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# Neurofilament medium polypeptide (NFM) protein concentration is increased in CSF and serum samples from patients with brain injury

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## Abstract

**Background:** Brain injury is a medical emergency that needs to be diagnosed and treated promptly. Several proteins have been studied as biomarkers of this medical condition. The aims of this study were to: 1) evaluate the selectivity and precision of a commercial ELISA kit for neurofilament medium polypeptide (NFM) protein; and 2) evaluate the concentration in cerebrospinal fluid (CSF) and serum of healthy individuals and patients with brain damage.

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**Methods:** An ELISA from Elabscience was used. The selectivity was evaluated using size-exclusion chromatography and mass spectrometry. Intra- and inter-batch coefficients of variation (CV) were also studied. Fifty-one CSF samples from 36 age-matched patients with hemorrhagic stroke (HS) (n=30), ischemic stroke (IS) (n=11) and healthy individuals (n=10) were assayed. In addition, serum samples from healthy volunteers (n=47), 68 serum samples from seven patients with HS, 106 serum samples from 12 patients with traumatic brain injury (TBI) and 68 serum samples from 68 patients with mild traumatic brain injury (mTBI) were also analyzed.

**Results:** NFM was identified in the chromatographic fraction with highest immunoreactivity. The intra- and inter-batch CVs were  $\leq 10\%$  and  $\leq 13\%$ , respectively. The CSF-NFM concentration in HS was significantly higher ( $p < 0.0001$ ) than in IS and controls. Serum NFM concentration ranged from 0.26 to 8.57 ng/mL in healthy individuals (median=2.29), from 0.97 to 42.4 ng/mL in HS (median=10.8) and from 3.48 to 45.4 ng/mL in TBI (median=14.7). Finally, 44% of patients with mTBI had increased NFM concentration, with significantly higher levels ( $p=0.01$ ) in patients with polytrauma.

**Conclusions:** To our knowledge this is the first study describing increased NFM levels in CSF and serum from patients with brain damage.

**Keywords:** biomarker; brain injury; cerebrospinal fluid; neurofilament; serum; stroke.

## Introduction

The term “brain injury” refers to a potentially lethal medical condition that includes two major types of brain damage: 1) spontaneous, non-traumatic brain injury; and 2) traumatic brain injury (TBI). The most common cause of brain injury is by accidental injury to the head. Approximately 1.7 million Americans suffer a TBI each year, and

75%–90% of those are classified as mild traumatic brain injury (mTBI) [1]. However, stroke is the most common form of non-traumatic brain injury, with 795,000 people in the US suffering a new or recurrent stroke each year. Ischemic stroke (IS) represents 87% of all stroke cases whereas hemorrhagic stroke (HS) accounts for 13% of cases [2].

A brain injury is usually a medical emergency that needs to be diagnosed and treated promptly, in order to improve the likelihood of patient survival and reduce the consequences of brain damage. For this purpose, several proteins such as S100 calcium-binding protein B (S100B), neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) have been proposed as diagnostic and prognostic biomarkers of TBI and stroke [3–6]. However, given the complexity and heterogeneity of brain injury etiology, none of these proteins seem to be useful in these patients as single biomarkers. Therefore, it has been suggested that integrated panels of biomarkers with specific and complementary characteristics may assist in the diagnosis, risk assessment, treatment selection and prediction of clinical outcomes of patients with brain damage.

In our previous study [7], we identified three novel biomarkers of brain injury: neurofilament medium polypeptide (NFM),  $\alpha$ -internexin ( $\alpha$ -Inx) and  $\beta$ -synuclein ( $\beta$ -Syn) in the cerebrospinal fluid (CSF) of patients with HS, by using a mass spectrometry-based assay. The aim of the current work was to perform a verification study of one of these biomarkers using a more sensitive analytical assay. The protein selected was NFM for three reasons: 1) given its novelty (to our knowledge this is the first study evaluating NFM as biomarker of brain injury); 2) the other two members of its family, neurofilament light and heavy polypeptides (NFL and NFH), have also been studied and identified as potential biomarkers of HS [8–11]; and 3) there was a commercial ELISA kit available, with enough sensitivity (low pg/mL level) to detect this protein in CSF and blood samples of patients with brain damage.

Before we assayed NFM protein in biological samples from patients with brain injury, the commercial ELISA kit was validated, since we and others have drawn attention to the rapid dissemination of ELISA kits of questionable quality from various manufactures [12, 13].

Therefore, the aims of this study were to: 1) evaluate the selectivity (using size-exclusion chromatography and mass spectrometry) and precision of a commercial ELISA kit for NFM protein; and 2) evaluate the concentration of this protein in biological fluids (CSF and serum) of healthy individuals and patients with brain injury (stroke, TBI and mTBI).

## Materials and methods

### ELISA kit for NFM protein

A commercial ELISA kit [Human Neurofilament 3 (NEF3); Catalog No: E-EL-H0740; from Elabscience Biotechnology Co., Ltd] was used to measure NFM protein in various biological fluids. The ELISA kit uses capture and secondary monoclonal antibodies from rat and whole protein (natural, purified protein) as standard material, according to the information provided by the manufacturer. The reported limit of detection is 9.4 pg/mL; with an analytical measurement range from 15.6 to 1000 pg/mL. The intra-batch coefficient of variation (CV) is below 10%. The assay was performed according to the manufacturer's instructions.

### Size exclusion high-performance liquid chromatography (SE-HPLC)

The SE-HPLC was performed using 0.1 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , 0.15 M NaCl (pH 7.0) buffer at a flow rate of 0.5 mL/min for 60 min with a gel filtration column TSKgel G3000SW, 7.5 mm (ID) x 60 cm (L), 10  $\mu\text{m}$  (Tosoh Bioscience, King of Prussia, PA, USA). Liquid chromatography was performed in a HPLC 1100 series (Agilent Technologies) with diode array detector (DAD), set at 254 nm (UV lamp). The system was controlled with ChemStation software (Agilent Technologies). One hundred microliters of each sample (100  $\mu\text{L}$  loop) were loaded into the column. Proteins contained in four different samples (NFM standard from ELISA kit, brain tissue extract from previous study [7], CSF sample from patient with HS and no blood contamination and serum sample from healthy individual) were subjected to chromatographic separation. Fractions (0.5 mL) were collected every minute, from 18 to 58 min, after the isocratic flow was started. The same volume of a Gel Filtration Standard (Catalog 151-1901, Bio-Rad Laboratories) was loaded before samples.

### Mass spectrometry identification

Three SE-HPLC fractions from brain tissue extract were analyzed in an LTQ Orbitrap XL (Thermo Scientific). Samples volume was reduced from 400 to 50  $\mu\text{L}$  using a SpeedVac concentrator (Savant SC250EXP, Thermo Scientific) and then processed as described previously [7]. Briefly, proteins were denatured, reduced, alkylated and trypsin digested. Peptides were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), and the resulting spectra were searched separately against the Human UniprotKB-SwissProt Database 2013\_10 (containing 88266 forward protein sequences) by Mascot software (version 2.2, Matrix Science).

### Intra- and inter-batch CV of NFM ELISA kit

Serum samples from six healthy volunteers were diluted 1:20 and used to study the precision of NFM ELISA kit. Four samples (named QC1 to 4) were analyzed six times with the same ELISA kit to calculate the CV intra-batch. The other two samples (named QC5 and 6) were measured eight times with five ELISA kits from three different lots to calculate the CV inter-batch.

## Sample collection

A total of 51 CSF samples from 36 individuals and 299 serum samples from 134 individuals were collected for this study (Table 1).

## CSF samples from patients with stroke and healthy controls

Fifty-one samples from 36 age-matched patients were retrospectively selected from a CSF biobank at the Department of Clinical Biochemistry, Hospital Universitario Central de Asturias (HUCA), Spain, including samples from individuals with HS (n=30), IS (n=11) and healthy individuals (n=10). Some of these samples (n=36) were used in our previous study [7] for the identification of proteins (including NFM) elevated in the CSF of patients with HS. CSF samples were collected a few days after symptoms onset [median, (minimum-maximum)]: 5 (0–9) days in HS group and 1 (0–3) day in IS group.

Sample aliquots were prepared and stored at  $-80^{\circ}\text{C}$  until assayed. Ethics approval for sample collection was obtained from the institutional review board of HUCA.

Briefly, patients with HS (n=15) were diagnosed with intracerebral hemorrhage (ICH) (n=7) or subarachnoid hemorrhage (SAH) (n=8). Control individuals (n=10) were diagnosed with benign headache, mild cognitive impairment, depression and other non-neurological diseases. CSF samples from controls and IS patients had  $\leq 3$  white blood cells (WBC)/ $\text{mm}^3$  (except for one IS patient with 6 WBC/ $\text{mm}^3$ ) and negative xanthochromia.

NFM protein was also measured in 19 serial samples (n=6, 3, 5 and 5 samples, respectively) from four patients with HS (ICH, n=2; SAH, n=2). These CSF samples were collected between 2 and 41 days after symptoms onset.

## Serum samples from healthy volunteers

Serum samples from healthy 47 volunteers, 26 women and 21 men, between 17 and 57 years old were collected to establish the reference range for NFM. Individuals were recruited at the University Health

Network and the Lunenfeld-Tanenbaum Research Institute (Toronto), with informed consent.

## Serum samples from patients with hemorrhagic stroke

Seventy-eight serum samples from seven patients admitted to St. Michael's Hospital (Toronto) with SAH were used for the analysis of NFM. Samples were obtained as soon as possible after admission of individuals to the intensive care unit and during therapy, for 4–13 days. Between 7 and 12 samples from each patient were analyzed, which were stored at  $-80^{\circ}\text{C}$  until assayed. These were samples from a previous study [14] for which a frozen aliquot was available. Ethics approval for the collection of samples was obtained from the Institutional Review Board of St. Michael's Hospital.

## Serum samples from patients with TBI

A total of 106 serum samples from 12 patients with severe TBI admitted to the surgical intensive care unit at National University Hospital (Singapore) for medical or emergency surgical management of their brain injury were used for analysis of NFM protein concentration. These were serum samples from patients (4 female and 8 male), aged 21–78 years, recruited for a previous study [15]. Outcome at 3 months after injury was assessed using the Glasgow Outcome Score (GOS). Research board ethics approval (Singapore DSRB project 2010/00286) was obtained before the study commenced.

## Serum samples from patients with mTBI

Serum samples were collected from 68 patients aged 15–84 years (27 females and 41 males), presenting at the emergency department of the Academic Hospital of Parma (Italy) with a history of mTBI in the previous 6 h, and Glasgow Coma Score (GCS) of 14–15 at presentation. Patients fulfilled the criteria for mTBI requiring computed tomography (CT) scanning according to local guidelines. The peritraumatic amnesia was determined by the patient's ability to recall events they can remember immediately before and after the injury. Inclusion criteria were the same as those described in a previous study [16]. Ethics approval for the collection of samples was obtained from the local committee.

## Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistical software, version 20. A p-value  $< 0.05$  was considered statistically significant. Normal distribution was evaluated using Shapiro-Wilk test and by inspection of Q-Q plots. Student's t- or Mann-Whitney U-tests were performed for comparisons between two groups. Kruskal-Wallis test was performed for comparisons between more than two groups. Associations between variables were assessed by Spearman's rank correlation coefficient. Reed's test was used for identification of outliers.

This study was conducted according to the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals.

**Table 1** CSF (n=51) and serum samples (n=299) collected from 170 individuals.

Samples, n	Patients, n	Diagnosis
CSF, n		
10	10	Control
11	11	Ischemic stroke (IS)
30	15 <sup>a</sup>	Hemorrhagic stroke (HS)
Serum, n		
47	47	Healthy individual
78	7 <sup>b</sup>	Subarachnoid hemorrhage (SAH)
106	12 <sup>c</sup>	Traumatic brain injury (TBI)
68	68	Mild traumatic brain injury (mTBI)

<sup>a</sup>11 patients had only one sample and 4 patients had 6, 3, 5 and 5 samples (see Figure 1). Only the first sample of these four patients is displayed in Figure 1; <sup>b</sup>See Table 2; <sup>c</sup>See Table 3.

## Results

### SE-HPLC

One hundred microliters of four samples (NFM standard from ELISA kit, brain tissue extract, CSF and serum) were injected. The SE-HPLC fractions from each sample (from 18 to 58 min) were analyzed with the ELISA kit for NFM protein. The four samples analyzed showed the highest immunoreactivity for NFM protein in fractions 23 and 24 (Supplemental Data, Figure S1, that accompanies the article <http://www.degruyter.com/view/j/cclm.2015.53.issue-10/cclm-2015-0908/cclm-2015-0908.xml?format=INT>), in agreement with the molecular weight of this protein (about 160 kDa).

### Mass spectrometry identification

Three SE-HPLC fractions from brain tissue extract (fractions 18, 23 and 34) were analyzed by LC-MS/MS. A tryptic peptide from NFM protein was exclusively identified in fraction 23 (Supplemental Data, Figure S2). These results agreed with the results obtained with the ELISA kit, confirming the presence of NFM protein in the fraction with the highest ELISA immunoreactivity.

### Linearity and intra- and inter-batch CV of NFM ELISA kit

The ELISA kit showed good linearity between 15.6 and 500 pg/mL (Supplemental Data, Figure S3). CSF and serum samples had to be diluted (from 1:1 to 1:1000) before analysis using the diluent provided in the ELISA kit. Samples were analyzed in duplicate but at two different dilutions (1:10 and 1:100 for CSF and serum from healthy volunteers and 1:50 and 1:100 for serum from patients with brain injury). If NFM concentration was above or below the linear range in both dilutions, samples were analyzed again with higher or lower dilutions.

Four samples (QC 1–4) with different concentrations of NFM protein were analyzed six times with the same ELISA kit. The concentration (mean±SD) and the intra-batch CV were: QC1: 20±2 pg/mL (CV=9%); QC2: 90±4 pg/mL (CV=5%); QC3: 198±16 pg/mL (CV=8%); and QC4: 405±39 pg/mL (CV=10%).

Two samples (QC 5–6) with low and high concentrations of NFM protein were analyzed eight times with five ELISA kits from three different lots. The concentration

(mean±SD) and the inter-batch CV were: QC5: 74±9 pg/mL (CV=12%); and QC6: 314±41 pg/mL (CV=13%).

### CSF samples from patients with stroke and healthy controls

The concentration of NFM protein in the CSF samples from patients with ICH (n=7), SAH (n=8), IS (n=11) and controls (n=10) was, respectively, (median and minimum-maximum): 10.6 (4.78–373) ng/mL, 5.66 (0.43–131) ng/mL, 0.21 (0.07–1.36) ng/mL and 0.23 (0.11–0.62) ng/mL. These results agreed with those obtained with the mass spectrometry-based assay, because both analyses showed statistically significant differences between the HS group and the other two groups (Figure 1A). When combined, the NFM concentration in the HS group was significantly higher than the concentration in the IS and control groups ( $p<0.0001$ ). There were no significant differences between the IS and control groups ( $p=0.86$ ). The p-values obtained with the NFM results from ELISA are lower because this assay has higher sensitivity than the mass spectrometry-based assay. The analysis of serial samples from four of these patients revealed that the highest NFM concentration was observed between 8 and 11 days after onset of symptoms (Figure 1B).

The seven patients with ICH showed secondary intraventricular hemorrhage (IVH). Four of these patients had an unfavorable outcome (GOS 1–2) and three had a favorable outcome (GOS 3–4). Five out of the eight patients with SAH had Fisher grade 4 scans [GOS 1 (n=2) and GOS 3 (n=3)] whereas the CT scan confirmed the absence of IVH in the other three patients [GOS 4 (n=1) and GOS 5 (n=2)]. The three patients with CSF NFM concentration >100 ng/mL (Figure 1A) were some of the patients who finally died (GOS 1). However, the three patients with NFM concentration lower than 1 ng/mL had a favorable outcome [GOS 3 (n=1) and GOS 5 (n=2)].

### Serum NFM concentration in healthy individuals

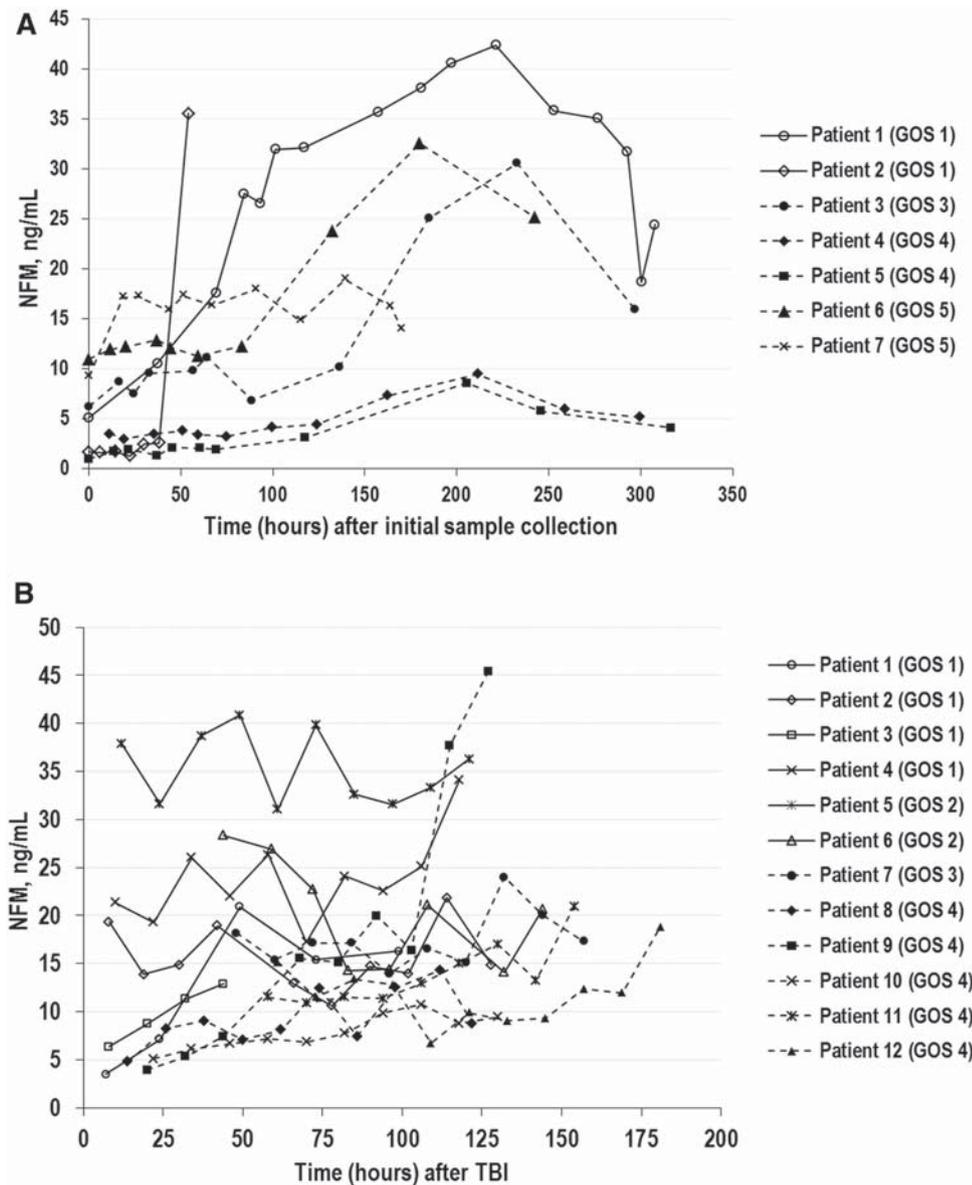
NFM concentration was analyzed in serum samples from 47 apparently healthy volunteers (26 women and 21 men). The NFM concentration was from 0.26 to 27.8 ng/mL. The highest concentration corresponding to a 35-year-old man (27.8 ng/mL) was identified as an outlier (according to the Reed's test) and excluded from subsequent analyses. Therefore, the NFM concentration



**Table 2** Time of blood sample collection and NFM concentration (ng/mL) in patients with SAH.

Patient	Gender	Age, years	Time of first sample, h	Time of final sample, h	Samples, n	Initial NFM value, ng/mL	NFM Median (minimum–maximum)	Outcome
1	Male	48	0	307	16	5.08	31.9 (5.08–42.4)	GOS 1
2	Female	50	0	54	7	1.66	1.69 (1.28–35.6)	GOS 1
3	Female	63	0	296	11	6.21	9.81 (6.21–30.6)	GOS 3
4	Female	50	11 <sup>a</sup>	299	12	3.48	3.93 (2.96–9.47)	GOS 4
5	Male	56	0	316	11	0.97	2.02 (0.97–8.57)	GOS 4
6	Male	68	0	242	10	10.96	12.2 (11.0–32.6)	GOS 5
7	Male	35	0	170	11	9.22	16.3 (9.22–19.0)	GOS 5

<sup>a</sup>There was not any serum left of the first sample of patient 4.



**Figure 2** Evolution of NFM concentration in serial serum samples from seven patients with SAH (A) and 12 patients with TBI (B). Patients with unfavorable outcome (GOS 1–2) are represented with continuous lines while patients with favorable outcome (GOS 3–5) are represented with broken lines.

Patient 1 showed the highest levels of NFM, with a peak of 42.4 ng/mL, a few hours after he presented with unreactive pupils and was diagnosed with brain death. NFM concentration in Patient 2 increased from 2.56 to 35.6 ng/mL in <16 h and just after she suffered an infarction of median brain artery.

### Serum samples from patients with severe TBI

Serial serum samples (n=106) from 12 patients with severe TBI were also measured. NFM levels were from 3.48 to 45.4 ng/mL (Table 3). The median of initial NFM concentration (13.3 ng/mL) for 12 patients with TBI was significantly higher ( $p < 0.001$ ) than the median NFM concentration in healthy individuals (2.29 ng/mL, n=46). All patients showed at least one sample with NFM concentration above the upper reference limit (Figure 2B). Patients with unfavorable outcome (GOS 1–2) showed slightly higher levels (medians from 10.0 to 34.8 ng/mL) than patients with favorable outcome (GOS 3–4) (medians from 7.45 to 15.5 ng/mL). Seven out of these 12 patients (4 with a favorable outcome and 3 with an unfavorable outcome) had an initial value higher than the upper reference limit. The median of initial NFM concentration (20.4 ng/mL) for six patients with unfavorable outcome (GOS 1–2) was higher but not statistically significant ( $p = 0.18$ ) than the median of initial NFM concentration (8.30 ng/mL) for six patients with favorable outcome (GOS 3–4).

### Serum samples from patients with mTBI

NFM protein concentration was also analyzed in 68 patients with mTBI. One sample from each patient was

collected within 6 h of accident. NFM levels were from 0.21 to 202.2 ng/mL. Median NFM concentration in patients with mTBI (7.89 ng/mL, n=68) was significantly higher ( $p < 0.001$ ) than median NFM concentration in healthy individuals (2.29 ng/mL, n=46).

In total 30 out of 68 (44%) of patients with mTBI had a NFM concentration higher than the upper reference limit (Figure 3). A total of 34% (14 out of 41) of patients with negative CT scan (for hemorrhage or any other suggestive brain abnormality) and no polytrauma (–/–) had a NFM concentration higher than the upper limit. However, 69% (11 out of 16) of patients with negative CT scan and polytrauma (–/+ ) had a NFM concentration higher than the upper reference limit. NFM concentration was significantly higher ( $p = 0.01$ ) in the group of patients with polytrauma (–/+ ) than in the group of patients without polytrauma (–/–). Finally, 45% (5 out of 11) of patients with positive CT scan (with and without polytrauma) (+/+ , +/-) had a NFM concentration higher than the upper reference limit. The median NFM concentration in these three groups of patients with mTBI (displayed below) was significantly higher than the median NFM concentration in healthy individuals [Kruskal-Wallis (four groups),  $p < 0.0001$ ; Mann-Whitney U, group (–/–): 5.59 ng/mL,  $p = 0.001$ ; group (–/+): 17.68 ng/mL,  $p < 0.001$ ; group (+/+ , +/-): 7.88 ng/mL,  $p = 0.002$ ].

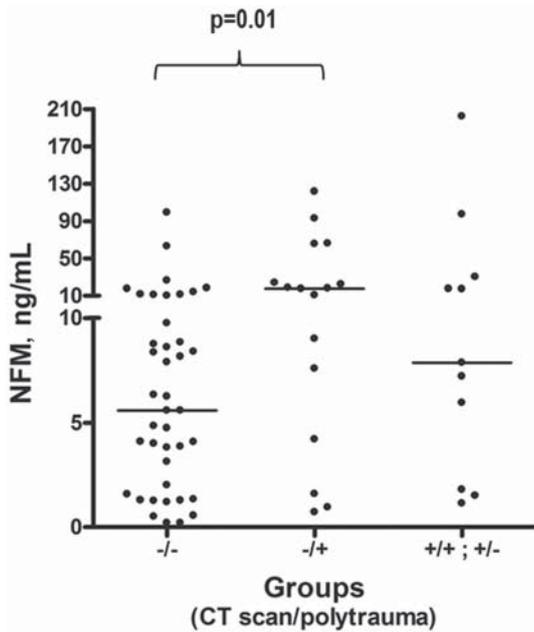
## Discussion

Human NFM is a 916 amino acid protein with a molecular mass of approximately 160 kDa, encoded by the *NEFM* gene, which is located on chromosome 8 (8p21) [17].

Neurofilaments (NFs) are type IV intermediate filaments (IFs) expressed under three different subunit

**Table 3** Time of blood sample collection and NFM concentration (ng/mL) in patients with severe TBI.

Patient	Gender	Age, years	Time of first sample, h	Time of final sample, h	Samples, n	Initial NFM value, ng/mL	NFM Median (minimum–maximum)	Outcome (3 months)
1	Male	39	7	99	5	3.48	15.4 (3.48–20.9)	GOS 1
2	Male	23	8	128	10	19.4	14.8 (10.6–21.9)	GOS 1
3	Male	24	8	44	4	6.35	10.0 (6.35–12.9)	GOS 1
4	Female	78	10	118	10	21.4	23.3 (17.3–34.1)	GOS 1
5	Female	68	12	121	10	37.8	34.8 (31.1–40.9)	GOS 2
6	Female	75	44	144	8	28.4	20.9 (14.1–28.4)	GOS 2
7	Female	47	48	157	10	18.2	17.2 (14.0–24.0)	GOS 3
8	Male	25	14	122	10	4.79	8.50 (4.79–14.3)	GOS 4
9	Male	62	20	127	9	3.94	15.5 (3.94–45.4)	GOS 4
10	Male	34	22	130	10	5.09	7.45 (5.09–10.7)	GOS 4
11	Male	21	58	154	9	11.5	13.0 (10.9–20.9)	GOS 4
12	Male	26	61	181	11	15.1	11.9 (6.70–18.8)	GOS 4



**Figure 3** NFM concentration in 68 serum samples from patients with mTBI.

Patients were divided into three groups: (-/-), negative CT scan and no polytrauma; (-/+), negative CT scan and polytrauma, (+/+, +/-); positive CT scan with and without polytrauma. Vertical lines represent the median NFM concentration.

proteins, NFL, NFM and NFH. NFs and  $\alpha$ -internexin are the major protein components of IFs in the central nervous system [18]. NFs (diameter: 8–10 nm) are exclusively expressed in neurons and act with microtubules (diameter: 24–26 nm) and microfilaments (diameter: 6–8 nm) to form and maintain the axonal structural integrity and neuronal shape, establishing a very stable tubular system of the neuronal cytoskeleton. NFs also participate to the axonal mechanisms of transport [17, 18].

NFs can be released in the interstitial fluid after axonal injury or degeneration due to the disruption of the axonal cytoskeleton, and diffuse into CSF, where they can be measured to monitor axonal degeneration. NFL and NFH proteins have been studied by many authors as markers of axonal degeneration in CSF and blood of patients suffering from various neurological diseases. Thus, axonal degeneration is thought to be an underestimated complication of SAH, which can continue for days after the primary injury, and extend into the period of delayed cerebral ischemia [17]. Several authors [8, 9] have reported increased CSF levels of these two proteins (NFL and NFH) in patients with SAH. Kuhle et al. reported that NFH levels in patients with SAH ( $n=20$ ) were from 8.4 to 997 pg/mL (median=345 pg/mL), while NFH concentration in controls ( $n=73$ ) was from 10 to 100 pg/mL. Similarly, Zanier

et al. reported increased NFL levels in patients with SAH ( $n=35$ ), from 60 to 2688 pg/mL (median=643 pg/mL), while NFL concentration in controls ( $n=13$ ) was below the limit of detection (<12 pg/mL).

More recently, Kuhle et al. [19] studied serum NFL as a predictive biomarker of clinical outcome in patients with spinal cord injury, showing a correlation between NFL concentration and acute severity and neurological outcome. Gaiottino et al. [20] found increased serum NFL concentration in various neurological diseases, with NFL levels more than 20-times higher in patients with amyotrophic lateral sclerosis than in controls.

Cai et al. [21] studied the correlation between plasmatic concentration of phosphorylated NFH (pNFH) and clinical outcome or early neurological deterioration of patients with ICH, showing that plasmatic values of pNFH were increased in ICH patients compared to healthy individuals (700 pg/mL,  $n=112$  vs. 26 pg/mL,  $n=112$ ), and that plasma pNFH levels predicted clinical outcomes and early neurological deterioration with high sensitivity and specificity. The pNFH protein has also been proposed as biomarker of axonal injury in multiple sclerosis [22].

To our knowledge this is the first study describing increased concentrations of NFM protein in CSF and serum samples from patients with brain damage (HS, SAH and mild and severe TBI).

Here, we have shown that NFM is released into the CSF compartment after HS, both in ICH and SAH. However, NFM concentration was not significantly elevated in the CSF of individuals with IS. Compared to previous studies where CSF levels of NFL and NFH proteins were measured [8, 9], NFM seems to be present at higher concentrations in patients with HS (0.43–373 ng/mL). Moreover, CSF-NFM levels of four patients with HS showed a concentration peak several days (between 8 and 11 days) after symptoms onset.

We also observed that NFM was released and detected in greater concentrations in serum samples from two patients with SAH that suffered a brain infarction and brain death few hours before serum sampling. However, NFM levels in patients with SAH seemed not to correlate with outcome.

Serum samples from patients with severe TBI showed also increased NFM levels, with 58% (7 out of 12) of patients (67% of patients with GOS 1–2 and 50% of patients with GOS 3–4) showing an initial NFM value higher than the upper reference limit (8.57 ng/mL). Moreover, 83% (10 out of 12) of patients (100% of patients with GOS 1–2 and 67% of patients with GOS 3–4) showed NFM concentrations higher than the upper reference limit (Table 3).

Finally, 44% of patients with recent mTBI (within 6 h of sampling) showed increased serum NFM levels with significantly higher concentrations in patients with polytrauma but not in patients with positive CT scan. These results may indicate that NFM protein is released from other parts of the body apart from the brain since patients with polytrauma showed very high serum concentrations. This is probably related to the specific expression of NFM in neuronal cells and ganglia. According to the information provided by the Human Protein Atlas (<http://www.proteinatlas.org>) [23], NFM expression at protein level (based on immunohistochemistry results) is specific of neuronal cells. This protein is highly expressed in central nervous system (about 0.2 µg/mg total protein in our brain tissue extracts) but it also shows expression in peripheral nervous system (peripheral neurons of peripheral nerve and ganglia). Therefore, the elevation of serum NFM concentration in polytrauma could be the consequence of a peripheral nerve injury (in neurons outside of the brain and spinal cord).

One of the limitations of this study is that paired CSF and serum samples from patients with brain injury were not collected and analyzed. The CSF samples used in our previous study [7] for identification of novel biomarkers of brain injury were reanalyzed with the NFM ELISA kit in order to confirm the results obtained with the mass spectrometry-based method. New prospective validation studies should be performed to elucidate the kinetics of NFM protein in central and peripheral nervous system after a brain injury. Another aspect that would be necessary to reproduce in an independent study is the differences observed in the serum NFM concentrations among genders.

## Conclusions

In the present study, we have shown that NFM protein concentration is increased in CSF and in blood circulation after a brain injury, with higher levels in patients suffering HS and polytrauma. More studies are needed to elucidate the potential of this protein in the diagnosis, prognostication and management of patients with brain injury and other neurological diseases with axonal degeneration.

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