Neurofilament changes in serum and cerebrospinal fluid after acute ischemic stroke

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ABSTRACT

Background Neurofilament light (NFL) is a well-validated biomarker for neuronal injury and neurodegeneration. Increased cerebrospinal fluid (CSF) levels have been shown after stroke, as well as in patients with a broad range of neurodegenerative and neuroinflammatory diseases. Neurofilament heavy (NFH) belongs to the same family of structural proteins but it is less extensively studied. The potential of phosphorylated NFH (pNFH) as a stroke biomarker and for the prediction of clinical outcome is unknown. In this study, we aimed to examine the temporal pattern of NFL and pNFH concentrations in serum and CSF after acute ischemic stroke.

Materials and Methods A quantitative Enzyme-Linked ImmunoSorbent Assay (ELISA) for pNFH was developed and tested on CSF and serum samples. NFL and pNFH were analysed in serum and CSF of acute ischemic stroke patients, who were followed over time (Day 0-1, Day 2-3, Day 7-9, three weeks, and 3-5 months after stroke).

Results NFL and pNFH concentrations in serum and CSF increased after stroke, peaked during the 3rd week, and then decreased back to almost baseline levels at 3-5 months. CSF-NFL and serum-NFL correlated to the outcome measured by Barthel Index after 3-5 months, whilst no such association was seen for pNFH.

Discussion These findings suggest that NFL and pNFH in both CSF and serum reflect the temporal pattern of the post ischemic axonal injury and that this process does not seem to progress after 3-5 months.

Conclusion NFL and pNFH in CSF and serum are promising biomarkers for axonal injury following stroke. Further studies in larger populations are needed to fully understand the progression of the neuronal damage after acute ischemic stroke and to evaluate if these biomarkers can provide additive information and how they relate to outcome.

Keywords:
Neurofilament light, neurofilament heavy, stroke, cerebrospinal fluid, serum.

Abbreviations:
Nfs, neurofilaments; NFT, neurofilament triplet; NFL, neurofilament light; NFM, neurofilament medium; NFH, neurofilament heavy; pNFH, phosphorylated neurofilament heavy; CSF, cerebrospinalfluid; ELISA, enzyme linked immunosorbent assay; IS, ischaemic stroke; HS, haemorrhagic stroke; ALS, amyotrophic lateral sclerosis; CT, computer tomography; MRI, magnetic resonance imaging; SSI, stroke scale index; BI, Barthel Index; PBS-T, phosphate buffer saline with tween; BSA, bovine serum albumin; LOD, limit of detection.
INTRODUCTION

Stroke can cause long-term disability, lasting brain damage or death making it a major public health problem [1]. In 2010, stroke was the third leading cause of disability [1]. Furthermore, the incidence of stroke has been rising in the younger population [2].

Stroke occurs when the blood supply to parts of the brain is blocked by a thrombosis or embolus (ischemic stroke; IS) or by a blockage due to blood vessel disruption (haemorrhagic stroke; HS). The neuronal damage after stroke leads to the release of neuronal proteins into the cerebrospinal fluid (CSF) and into the blood [3, 4]. Monitoring neuronal proteins longitudinally may thus provide an indication for the extent of brain damage during stroke process.

Neurofilaments (Nfs) are the main structural component of the intermediate filaments in neurons and are composed of four different protein subunits; α-internexin which is central nervous system-specific and the neurofilament triplet (NFT) consisting of neurofilament light (NFL), medium (NFM) and heavy (NFH) [5, 6]. These proteins are synthesized in the nerve cell body and assemble into intermediate filaments in the axon where they provide stability [7].

Since the first description of an NFL assay with sensitivity high enough to analyse CSF [8], high CSF levels of NFL have consistently been found in various neuroinflammatory and neurodegenerative diseases, such as multiple sclerosis [9], amyotrophic lateral sclerosis (ALS) [10], different types of dementia [11] and in parkinsonian disorders [12]. CSF-NFL has been reported high in Alzheimer’s disease patients, where its levels correlate with disease progression [13] and to the levels found in blood [14, 15].

High CSF levels of NFL are also found after acute damage to the brain, such as stroke, traumatic brain injury and subarachnoid haemorrhage [3, 16, 17]. The levels also correlate with the severity of the brain damage reflecting the amount of axonal degeneration [3, 18].

Although not as extensively studied as NFL, the phosphorylated neurofilament heavy (pNFH) levels have also been examined in stroke, traumatic brain injury and ALS [19-21]. Interestingly, it has been reported that the ability of pNFH to differentiate ALS patients from other diseases mimicking ALS symptoms and controls was better than NFL [21]. Therefore, the study of pNFH is becoming essential to help understand how we can differentiate between diseases, as well as to comprehend how the neuronal damage may evolve over time.

Even though it previously has been shown that NFL is a good biomarker for several neurodegenerative diseases and conditions, with a good correlation between CSF and blood, the relationship between the different neurofilament proteins is less well understood. In an acute event such as stroke little is known about the temporal evolution of these markers, how they compare to each other, and how that information may provide information about the recuperation progress.
In this study, we aimed to examine the temporal pattern evolution of pNFH and NFL concentrations in serum and CSF after acute ischemic stroke. To this end, we developed a quantitative Enzyme-Linked ImmunoSorbent Assay (ELISA) for pNFH and employed already established assays for NFL.

MATERIAL AND METHODS

Sampling

Paired CSF and serum samples were collected longitudinally between September 1992 and January 1994 from 30 patients with acute ischemic stroke. The CSF and serum samples were collected on five occasions, when possible (Table 1). CSF was collected through a lumbar puncture between L3/L4 or L4/L5 interspace. To avoid possible gradient effects [3] the first 12mL of CSF were collected in polypropylene tubes and gently mixed. Then CSF samples were centrifuged at 2000g for 10 min to eliminate cells and other insoluble material. Blood samples were collected at the same time as CSF into serum tubes. Aliquots of all the samples were stored at -80°C until biochemical analysis. There were no freeze-thaw cycles and the temperature of the freezers was monitored, verifying an unbroken freezing time.

To evaluate the extent and localisation of the lesions, neuroimaging was performed 3-5 months after onset of stroke. The late time point was chosen to get a better definition of the permanent damage. The CT scans were routinely performed parallel to the canthomeatal plane (i.e., a gantry tilt about + 10° from Reid’s baseline) with 5-mm (posterior fossa) and 10-mm (supratentorial) slice thickness. The MRI examinations (Philips Gyroscan T5-II) were performed with axial proton density and T2-weighted images of the brain. The relative size of the infarcts was calculated as the product of the maximal sagittal and transversal diameters of the area of the infarctions as measured by computer tomography (CT) or magnetic resonance imaging (MRI). The MRI volume measurements were calculated by means of a 3D reconstruction program in the workstation environment (Philips Gyroview).

All patients were evaluated as described previously [22]. A modified Scandinavian Stroke Scale index (SSI) was used to evaluate the patient status on Day 0-1, Day 2-3, Day 7-9, at 3 weeks and 3-5 months after onset. The performance in activities of daily living was scored with the Barthel Index (BI) 3-5 months after stroke.

An age and gender matched contrast group of control samples was collected during August 2016 until February 2017 from patients who sought medical advice for suspected neurological disease. The selected samples did not show signs of ongoing neurodegeneration according to established CSF biomarkers [23] (Table 2).

Ethical approval for the study was obtained from the Gothenburg Regional Ethics Committee (Dnr 172-92). Prior to inclusion, informed consent was obtained from each subject.

pNFH
CSF and serum pNFH concentrations were determined with an in-house developed sandwich ELISA. In brief, F16 black MaxiSorp FluoroNunc plates (NUNC) were coated with 6µg/mL of NF-01 an antibody specific to a phospho-epitope of NFH (Abcam, cat no ab7795) and incubated overnight in +4°C. Blocking was performed by adding 200µL 1% bovine serum albumin (BSA) in phosphate-buffered saline with Tween (0.05%) (PBS-T). Calibrants were prepared by diluting NFH recombinant protein (Origene) in PBS-T. One-hundred microliters of calibrants and non-diluted CSF and serum samples were incubated in +4°C overnight. To each well 100µL of biotinylated detection antibody NF-05 (Abcam, cat no ab118812) was added at a concentration of 250ng/mL followed by incubation for 1 hour at room temperature. Finally, 100µL SuperSignal™ ELISA Femto Substrate (ThermoFisher) was added and then the plates were immediately read with Viktor X4 2030 Multilable Reader (Perkin Elmer). Four washes with PBS-T were performed in between all steps. A fitted 4-parameter logistic model was used to generate the calibration curve and the blank was included as zero concentration of pNFH. The phosphorylation specificity was assessed by incubating 3ng/mL of the recombinant protein used as calibrant with 1µL of 0.2U/mL Alkaline phosphatase from Escherichia coli (Sigma-Aldrich) during 3 hours at 37°C and measured using the assay previously described, a control with no Alkaline phosphatase was also included. A validation was performed by evaluating assay range, limit of detection (LOD), inter-plate precision and linear dilution, during three separate analyses on different days.

NFL

CSF-NFL from controls was analysed with UmanDiagnostics kit ELISA described previously [24]; CSF-NFL from patients was determined using an in-house developed ELISA as described previously [8] and serum-NFL from both patients and controls was measured using single molecule array (Simoa) previously described [25].

Statistical analyses

To have both control and patient samples consistently analysed with one method and therefore comparable, the in-house CSF-NFL data from patients was transformed into UmanDiagnostics CSF-NFL data by re-analysing all the left samples with the UmanDiagnostics kit and performing a Passing-Bablok regression to transform the whole data set. A mix model analyses was performed on each biomarker repeated measurements. Subjects were included as random factors and age and gender as covariates. The Mann-Whitney test was used for comparisons between controls and the other time points. Spearman’s non-parametric test was used to calculate the correlations between groups (r). All tests were 2-sided and significance was set at p≤0.05. Statistical analyses were performed using SPSS software, version 23.0.

Samples below the limit of detection (LOD) were included in the statistical analysis as half the value of limit of LOD (0.033ng/mL).
RESULTS

The novel pNFH ELISA had a measurement range between 0.077ng/mL to 50ng/mL and the LOD was 0.066ng/mL. The inter-plate precision was 8.8%. The samples did not dilute in a linear way, hence all samples were analysed neat. The signal of the treated calibrant with Alkaline phosphatase at 37°C decreased 19% after 3 hours of incubation whereas the non treated calibrant signal decreased only 4%; hence, we conclude that our assay is specific for phosphorylated neurofilament heavy since when NFH is dephosphorylated the signal is reduced.

Median CSF and serum levels of NFL and pNFH after acute ischemic stroke are shown in Figure 1. CSF-NFL (Figure 1a) was lowest on Day 0-1 (median, IQR; 0.70, 0.38-1.2ng/mL) and increased over time until the third week, Day 2-3 (2.2, 0.46-5.2ng/mL), Day 7-9 (8.9, 3.7-13ng/mL) and week 3 (14, 7.9-21ng/mL). The concentrations had decreased at the month 3-5 time point (5.8, 3.1-12ng/mL). Similarly, serum-NFL (Figure 1b) followed the same pattern, with the lowest level on Day 0-1 (0.028, 0.011-0.046ng/mL) followed by increased concentrations during Day 2-3 (0.038, 0.023-0.082ng/mL) and Day 7-9 (0.13, 0.088-0.29ng/mL), a peak at the third week (0.31, 0.11-0.60ng/mL) and a return to normal values after 3-5 months (0.076, 0.033-0.21ng/mL).

The lowest level of CSF-pNFH (Figure 1c) was found on Day 0-1 (median, IQR; 0.13, 0.10-0.21ng/mL), while an increase was evident on Day 2-3 (0.23, 0.14-0.90ng/mL) continuing on the Day 7-9 (0.31, 0.19-1.4ng/mL). After three weeks, the concentrations peaked (0.45, 0.25-0.73ng/mL) and returned almost back to normal after 3-5 months (0.21, 0.21-0.45ng/mL). The CSF-pNFH concentrations in controls were significantly higher than in the patients on Day 0-1 (p ≤ 0.01).

A similar distribution was observed for serum-pNFH (Figure 1d). The concentration of serum-pNFH was lowest on Day 0-1 (0.033, 0.033-0.062ng/mL) with a slight increase over the next two time points, Day 2-3 (0.20, 0.13-4.9ng/mL) and Day 7-9 (0.17, 0.12-0.39ng/mL). Serum-pNFH reached the maximum concentration on the 3rd week (0.81, 0.32-2.5ng/mL) before decreasing after 3-5 months (0.20, 0.093-4.3ng/mL).

The best correlation between CSF-NFL and serum-NFL concentrations was at 3-5 months (r=0.782; p≤0.0001) (Figure 2), the other time points correlated significantly as well at the exception of Day 7-9 (Figure S1). There was no correlation between CSF-pNFH and serum-pNFH at any time point (Figure S2). A low, but still significant correlation was observed between CSF-pNFH and CSF-NFL at Day 2-3 (r=0.513; p≤0.05) (Figure 2). No other correlations were significant between CSF-pNFH and CSF-NFL or serum-NFL and serum-pNFH.

We found significant correlations between size (cm²) of the infarct and CSF-NFL on Day 7-9 (r=0.44; p≤0.05), at three weeks (r=0.72; p≤0.0001) (Figure 3a) and at 3-5 months (r=0.63; p≤0.001) (Figure S3). Size and serum-NFL correlated on Day 7-9 (r=0.91; p≤0.0001) (Figure 3b), at three weeks (r=0.81; p≤0.001) and at 3-5 months (r=0.69; p≤0.001) (Figure S4). When the
outlier shown in Figure 3b was removed the correlation between serum-NFL and size at Day 7-9 was still significant ($r=0.880; p≤0.0001$). CSF-pNFH and serum-pNFH did not correlate with the size of the infarction at any time point. CSF-NFL correlated significantly to the volume (mL) of infarction at three weeks ($r=0.76; p≤0.001$) and 3-5 months ($r=0.65; p≤0.01$) whereas serum-NFL only correlated on Day 7-9 ($r=0.72; p≤0.05$). CSF-pNFH and serum-pNFH did not correlate with the volume of the infarction at any time point.

The patient status was evaluated periodically with the SSI. We found a correlation between SSI and CSF-NFL at three weeks ($r=0.60; p≤0.01$) and 3-5 months ($r=0.43; p≤0.05$). SSI correlated to the size of infarction on Day 7-9 ($r=0.43; p≤0.05$) and at three weeks ($r=0.45; p≤0.05$), but did not correlate to the volume of infarction at any time point (data not shown).

We observed negative correlations between CSF-NFL and BI at three weeks ($r=-0.72; p≤0.0001$) (Figure 4a) and 3-5 months ($r=-0.67; p≤0.0001$) (Figure S5). Serum-NFL and BI correlated on Day 2-3 ($r=-0.79; p≤0.001$) (Figure 4b), Day 7-9 ($r=-0.77; p≤0.01$), at three weeks ($r=-0.77; p≤0.001$) and 3-5 months ($r=-0.72; p≤0.0001$) (Figure S6).

BI 3-5 months after stroke correlated to the patient status (evaluated by SSI) on Day 2-3 ($r=-0.45; p≤0.05$), Day 7-9 ($r=-0.59; p≤0.01$), at three weeks ($r=-0.59; p≤0.01$) and 3-5 months ($r=-0.67; p≤0.0001$).

**DISCUSSION**

Stroke is a major public health problem that may cause long-lasting brain damage or death. In this study, we aimed to examine the temporal pattern of pNFH and NFL concentrations in serum and CSF after acute ischemic stroke.

Our data show a progressive increase over time of NFL and pNFH in both CSF and serum after acute IS, with a peak at the third week after onset and reaching back to almost normal values after 3-5 months, as well as a correlation between CSF-NFL and serum-NFL and CSF-NFL and CSF-pNFH. The lack of a correlation between CSF and serum pNFH may be due to that the biological half-life of pNFH in CSF differs from blood. In addition, the samples did not display a linear dilution suggesting that interference from matrix and/or conformation of pNFH differs between the two sample types.

Singh et al. (2011) have previously reported the progression of serum-pNFH after acute IS. They described increasing concentrations of pNFH from Day 0-1 to the 3rd week and a correlation between the volume of the infarcts and the levels of pNFH at three weeks [20]. Our results agree well with their finding of increasing serum-pNFH levels during the first three weeks, but also adds the novel information that after 3-5 months, levels are returned back to almost normal, showing that axonal breakdown is no longer prevailing. While we did not see any correlation between size or volume and serum-pNFH at three weeks, serum-NFL as well as CSF-NFL correlated with size at that time point, as well as on Day 7-9 and after 3-5 months, and with volume at three weeks and 3-5 months. In general, correlations between NFL and
size were better than between NFL and volume, probably due to the lower number of volume determinations consequent to MRI contraindication in some patients (e.g., cardiac pacemaker).

The concentrations of CSF-pNFH were significantly higher in controls than in patients on Day 0-1. This has previously been described regarding serum-pNFH where levels also were found higher in controls compared to stroke patients [4]. It was hypothesised that it was the result of an intravenous fluid–related dilution due to the treatment of patients with stroke. In addition, in the present study the time gap between the collection and analysis of the patient samples was 22 to 24 years, while for the controls it was only a few months. Since there are no studies about pNFH stability over time when frozen, a possible explanation for the higher concentrations of pNFH in the CSF of controls might be the difference in storage time.

Total tau (T-tau) has been reported as an important biomarker for neurodegeneration [26]. Interestingly, its CSF concentrations have previously been described in the same cohort of stroke patients as in the present study [22]. Normal concentrations of CSF T-tau were found on Day 0-1, and then levels were increased approximately five times reaching a plateau the three following sampling times with slightly higher levels on Day 7-9. After 3-5 months, levels were normalised. Note that the time course of the increase of neurofilament proteins is more extended than and not as abrupt as for T-tau. The different kinetics may reflect the subcellular localisation of the proteins. Neurofilaments are abundant in large myelinated axons susceptible to Wallerian post-stroke degeneration and the time profile of immunohistochemical as well as radiological findings after stroke showing a delayed and longstanding degenerative process fit the release pattern of the neurofilament proteins in this study [27, 28]. Tau is a microtubule-associated protein, present in axons together with tubulin, particularly enriched in thin unmyelinated axons of the neuropil of central nervous tissue but less prevalent in peripheral neurofilament rich neurites [29, 30]. Thus, it could be speculated that post-ischemic tau release to a higher extent is due to degradation of non-myelinated tubulin rich axons of the neuropil, but this remains to be further studied. Another explanation could be that the two biomarkers reflect different degeneration processes [31, 32].

Outcome measured as BI correlated well with the levels of NFL in serum from all time points except Day 0-1, while CSF-NLF levels only correlated significantly at three weeks and 3-5 months. The reasons why NFL in CSF at the early time points or pNFH from both serum and CSF did not correlate with outcome is obscure. However, little is known about how these biomarkers are transported from the damaged tissue to serum. Some possibilities could be by the ordinary rout for CSF drainage or directly from the tissue to blood. Different pathways would probably implicate disparate kinetics. Also, regarding pNFH, the antibody-antigen interaction is dependent on the phosphorylated epitopes of the molecule. The pNFH protein released from degraded neurofilaments in anoxic tissue might possible to some extent be dephosphorylated thus rendering less signal in the ELISA. This could be one reason for the overall lower values for pNFH compared to NFL observed in this study. It might also explain why the samples did not dilute in a linear way in the testing of the pNFH ELISA. The NFL
protein, on the other hand is only marginally phosphorylated and dephosphorylation of the antigen did not change the antigen-antibody interaction of the antibodies used in the assay system in this study [8].

The patient status, periodically evaluated with the SSI on Day 7-9 and at three weeks correlated to the size of infarction. SSI on Day 2-3, Day 7-9, at three weeks and after 3-5 months also correlated to outcome. Interestingly, both CSF and serum NFL correlated to the size of the infarction and outcome at the same time points as SSI with better correlation values, suggesting that the biomarkers reflect better the extension of the damage in the brain and that can predict for outcome more precisely than the clinical scoring. Still, using other clinical scores such as NIHSS for evaluation of status and modified Rankin scale for outcome might give other results, as well as the use of other biomarkers such as tau in serum.

We intended to compute at which time point after stroke the biomarkers best would predict bad outcome, but it became evident that in our data set there were not enough patients classified as bad outcome to build ROC curves. Therefore, studies in larger populations are needed. Still, using Spearman’s rank correlation it is very encouraging that the best correlation to outcome was observed for serum-NFL on Day 2-3, suggesting that already at this short time after the stroke it could be possible to predict final outcome. Obviously, a simple blood sample taken early after the stroke and no need of a lumbar puncture will suit clinicians working in stroke units.

This study has several limitations. First, the size of the study was limited. Second, the samples were taken a long time ago and we do not know the effect of long-term storage (several decades) at -80°C on NF concentrations. However, the longitudinal samples from the same individuals all had this long storage time, which mitigates the potential problem. Third, two different assays were used to analyse CSF-NFL controls and patients, although this problem was solved by transforming the data. The strengths of this study are first, the long-term follow up of the patients 3-5 months after stroke to evaluate outcome. Second, the longitudinal sampling of both CSF and serum at different time points after the stroke. Third, no patients underwent thrombolysis. Finally, the thorough evaluation of the patient status with the SSI at each time point.

In summary, our study shows that both CSF and serum NFL and pNFH concentrations reflect neuronal injury after acute stroke with the highest levels during the 3rd week and a decrease after 3-5 months. These data suggest that neurofilament proteins are biomarkers reflecting the temporal dynamics of post ischemic Wallerian degeneration of axons following stroke. Further studies in larger populations are needed to fully understand the kinetics of these and other biomarkers after acute ischemic stroke, as well as to establish serum measurements of neurofilament proteins as a mean to early forecast outcome and thus the need of rehabilitation arrangements for the individual stroke patient.
CONFLICT OF INTEREST

Henrik Zetterberg has served at advisory boards of Roche Diagnostics and Eli Lilly, has received travel support from Teva and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg.

Kaj Blennow has served as consultant at advisory boards for Alzheon, BioArctic, Biogen, Eli Lilly, Fujirebio Europe, IBL International, Merck, Novartis, Pfizer, and Roche Diagnostics, and he is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg.

ACKNOWLEDGEMENTS

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REFERENCES

Table 1. Number of samples per each type of variable at the different time points.

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Table 2. Inclusion criteria for controls.

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Figure 1. Biomarkers evolution over time after acute ischemic stroke. Represented as median with inter quartile range, * = outlier, ** = extreme value. Statistical significance comparing to group control *= p≤0.05; **= p≤0.01; ***= p≤0.001; ****= p≤0.0001. Statistical significance comparing to Day 0-1 #= p≤0.05; # #= p≤0.01; # # #= p≤0.001; # # # #= p≤0.0001.
Figure 2. Correlation between biomarkers. a) CSF-NFL and Serum-NFL at 3-5 months, N=21; r= 0.782; p≤0.0001. b) CSF-NFL and CSF-pNFH at Day 2-3, N=22; r=0.513; p≤0.05.
Figure 3. Correlation between size of infarct and NFL. a) Cerebrospinal fluid NFL at 3 weeks correlated to size of infarcts as calculated by CT at 3 months, n=21; r=0.72; p≤0.0001. b) Serum-NFL levels at Day 7-9 correlated to size of infarcts, n=13; r=0.91; p≤0.0001.
Figure 4. Correlations between final outcome and NFL. a) Cerebrospinal fluid NFL at 3 weeks correlated to Barthel Index, n=22; r=-0.72; p≤0.0001. b) Serum-NFL at Day 2-3 correlated to Barthel Index, n=8; r=-0.79; p≤0.05.