Clinical Interventions in Aging

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ORIGINAL RESEARCH

Expression of Acidic Fibrillar Protein and Neuroglobin in Thrombolytic Patients in Ischemic Stroke

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Purpose: Glial fibrillary acidic protein (GFAP) and neuroglobin (NGB) are important biomarkers of cerebral hypoxia. For this reason, an attempt was made to assess their concentrations in various time intervals and their impact on the severity of neurological symptoms and functional prognosis of thrombolytic ischemic stroke patients.

Patients and Methods: The study involved 94 patients reporting to the emergency department of the Collegium Medicum University Hospital in Bydgoszcz within < 4.5 hours of the onset of stroke symptoms. GFAP and neuroglobin levels were measured in plasma at indicated times using a commercial ELISA kit.

Results: Based on the data gathered, statistically significant differences were found between the concentration of biomarkers in stroke patients and the control group. The concentrations of both biomarkers, GFAP and NGB, were elevated in patients after ischemic stroke and the changes in their concentrations in the subsequent stages of stroke may suggest their prognostic value strictly dependent on time. NGB was determined on the 7th day, and mRS - after a year (0.35). GFAP measured after 24 h and on day 7 could be a promising biomarker of functional outcome after one year (cut-off point ≤ 0.231 ng/mL, sensitivity 75.0%, specificity 61.2%, cut off point ≤ 0.235 ng/mL, sensitivity 75.0%, specificity 73.9%, respectively) and the severity of the patient's neurological condition. At GFAP concentrations above 0.25 ng/mL, measured within 24 hours, a sharp increase in mortality was observed in stroke patients. In the case of NGB, at the time of stroke occurrence (14 ng/mL) and after 24 hours (10–60 ng/mL). Differences in the concentrations of these biomarkers have been demonstrated in different stroke subtypes.

Conclusion: NGB and GFAP are important biomarkers of ischemic brain injury and may also participate in predicting neurological outcomes.

Keywords: GFAP, NGB, stroke, oxidative stress, inflammation, thrombolysis

Introduction

Worldwide, stroke is recognised as the leading cause of serious, long-term disability and even death. Approximately 85% of all strokes are ischemic strokes (AIS) caused by arterial occlusion, while the remaining 15% lead to intracerebral haemorrhages. In terms of aetiology, stroke is characterized by high heterogeneity. The most commonly used division of ischemic strokes is according to The Trial of Org 10172 in Acute Stroke Treatment (TOAST). This classification is based on clinical features and diagnostic test results. According to this classification, the following etiological categories of cerebral infarction are distinguished: stroke caused by atherosclerosis of large vessels in the brain, stroke caused by small

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vessels (lacunar stroke), cardioembolic stroke, ischemic stroke of another specific aetiology, and stroke of unknown aetiology.[1]

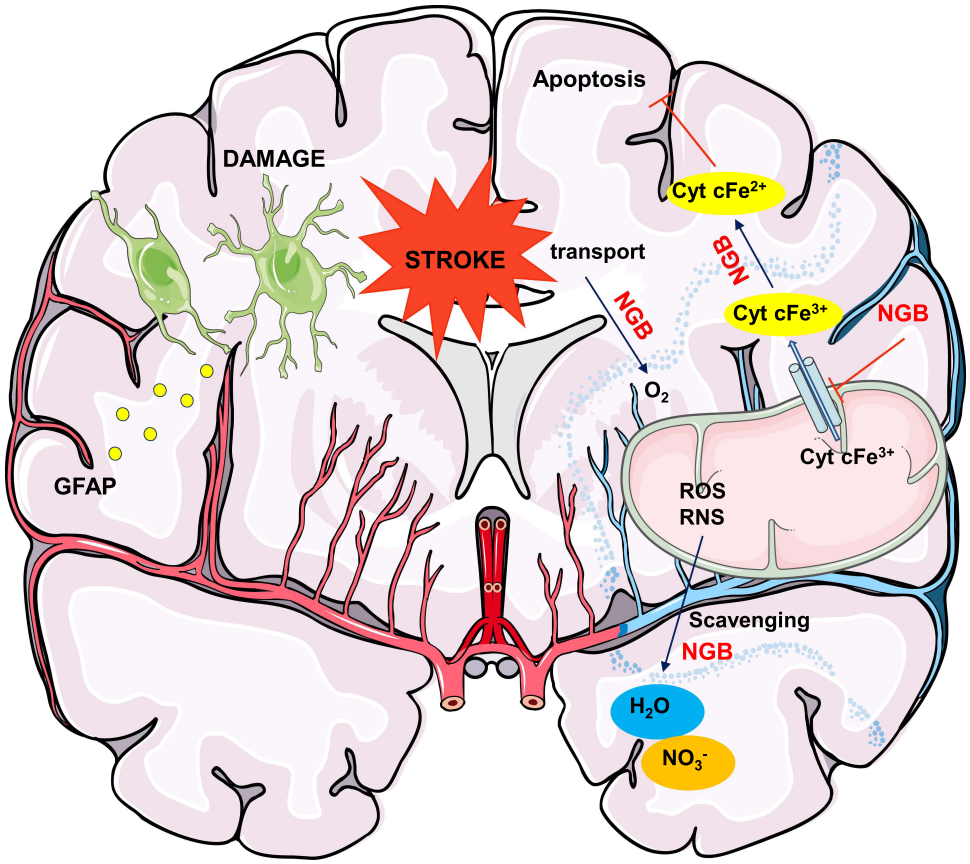
The mechanisms underlying ischemic stroke's pathophysiology are not fully understood. Still, there is more and more evidence indicating the involvement of inflammation or oxidative stress in the drastic changes that occur during and after cerebral ischemia.[2–5]

Glial fibrillary acidic protein (GFAP) is considered highly brain-specific. As a structural protein, it is not released from cells under physiological conditions, and healthy people do not have detectable levels of GFAP in the blood. However, many studies suggest that GFAP is associated with intracerebral haemorrhage (ICH), a traumatic brain injury.[6,7]

Recent findings have suggested that serum GFAP concentrations recorded soon after the onset of symptoms may be a sensitive and specific biomarker not only for differentiating ICH and AIS[8–11] but also for distinguishing strokes from other acute neurological disorders (Scheme 1).[12]

So far, it has been established that GFAP can not only be a prognostic and diagnostic parameter but also a biomarker in screening tests.[13–16] This may be a molecule that predicts stroke recurrence and risk of intracerebral hemorrhage.[17]

In turn, neuroglobin (NGB) is considered an important parameter exhibiting neuroprotective effects by modulating mitochondrial function, such as ATP production, removal of reactive oxygen and nitrogen species (ROS/RNS), promoting mitochondrial dynamics and controlling apoptosis.[18] Nevertheless, the precise interactions between neuroglobin and proteins with biological function in mitochondria have not been fully explored. Neuroglobin has a direct antioxidant effect by scavenging not only nitrogen oxide (NO) but also hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical ($HO\cdot$) and 2.2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.[19,20] Studies have shown that NGB inhibits the intrinsic apoptosis pathway in human neuroblastoma SH-SY5Y cells by interrupting pro-caspase 9 activation through interaction with Cytc.[21] The neuroprotective effect of NGB with Cytc is probably related to the



Scheme 1 The role of GFAP and NGB in stroke. This figure was created using Servier Medical Art (available at https://smart.servier.com/) (accessed on 12 April 2023).

sequestration of Cyt c(Fe^{3+}) through the formation of the NGB(Fe^{2+})-Cyt c(Fe^{3+}) complex. NGB(Fe^{2+}) reduces Cyt c(Fe^{3+}) to Cyt c(Fe^{2+}) and thus decreases the release of pro-apoptotic Cytc (Scheme 1).[21,22]

NGB is thought to protect neurons against hypoxic-ischemic damage and is upregulated in ischemic stroke.[23] Its level depends on both the type of stroke, severity and time of onset of stroke symptoms.[24–26]

The basic therapy of acute AIS is based on the administration of intravenous tissue plasminogen activator (rt-PA) within the first 4.5 hours.[3–5] In selected patients with intracranial vascular occlusion, endovascular therapies are also used to remove the clot.[27] Currently, however, the diagnosis of stroke subtype is based on brain neuroimaging data using CT (Computed Tomography) and MRI (Magnetic Resonance Imaging). The application of molecular biomarkers in diagnostics can help accelerate the process of assessing a stroke subtype. Additionally, these biomarkers may be considered as potential tools providing valuable information on the risk, aetiology, prognosis, and diagnosis of stroke so that appropriate treatment can be quickly implemented.

Therefore, we hypothesize that both serum GFAP and neuroglobin levels may be associated with future prognosis related to functional outcomes after IS. Modulation of mitochondrial dynamics by NGB may also open new opportunities not only in the field of neuroglobins but also in neurological diseases. Designing new therapies using these biomolecules to improve the treatment of neurological disorders may also be considered, but this requires further clinical studies.

Material and Methods

Study Group

The study involved patients reporting to the emergency department of the Collegium Medicum University Hospital in Bydgoszcz within < 4.5 hours of the onset of stroke symptoms. The material for the study was collected over 3 years, and the clinical evaluation of most patients continued one year after the stroke. All patients underwent extensive medical assessments and were classified according to the clinical diagnosis. AIS was defined according to the recommendations of the World Health Organization (stroke is defined as "neurological deficit of cerebrovascular origin that persists for more than 24 hours or is terminated by death within 24 hours"). Clinical diagnoses were confirmed by computed tomography (CT) and/or magnetic resonance imaging (MRI).

Medical information was collected from study participants. Demographic data, body mass index (BMI), and history of risk factors (hypertension, diabetes, atrial fibrillation, hyperlipidaemia, smoking habit, and alcohol abuse) were obtained at admission. Before the stroke, the selected patients were treated with anticoagulants, antiplatelet and antihypertensive drugs. Exclusion criteria from this research included malignancy, ICH (including initially ischemic strokes that later became haemorrhagic), severe oedema, renal failure, head trauma, febrile disorders, and autoimmune diseases. Patients on combined therapy (intravenous and endovascular therapy) with concomitant myocardial infarction after stroke were excluded from the analysis. The need for informed consent for additional blood sampling for biomarker determination led to the exclusion of patients with aphasia or impaired consciousness. Therefore, a group of patients with mild and moderate neurological deficits were included in the study. This study was approved by the local Bioethics Committee (no. KB 637/2016) of Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, in accordance with the Helsinki Declaration. Blood samples were collected from patients only after obtaining written informed consent from them or their relatives.

Clinical severity was assessed using the National Institutes of Health Stroke Scale (NIHSS; NIHSS scale ≤ 3 points (mild stroke)/NIHSS scale ≤ 3 points (moderate stroke)), and functional impairment was assessed using the modified Rankin Scale (mRS). The good functional outcome of a patient after stroke was defined as a result on the mRS scale of 0–2 points, while the poor functional result was in the range of 3–6 points on the mRS scale.

Participants' health conditions were monitored for 1 year using a standardized questionnaire and they were contacted via telephone or home visits for 3 months after enrolment.

A selected, healthy group of people recruited through a "volunteer panel" also took part in the study. The control group was a random group of healthy people aged 27 to 66 years, assessed based on interviews and clinical examination.

Biochemical Testing

Peripheral blood was collected in EDTA tubes upon admission to the hospital (< 4.5 h), before any therapeutics were administered, and after 12 h and 168 h in hospital conditions. Plasma was obtained by centrifugation at 3000 g for 15 min at 4°C and stored at −80°C until further use. All immunoassays in this study were performed with plasma samples according to the manufacturer's instructions and without clinical data. GFAP and neuroglobin levels were measured in plasma at indicated times using a commercial ELISA kit (Cloud Clone Corp). Each GFAP and neuroglobin measurement was performed in full calibration mode.

Statistical Methods

The R (v. 4.2.3) and the Python programming languages (3.8.10) were used to analyse the gathered data. The statistical analysis employed the Fisher, the Shapiro–Wilk normality, and the Mann–Whitney *U*-tests. Spearman correlation coefficients were used to examine the relationship between biomarkers and the values of the mRS and NIHSS scales. The receiver operating characteristic (ROC) curves were produced to assess the impact of biomarker values on the patient's functional status. All analyses were performed at a significance level of 0.05.

To verify differences between measurements of biomarkers at each time point, we used the Friedman test, which is a nonparametric version of repeated measure ANOVA. We used the Wilcoxon test for paired observation as a post hoc test to determine the groups where the differences occur. To verify multivariable dependencies we computed logistic regression models.

Results

The biological material was collected from 94 patients at the time they were admitted to the Neurology Department of the University Hospital in Bydgoszcz. The studies included patients who had a stroke and were classified according to the Oxfordshire Community Stroke Project (OCSP) into stroke severity: lacunar cerebral infarction (LACI), posterior circulation infarction (POCI) and partial anterior circulation infarction (PACI). We did not compare patients who had anterior cerebral artery stroke with those who had a middle cerebral artery stroke.

The size of the study group at the time of discharge was 91 patients (84 patients after 3 months and 70 patients after a year post-discharge). Favourable mRS scores were observed for 65 (77%) at 3 months, and 60 (86%) after 1 year. A total of 12 patients died, including 7 within 3 months of the stroke. After three months, 14 (16.7%) patients had coronary artery disease, 33 (39.3%) diabetes, 9 (10.7%) atrial fibrillation, 5 (5.9%) gout, and 3 (3.6%) renal failure. Before the stroke, 1 (1.2%) patient was using anticoagulants, 26 were using ASA (31.0%) and 24 (28.6%) were using statins. Patients with a favourable Rankin scale score were younger ($p = 0.045$) and had lower scale values after 3 months and 1 year since discharge ($p < 0.001$, $p = 0.003$, respectively). This study group presented fewer neurological changes assessed according to the NIHSS scale both at admission and discharge ($p < 0.001$ and $p < 0.001$). In addition, infections were observed less frequently ($p = 0.001$) and antibiotics were also less frequently administrated ($p = 0.001$). Moreover, younger patients had lower systolic blood pressure values ($p = 0.089$). The clinical characteristics of the subgroups of thrombolytic stroke patients with favourable and unfavourable functional outcomes after three months are shown in Table 1.

The functional and neurological status of patients was assessed according to the mRS scale after three months and one year. In addition, the same conditions were evaluated according to the NIHSS scale at admission and discharge. Patients were divided into two subgroups according to the favourable (mRS 0–2 points) and unfavourable (mRS 3–6 points) outcomes. The GFAP concentration levels for patients with a favourable functional outcome (mRS 0–2 points) were lower compared to patients with an unfavourable functional outcome (Table 2). However, no such difference was observed for GFAP concentration after 24 hours according to the mRS scale as well as after three months and after one year. Similar observations were seen according to the NIHSS scale both at admission and at discharge (Table 3). However, these were not statistically significant differences.

In turn, a statistically significant difference was demonstrated in the GFAP concentration determined < 4.5 h for patients with NIHSS ≤ 3 and NIHSS > 3 at admission (0.21 ng mL^{-1} with IQR 0.17–0.28 vs 0.30 ng mL^{-1} with IQR 0.19–0.50, $p = 0.031$).

Table 1 The Clinical Characteristics of the Subgroups of Stroke Patients with Favourable and Unfavourable Outcomes After Three Months

Clinical Parameters		Favourable (N = 65) (mRS 0–2 pts)	Unfavourable (N = 19) (mRS 3–6 pts)	p-value
Age	Median [Q1, Q3]	65.0 [56.0,70.0]	70.0 [64.0,80.0]	0.045
Gender (male)	n (%)	41 (63.08)	11 (57.89)	0.888
Current smoking	n (%)	21 (32.31)	6 (31.58)	1
BMI	Median [Q1, Q3]	25.80 [23.44,31.01]	26.03 [22.62,28.62]	0.395
SBP on admission	Median [Q1, Q3]	151.0 [140.0,170.0]	161.0 [145.0,179.0]	0.089
DBP on admission	Median [Q1, Q3]	83.0 [75.0,93.0]	90.0 [77.5,102.0]	0.195
Impaired renal function	n (%)	2 (3.08)	1 (5.26)	0.542
Gout	n (%)	5 (7.69)	0 (0.00)	1
Diabetes Mellitus	n (%)	23 (35.38)	10 (52.63)	0.277
Coronary heart disease	n (%)	10 (15.38)	4 (21.05)	0.727
Atrial fibrillation	n (%)	5 (7.69)	4 (21.05)	0.199
Statin therapy before stroke	n (%)	18 (28.57)	6 (31.58)	1
Statin therapy after stroke	n (%)	63 (96.92)	15 (93.75)	1
Anticoagulant therapy before the stroke	n (%)	1 (1.54)	0 (0.0)	1
ASA before the stroke	n (%)	19 (29.69)	7 (36.84)	0.757
Infection	n (%)	5 (7.69)	8 (42.11)	0.001
Antibiotic	n (%)	5 (7.69)	8 (42.11)	0.001
NIHSS on admission	Median [Q1, Q3]	4.00 [3.00,5.00]	12.50 [9.25,16.00]	< 0.001
NIHSS on discharge	Median [Q1, Q3]	1.00 [0.00,2.00]	10.00 [3.00,13.50]	< 0.001

Abbreviations: ASA, acetylsalicylic acid; BMI, body mass index; IQR, interquartile range (Q1-Q3); mRS, modified Rankin Scale; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 2 Biomarkers Level in Subgroups of Stroke Patients with Favourable and Unfavourable Functional Status According to mRS

Biomarker		Favourable (mRS 0–2 pts)	Unfavourable (mRS 3–6 pts)	p-value
		(N = 63)	(N = 30)	
GFAP (0) Median[Q1, Q3] [ng/mL]	after three months	0.26 [0.17,0.42	0.28 [0.16,0.51]]	0.938
GFAP (1) Median[Q1, Q3] [ng/mL]		0.25 [0.22,0.30]	0.23 [0.19,0.25]	0.525
GFAP (2) Median[Q1, Q3] [ng/mL]		0.21 [0.17,0.25]	0.24 [0.21,0.25]	0.450
NGB (0) Median[Q1, Q3] [ng/mL]		22.6 [15.6,38.6]	16.4 [14.2,22.7]	0.101
NGB (1) Median[Q1, Q3] [ng/mL]		18.0 [11.4,40.2]	25.2 [10.8,39.0]	0.805
NGB (2) Median[Q1, Q3] [ng/mL]		23.1 [16.9,33.9]	18.5 [14.3,30.8]	0.500

(*Continued*)

Table 2 (Continued).

Biomarker		Favourable (mRS 0–2 pts)	Unfavourable (mRS 3–6 pts)	p-value
		(N = 59)	(N = 34)	
GFAP (0) Median[Q1, Q3] [ng/mL]	after a year	0.26 [0.17,0.42]	0.37 [0.21,0.51]	0.522
GFAP (1) Median[Q1, Q3] [ng/mL]		0.25 [0.22,0.30]	0.23 [0.21,0.25]	0.468
GFAP (2) Median[Q1, Q3] [ng/mL]		0.21 [0.18,0.24]	0.25 [0.23,0.35]	0.142
NGB (0) Median[Q1, Q3] [ng/mL]		22.8 [16.2,38.9]	22.1 [16.4,25.9]	0.721
NGB (1) Median[Q1, Q3] [ng/mL]		14.4 [11.2,38.7]	36.4 [29.,42.2]	0.210
NGB (2) Median[Q1, Q3] [ng/mL]		21.6 [14.3,32.6]	29.8 [20.9,34.2]	0.722

Abbreviations: GFAP (0), glial fibrillary acidic protein within 4.5 hours; GFAP (1), glial fibrillary acidic protein within 24 hours; GFAP (2), glial fibrillary acidic protein within 7 days; NGB (0), neuroglobin within 4.5 hours; NGB (1), neuroglobin within 24 hours; NGB (2), neuroglobin within 7 days; IQR, interquartile range (Q1-Q3); mRS, modified Rankin.

Table 3 Biomarkers Level in Subgroups of Stroke Patients with Favourable and Unfavourable Functional Status According to NIHSS

Biomarker		NIHSS ≤ 3 pts	NIHSS > 3pts	p-value
		(N = 25)	(N = 65)	
GFAP (0) Median[Q1, Q3] [ng/mL]	on admission	0.21 [0.17,0.28]	0.30 [0.19,0.50]	0.031
GFAP (1) Median[Q1, Q3] [ng/mL]		0.25 [0.22,0.30]	0.23 [0.19,0.29]	0.245
GFAP (2) Median[Q1, Q3] [ng/mL]		0.20 [0.17,0.22]	0.22 [0.15,0.25]	0.499
NGB (0) Median[Q1, Q3] [ng/mL]		21.02 [14.16,27.15]	28.59 [18.20,39.64]	0.031
NGB (1) Median[Q1, Q3] [ng/mL]		24.45 [11.27,38.99]	16.57 [12.49,40.59]	0.947
NGB (2) Median[Q1, Q3] [ng/mL]		19.54 [14.07,34.00]	23.78 [20.63,33.53]	0.261
		(N = 71)	(N = 22)	
GFAP (0) Median[Q1, Q3] [ng/mL]	on discharge	0.24 [0.17,0.40]	0.33 [0.21,0.53]	0.259
GFAP (1) Median[Q1, Q3] [ng/mL]		0.24 [0.21,0.29]	0.23 [0.15,0.29]	0.525
GFAP (2) Median[Q1, Q3] [ng/mL]		0.20 [0.15,0.23]	0.25 [0.22,0.28]	0.089
NGB (0) Median[Q1, Q3] [ng/mL]		21.36 [15.10,26.73]	22.45 [14.52,34.48]	0.883
NGB (1) Median[Q1, Q3] [ng/mL]		27.12 [14.20,35.08]	18.82 [11.86,40.96]	0.805
NGB (2) Median[Q1, Q3] [ng/mL]		25.88 [17.95,34.72]	21.57 [16.58,32.59]	0.660

Abbreviations: GFAP (0), glial fibrillary acidic protein within 4.5 hours; GFAP (1), glial fibrillary acidic protein within 24 hours; GFAP (2), glial fibrillary acidic protein within 7 days; NGB (0), neuroglobin within 4.5 hours; NGB (1), neuroglobin within 24 hours; NGB (2), neuroglobin within 7 days; IQR, interquartile range (Q1-Q3); NIHSS, National Institutes of Health Stroke Scale.

Differences in NGB concentration were found at different time points (Table 2 and Table 3). In patients with a favourable functional outcome, the highest concentration was observed at the onset of symptoms and 7 days on the mRS scale after one year and three months, respectively. However, in the group of patients with an unfavourable outcome – 24 hours, after a year. On the NIHSS scale for patients with NIHSS > 3 pts at onset on admission and with NIHSS ≤ 3 in the first 24 hours on

discharge. Statistically significant differences were found between the NGB level determined < 4.5 h in the subgroups of patients depending on stroke severity (NIHSS ≤ 3 points and NIHSS > 3 points) at admission (21.02 ng mL^{-1} with IQR 14.16–27.1 vs 28.59 ng mL^{-1} with IQR 18.20–39.64, p = 0.031). Based on the multivariate logistic regression model taking into account the age of the subjects, infection during stroke and NGB level determined < 4.5 h, it can be concluded that the occurrence of infection reduces the chance of a positive result after three months tenfold compared to the absence of infection (0.101;[0.025, 0.412]; AOR [95% CI]; p = 0.001). An increase in the NGB value (< 4.5 h) by 1 unit increases the chance of a positive result after 3 months (1.061;[1.01, 1.114]; AOR [95% CI]; p = 0.019).

Statistically significant differences were observed between the values of GFAP and NGB concentrations in the group of stroke patients compared to the control group. The median GFAP concentrations were 0.25 (p < 0.001), 0.23 (p < 0.001), and 0.21 (p < 0.001) vs 0.08 ng mL^{-1}, respectively, and the median NGB concentrations were 22.15 (p < 0.001), 19.28 (p < 0.001), 22.51 (p < 0.001) vs 7.5 ng mL^{-1}, respectively.

Friedman test for repeated measures for GFAP and NGB was computed. Statistically significant differences between measurements were found (p = 0.019 for NGB and p < 0.001 for GFAP). For both markers, a tendency for the values to decrease was observed, with more scattered values for NGB in the second measurement. Both markers, grouped by the time of measurement are presented in Figure 1.

There were statistically significant differences between genders for GFAP concentrations determined for 24 hours and 7 days, which were 0.22 vs 0.26 ng mL^{-1} and 0.17 vs 0.23 ng mL^{-1} for women and men, respectively. Similarly, NGB concentration was higher in men than in women, but these differences were not statistically significant.

Moderate correlations were observed between GFAP at 1 week and the NIHSS score at admission and discharge (0.51, 0.36, respectively). NGB was determined on the 7th day, and mRS - after a year (0.35). There was no effect of statins on the level of the tested biomarkers.

Statistically significant differences were found in the LACI and PACI subtypes for the GFAP value determined during onset (0.228 with IQR 0.218 vs 0.400 with IQR 0.417, p = 0.005). NGB concentration was higher in patients with POCI stroke compared to patients after LACI stroke. However, statistically significant differences were observed only for the value of this parameter determined after 7 days (19.03 with IQR 7.97 vs 34.20 with IQR 10.79, p = 0.012). The obtained results are presented Figure 2.

Additionally, correlation values between the tested biomarkers were determined. Statistically significant positive correlations were found between the GFAP concentration value determined below 4.5 h and on the first day and the NGB concentration after 24 h, as well as moderate correlations between GFAP after 24 h and NGB on the first day. Dependencies between pairs of variables are presented on Figure 3.

To assess the impact of GFAP and NGB concentrations on the neurological functional status, ROC curves were produced. For GFAP, the AUCs were found to be 0.625, 0.613 and 0.734 for favourable functional outcomes assessed on the mRS scale three months and one year after the stroke, respectively (Figure 4). The cut-off points for these curves were 0.253 (sensitivity 51.6%, specificity 77.8%), 0.231 (sensitivity 75.0%, specificity 61.2%), 0.235 (sensitivity 75.0%,

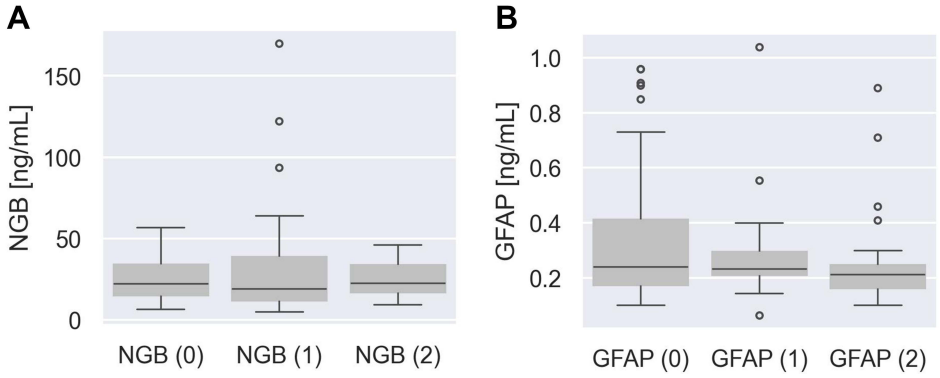


Figure 1 The change of concentration of (**A**) NGB and (**B**) GFAP in time.

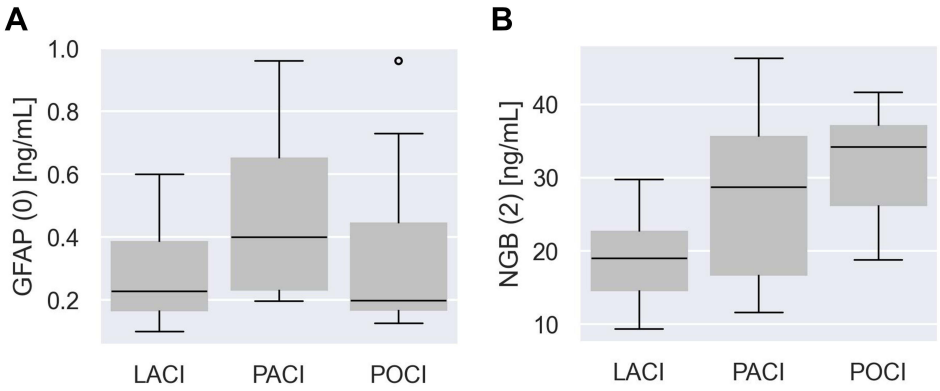


Figure 2 Change in (**A**) GFAP and (**B**) NGB levels by stroke subtypes.

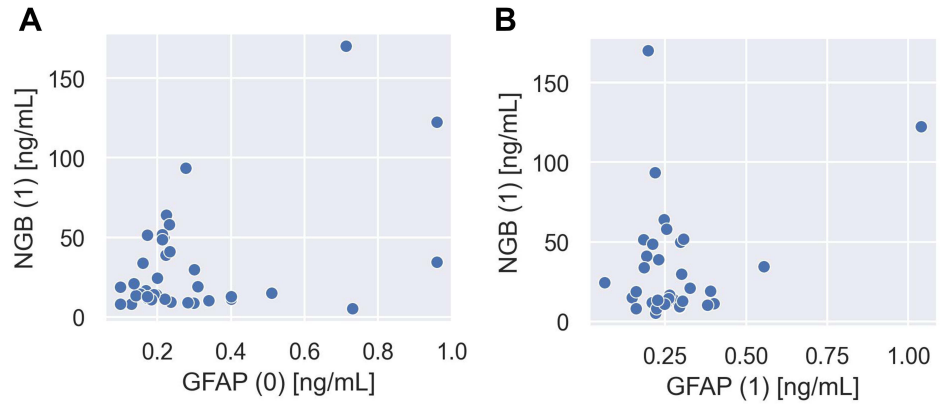


Figure 3 Scatter plots between the level of NGB (1) and GFAP (0) (**A**) and GFAP (1) (**B**).

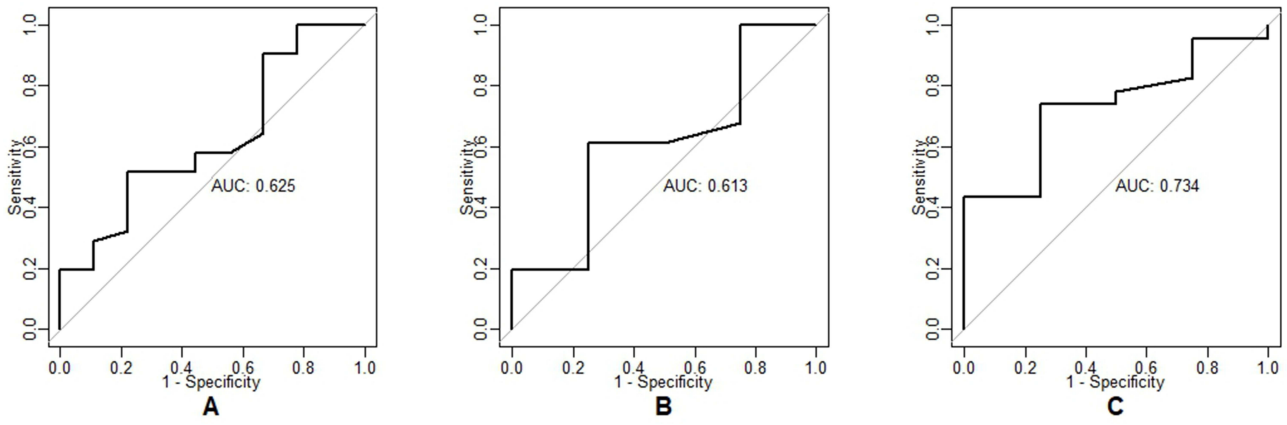


Figure 4 ROC curves showing sensitivity and specificity of GFAP measurements for good functional outcome: (**A**) GFAP within 24 h; after three months (cut-off point ≤ 0.253 ng/mL, sensitivity 51.6%, specificity 77.8%), (**B**) GFAP within 24 h; after a year (cut-off point ≤ 0.231 ng/mL, sensitivity 75.0%, specificity 61.2%), (**C**) GFAP within 7 days; after a year (cut-off point ≤ 0.235 ng/mL, sensitivity 75.0%, specificity 73.9%).

specificity 73.9%). For NGB, the AUCs were determined at 0.631, 0.639 and 0.590, respectively. The cut-off points for these curves were 17.28 (sensitivity 53.4%, specificity 64.7%), 38.94 (sensitivity 59.2%, specificity 100%), 15.79 (sensitivity 66.7%, specificity 71.4%), respectively (Figure 5).

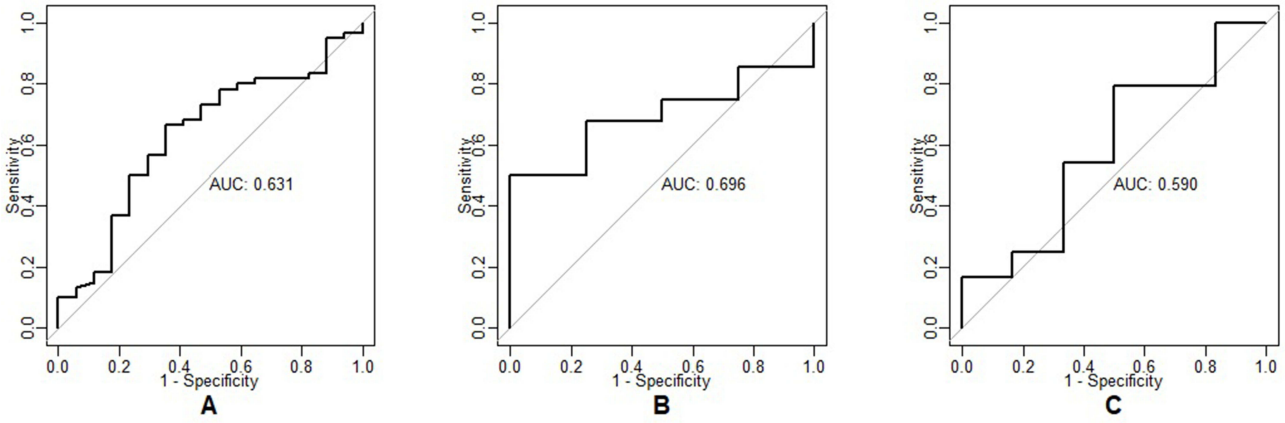


Figure 5 ROC curves showing sensitivity and specificity of NGB measurements for good functional outcome: (**A**) NGB < 4.5 h; after three months (cut-off point ≤ 17.28 ng/mL, sensitivity 53.4%, specificity 64.7%), (**B**) NGB within 24 h; after a year (cut-off point ≤ 38.94 ng/mL, sensitivity 59.2%, specificity 100%) (**C**) NGB within 7 days; after three months (cut-off point ≤ 15.79 ng/mL, sensitivity 66.7%, specificity 71.4%).

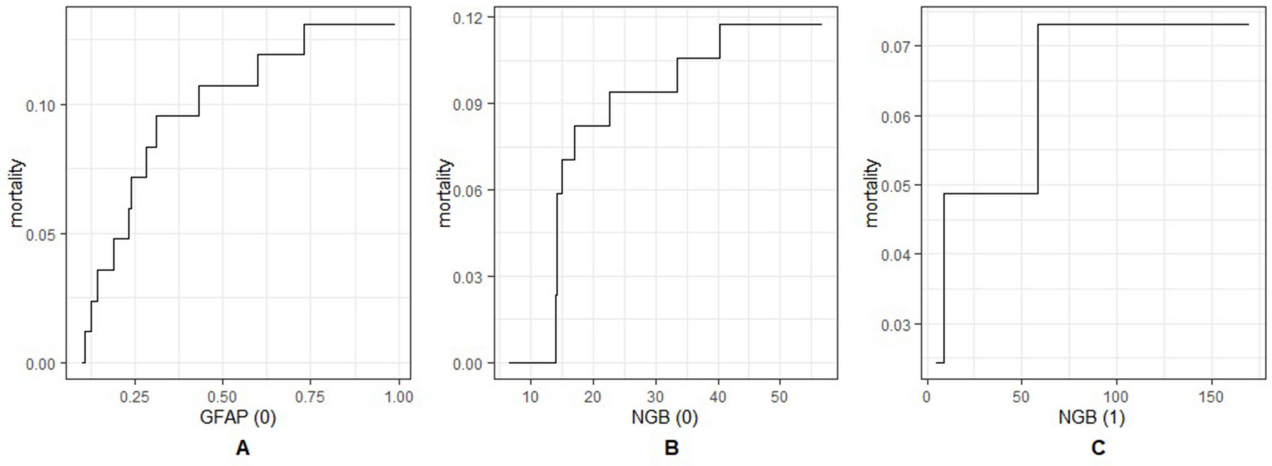


Figure 6 The percentage of deaths among patients after stroke based on the value of GFAP and NGB. (**A**) the impact of GFAP concentration assessed after 24 hours on the probability of death, (**B**), the impact of NGB concentration assessed after < 4.5 h on the probability of death (**C**) the impact of NGB concentration assessed after 24 hours on the probability of death.

The impact of GFAB and NGB concentrations on the patients' probability of death was also assessed (Figure 6). At GFAP concentrations above 0.25 ng mL^{-1}, determined over 24 hours, a sharp increase in mortality in stroke patients was observed. A similar relationship was also demonstrated for NGB assessed during onset (value above 14 ng mL^{-1}) and on the first day of stroke (10–60 ng mL^{-1}).

In our previous studies, the involvement of IL-6 and TNF-α[3,4] in AIS was evaluated and therefore the correlation between those inflammatory parameters (IL-6, TNF-α) and GFAP, NGB was also investigated. There is a statistically significant correlation between GFAP concentration on the seventh day and IL-6 assessed on the first day (0.54), and a moderate correlation between IL-6 after seven days (0.46) and TNF- α after 24 h (0.39). Correlation was performed analogously for NGB. The NGB level assessed in less than 4.5 h correlates with the concentration of IL-6 and TNF-α after seven days and on the first day (0.41; 0.47), respectively.

Discussion

Since ischemic stroke (AIS) is one of the most serious clinical problems, it seems reasonable to search for molecular biomarkers that would allow for the early diagnosis of patients in the acute phase and would also enable the assessment of the degree of damage to the nervous tissue. Currently, the only methods used to treat AIS are thrombolysis and

endovascular treatments caused by large vessel occlusion. Therefore, we have been looking for biomarkers that can be used in conjunction with clinical treatment. This would allow predicting the need for rehabilitation and tailored patient care. The development of an objective, quantifiable biomarker may be important not only for improving existing clinical assessment approaches but may also be a valuable tool in developing further therapy and appropriate patient management strategies.

Two of the possible markers expressed during ischemic brain damage are GFAP and NGB.[20,28,29]

GFAP is a cytoskeletal component that plays a key role in the process of reactive astrogliosis of the central nervous system (CNS) to ischemia. Overexpression or suppression of this biomarker reflects modifying astrocyte function and metabolic abnormalities.[28] In turn, neuroglobin is expressed in neurons of the central and peripheral nervous system and in some endocrine tissues. The function of neuroglobin is to promote cell viability during hypoxia as well as oxidative stress.[20]

In this study, we present the concentrations of NGB and GFAP in blood in patients with ischemic stroke treated with thrombolysis. The concentrations of both biomarkers, GFAP and NGB, were elevated in patients after ischemic stroke and the changes in their concentrations in the subsequent stages of stroke may suggest their prognostic value strictly dependent on time. The study found a direct relationship between the level of GFAP and NGB and the degree of neurological deficit assessed according to the NIHSS scale. Biomarkers can be indicators differentiating stroke subtypes and predictors of mortality.

Our study illustrated statistically significant differences between the values of GFAP and NGB levels in the group of stroke patients compared to the control group.

According to some reports, the concentration of GFAB is very low in the blood of healthy people because under physiological conditions GFAP is not actively secreted from cells.[30] Glial fibrillary acidic protein is a highly specific marker for the brain, a protein that maintains the structure of astroglia cells and influences their migration.[31] Exploratory studies have shown that GFAP is rapidly released in acute haemorrhagic stroke, perhaps due to sudden damage to astroglia cells and disruption of the blood-brain barrier. Therefore, GFAP levels can differentiate ICH and AIS in patients from the onset of stroke symptoms.[8] Similarly, in the case of NBG, low levels of this marker are observed in healthy people, which constitute the control group, while its overexpression occurs after ischemia.[23–26]

Literature data show that high concentrations of these parameters occur in different time intervals. M.T. Wunderlich et al showed that serum GFAP concentrations at the time of hospital admission were higher than in the control group and peaked 48 hours after the onset of ischemic stroke. GFAP high levels remained until the 5th day after AIS.[32] However, it should be noted that in the examined patients, serial venous blood samples were collected from the moment of admission only 6 hours after the stroke, and analyses were repeated cyclically after 12, 18, 24, 48, 72, 96 and 120 hours after the occurrence of the stroke. Others have observed a peak in GFAP concentration at 24 h and its changes over time. Dynamic change in GFAP level may be related to prognosis at different stages of the disease.[13] According to the results obtained, the peak concentration of this protein had already been observed during onset. This could have indicated that this parameter can be a very early prognostic indicator, which is confirmed by studies conducted on another population,[33] and for screening tests.[15] The additional analyses were performed after the first day and the seventh day and these were statistically significantly higher values compared to the control group. Necrotic cell death and cell lysis typically occur within 6 to 12 hours. In turn, Fani Pujol-Calderón et al found an increase in the concentration of this biomarker in AIS patients during the first 3 days and then a decrease after 3 months. After 48 hours, GFAP increased 43 times compared to its concentration when the patient was admitted to the hospital.[33] In our studies, the value of this parameter increased approximately 3 times already during onset, even though we used a 100-fold less sensitive conventional ELISA method. Therefore, it can be assumed that GFAB may reflect ischemia-induced focal brain damage.

Neuroglobin concentration peaks at different time points, 24 h, 48 h and 72 h after stroke.[24,25,34] In the following hours, a gradual decrease in the concentration of this biomarker is observed. Interestingly, in our study, the peak of NGB was observed already during the first hours after the onset of stroke in patients with favourable prognoses assessed according to the OCSP scale. In turn, in patients with unfavourable prognosis, the peak of NGB concentration occurs on the first day after stroke, which confirms a previous study.[24] Like us, the authors observed an increase in NGB from the first to the sixth hour in the serum of patients with acute cerebral infarction ACI (Anterior Circulation Infarction), a peak

after 24 hours, and then a gradual decrease in concentration.[24] It should be emphasized, however, that the researchers did not divide the study population into patients with favourable and unfavourable functional and functional results.[24] We also cannot comment on the kinetics of this marker because our studies were carried out in different time periods. The sharp increase in this marker in the first hours in patients with a favourable functional result may result from the body's immediate reaction to hypoxia, which may indicate that the level of NGB in serum of acute cerebral infarction patients is correlated with infarct time.[2,24] This overexpression of NGB to hypoxia in patients with a favourable outcome mainly occurred in younger subjects with small vessel strokes on admission.

There are also reports indicating that the level of GFAP and NGB in the blood depends on both the type and severity of stroke and the time of symptom onset.[24–27,35] Different GFAP kinetics may be due to the location of the ischemic area (grey or white matter). As a result of ischemia in the necrotic tissue, the breakdown of nerve cells occurs, especially astroglia.[33] This is confirmed by our research, as we found an increase in this marker in patients with unfavourable functional results and moderate neurological deficit (Table 2 and Table 3). This parameter differentiates patients depending on neurological scales. We demonstrated statistically significant differences in GFAP at admission to hospital, according to NIHSS scales, during onset. Similarly, we observed an increase in NGB levels in patients with neurological disorders assessed on the NIHSS scale above 3, which was confirmed by other researchers.[25] We found statistically significant differences in these patients at admission, assessed according to NIHSS scales, in less than 4.5 h (Table 3). We also showed a positive, statistically significant correlation between the GFAP concentration in less than 4.5 h and 24 h and the NBG concentration assessed on the first day, which proves the expression of these parameters after hypoxia/ischemia. As stroke progresses, GFAP is expressed and is associated with structural and functional brain damage and stroke severity, which results from sudden disruption of the blood-brain barrier and astrocyte damage. Demonstration of a positive correlation with NGB may complement clinical assessment in predicting the degree of brain damage due to its importance in hypoxia. As nerve cell damage increases, acidic fibrillary protein and neuroglobin concentrations increase. NGB plays an important role in the pathophysiology of stroke, has neuroprotective effects, and modulates the function and dynamics of mitochondria.[18]

Interestingly, we also found differences in stroke subgroups according to the OCSP classification. We demonstrated statistically higher GFAP levels assessed in less than 4.5 hours in PACI compared to LACI small vessel stroke (Figure 2). In turn, we found differences between LACI and POCI subtypes for NGB determined after 7 days. Other authors also suggest that the time window of 2 to 6 hours after stroke onset is the optimal time for GFAP measurement to distinguish stroke types. They believe that GFAP provides the highest diagnostic accuracy for distinguishing between haemorrhagic and ischemic stroke.[9,36]

Interestingly, we observed moderate correlations between GFAP measured at 1 week and the NIHSS score at admission and discharge. In turn, NGB was evaluated on the seventh day and the mRS scale was determined after a year. The impact of the tested biomarkers on the functional status is confirmed by the ROC and AUC curves (Figures 4, 5). Based on these results, it can be suggested that GFAP levels measured in serum at 24 hours and on the seventh day may be a promising biomarker of functional outcome at 1 year and the severity of the patient's neurological condition.

The results of previous studies have shown a positive correlation between serum GFAP and NGB levels and the degree of neurological deficit based on the NIHSS score in patients with ischemic stroke.[25,29,34] A correlation of GFAP with infarct volume, NIHSS after 24 hours and the degree of disability defined by mRS after 3 months was also found.[13,33] In haemorrhagic stroke, a positive correlation of NGB with the Rankin scale after 12 months and with the results of neuropsychological tests indicating cognitive impairment was also observed.[37] Moreover, in studies in patients with TBI (Traumatic Brain Injury), the increase in NGB concentration was correlated with the stroke volume and the GSC scale (Glasgow Coma Scale).[26] Based on the above literature data, it can be concluded that the expression of GFAP and NGB levels is associated with the severity of the neurological deficit and may be a marker of poor neurological outcomes in patients after ischemic or hemorrhagic stroke.[25,38]

On the other hand, there are studies conducted on patients with AIS that did not show a correlation between GFAP and functional status.[9,39] There are also reports in which the NGB concentration does not correlate with the GCS score in patients with haemorrhagic stroke, which suggests that in humans, plasma neuroglobin can be used as a biomarker for initial diagnosis, but not for the short-term prognosis of acute stroke.[34]

In our research, we showed a relationship between gender and the concentration of the tested parameters. We found a statistically significantly higher concentration in men for GFAP and a statistically non-significantly higher concentration for NGB. Similarly, other authors showed[40] that NGB expression in neurodegenerative disease was lower in women compared to men. In turn, in haemorrhagic stroke, no such relationship was found.[34] Although we demonstrated consistency between GFAP and gender, previous reports did not confirm our research results.[12] This may be because the male gender dominated our research.

In turn, age had no effect on the tested biomarkers. The patients participating in the studies were of similar age. There is little information in the literature describing such consistencies, especially when it comes to AIS. Similarly, to our studies, there was no correlation with age between GFAP values in ischemic stroke or NGB in haemorrhagic stroke.[12,34] In experimental studies on rats, Sun[41] et al noticed a decrease in neuroglobin expression in the brain depending on the age of the animals. The fact that our results differ from those of Sun et al is likely since in humans, cerebral NGB expression is a function of the brain's hypoxic-ischemic tolerance areas. Moreover, under physiological conditions and depending on age, there are regions of the central nervous system with different tolerance to ischemia.[42–51]

We also attempted to investigate the influence of GFAP and NGB concentrations on the mortality of patients with AIS. We observed that the mortality rate of patients increased when the GFAP concentration measured within 24 hours was close to 0.25 ng mL^{-1}. For neuroglobin, this value is 14 ng during onset and on the first day, it ranges from 10 to 60 ng mL^{-1}. This may indicate the predictive ability of GFAP and NGB measured in serum to predict patient death. Liu et al confirmed our observations and concluded that GFAP may be an important predictor of mortality. People who died had twice the concentration of acidic fibrillary protein.[14] However, in the case of NGB, no relationship was found between its level at admission and short-term mortality in the case of haemorrhagic stroke.[34] In turn, other experimental studies have shown that NGB expression is associated with the improvement of ischemic damage and functional regeneration in rodents[52–56] and that high neuroglobin concentration may lead to increased apoptotic activity and cell necrosis.[52–54]

Inflammatory mediators and acute phase markers appear to identify the ischemic nature of stroke.[39] Reactive astrogliosis occurs during stroke and is characterized by astrocytic hypertrophy and overexpression of GFAP. After undergoing reactive astrogliosis, astrocytes produce and release pro-inflammatory mediators, such as IL-6, TNF-α, and IL-1, and IFNγ and free radicals such as NO, superoxide and peroxynitrite.[57] In turn, NGB contributes to the regulation of the inflammatory process.[58] Therefore, we tried to examine the relationship of previously determined inflammatory markers in AIS[3,4] with the newly tested parameters. We showed a positive correlation between indicators of inflammation (IL-6, TNF- α) and GFAP and NGB. This is innovative research. A statistically significant relationship is observed on the seventh day for GFAP vs IL-6 determined after 24 h and a moderate relationship for IL-6 (7 days). Interestingly, the concentration of NGB determined in the first 4.5 h also correlates with IL-6 (7 days) and TNF α (24 h). Such multi-parameter analysis in AIS may have better clinical value and provide additional information that can be used in stroke diagnosis. It will also allow us to determine the aetiology of this disease more precisely.

Our report has some limitations: 1) The studies were conducted at a single centre, which limited the number of subjects, 2) Obtaining informed consent for additional blood sampling for biomarker determination resulted in the exclusion of patients with aphasia and impaired consciousness, 3) Recruitment mainly concerned patients with mild and moderate neurological deficits, 4) determination of biomarkers in different time intervals was not always possible. The above limitations provide a basis for continuing the studies both on a larger population and in multicentre.

Conclusion

The pathophysiology of stroke is a multi-step process in which sensitive and specific biomarkers may play an important role.

Their measurement in various time intervals from the initial phase of hypoxia may be important in predicting the outcome of neurological severity or functional prognosis. We can therefore assume that NGB and GFAP are important biomarkers of ischemic brain injury and may also participate in predicting neurological outcomes. GFAP concentration measured in serum at 24 hours and on the seventh day may be a promising biomarker of functional outcome at 1 year and the severity of the patient's neurological condition. Demonstration of a positive correlation of GFAP with NGB may

complement clinical assessment in predicting the degree of brain damage due to its importance in hypoxia. Their increased value in blood serum may be a negative predictor of clinical effectiveness or mortality.

They can be used in the development of new pharmacological strategies. There are already reports that injection of exogenous NGB into the bloodstream may be a good therapeutic option for some neurological diseases, including stroke.[59] It has been proven that it is a good therapeutic tool in the treatment of stroke, provided, however, that its concentration in the infarcted area increases. Therefore, it should be considered to introduce such therapy in future clinical trials.

The Banyan Brain Trauma Indicator (BTI) blood test conducted by the US Food and Drug Administration (FDA) as part of the Breakthrough Devices program has already successfully used GFAP to assess patients with acute and mild TBI. Then, in 2021, Abbott received approval to implement GFAP as a diagnostic tool to exclude evidence of intracranial trauma on head computed tomography.[60] In turn, Chen and colleagues confirmed the efficacy of GFAP as a marker reflecting disease severity and risk of future bleeding in CADASIL (Cerebral autosomal dominant arteriopathy with subcortical infarcts and white matter degeneration).[17]

Design of studies including these biomarkers in the very elderly should be considered, taking into account demographic changes and risk factors. In those over 85 years of age, atrial fibrillation, greater focal neurological impairment suggest cardioembolic mechanisms.[61]

It is also possible to consider the introduction of experimental and clinical studies in the future that will allow for the combination of molecular information with genomic, epigenomic, transcriptomic, proteomic. Understanding the detailed mechanisms in acute ischemic stroke, and mainly in its various subtypes, may lead to the creation of effective therapeutic plans to prevent neuronal death and improve the patient's functioning after neuronal damage. Enable their rapid recovery. Having such information will also provide the opportunity to search for new therapeutic biomolecules in the field of improving cerebral reperfusion, neuroprotection and secondary prevention.

Ethics Statement

The research is consistent with the Declaration of Helsinki.

Disclosure

The authors report no conflicts of interest in this work.

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