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Plasma neurofilament light chain is associated with mortality after spontaneous intracerebral hemorrhage

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Abstract

Background: Neurofilament light chain (NfL) is a neuron-specific biomarker with prognostic ability in several types of central nervous system injuries. This study investigates if plasma NfL (pNfL) is elevated early after spontaneous intracerebral hemorrhage (ICH) and whether such elevation reflects disease severity and day-30 outcome.
Methods: pNfL was quantified by single molecule array analysis in 103 reference subjects (RS) and in samples from 37 patients with ICH obtained on admission to hospital and at 24-h follow-up. The primary outcome was day-30 mortality. Clinical status on admission was evaluated by standardized scoring systems.
Results: Median pNfL among RS was 9.6 (interquartile range [IQR] 6.2) pg/mL. Upon admission, ICH patients had pNfL of 19.8 (IQR 30.7) pg/mL increasing to 35.9 (IQR 44.5) pg/mL at 24 h (all, $p<0.001$). On admission, pNfL was higher among ICH non-survivors than survivors (119.2 [IQR 152.6] pg/mL vs. 15.7 [IQR 19.6] pg/mL, $p<0.01$) and this difference was observed also on 24 h follow-up (195.1 [IQR 73.9] pg/mL vs. 31.3 [IQR 27.8] pg/mL, $p<0.01$). The area under the receiver operating characteristic curve (ROC AUC) for discrimination of day-30 mortality was significant on admission (AUC = 0.83, 95% confidence interval [CI]: 0.56–1.0) and increased on 24-h follow-up (AUC = 0.93, 95% CI: 0.84–1.0). The odds ratio (OR) for death, by each quartile increase in pNfL was significant both on admission (OR = 4.52, 95% CI: 1.32–15.48) and after 24-h follow-up (OR = 9.52, 95% CI: 1.26–71.74).
Conclusions: PNfL is associated with day-30 mortality after spontaneous ICH when early after the ictus.

Keywords: biomarkers; cerebral hemorrhage; neurofilament proteins; prognosis.

Introduction

Spontaneous intracerebral hemorrhage (ICH) is associated with a high morbidity and mortality [1, 2]. Risk factors for poor outcome such as patient age, hematoma volume and intra-ventricular hemorrhage are well established [3, 4], but it remains difficult to accurately quantify severity and predict outcome for the individual patient [5]. The characterization of novel biomarkers may have the potential to add valuable information on disease severity and to enhance outcome prediction which could ultimately aid clinical decision-making.

Neurofilaments are neuron-specific, cytoskeletal proteins abundant in myelinated axons [6]. They are released to the extracellular fluid upon acute as well as chronic axonal injury depending on the extent of the damage [7–9]. Previous investigations have uncovered a solid biomarker capacity of neurofilaments to quantify disease severity and prognosticate outcome in a diverse range of neurological diseases [8–11]. Neurofilaments have been intensively studied as biomarkers in ischemic stroke [12–18] but their biomarker capacity in hemorrhagic stroke is less investigated [19–25]. The available literature on hemorrhagic stroke studied neurofilament levels in patients suffering from subarachnoid hemorrhage (SAH) [20–22, 25] and only few studies on ICH patients are available [19, 23, 24]. Most of these studies found neurofilament heavy chain (NfH) levels to be correlated with clinical disease

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severity and outcome, but the neurofilaments were commonly measured in the cerebrospinal fluid (CSF) and only days after the hemorrhagic stroke [20–23, 25]. Thus, only sparse evidence exists on the value of plasma measurements, and especially the predictive value of early neurofilament measurement remains unaddressed.

The recent development of ultra-sensitive technologies such as the Single Molecule Array (Simoa) now allows the detection of neurofilaments also in the peripheral circulation [26, 27]. By the use of this technology, close correlations and similar biomarker capacities have been found between CSF and circulating levels of neurofilaments in a broad range of neurologic diseases [9, 10, 26, 28]. This opens for the investigation of neurofilaments as easily accessible blood-based biomarkers that may be used for repeated measurements and in patients where sampling of CSF is not done routinely.

The biomarker potential of plasma neurofilament light chain (pNfL) in ICH has not been investigated previously. The present study was undertaken to investigate if pNfL levels are elevated in the acute phase following ICH and whether such elevation reflects clinical disease severity and day-30 outcome.

Materials and methods

Study populations

The present study analyzed material from a cohort of prospectively included patients admitted with ICH to the Departments of Neurosurgery and Neurology, Aarhus University Hospital between June 18th 2014 and August 31st 2016. We have previously published investigations on this cohort [29, 30]. Of the original 41 patients included in the cohort, plasma samples from 37 patients were available for inclusion. Plasma samples collected upon admission and 24 h after the ictus were included in the present study. The in- and exclusion criteria for the cohort have been published previously [29, 30]. Briefly, patients above 18 years of age with a computer tomography (CT) or magnetic resonance imaging (MRI) verified ICH, that could be enrolled within 6 h of ictus, were considered eligible for inclusion. The exclusion criteria were: pre-existing bleeding disorder, treatment with any antithrombotic drugs, ongoing treatment with antibiotics, pregnancy, active cancer or chemotherapeutic treatment within the last 3 months, liver cirrhosis, ischemic or hemorrhagic cerebral infarction within the last 3 months, and structural cause of the ICH (such as arterio-venous malformation, tumor or trauma).

On admission, patients were scored by the Glasgow Coma Scale (GCS) and National Institutes of Health Stroke Scale (NIHSS) by the attending physician. In comatose patients, NIHSS was set to 38. The remaining information was collected by review of the medical charts. Diabetes was defined as a specified diagnosis in the medical chart or pharmacological anti-diabetic treatment; hypertension was defined as antihypertensive treatment prior to hospital admission; renal or hepatic insufficiency by elevated plasma biomarker levels or a specified diagnosis in the medical chart; smoking and alcohol abuse as specified in the medical chart; and the use of statins registered as a surrogate marker of hypercholesterolemia. The CT and MRI scans were evaluated by a consultant in neuroradiology and ICH volume was estimated by the ABC/2 method [31]. A rescan was performed only at the discretion of the attending physician. Follow-up on day 30 after ictus was performed by chart review with registration of death and the modified Rankin Scale (mRS) score.

A total of 108 aged-matched reference subjects (RS) were included. Of these, 61 were recruited among blood donors in the blood bank at Aarhus University Hospital. Blood donors are subjected to very strict health requirements and are excluded from donation at the age of 67 years. We included, volunteers and anonymous (only sex and age known) blood donors aged 18–65 years were included. The remaining 47 RS were recruited among outpatients in the blood sampling unit at the Department of Clinical Biochemistry, Aarhus University Hospital. This unit services all medical specialties but based on systematic interview, patients were excluded if they suffered from diabetes, dementia, current or previous stroke or if they were referred to blood sampling from the Departments of Neurology and Neurosurgery or the Dementia Clinic. In this study, volunteer and anonymous (only sex and age known) outpatients aged 60–87 years were included.

Statement of ethics

The present study was conducted in accordance with the World Medical Association Declaration of Helsinki. The study was approved by the Regional Ethical Committee of Central Denmark Region (1-10-72-95-14, version 7, 05102017 and 1-10-72-94-14, version 7, 05102017), the National Data Protection Agency (1-16-02-225-14 and 1-16-02-224-14). All patients or their legal representatives provided written informed consent.

Laboratory analysis

Blood samples were drawn into ethylenediaminetetraacetic acid (EDTA) anti-coagulated tubes (BD Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) from the antecubital vein or from an arterial cannula. The reference samples were immediately processed at room temperature, centrifuged at $3000 \times g$, 10 min, at 22–24 °C and the patient samples were centrifuged at $3100 \times g$, 25 min, 22–24 °C and frozen at −80 °C until further analysis. The patient samples analyzed were subjected to a maximum of two freeze-thaw cycles.

For the measurement of pNfL levels, the NF-light® assay was established on the ultra-sensitive Simoa™ HD-1 platform (Quanterix©, Lexington, MA, USA). This is a digital immunoassay with dedicated hard- and software that quantify analyte concentrations by singulated capture and reading of immunocomplexes on microbeads. This assay is at least 125 times more sensitive than conventional enzyme-linked immunosorbent assay (ELISA) while maintaining high analytical performance [32]. According to the manufacturer, the limit of detection (LoD) and limit of quantification (LoQ) for NfL are 0.038 pg/mL and 0.174 pg/mL, respectively. The calibrator range is 0–500 pg/mL with linearity from 4 to 128 times dilution. On the level

10–20 pg/mL, the intra- and inter-assay coefficients of variation (CV) in our laboratory are 4.3% and 6.4%, respectively.

All samples were analyzed in duplicate and only duplicate CVs <12% were accepted. We have confirmed plasma NfL levels to be stable through at least three freeze-thaw cycles. Plasma samples were thawed on ice before being batch analyzed in duplicate and the results are reported as mean of the duplicates.

Statistics

A power calculation was done by Satterthwaite's test on the endpoint of elevated pNfL levels among patients compared to RS. Applying a two-sided α level of 0.05 with the included study groups, the power to detect a 1.5-fold increase among patients was 97%.

The data distribution was evaluated by inspection of inverse QQ-plots. Data are displayed as mean with standard deviation (SD) or median and interquartile range (IQR) as appropriate. Categorical variables were compared by the chi-squared (χ^2) test. For continuous data, crude bivariate analysis was done by Student's t-test, the Mann-Whitney U-test or the Wilcoxon signed-rank test as appropriate and adjusted bivariate analysis was done by logistic regression analysis. The comparison of more than two continuous variables was done by one-way analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate. Spearman's rank test was used for correlation analysis. Diagnostic performance was tested by receiver operating characteristic curve (ROC) analysis. Areas under the ROC curves (AUROC) were compared by the algorithm suggested by DeLong et al. [33]. The optimal cutoff point was chosen as the pNfL value with maximum correct classification and the positive/negative likelihood ratio for that point was calculated. To explore the combined discriminative performance of hematoma volume and pNfL, a probability score for death was estimated for each individual, by the use of the logistic regression coefficients. This score was subsequently used to perform the AUROC analysis. Outlier detection in the reference group was done by Tukey's fences [34] using a constant of 2 times IQR. A poor functional outcome was defined as mRS score of 3–6 whereas mRS scores 0–2 was considered a good functional outcome. For all analysis, the significance level was set to 0.05 and the statistical analyses were performed in STATA 15.1 for Windows.

Results

RS

After exclusion of outliers, 103 RS aged 60 ± 13 years were included in the data analysis. Fifty-nine were males with an average age of 59 ± 11 years, while 44 were females aged 61 ± 15 years ($p = 0.62$). The median pNfL level in the reference group was 9.6 (IQR 6.2) pg/mL and no difference was observed between pNfL levels in males and females ($p = 0.14$). A strong positive correlation was observed between age and pNfL levels, Spearman's $\rho = 0.80$ ($p < 0.0001$).

ICH patients

The demographic and clinical characteristics of the ICH patients are presented in Table 1. There was no difference in age between patients and RS ($p = 0.33$) but relatively more females among the ICH patients than the RS ($p = 0.02$). The level of pre-existing comorbidity was low except for hypertension, which was present in almost half of the patients. On admission, 11 patients (30%) had an NIHSS score above 20 and five (14%) were admitted with a GCS below 9. The median time from ictus to the first cerebral scan was 2 h and 7 min (IQR 1 h, 32 min) and median time to first blood sampling was 3 h 55 min (IQR 1 h 42 min). The majority of

Table 1: Demographic and clinical characteristics of the 37 patients with spontaneous ICH.

Variable	Result
Age, years (mean ± SD)	62 ± 16
Sex (M/F)	13/24
Comorbidity	
Diabetes, n (%)	1 (3%)
Hypertension, n (%)	17 (46%)
Kidney failure, n (%)	1 (3%)
Liver failure, n (%)	0 (0%)
Current smoking, n (%)	9 (24%)
Use of statins, n (%)	3 (8%)
Clinical status at admission	
Glasgow Coma Scale, n (%)	
15–14	20 (54%)
13–9	12 (32%)
8–3	5 (14%)
NIH Stroke Scale, n (%)	
1–4	7 (19%)
5–15	8 (21%)
16–20	11 (30%)
21–42	11 (30%)
Hemorrhage and scanning	
Location of bleeding, n (%)	
Hemisphere	34 (92%)
Brainstem	3 (8%)
Volume of bleeding on first scan, median (IQR), mL	22.5 (35.1)
Intraventricular bleeding, n (%)	14 (38%)
Microbleeds (MRI scanned patient only, n = 25)	11 (44%)
Day 30 outcome	
Dead, n (%)	7 (19%)
Modified Rankin Scale, n (%)	
1	4 (11%)
2	5 (14%)
3	6 (16%)
4	12 (32%)
5	3 (8%)
6	7 (19%)

Data presented as number (%), mean ± SD, or median (IQR). NIH Stroke Scale, National Institutes of Health Stroke Scale.

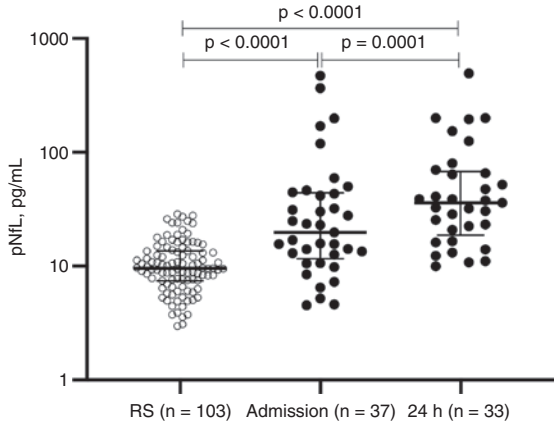

Figure 1: pNfL levels in RS and patients with spontaneous ICH on admission to hospital and at 24 h follow-up.
Individual levels displayed with median and IQR.

patients suffered from hemisphere bleeding (92%) and the median hematoma volume was 22.5 mL (IQR 32.1) at admission. Eleven patients were rescanned within the first 48 h due to clinical deterioration and significant hematoma expansion was detected in one patient who subsequently died. Two patients underwent surgical intervention; one by craniotomy with hematoma evacuation, the other by extraventricular drainage, and both patients survived.

The 24 h blood sample was lost from four patients, two of whom died before 24 h and two who survived but were transferred to their local hospital before sampling. On day-30 follow-up, seven (19%) patients had died and 28 were in mRS group 3–6.

Plasma NfL levels and clinical status

Median pNfL levels were 19.8 pg/mL (IQR = 30.7) on admission and increased to 35.9 pg/mL (IQR = 44.5) 24 h after the ictus, Figure 1. Median pNfL measured upon admission was 170.5 pg/mL (IQR = 154.5) among patient with a GCS < 9 compared to 16.4 pg/mL (IQR = 20.0) among patients with GCS ≥ 9 (p = 0.06). Patients with a NIHSS > 20 had median pNfL levels of 43.9 pg/mL (IQR: 145.6) on admission compared to 15.7 pg/mL (IQR 19.6) among patients with NIHSS ≤ 20 (p = 0.06). The correlation between pNfL levels on admission and hematoma volume on the first scan was significant, though weak (ρ = 0.33; p = 0.04).

Plasma NfL levels are associated with day-30 mortality

The characteristics of the seven patients succumbing to the ICH are presented in Table 2.

Table 2: Non-survivors and survivors of ICH.

	Non-survivors, n = 7	Survivors, n = 30	p-Value
M/F, n	3/4	10/20	0.64
Age, years	64 (43)	64 (18)	0.86
Hematoma volume on admission, mL	134.1 (185.1)	17.8 (25.5)	0.01

Data presented as absolute numbers or median with IQR.

Non-survivors had significantly higher hematoma volume than survivors but were comparable in respect to age and sex. Non-survivors had significantly higher circulating pNfL levels than those surviving on admission as well as 24 h following ictus (Figure 2A and B).

The circulating pNfL levels differentiated non-survivors from survivors with an AUC of 0.83 on admission and 0.93 24 h after admission (Figure 2). The sensitivity and specificity were above 80% at both time points (Figure 2). The AUC of hematoma volume and NfL combined was 0.84 (95% confidence interval [CI]: 0.62–1.0) on admission and 0.81 (95% CI 0.51–1.0) 24 h after ictus neither of which was significantly different from pNfL alone (p, admission = 0.81; p, 24 h = 0.44).

In a crude analysis, each quartile increases in pNfL levels on admission and 24 h after ictus were associated with increased odds ratio (OR) for dying (OR, admission = 4.52, 95% CI: 1.32–15.48 and OR, 24 h = 9.52, 95% CI: 1.26–71.74).

Patients with poor functional status (mRS ≥ 3) on day-30 presented with higher pNfL than patients with good functional status (mRS ≤ 2) on admission (24.3 pg/mL [IQR 30.7] vs. 15.6 pg/mL [IQR 12.4], p = 0.42) and at 24 h follow-up (38.7 pg/mL [IQR 44.6] vs. 19.9 pg/mL [IQR 27.7], p = 0.22) but this difference did not reach statistical significance.

Discussion

The present study demonstrates that pNfL levels measured on admission to hospital and 24 h after the ictus are associated with day-30 mortality in patients with spontaneous ICH. The measured pNfL levels were highly elevated among ICH patients and moderately correlated to the volume of the hematoma but it did not reflect the symptom severity on admission.

The ROC curve analysis demonstrated a capacity of pNfL to separate survivors from non-survivors that was evident just few hours after the ictus and increased during the first 24 h. Previous studies on neurofilaments in

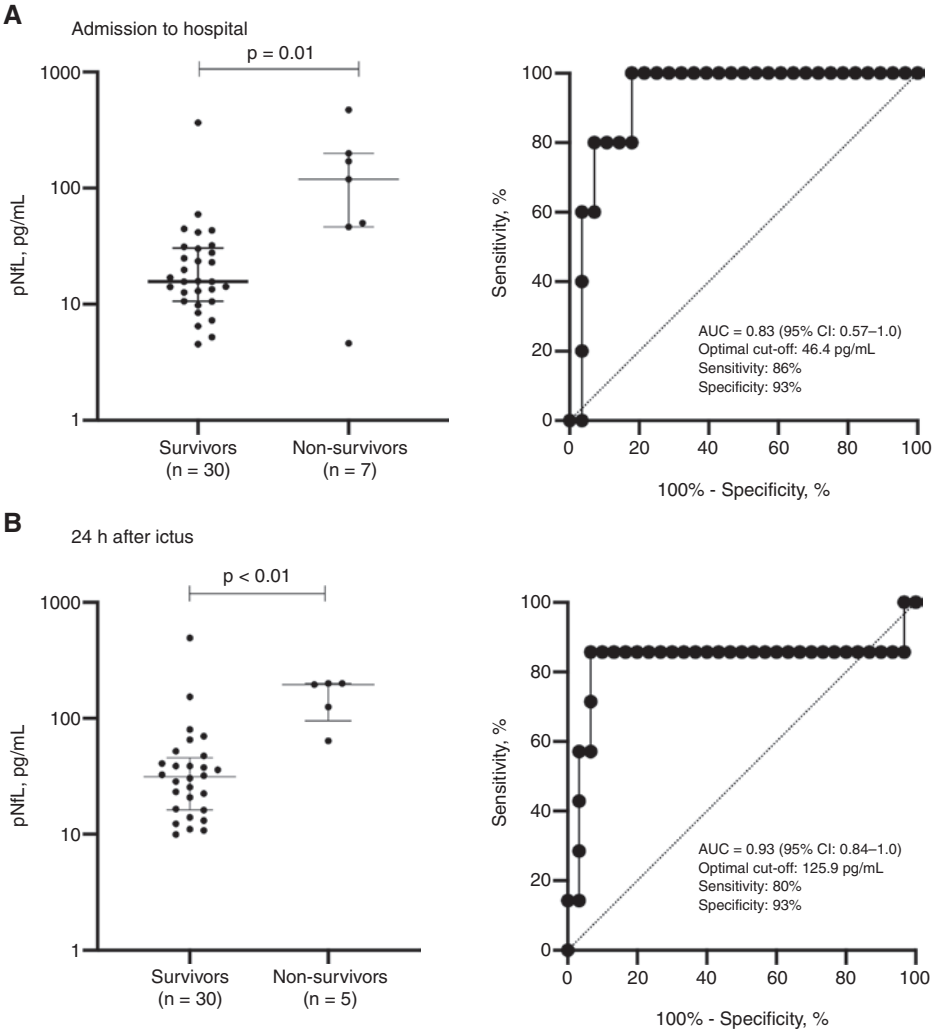

Figure 2: Separation of ICH survivors and non-survivors by pNfL.
pNfL levels in survivors and non-survivors of spontaneous ICH on admission to hospital (A) and at 24 h follow-up (B) displayed with the corresponding ROC curve for prediction of death. Individual pNfL levels displayed with median and IQR.

hemorrhagic stroke have demonstrated elevations of NfH in ICH [19, 23, 24] and SAH [20–22, 25] days after the ictus. Though not completely uniform, the studies have provided compelling insight that measurement of neurofilaments may be an independent predictor for outcome after hemorrhagic stroke [19–22]. Most of these studies measured NfH in the CSF but Cai et al. investigated the predictive value of plasma NfH (pNfH) in 112 ICH patients [19]. They reported an AUC for prediction of 6-months mortality, unfavorable outcome and early neurological deterioration comparable to that of our study. Cai et al. also applied a regression model to identify pNfH as an independent predictor of outcome [19]. While our sample size precluded multivariate regression, the results of our univariate analysis agree with that of Cai et al., which supports an association also between pNfL and mortality after ICH.

While it is acknowledged that differences between the biomarker capacity of NfH and NfL may exist [35], our results clearly indicate that pNfL may be a promising biomarker for outcome prediction in spontaneous ICH. Furthermore, our results suggest that such prognostic capacity may be present very early following ictus.

To the best of our knowledge, none of the previous studies on neurofilaments in hemorrhagic stroke investigated its association to hematoma volume. The correlation observed in our study is in line with the understanding that NfL levels reflect the amount of axonal damage [36–38]. As the hematoma volume is an established predictor of outcome in spontaneous ICH [3, 4], this may explain that the predictive value of hematoma volume and pNfL combined did not significantly exceed that of each parameter individually in our ROC analysis. Still,

the correlation between hematoma volume and pNfL was weak and further studies are needed to clarify whether this is because pNfL reflects only white matter damage, is due to inaccuracy of the ABC/2 technique or a matter of sample size. Nevertheless, our results demonstrate that Simoa is a sensitive and accurate method to detect pNfL and indicate that this biomarker may improve monitoring of patients with ICH by reflection of subclinical hematoma progression.

Altogether, our data are encouraging for further investigations on the usefulness of circulating NfL in outcome prediction early in the disease course of ICH. This topic has received much attention and several scoring systems have been developed to support clinical decision-making [39, 40]. While these systems work well on the population level, it remains challenging to predict the disease course for the individual. The inclusion of biochemical biomarkers in these scoring systems might provide valuable additional information to increase their predictive value.

The present study has some limitations. It is limited by its rather small sample size but despite this, it strongly suggests that pNfL possess biomarker capabilities in spontaneous ICH which is encouraging for further large-scale studies. The investigations were conducted using biobank material not specifically collected for the purpose of the study. This may have led to the inclusion of patients suffering from conditions known to affect pNfL levels and interfered with the results. However, the present study mimics the routine use of any biomarker more closely than a trial performed in a highly selected cohorts and therefore strengthens the presented results.

Conclusions

In conclusion, this explorative study suggests that pNfL levels measured shortly after the ictus is associated with day-30 mortality in patients with spontaneous ICH. It provides implications that pNfL levels measured upon admission to hospital may reflect hematoma volume and encourage further investigations on the biomarker potential of pNfL in ICH.

Author contributions: Conception and design of the study: CVBH, AMH, TP. Acquisition and analysis of data: CVBH, TG, SVL, NH, TP, AMH, TP. All authors drafted/revised the manuscript for its intellectual content, approved the final version, and agreed to be accountable for all aspects of the work. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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