 pg/mL, AUC: 0.895) or β-synuclein (with cutoff 19.0 pg/mL, AUC: 0.854), whereas a 3-biomarker combination was not better (Table S2). Patients with, at baseline, at least one biomarker level above the chosen cutoff value had a mRS ≥3 in more than 90% of cases (Figure 1d).

Serum biomarkers and all-cause mortality within 3 months

Patients who did not survive ($n = 80$, 37.6%) had higher serum NfL (Cohen's d: 0.20, $p < 0.001$), GFAP (Cohen's d: 1.19, $p < 0.001$) and β-synuclein (Cohen's d: 0.13, $p < 0.001$) concentrations at baseline than patients who survived ($n = 133$, 62.4%) (Figure 2a). ROC analysis showed moderate accuracy of serum biomarkers when considered alone (AUC <0.72) (Figure 2b, Table 3). By maximizing the Youden index, we found the following best cutoff values for 3-months mortality: NfL ≥78.0 pg/mL, GFAP ≥3.0 ng/mL, β-synuclein ≥86.0 pg/mL. After accounting for covariables (see Methods), higher serum biomarker levels were significantly associated with 3-months mortality (Table 3). Overall, models including serum biomarker cutoff

TABLE 1 Demographic, clinical, radiological and biochemical data of the study population.

	whole cohort ($n=213$)	3-month mRS 0–2 or unchanged to pre-event mRS ($n=55$)	3-month mRS 3–6 ($n=158$)	*p*-value
Age	79 (69–85)	78 (67–82.5)	79.5 (69–86)	0.076
Female sex [*n* (%)]	100 (46.9)	31 (56.4)	69 (43.7)	0.142
AIS characteristics				
NIHSS on admission	13 (9–17)	10 (8–14.5)	14 (10–17)	**<0.001**
ASPECTS on admission	7 (6–9)	8 (8–9)	7 (6–8)	**<0.001**
Wake-up [*n* (%)]	69 (32.4)	12 (21.8)	57 (36.1)	0.075
Etiology [*n* (%)]				0.335
LAA	26 (12.2)	8 (14.5)	18 (11.8)	
CE	98 (46.0)	30 (54.5)	68 (42.9)	
Other determined	11 (5.2)	3 (5.5)	8 (5.0)	
Cryptogenic	75 (35.2)	14 (25.5)	61 (38.5)	
Concurrent etiology	3 (1.4)	–	3 (1.8)	
Site of occlusion [*n* (%)]				**0.048**
ACA	16 (7.5)	7 (12.7)	9 (5.7)	
MCA	149 (70.0)	41 (74.6)	108 (68.4)	
ICA	48 (22.5)	7 (12.7)	41 (25.9)	
Acute therapy [*n* (%)]				**0.003**
None	27 (12.7)	4 (7.3)	23 (14.6)	
Only IVT	28 (13.1)	15 (27.3)	13 (8.2)	
Only MT	101 (47.4)	25 (45.4)	76 (48.1)	
MT + IVT	57 (26.8)	11 (20.0)	46 (29.1)	
Serum biomarkers				
NfL (pg/ml)	95.8 (51.1–223.0)	49.7 (30.0–68.9)	137.5 (65.6–288.8)	**<0.001**
GFAP (ng/ml)	5.7 (1.6–21.8)	0.8 (0.4–2.7)	9.9 (3.1–37.2)	**<0.001**
β-synuclein (pg/ml)	27.2 (8.7–85.3)	7.6 (4.2–16.8)	418.0 (16.0–117.6)	**<0.001**

Note: Continuous data are reported as median (IQR) and categorical data as number of cases (%). Values in bold are statistically significant at $p<0.05$.

Abbreviations: ACA, anterior cerebral artery; AIS, acute ischemic stroke; ASPECTS, Alberta Stroke Program Early CT Score; CE, cardioembolism; GFAP, glial fibrillary acidic protein; ICA, internal carotid artery; IVT, intravenous thrombolysis; LAA, large-artery atherosclerosis; MCA, middle cerebral artery; mRS, modified Rankin Scale; MT, mechanical thrombectomy; NfL, neurofilament light chain; NIHSS, National Institute of Health Stroke Scale.

values showed AUC values ≥0.80 and ORs ≥5, which were significantly more accurate than models not including biomarkers (Table 3, Table S3). Patients with any biomarker concentrations higher than the found cutoff value died in ≥50% of cases at 3-months follow-up (Figure 2c). Biomarker combinations did not perform better than biomarkers taken individually for predicting mortality (Table S3).

Serum markers and clinical/radiological variables

Multivariable linear regression models accounting for age, sex, GFR, time from onset to sampling, NIHSS at admission and ASPECTS at admission showed that age, sex and time from symptom onset to blood sampling were not associated with serum NfL, GFAP and

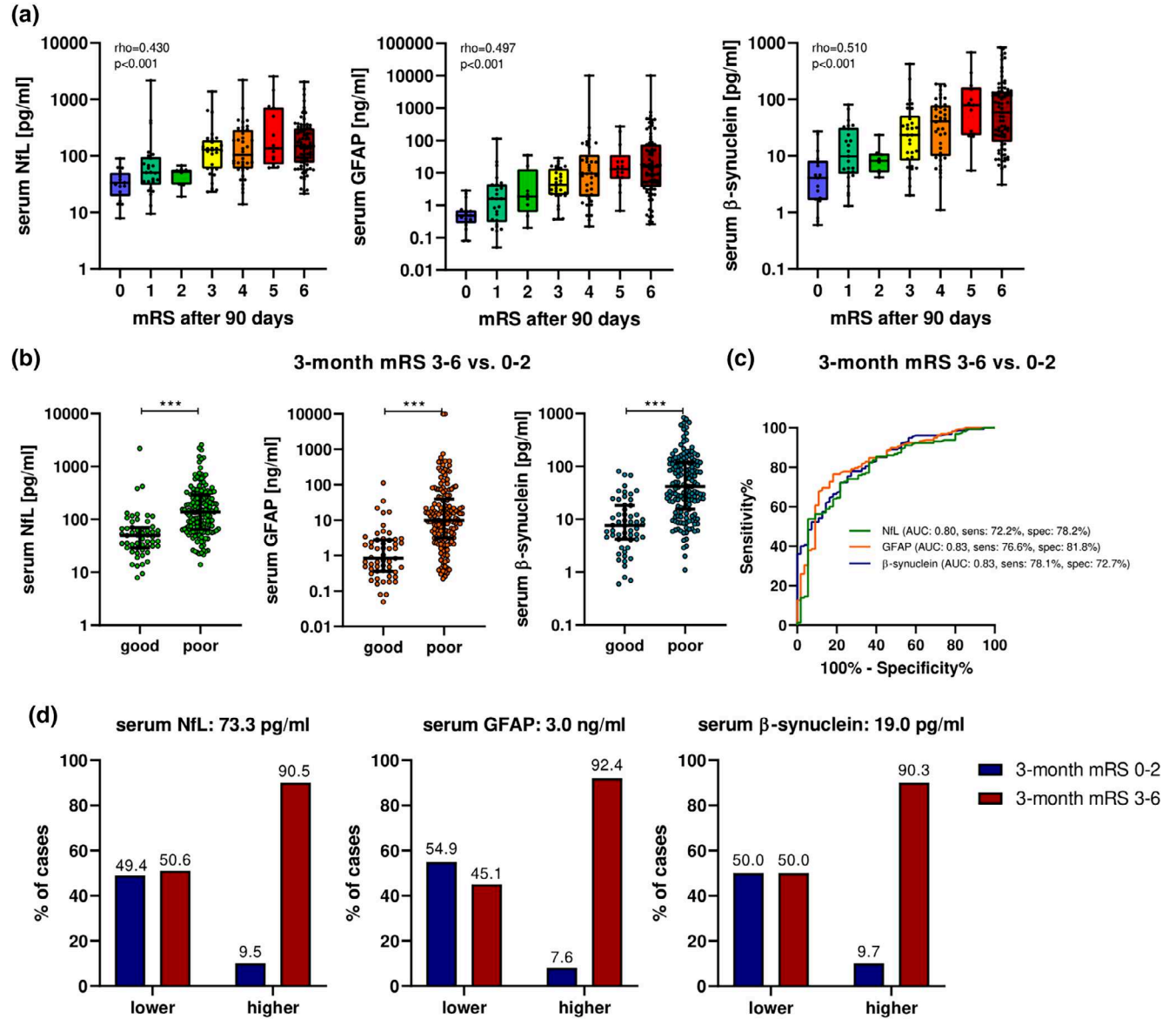

FIGURE 1 Association between serum biomarkers and 3-month mRS scores. (a) Spearman's correlations between serum biomarker levels and 3-month mRS scores. (b) Serum biomarker levels in patients with good (mRS 0–2 or mRS unchanged compared to pre-event mRS) vs. poor (mRS 3–6) functional outcome at 3-month follow-up. (c) Receiver operating characteristics (ROC) analysis for the discrimination between patients with good vs. poor 3-month functional outcome assessing serum biomarkers. (d) Distribution of AIS patients with good vs. poor 3-month functional outcome according to best biomarker cutoff values (found by maximizing the Youden index at ROC analysis). Patients were distinguished into two groups depending on whether the serum biomarker levels were above or below the cutoff value. ***$p < 0.001$.

β-synuclein concentrations (Table S4). Instead, lower GFR values were significantly associated with higher serum NfL (in pg/ml) [β: −3.47 (95% confidence interval, 95%CI: −5.60 – −1.34), $p = 0.002$] and GFAP concentrations (in ng/ml) [β: −0.65 (95%CI: −1.18 – −0.12), $p = 0.018$], but not with serum β-synuclein ($p = 0.21$) (Table S4). A lower ASPECTS on hospital admission was associated with higher serum β-synuclein [β: −16.97 (95%CI: −28.86 – −4.89), Cohen's d: 0.48, $p = 0.007$] and GFAP level [β: −16.78 (95%CI: −24.53 – −9.03), Cohen's d: 0.43, $p < 0.001$] while not with NfL (Cohen's d: 0.27, $p = 0.12$) (Figure 3a,b, Table S4). We did not find significant differences of serum β-synuclein, NfL and GFAP concentrations in AIS patients depending on anticipated stroke etiology at discharge or IVT/MT use (Table 1).

Serum levels of NfL, GFAP and β-synuclein correlated with NIHSS scores at all timepoints recorded (complete data in Table S1–S7). Higher serum biomarker levels were negatively correlated with the NIHSS change within 24 hours (admission NIHSS–NIHSS at 24 h) (NfL rho = −0.416, GFAP rho = −0.502, β-synuclein $p = -0.516$, $p < 0.001$ for all, Figure 3c, Table S1–S7), independently from baseline NIHSS (Table S5). Patients with ENI ($n = 78$, 36.6%) had significantly lower serum levels of β-synuclein (Cohen's d: 0.83, $p < 0.001$), NfL (Cohen's d: 0.42, $p < 0.001$) and

TABLE 2 Accuracy of serum biomarkers for predicting 3-month mRS 3–6 vs. 0–2 (or unchanged to pre-event mRS).

Model	Variable	Best cutoff	AUC (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	OR (95%CI)	*p*	DeLong p for continuous vs. categorical
No biomarker								
Multivariable	–	–	0.624 (0.517–0.730)	89.9 (37.4–96.0)	41.9 (25.6–81.5)	–	–	–
Serum NfL								
Univariable	in pg/ml (continuous)	73.3	0.799 (0.731–0.867)	72.2 (64.7–78.6)	78.2 (65.6–87.1)	1.004 (1.001–1.007)	**0.007**	–
Multivariable	in pg/ml (continuous)	–	0.709 (0.614–0.805)	88.9 (44.4–96.0)	53.5 (39.5–90.7)	1.002 (0.9997–1.005)	0.088	–
	≥73.3 pg/mL (categorical)	–	0.804 (0.726–0.881)	75.8 (53.5–89.9)	79.1 (60.5–95.4)	12.49 (4.50–34.70)	**<0.001**	**0.024**
Serum GFAP								
Univariable	in ng/ml (continuous)	3.0	0.832 (0.770–0.894)	76.6 (69.4–82.5)	81.8 (69.7–89.8)	1.06(1.02–1.11)	**0.004**	–
Multivariable	in ng/ml (continuous)	–	0.784 (0.703–0.866)	74.8 (53.5–86.9)	79.1 (60.5–95.4)	1.04 (1.01–1.08)	**0.022**	**–**
	≥3.0 ng/mL (categorical)	–	0.849 (0.781–0.917)	85.9 (60.6–92.9)	79.1 (65.1–97.7)	19.16 (6.61–55.53)	**<0.001**	**0.027**
Serum β-synuclein								
Univariable	in pg/ml (continuous)	19.0	0.830 (0.771–0.888)	72.3 (64.7–78.7)	78.2 (65.6–87.1)	1.05 (1.02–1.07)	**<0.001**	**–**
Multivariable	in pg/ml (continuous)	–	0.812 (0.741–0.883)	68.7 (51.5–91.9)	86.1 (60.5–97.7)	1.04 (1.02–1.06)	**<0.001**	–
	≥19.0 pg/mL (categorical)	–	0.768 (0.683–0.853)	69.7 (50.5–89.9)	79.1 (53.5–93.0)	6.51 (2.74–15.48)	**<0.001**	0.058

Note: Covariables were age, sex, GFR, time-to-sampling, NIHSS at admission and acute therapy with IVT and/or MT. Values in bold are statistically significant at $p<0.05$.

Abbreviations: AUC, area under the curve; GFAP, glial fibrillary acidic protein; GFR, glomerular filtration rate; IVT, intravenous thrombolysis; MT, mechanical thrombectomy; NfL, neurofilament light chain; NIHSS, National Institute of Health Stroke Scale; OR, odds ratio.

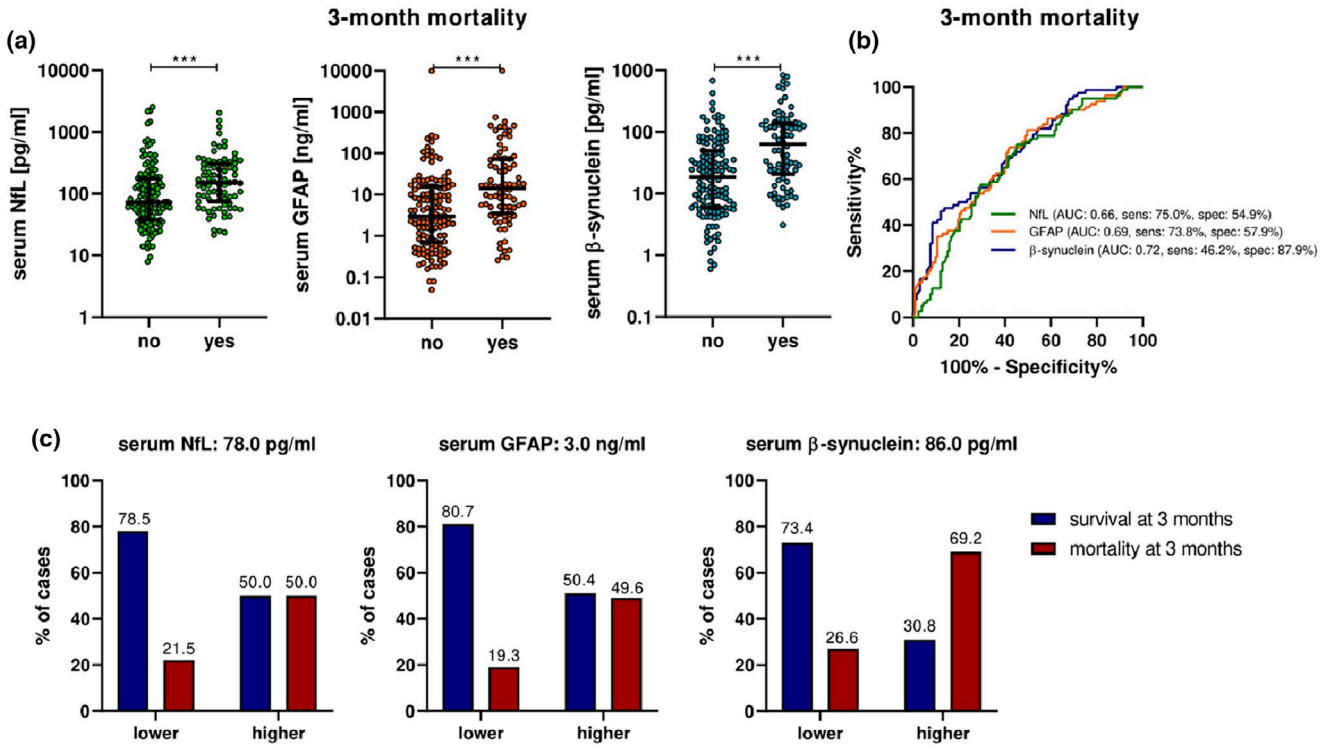

FIGURE 2 Association between serum biomarkers and 3-month all-cause mortality. (a) Serum biomarker levels in AIS patients who survived vs. who did not survive at 3 months. (b) Receiver operating characteristics (ROC) analysis for the discrimination between patients who survived vs. who did not survive at 3 months by assessing serum biomarkers. (c) Distribution of patients who survived vs. who did not survive at 3 months according to best biomarker cutoff values (found by maximizing the Youden index at ROC analysis). Patients were distinguished into two groups depending on whether the serum biomarker levels were above or below the cutoff value. ***$p < 0.001$.

GFAP (Cohen's d: 0.004, $p < 0.001$) compared to patients without ENI ($n = 135$, 63.4%) (Figure 3d), also when accounting for covariables (Table S6). Models including any serum biomarker performed significantly better (AUC ≥0.72) than the model without biomarkers (AUC: 0.62, $p < 0.001$), and β-synuclein showed the highest accuracy either alone (AUC: 0.81) or in combination with NfL/GFAP (AUC ≥0.82) (Table S7).

DISCUSSION

In this study, we investigated the prognostic value of a serum biomarker of synaptic damage, β-synuclein, in comparison to established biomarkers of neuroaxonal (NfL) and glial injury (GFAP) in a well-characterized prospective cohort of patients with moderate-to-severe AIS of the anterior circulation. Overall, our data suggest that: (1) serum β-synuclein and GFAP concentrations, but not NfL, measured within few days from clinical onset are associated with the ASPECTS on hospital admission; (2) NfL, GFAP and β-synuclein serum concentrations are correlated with the NIHSS scores at different timepoints during hospitalization; (3) higher β-synuclein, GFAP and NfL serum levels may accurately identify patients with risk of 3-month mortality; (4) serum β-synuclein, GFAP and NfL may be used either alone or in combination for predicting poor 3-month functional outcomes with good accuracy.

Overall, our results extend previous findings [4, 11, 17–19] and support the use of serum biomarkers in addition to other clinical variables for prognostic purposes in moderate-to-severe AIS. Moreover, we provided biomarker cutoff values for predicting 3-months clinical outcomes after stroke (i.e., all-cause mortality and mRS 3–6 vs. 0–2), which may ease the clinical implementation of serum biomarker quantification for prognosticating AIS patients.

As a novel biomarker of synaptic disruption/dysfunction, β-synuclein has been assessed in blood samples only in neurodegenerative disorders and traumatic brain injury (TBI) [5, 10, 20]. Here, we confirmed previous data [11] on the associations between elevated serum β-synuclein concentrations with lower ASPECTS values, higher NIHSS scores at different timepoints as well as with poorer clinical outcomes, suggesting that the extent of the synaptic damage may reflect the severity of the neurological impairment. Indeed, synapse loss after AIS is a multifactorial process which involves ischemic and inflammatory mechanisms of both pre- and post-synaptic compartments [21]. However, even though previous study on β-synuclein suggested that higher biomarker levels reflect greater degrees of synapse loss [5, 22], it remains unclear whether this may apply also to AIS. In fact, synaptic dysfunction and recovery after AIS are tightly related to the individual neuroplasticity abilities [23], but it has not been explored yet whether blood synaptic protein changes may also reflect such mechanisms. In a similar way, serum GFAP

TABLE 3 Accuracy of serum biomarkers for predicting 3-months all-cause mortality. Covariables were age, sex, GFR, time-to-sampling, NIHSS at admission and acute therapy with IVT and/or MT.

Model	Variable	Best cutoff	AUC (95%CI)	sensitivity (95%CI)	specificity (95%CI)	OR (95%CI)	*p*	DeLong p for continuous vs. categorical
No biomarker								
Multivariable	–	–	0.752 (0.671–0.834)	79.6 (59.1–91.8)	50.5 (44.1–67.7)	–	–	–
Serum NfL								
Univariable	in pg/ml (continuous)	78.0	0.663 (0.589–0.736)	75.0 (64.5–83.2)	54.9 (46.4–63.1)	1.0004 (0.9997–1.001)	0.288	–
Multivariable	in pg/ml (continuous)	–	0.774 (0.696–0.852)	79.6 (57.1–95.9)	71.0 (48.4–88.2)	1.001 (0.999–1.003)	0.213	–
	≥78.0 pg/mL (categorical)	–	0.825 (0.755–0.894)	87.8 (71.4–98.0)	72.0 (55.9–87.1)	6.20 (2.37–16.21)	**<0.001**	0.745
Serum GFAP								
Univariable	in ng/ml (continuous)	3.0	0.692 (0.620–0.764)	81.3 (71.3–88.3)	50.4 (42.0–58.7)	1.0001 (0.9998–1.0004)	0.449	–
Multivariable	in ng/ml (continuous)	–	0.780 (0.703–0.858)	69.4 (53.1–91.8)	79.6 (51.6–90.3)	1.010 (1.003–1.018)	**0.009**	–
	≥3.0 ng/mL (categorical)	–	0.832 (0.763–0.901)	85.7 (61.2–95.9)	73.1 (59.1–91.4)	8.25 (3.04–22.39)	**<0.001**	0.079
Serum β-synuclein								
Univariable	in pg/ml (continuous)	86.0	0.717 (0.647–0.787)	46.2 (35.5–57.1)	87.9 (81.2–92.4)	1.006 (1.003–1.010)	**<0.001**	–
Multivariable	in pg/ml (continuous)	–	0.791 (0.717–0.865)	87.8 (71.4–98.0)	64.5 (49.5–80.7)	1.006 (1.002–1.010)	**0.006**	–
	≥86.0 pg/mL (categorical)	–	0.836 (0.767–0.905)	81.6 (55.1–91.8)	77.4 (64.5–96.8)	10.17 (3.43–30.10)	**<0.001**	**0.034**

Note: Values in bold are statistically significant at $p<0.05$.

Abbreviations: AUC, area under the curve; GFAP, glial fibrillary acidic protein; GFR, glomerular filtration rate; IVT, intravenous thrombolysis; MT, mechanical thrombectomy; NfL, neurofilament light chain; NIHSS, National Institute of Health Stroke Scale; OR, odds ratio.

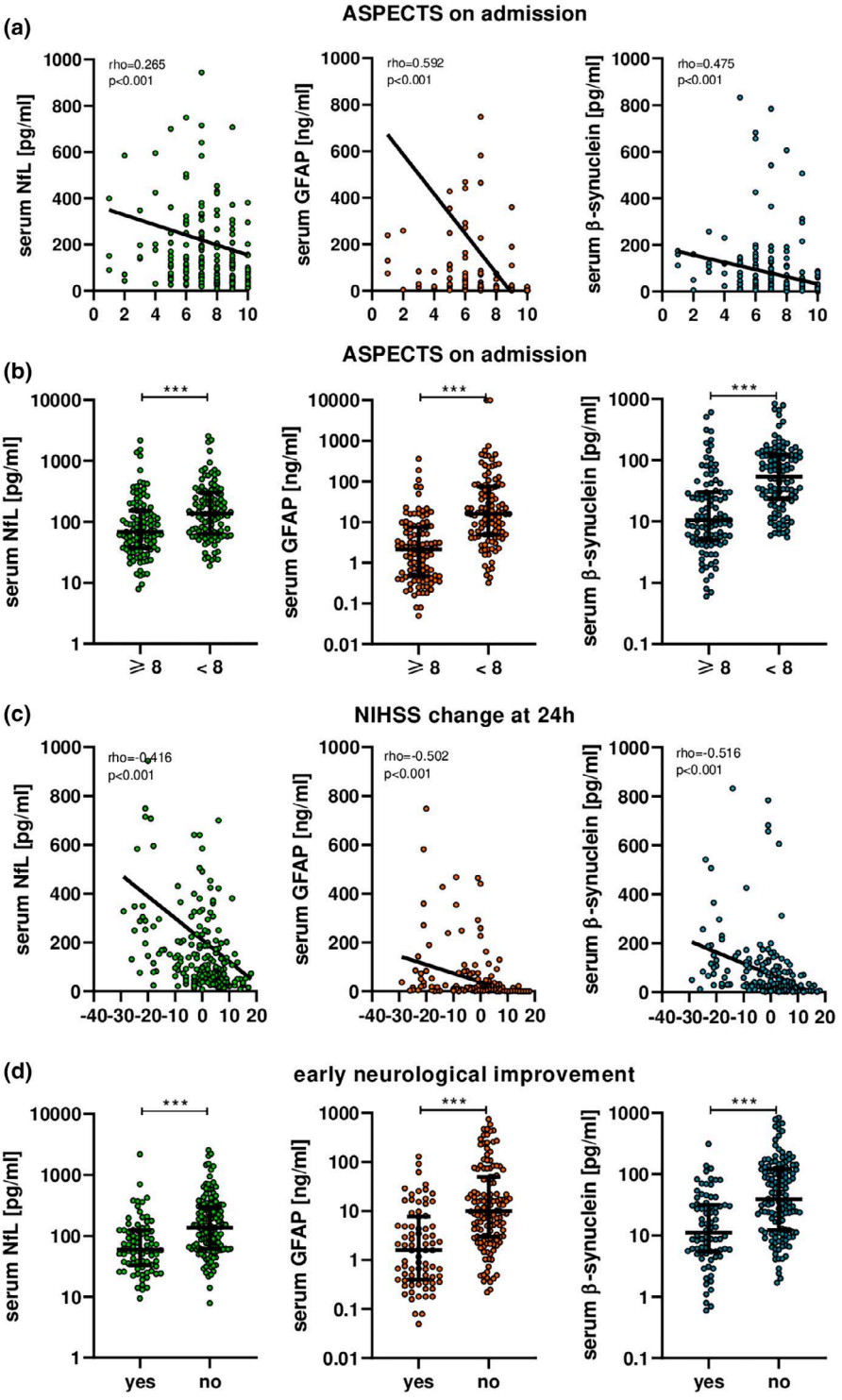

FIGURE 3 Association between serum biomarerks and clinical/radiological variables. (a) Spearman's correlations between serum biomarker levels and ASPECTS values on admission. (b) Serum biomarker levels in patients with admission ASPECTS <8 vs. ≥8 points. (c) Spearman's correlations between serum biomarkers and NIHSS score change within 24 h (admission NIHSS–NIHSS at 24 h). (d) Biomarker levels in patients with vs. without early neurological improvement (ENI) within 24 h (NIHSS change ≥4). ***$p < 0.001$.

concentrations are supposed to reflect glial activation in CNS [3], which was observed to occur extensively in the infarcted area soon after AIS [24]. In our cohort, serum GFAP had a strong prognostic value in AIS, either alone or in combination with NfL/β-synuclein, consistently to previous reports [25]. Interestingly, we found the same best biomarker cutoff value (3.0 ng/mL) for all-cause mortality and poor functional outcome (mRS 3–6) at 3 months, which showed a high accuracy in models combined with other demographic and clinical variables (AUC ≥0.83). Future studies on independent cohorts should better evaluate whether this biomarker cutoff could be used in real-life clinical settings and whether the biomarker quantified on different platforms or with different assays shows a similar accuracy. In comparison to β-synuclein and GFAP, serum NfL had a slightly worse prognostic performance and was not associated with admission ASPECTS or NIHSS scores, which is not surprising by taking into account previous literature evidence [4, 11, 18]. Indeed, these results may be due to the slower temporal kinetics of serum NfL increase after

AIS, which occurs progressively up to 3–12 weeks from clinical onset [17–19]. Elevated serum NfL level within day 1 after onset was associated with worse clinical outcomes, but its accuracy may be improved by considering cutoff values (Tables 2 and 3), by using biomarker combinations with GFAP and/or β-synuclein or by repeating the measurement at later timepoints for tracking the temporal concentration changes.

Within this frame, the choice of the best biomarker for clinical purposes in AIS bases on different aspects, such as measurement availability/accessibility as well as pre-analytical factors. On the one hand, much efforts have been put on the inter-laboratory validation of reliable NfL and GFAP quantification methods with already available point-of-care platforms for GFAP [3, 26]. If validated in AIS, the prompt quantification of such biomarkers could aid clinicians in decision making since the pre-hospital phases [27]. Moreover, even though the accuracy was similar between β-synuclein and GFAP in predicting mortality and 3-months mRS, the two biomarkers showed could be useful for distinct target populations, also considering pre-analytical and analytical factors that may influence their interpretation. In fact, β-synuclein may have advantages in comparison to GFAP, such as a less strong association with age and reduced renal function as well as its selective expression in neurons. On the other hand, the quantification of blood β-synuclein concentration is still not widely available and needs further validation in large scale studies.

As a strength of this study, we investigated a well-characterized AIS cohort with available clinical, biochemical and neuroimaging data during hospitalization as well as 3-months follow-up data. Moreover, for assessing the value of serum biomarkers, we took several variables into account which are often neglected in previous studies, such as the renal function which impacts on NfL and GFAP levels [2, 3]. We acknowledge the lack of brain MRI data and of a control group as major limitations of our study. Further studies should assess the value of β-synuclein and GFAP in other types of AIS, such as small vessel disease, and their predictive value for novel (sub-)clinical MRI lesions as described for NfL [18]. Further, the study cohort included patients with NIHSS score on admission ≥6 and/or indication for MT. The prognostic value of serum biomarker level and alteration of values during clinical course in AIS should be assessed in comparison to healthy controls in further studies. Indeed, it is unknown so far how long biomarker level remain increased after AIS considering the more rapid normalization after traumatic brain injury [10]. Moreover, it is unclear which therapeutic consequences the identification of AIS patients at high risk of poor clinical outcome may have during the post-acute phases. Robust and easily accessible prognostic blood-based biomarkers may help clinicians to tailor therapeutic strategies on an individual level in a rapid and precise fashion, which turns particularly important especially in severe AIS [28]. However, large-scale studies are needed to support routine clinical use. Lastly, we quantified biomarker concentrations at a single timepoint post-therapy after AIS and could not investigate their temporal dynamics longitudinally.

In conclusion, higher serum levels of β-synuclein, GFAP and NfL are associated with poor short-term and 3-months functional outcome in patients with moderate-to-severe AIS. Those biomarkers can be used for prognostic purposes to identify patients at risk of worse clinical course. Biomarker cutoff values may encourage clinical implementation but need further validation in large AIS cohorts.

AUTHOR CONTRIBUTIONS

Lorenzo Barba: Conceptualization (equal); Formal analysis (lead); Data curation (equal); Investigation (equal); Methodology (equal); Writing–original draft (lead); Writing–review & editing (equal). **Christoph Vollmuth**: Conceptualization (equal); Formal analysis (equal); Data curation (equal); Investigation (equal); Methodology (equal); Writing–original draft (lead); Writing–review & editing (equal). **Steffen Halbgebauer**: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Kathrin Ungethüm**: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Christian Hametner**: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Fabian Essig**: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Alexander M. Kollikowski**: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Mirko Pham**: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Michael K. Schuhmann**: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Peter U. Heuschmann**: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Patrick Oeckl**: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Petra Steinacker**: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Michele Romoli**: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Lucio D'Anna**: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Samir Abu-Rumeileh**: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Karl Georg Haeusler**: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Guido Stoll**: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing–review & editing (equal). **Hermann Neugebauer**: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Resources (lead); Supervision (lead); Writing–original draft (equal); Writing–review & editing (lead). **Markus Otto**: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Resources

(lead); Supervision (lead); Writing–original draft (equal); Writing–review & editing (lead).

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CONFLICT OF INTEREST STATEMENT

MO gave scientific advice for Fujirebio, Roche, Biogen, and Axon; MO, SH and PO are co-inventors of a patent handed for the measurement of beta-synuclein. KGH reports speaker's honoraria, consulting fees, lecture honoraria and/or study grants from Abbott, Alexion, Amarin, AstraZeneca, Bayer Healthcare, Biotronik, Boehringer Ingelheim, Boston Scientific, Bristol-Myers Squibb, Daiichi Sankyo, Edwards Lifesciences, Medronic, Novartis, Pfizer, Portola, Premier Research, Sanofi, SUN Pharma, and W.L. Gore and Associates. PUH reports grants from German Ministry of Research and Education, German Research Foundation, European Union, Federal Joint Committee (G-BA) within the Innovationfond, Berlin Chamber of Physicians, German Parkinson Society, University Hospital Würzburg, Robert Koch Institute, German Heart Foundation, University Göttingen (within FIND-AF [A Prospective, Randomised, Controlled Study to Determine the Detection of Atrial Fibrillation by Prolonged and Enhanced Holter Monitoring as Compared to Usual Care in Stroke Patients], supported by an unrestricted research grant to the University Göttingen from Boehringer-Ingelheim), University Hospital Heidelberg (within Registry of Acute Stroke Under Novel Oral Anticoagulants [RASUNOA]-prime, supported by an unrestricted research grant to the University Hospital Heidelberg from Bayer, Bristol-Myers Squibb, Boehringer-Ingelheim, Daiichi Sankyo), Charité–Universitätsmedizin Berlin (within MonDAFIS, supported by an unrestricted research grant to the Charité from Bayer), all outside the submitted work. PO received research support from the Cure Alzheimer Fund, ALS Association (24-SGP-691, 23-PPG-674-2), ALS Finding a Cure, the Charcot Foundation, the DZNE Innovation-to-Application program and consulting fees from LifeArc and Fundamental Pharma. The other authors have nothing to declare.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The present study was conducted according to the Declaration of Helsinki and its recent modifications. All participants or their legal representatives gave written informed consent to research and the local Ethics Committee of the University of Würzburg approved the study protocol (Reference No 05/20-am, registry number: DRKS00022064).

ORCID

Lorenzo Barba https://orcid.org/0000-0003-1328-3620
Christoph Vollmuth https://orcid.org/0000-0002-5893-1196
Mirko Pham https://orcid.org/0000-0003-1295-2685
Samir Abu-Rumeileh https://orcid.org/0000-0003-0631-8506
Markus Otto https://orcid.org/0000-0003-4273-4267

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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