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ORIGINAL PAPER

Hyperacute Detection of Neurofilament Heavy Chain in Serum Following Stroke: A Transient Sign

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Abstract Serological biomarkers which enable quick and reliable diagnosis or measurement of the extent of irreversible brain injury early in the course of stroke are eagerly awaited. Neurofilaments (Nf) are a group of proteins integrated into the scaffolding of the neuronal and axonal cytoskeleton and an established biomarker of neuroaxonal damage. The Nf heavy chain (NfH^{SMI35}) was assessed together with brain-specific astroglial proteins GFAP and S100B in hyperacute stroke (6 and 24 h from symptom onset) and daily for up to 6 days. Twenty-two patients with suspected stroke (median NIHSS 8) were recruited in a prospective observational study. Evidence for an ischaemic or haemorrhagic lesion on neuroimaging was found in 18 (ischaemia $n = 16$, intracerebral haemorrhage $n = 2$). Serum NfH^{SMI35} levels became detectable within 24 h post-stroke ($P < 0.0001$) and elevated levels persisted over the study course. While GFAP was not detectable during the entire course, S100B levels peaked at the end of the observation period. The data indicate that significant in vivo information on the pathophysiology of stroke may be obtained by the determination of NfH^{SMI35}. Further studies are required to evaluate whether NfH^{SMI35} in hyperacute stroke reflects the extent of focal ischaemic injury seen on neuroimaging or is a consequence of more diffuse neuroaxonal damage.

Keywords Neurofilament · Biomarker · Ischaemic stroke · Blood–brain barrier · Pathophysiology

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Introduction

Stroke is a serious condition resulting in significant mortality and long-term morbidity. Ischaemia is associated with reduction in oxygen and glucose supply within the central nervous system (CNS). When blood flow falls below a critical threshold, a cascade of biochemical reactions generating excitotoxicity, oxidative stress, inflammation, and cell death is triggered, leading to tissue infarction [1]. This detrimental process is associated with release of different cell-type specific proteins including calcium-binding protein S100B, glial fibrillary astrocytic protein (GFAP) and neurofilaments (Nf) into extracellular fluids (ECF). From the ECF, these proteins diffuse to the CSF and peripheral blood, where they can be quantified

[2]. Increased attention has been directed to establishing the measurement of these molecules as a tool to assist in diagnosis, selection of treatments, assessment of the efficacy of treatments, and prediction of outcome of stroke [3].

In brief, GFAP is a brain-specific astrocytic intermediate filament protein, which is released upon cellular disintegration and degradation of the cytoskeleton. The determination of GFAP in the time window of 2 and 6 h after stroke may be helpful in the distinction of ischaemic from haemorrhagic stroke. Dvorak et al. reported that patients with intracerebral haemorrhage had significantly elevated GFAP levels in this period, whereas GFAP levels remain below the detection limit in ischaemic stroke [4]. S100B is mainly found in astrocytes and other sources include oligodendrocytes, microglia and neurons. Concentrations in cerebrospinal fluid (CSF) were found to be up to 60 times higher than in serum [5]. Importantly, previous reports indicated that S100B serum values correlate with final infarct volume when determined beyond 24 h after symptom onset [6–8]. In contrast to the multiple sources of S100B, neurofilaments, a group of proteins integrated into the scaffolding of the neuronal and axonal cytoskeleton, are specific to and abundant in the neuro-axonal compartment [9]. Both neurofilament light (NfL, 68 kDa) and heavy chains (NfH, 190–210 kDa) can be measured in CSF [10]. So far, quantification from serum is possible for NfH only. Significant positive associations were found for blood NfH levels and clinical severity, volume of the ischaemic territory and outcome when assessed 3 weeks after symptom onset [11]. There is no linear correlation for CSF and serum concentration. Assessment of S100B and NfH concentrations in ischaemic stroke revealed that CSF but not serum concentrations correlated positively with outcome on the modified Rankin scale (mRS) [5]. But samples were taken relatively late in this study (median 3 days, range 1–15 days).

An important question for future treatment approaches is the time course of irreversible neuronal damage and the potential to quantify the extent. Not all incidents of oxygen and glucose deprivation will lead to irreversible brain injury [12]. Indeed, ischaemic lesions visualized by CT and even multimodal magnetic resonance imaging (MRI) in the acute phase (<24 h of symptom onset) do not necessarily reflect the final infarct volume [13]. Moreover, milder ischaemic events will only lead to temporary neuronal dysfunction and may be even associated with preconditioning and increase resistance towards irreversible damage [14]. Hence, a biomarker identifying the extent of irreversible neuro-axonal injury in the critical early phase is of utmost importance. Here, we aimed at evaluating the earliest consequences of stroke by studying sequential serum samples with a panel of three biomarkers within 24 h of symptom onset and the subsequent period of 6 days.

Materials and Methods

Patients and Sample Collection

The study was approved by the local ethics committee. Written informed consent was obtained from the patients and when this was not possible from the next family member.

Patients admitted to the Accident and Emergency Department of University College London Hospital (UCLH) with a suspected acute cerebrovascular event with onset of symptoms <24 h prior to assessment were evaluated prospectively over an 8 month period. Twenty-two patients were recruited and peripheral-venous blood was obtained upon arrival and daily between 9:00 and 10:00 a.m. over a 6-days period. The tube was left standing at room temperature for 30 min to allow blood clotting. The sample was then centrifuged at 1,500*g* for 10 min, and serum was removed and coded aliquots were stored at −80°C. The clinical assessment consisted of the NIH stroke scale (NIHSS), the Glasgow coma scale and basic vital signs including blood pressure, temperature and peripheral oxygen saturation. Further parameters monitored were haematocrit and blood pressure. All patients were examined with a brain CT scan. Twenty patients presenting to the same Accident and Emergency Department without evidence for neurological disease were recruited as controls.

Quantification of NfH, S100B and GFAP Levels

Serum NfH^{SMI35} levels were measured using an enzyme-linked immunoassay, as described previously [10, 15]. The mouse monoclonal antibody SMI35 (IgG) was originally purchased from Sternberger Monoclonals Inc., and is now available through Covance Research Products (Berkeley, CA, USA). The secondary and tertiary antibodies used were the rabbit polyclonal anti-NfH IgG (Sigma, St. Lous, MO; N 4142) and the horseradish peroxidase (HRP)-labelled swine polyclonal anti-rabbit IgG (DAKO, Copenhagen, Denmark). Adhering to the nomenclature previously set up, we indicate the captured antibodies used for NfH quantification in superscript (NfH^{SMI35} for SMI35). Serum S100B and $GFAP^{SMI26}$ levels were determined with an ELISA as described before [16, 17]. The detection limit for the NfH^{SMI35} assay is 0.01 ng/mL. The detection limits for $GFAP^{SMI26}$ is 5 pg/mL and for S100B

7 pg/mL. The analyst was blinded to all clinical information and measurements were done in duplicates.

Statistical Analysis

Statistical analysis was carried out using Graph Pad Prism Software Version 5.0 (San Diego, CA, USA). The non-parametric Mann–Whitney test was used for comparison of controls and stroke at baseline (<24 h). The Kruskal–Wallis test with Dunn's multiple post-hoc comparison was used to compare dynamics of NfH^{SMI135}, S100B and $GFAP^{SMI26}$ serum levels in the first 6 days after stroke onset. Two sided tests were used and a P value <0.05 was accepted as significant.

Results

Stroke Patients and Controls

Radiological evidence for stroke was found in 18 patients. Ischaemic stroke was diagnosed in the majority ($n = 16$), whereas haemorrhagic stroke was the cause of neurological symptoms in two. In the remaining four subjects no vascular or structural pathology was seen on MRI and lead to exclusion from further analysis. None of the patients with ischemic stroke was treated with intravenous alteplase. The baseline characteristics of the 18 stroke patients and controls are shown in Table 1.

Table 1 Baseline characteristics of the control and stroke cohort

Characteristic	Control	Stroke	Stroke subtype	
			Ischaemic	Haemorrhagic
Age, year (mean)	40.2	65.5	69.5	59.5
Sex (F:M)	10:10	10:8	10:6	0:2
NIHSS[a]	–	8	8	–
GCS[a]	15	15	14	15
No.	20	18	16	2

SD standard deviation

[a] On admission

Serum Levels of NfH^{SMI35}, S100B and $GFAP^{SMI26}$ in Acute Stroke and Controls

Hyperacute stroke was defined as onset of stroke symptoms <24 h before assessment and samples from 15 patients were available within this time period. NfH^{SMI35} levels were significantly higher in hyperacute stroke (median 0.13 ng/mL, range 0–0.17) compared to the control group (median 0 ng/mL, range 0–0.05; $P < 0.0001$) (Fig. 1). Median S100B levels in hyperacute stroke were (0.03 ng/mL, range 0–0.33) were lower than in controls (0.09 ng/mL, range 0.05–0.32). The S100B levels in stroke patients were not statistically different from controls. $GFAP^{SMI26}$ serum levels were below the detection limit in all the samples. Neither NfH^{SMI35} nor S100B levels correlated with age or NIHSS scores.

Time Course of NfH^{SMI35} and S100B in Acute Stroke

The time course of the three biomarkers levels over 6 days following stroke onset is shown in Table 2. Patients with confirmed stroke on neuroimaging had increased NfH^{SMI35} levels already within 6 h of symptom onset. The increase persisted over the 6 days course and peaked at day 4 but changes did not reach statistical significance compared to other time points. The increase of S100B, however, was delayed and started 3 days and peaked at 6 days post stroke. Differences were not statistically significant. GFAP remained below the detection limit in all stroke patients over the entire time course.

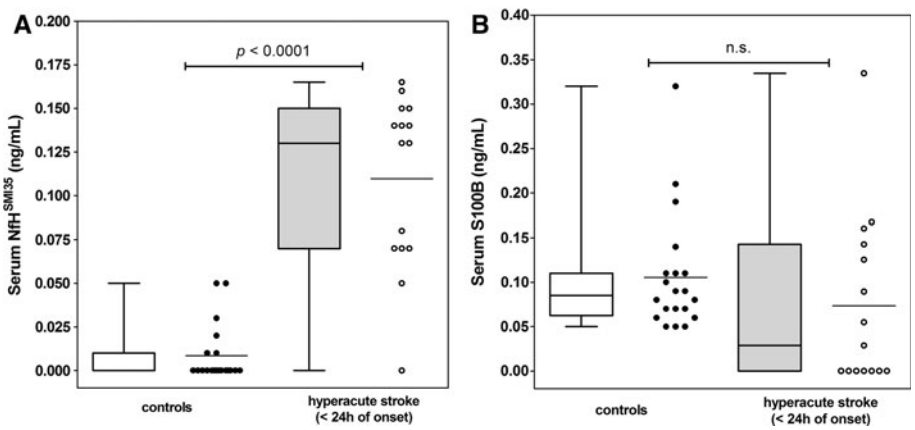

Fig. 1 Serum NfH^{SMI35} and S100B in hyperacute stroke (<24 h from onset of symptoms). *Box-whisker plots* indicate minimum, median, max and the 25th and 75th percentiles. The *horizontal bar* in the *dot-plot diagrams* depicts the mean. *n.s.* not significant

Table 2 The longitudinal profile of serum NfH^{SMI35} (ng/mL) and S100B (ng/mL) levels in stroke patients

	Time (hours) after stroke						
	<6 h	6–24 h	2 days	3 days	4 days	5 days	6 days
No.	8	7	10	7	8	11	10
NfH^{SMI35}	0.14 (0–0.14)	0.14 (0–0.07)	0.13 (0–0.13)	0.09 (0–0.09)	0.14 (0–0.14)	0.13 (0–0.14)	0.13 (0–0.13)
S100B	0.01 (0–0.17)	0.02 (0–0.34)	0.01 (0–0.33)	0.03 (0–0.38)	0.03 (0–0.6)	0.04 (0–0.59)	0.05 (0–0.15)
GFAP	–	–	–	–	–	–	–

NfH^{SMI35} becomes detectable already after 6 h from symptom onset. GFAP was below the detection limit at all time points. Median and range (brackets) are shown. Statistical analysis did not reveal significant changes over time

Discussion

This study found an extracranial increase in venous blood of NfH^{SMI35}, a specific marker for axonal injury, degeneration and neuronal loss, already present in the very early course of acute stroke. Serum levels of NfH^{SMI35} became detectable within 24 h of symptom onset, an observation which was also verified within in the 6 h post-stroke time frame. These findings corroborate recent data in an experimental piglet model of global brain ischaemia due to circulatory arrest. The peak of NfH^{SMI35} serum level was already detected within 30 min after a 60 min course of global hypoxia [14]. The findings also extend the observation of reduced and disrupted NfH staining (NfH^{SMI31} and silver staining) in the ischaemic hemisphere in experimental focal ischaemia starting at 1–3 days post insult [18]. Indeed, NfH^{SMI35} was shown to be a sensitive marker and even subtle brain injury due to symptomatic carotid stenosis was associated with 2.4-fold higher NfH^{SMI35} serum levels compared to asymptomatic carotid stenosis [15]. Hence, it is tempting to speculate that increased serum levels of NfH^{SMI35} reflect the respective acute neuro-axonal damage evidenced on neuroimaging. Such information would also be important for targeted management of stroke patients in future treatment trials. Hence, a correlation of early NfH^{SMI35} levels with a sensitive neuroimaging measure would be one of the next steps.

Final infarct size is determined by a combination of the initial ischaemic injury and delayed responses to the ischaemia. The latter include a number of mechanisms such as inflammatory reactions and apoptosis, which contribute to the overall brain injury [19]. This process evolves over several days and even weeks post-stroke. In keeping with this concept, Foerch and coworkers reported that serum 100B levels assessed within the early stages of stroke onset did not reflect infarct size [20]. This finding is also supported by several reports that S100B levels only correlate with final infarct volume if determined >24 h after symptom onset [6, 7, 21]. Thus, Foerch et al. argued that early S100B levels may be regarded more as qualitative measure of blood–brain barrier (BBB) leakage. We are hesitant to subscribe the findings to a leakage of the BBB since this is not a precondition of increased blood concentrations of biomarkers for brain damage [22]. We previously reported a median NfH^{SMI35} concentration of 0.21 ng/mL in CSF of a biologically representative population recruited from the UCLH Emergency and Accident Department. This exceeds the amount found in serum by far, where NfH^{SMI35} levels were below the detection limit in a group of healthy volunteers [23]. Further knowledge on release, kinetics and clearance of NfH across the body fluid compartments is required.

Singh et al. did not find significantly increased NfH values on day 1 compared to controls [11], but did not report hourly timing from stroke onset and their samples may have been taken later than in the present study. Additionally, methodological differences may apply as the analytical sensitivity in not known. Finally, our study comprised more severely affected patients with a median NIHSS of 8 compared to the 6 in the Singh et al. study. Therefore our patients are likely to have more substantial brain damage as the source for the serum NfH levels we measured. These issues may have accounted for the detectability of NfH^{SMI35} in the hyperacute phase of stroke. Compared to healthy controls, Singh reported a 2.9- and 8.5-fold increase after 1 and 3 weeks, respectively. The further increase at the 3 weeks time point is particularly intriguing since the observation may correspond to additional neuro-axonal injury induced by post-stroke immune and inflammatory processes. We confirm previous studies that S100B is detectable very early in the course of stroke and peaks within 1 week in nonfibrinolytic-treated stroke patients [7, 24, 25]. Yet, S100B in our study did not differ from the control group, which could be traced back to the small sample size. In contrast, GFAP could not be detected over the entire study period. In this regard, Herrmann and coworkers reported that GFAP becomes detectable 24–72 h post-stroke, and steadily increases and peaks between 48 and 96 h [24].

In conclusion, this pilot study demonstrated that NfH^{SMI35} levels become detectable in the hyperacute phase of stroke. Differences in the early NfH^{SMI35} release pattern

in ischaemic and haemorrhagic stroke could not be evaluated due to only two patients with the latter stroke entity. Further limitations include the small sample size, and lack of stratification for stroke localization (e.g. vascular territory, cortical vs. subcortical) and cause of ischaemic stroke. Indeed, biomarkers released by cortical pathology are likely to be found quicker and at higher concentration in the bloodstream due to diffusion through the cortical arachnoid villi [22]. Potential factors influencing NfH release include systemic blood pressure, haemodilution and brain temperature [26]. Glucose metabolism and glutamate toxicity are additional factors which could influence NfH release in the setting of cerebral ischemia. While no post-hoc adjustments were possible due to the small sample size, these factors were closely monitored and variations kept at a minimum. The issues should be considered for the set-up of further studies aimed at refining the potential value and different time windows of serum NfH^{SMI35} in future stroke management.

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