Phosphorylated neurofilament heavy: A potential blood biomarker to evaluate the severity of acute spinal cord injuries in adults

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ABSTRACT

Aims: The aim of this study is to analyze the serial estimation of phosphorylated neurofilament heavy (pNF-H) in blood plasma that would act as a potential biomarker for early prediction of the neurological severity of acute spinal cord injuries (SCI) in adults.

Settings and Design: Pilot study/observational study.

Subjects and Methods: A total of 40 patients (28 cases and 12 controls) of spine injury were included in this study. In the enrolled cases, plasma level of pNF-H was evaluated in blood samples and neurological evaluation was performed by the American Spinal Injury Association Injury Scale at specified period. Serial plasma neurofilament heavy values were then correlated with the neurological status of these patients during follow-up visits and were analyzed statistically.

Statistical Analysis Used: Statistical analysis was performed using GraphPad InStat software (version 3.05 for Windows, San Diego, CA, USA). The correlation analysis between the clinical progression and pNF-H expression was done using Spearman’s correlation.

Results: The mean baseline level of pNF-H in cases was 6.40 ± 2.49 ng/ml, whereas in controls it was 0.54 ± 0.27 ng/ml. On analyzing the association between the two by Mann–Whitney U–test, the difference in levels was found to be statistically significant. The association between the neurological progression and pNF-H expression was determined using correlation analysis (Spearman’s correlation). At 95% confidence interval, the correlation coefficient was found to be 0.64, and the correlation was statistically significant.

Conclusions: Plasma pNF-H levels were elevated in accordance with the severity of SCI. Therefore, pNF-H may be considered as a potential biomarker to determine early the severity of SCI in adult patients.

Key Words: Biomarkers, phosphorylated neurofilament heavy subunit, spinal cord injuries

INTRODUCTION

Spinal cord injury (SCI) is an insult to the spinal cord resulting in a change either temporary or permanent, in its normal motor, sensory, or autonomic function. Patients with traumatic SCI are often in spinal shock, and hence it is difficult to have an idea about the true status of injury (cord damage). Currently, the only practical real-time method to detect a change in neurological status is by repeated clinical examination. Despite enormous advancement in the field of medicine, it is still a challenge...
to prognosticate patients presenting with traumatic SCI. One of the major problems against the innovation of novel therapies for traumatic SCI is the difficulty in finding out the baseline severity of the injury.

It is well evident from the literature available that there are a number of proteins and enzymes (neuron-specific enolase, S100 beta, interleukin-6 and interleukin-8, serum-soluble CD95 ligand, phosphorylated neurofilament heavy [pNF-H] etc.) which are released into the cerebrospinal fluid (CSF) and/or serum/plasma as a result of spinal cord injury.[3-7] The level of these proteins if precisely detected can guide us about the nature and probably the severity of injury. Hence, detection and validation of the so-called SCI biomarkers may provide a quick and simple clinical diagnosis and prognosis of traumatic SCI in human. However, to qualify as an ideal or near ideal biomarker, the substance released in response to injury to spinal cord should be specific to axons, and it should be released in quantity sufficient enough to be readily detectable in plasma even after the significant dilution that occurs following release into the blood. It should be resistant to proteases so that it is not broken down before or following release and normally should not be detectable in plasma and hence that its presence even in minimum quantity can be a justification for proving the ailment.

In this study, we have selected pNF-H as a biomarker to study patients with SCI because most of the other neuronal injury markers achieve too early a peak after SCI or they wane off rapidly, whereas pNF-H can be stably detected at a later point of time.[8,9] pNF-H is one of the structural proteins of axon fibers which is not detectable in the blood of healthy animals, the elevation of this protein in blood implies the breakdown of axons.[10]

Autoantibodies have been shown to be present against pNF-H in a vast majority of neurodegenerative diseases. pNF-H level in plasma gets elevated in patients with traumatic brain injury (TBI), subarachnoid hemorrhage, and amyotrophic lateral sclerosis.[9,11,12] Prolonged elevation of plasma pNF-H in patients with central nervous system disorders further suggests that it is due to the continuous axonal degeneration, such as Wallerian degeneration or secondary axonal damage. pNF-H is stable on release from neurons and can be captured and detected with exceptionally high avidity due to its exotic multi-epitope nature. Since pNF-H is only found in axons, its detection in CSF, blood, or other bodily fluids points unambiguously to release of this protein from axons.

We performed this study hypothesizing that pNF-H protein would be released from damaged and degenerating neurons of patients with acute traumatic SCI in amounts large enough to allow its detection in blood and that the levels detected would reflect the degree of injury severity. Our aim was to analyze the serial estimation of pNF-H in blood plasma that would act as a potential biomarker for early predicting the neurological severity of acute SCI in adults.

**SUBJECTS AND METHODS**

This was a pilot study conducted in the Department of Orthopaedic Surgery and Department of Biochemistry, King George’s Medical University, Lucknow. After obtaining ethical clearance from the Institutional Ethics Committee, 40 patients with SCIs were enrolled in the study. Of whom, there were 28 cases and 12 controls. All the patients of either sex between 19 and 65 years of age sustaining SCI from dorsal 4th to lumbar 2nd vertebrae and having neurological deficit (American Spinal Injury Association Injury Scale [AIS]-A/B/C/D) with a thoracolumbar injury classification and severity score of ≤4 (i.e., those who can be treated conservatively) or >4 who refused to undergo surgery and presenting to us within 3 days were included as cases, and patients with similar parameters, but without neurological deficit (AIS E) were taken as controls.[13,14] The patients beyond this age group, suffering from any type of neurological ailment or having a history or family history of the same, head injury, with injury levels beyond dorsiolumbar region (D4 to L2) and those not willing to give an informed consent were excluded from the study.

Clinical, biochemical, and radiological evaluation was performed for both the cases and controls at enrollment. Subsequent clinical and biochemical evaluation was performed as per protocol described in methodology. Radiological evaluation by means of X-rays and magnetic resonance imaging was performed to justify inclusion and exclusion criteria to minimize bias. After obtaining all the above mentioned data, the pNF-H levels were serially quantified and correlation with the severity of neurological status of the patient with SCI was evaluated in all patients [Figure 1].

The pNF-H protein level estimation in blood plasma was done using 3 ml of venous blood sample which was centrifuged at ×2000 g for 10 min. The plasma samples thus obtained were stored at −20°C, which were further analyzed for plasma pNF-H protein level by ELISA (Enzyme-linked immunosorbent assay) Kits (Human pNF-H ELISA; BioVendor, Modrice, Czech Republic) as per manufacturer’s protocol in duplex.[8]

**Statistical analysis**

Statistical analysis was performed using GraphPad InStat software (version 3.05 for Windows, San Diego, CA, USA). The demographic characteristics were compared between both groups using Student t-test (Unpaired), Fisher’s exact test, or Chi-square test. All values are expressed as mean ± standard deviation to facilitate the statistical
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As per inclusion/exclusion criteria, all patients of age 19-64 years, of either sex, presenting to us with traumatic SCI were enrolled in this study (28 cases and 12 controls). Statistical analysis, AIS score was converted into numerical values (i.e., AISA = 5; B = 4; C = 3; D = 2; and E = 1). Statistical mean significant difference of pNF-H expression in both groups was determined using Mann–Whitney U-test. The correlation analysis between the clinical progression and pNF-H expression were performed by Spearman’s correlation. At 95% confidence interval, values of $P < 0.05$ were considered statistically significant.

### RESULTS

A total of 40 subjects with spinal injuries were enrolled in the study (28 cases and 12 controls). All the cases and controls in the study population were matched for their age and sex [Table 1]. Among the cases, nine patients (32.1%) were with AIS A, 12 (42.9%) with AIS-B, and rest 7 (25%) were with AIS-C. Considering the mode of injury, fall from height was the most common reason in both cases ($n = 11$; 39.3%) and controls ($n = 05$; 41.6%). Plasma pNF-H level was estimated in all the cases and controls at specified intervals. The mean baseline levels of pNF-H calculated on the 3rd day showed a statistically significant difference between cases and controls [Table 2]. In all cases, the levels of pNF-H on the 28th day were high in comparison to mean level in controls on the 3rd day and the difference was statistically significant. Of 28 cases, the peak value of pNF-H was seen in 25 cases (89.3%) on the 3rd postinjury day, whereas in 2 cases (7.14%), the peak levels were on the 7th day and in 1 case (3.6%) on the 14th day after injury [Figure 2]. The AIS grading evaluation was further performed at specified days [Figure 3].

**Table 1: Baseline characteristics of the patient**

| Patient Characteristics | Cases ($n = 28$) | Controls ($n = 12$) | $P$ |
|-------------------------|-----------------|-------------------|-----|
| Mean age ± SD (range) in years | | | |
| Male | 18 (64.3%) | 08 (66.6%) | NS |
| Female | 10 (34.7%) | 04 (33.6%) |

**Table 2: Association between Phosphorylated Neurofilament H levels with severity of spinal cord injury patient**

| Follow up interval | Mean AIS Score at different follow up | Mean pNF-H level at different follow up intervals (ng/mL) | Significance of Difference |
|--------------------|--------------------------------------|--------------------------------------------------------|---------------------------|
| Cases ($n = 28$) | Controls ($n = 12$) | $P < 0.001^*$ |
| 3rd Day | 6.40 ± 2.49 | 0.54 ± 0.27 |
| 7th Day | 6.38 ± 2.49 | 0.56 ± 0.27 |
| 14th Day | 4.07 ± 0.76 | 5.60 ± 3.33 |
| 21st Day | 4.03 ± 0.74 | - |
| 28th Day | 3.89 ± 0.87 | 4.91 ± 3.12 |
| 35th Day | 3.57 ± 0.95 | - |
| 42nd Day | 3.17 ± 1.02 | - |
| 90th Day | 3.00 ± 1.38 | - |

*Significant; ASIA Score A = 5; B = 4; C = 3; D = 2; E = 1

We had 9 cases who presented to us with AIS-A. Of these, 2 cases (22.2%) had no change in neurological status till last follow-up visit at 90 days. In these 2 cases, the pNF-H levels increased till the 7th day and thereafter showed a decline in levels by 28th day follow-up visit. Remaining 7 cases (77.8%) showed a partial neurological recovery during subsequent follow-up visits in the following manner: three (33.4%) of them progressed to AIS-C and rest 4 (44.4%) progressed to AIS-B, but none...
of them had progressed to AIS-E till the last follow-up visit (3 months).

Twelve cases presented to us with AIS-B. Of these, there was only 1 case (8.3%) whose neurological status neither deteriorated nor improved. The pNF-H levels in this case increased till the 14th day and thereafter decreased to the initial levels, that is, levels on 3rd day [Figure 3]. Three cases (25%) of AIS-B showed neurological deterioration to AIS-A at the final follow-up visit. Out of these cases, 2 (16.7%) cases showed deterioration at the last follow-up visit (i.e., at 90 days) and they had their peak pNF-H levels at 14th day, which probably was indicating toward some on-going secondary damage. One case (8.3%) had deterioration on the 35th day and this case too had a peak pNF-H level on 14th day. On evaluating pNF-H levels and AIS of these patients on common follow-up dates (day 14th and 28th), there was not much of difference between levels of pNF-H, except for a slight increase in levels as their AIS remained constant. This may be as a result of secondary insult and was also reflected in pNF-H levels which showed increasing levels on consecutive estimations [Figure 4]. In the remaining 8 cases (66.7%), 4 cases (33.3%) showed neurological recovery to AIS-C, 2 cases (16.6%) recovered to AIS-D and rest 02 (16.6%) moved to AIS-E at the last follow-up visit [Figure 3]. In all the cases of AIS-B, there was a decrease in pNF-H levels from its peak in subsequent follow-up, but none reached to mean level of controls.

Seven cases presented with AIS-C. Three cases (42.8%) had a complete neurological recovery to AIS-E, whereas 4 (57.1%) had a partial recovery to AIS-D. In all of these seven cases, the pNF-H levels showed a decreasing trend from its peak level on 3rd day. None of them also reached to mean level of controls.

In the present study, we observed a statistically significant association between the mean AIS score of the cases and mean pNF-H levels at different follow-up intervals [Table 2]. A mild correlation between baseline pNF-H levels and AIS was observed, which was also significant statistically ($P < 0.05$) [Table 3].

### DISCUSSION

The prediction of neurological outcomes of traumatic SCI is not possible during the early stages of injury; therefore, novel biomarkers other than neurological symptoms would be in demand to increase the accuracy of evaluating the initial severity of injury as well as the progression of neurological status.

In this study, we observed different peak expression of pNF-H at different points of time, and this may be due to the severity of neural damage/secondary insult. However overall, we were able to find a decreasing trend (from its peak) of pNF-H levels at subsequent follow-up

| Correlation Coefficient | r-Squared | Standard Deviation | P |
|-------------------------|-----------|--------------------|---|
| 0.64                    | 0.41      | 3.2                | 0.046* |

*Significant

Figure 2: Mean plasma phosphorylated neurofilament heavy level of patients

Figure 3: Association Injury scale scoring of each case at different follow-up

Figure 4: Mean plasma phosphorylated neurofilament heavy level in relation to ASIA score at different follow-ups. The above graphical representation shows that cases who have shown a deterioration or no improvement in their Association Injury scale grades in consecutive visits have an increasing trend or a relatively constant level of phosphorylated neurofilament heavy, whereas those who have an improvement in their Association Injury Scale grade have a decreasing trend of phosphorylated neurofilament heavy levels in subsequent follow up visits.

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Table 3: Correlation between baseline Phosphorylated Neurofilament H levels and American Spinal Injury Association scores at different follow up intervals (Spearman’s rank coefficient)

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visits [Figure 4]. Moreover, the mean baseline levels of pNF-H calculated on the 3rd day showed a statistically significant difference (P < 0.001) between all cases and controls. We also observed a statistically significant association and correlation of mean baseline pNF-H level with the AIS score at different follow-up intervals after injury [Table 3]. Majority of cases (89.3%) showed mean pNF-H peak level at baseline (3rd day of postinjury) which also showed a positive correlation with AIS score. Thus, it can be postulated that plasma pNF-H is a good predictable marker for early evaluation in patients with SCI.

In the past, a few studies have been carried out on animals as well as humans. A study was carried out on rat models by Shaw et al.\(^1\) in 2005 with serum estimation of pNF-H levels. They observed that the levels of this biomarker returned to normal levels by 14th day. We cannot compare our results with above study as this was conducted on animal model. Although we conducted our study on serum samples, we find support in our conclusions from St. Iencean et al.,\(^5\) who in their study on two cases with cervical spine injury (tetraplegia) studied PNF-H levels on CSF samples and concluded that its increased levels in CSF samples are consistent with an unfavorable outcome. Ungureanu et al.,\(^6\) did serial estimation of pNF-H in CSF of 15 patients with SCI (both cervical and dorsal) and concluded that it is an important predictor of severity of SCI and is sensitive to secondary lesions.

Ahadi et al.,\(^9\) concluded that serum levels of pNF-H, NSE, and GFAP, combined with neurological testing is a good predictor of severity of SCI in initial 48 h. They further inferred that the mean serum levels of pNF-H were significantly higher during the first and second 24 h in patients with SCI than in the control group (P < 0.001), but there was no significant difference compared with the control group during the third 24 h after injury, whereas in our study, the levels showed a persistence elevation of pNF-H levels in a good number of cases even after 72 h of injury in comparison to the control group. A study conducted by Hayakawa et al.,\(^8\) on human blood plasma correlates with the present study findings. They showed that the serum pNF-H levels were elevated in accordance with the severity of acute SCI patients. However, unlike to Hayakawa that quantified the pNF-H early on 6th h and ended on 21st day of injury, we evaluate the pNF-H level early on 3rd day. The reason behind our protocol was that our institution is a tertiary care center, where most of the patients are referred and are thus not able to come immediately after the injury.

The study is a pilot study conducted with small sample size, and as we have adopted strict inclusion/exclusion criteria, it is difficult to find equal number of age/sex match case–controls. So, further multicentric studies with larger sample size having adequate case–control ratio are recommended to validate the above results.

There is some direct proteomic evidence of the presence of low levels of peptides derived from NF-H and other neurofilament subunits in normal and TBI patient blood samples,\(^19\) and in normal and multiple sclerosis patient blood samples.\(^19\) The origin of these peptides in the blood of healthy individuals is currently unknown. This is therefore another area that requires more study to elaborate our understanding and the significance of the pNF-H blood biomarker.

**CONCLUSIONS**

In this study, we found a statistically significant difference in the baseline pNF-H levels of cases and controls. Even in subsequent follow-up visits, the difference in level was found to be statistically significant and this difference positively correlates with the patients’ AIS. Thus, we conclude that this biomarker is elevated in response to SCI and varies with respect to the improvement or deterioration of his neurological status and thus can be a useful biomarker to prognosticate the case.

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**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**

1. McDonald JW, Sadowsky C. Spinal-cord injury. Lancet 2002;359:417-25.
2. Bach-y-Rita P, Illis LS. Spinal shock: Possible role of receptor plasticity and non synaptic transmission. Paraplegia 1993;31:82-7.
3. Loy DN, Sroufe AE, Pelt JL, Burke DA, Cao QL, Talbott JF, et al. Serum biomarkers for experimental acute spinal cord injury: Rapid elevation of neuron-specific enolase and S-100beta. Neurosurgery 2005;56:391-7.
4. Cao F, Yang XF, Liu WG, Hu WW, Li G, Zheng XJ, et al. Elevation of neuron-specific enolase and S-100beta protein level in experimental acute spinal cord injury. J Clin Neurosci 2008;15:541-4.
5. Pouw MH, Hosman AJ, van Middendorp J, Verbeek MM, Vos PE, van de Meent H. Biomarkers in spinal cord injury. Spinal Cord 2009;47:519-25.
6. Kwon BK, Stammers AM, Belanger LM, Bernardo A, Chan D, Bishop CM, et al. Cerebrospinal fluid inflammatory cytokines and biomarkers of injury severity in acute human spinal cord injury. J Neurotrauma 2010;27:669-82.
7. Kochanek PM, Berger RP, Bayir H, Wagner AK, Jenkins IW, Clark RS. Biomarkers of primary and evolving damage in traumatic and ischemic brain injury: Diagnosis, prognosis, probing mechanisms, and therapeutic decision making. Curr Opin Crit Care 2008;14:135-41.
8. Hayakawa K, Okazaki R, Ishii K, Ueno T, Izawa N, Tanaka Y, et al. Phosphorylated neurofilament subunit NF-H as a biomarker for evaluating the severity of spinal cord injury patients, a pilot study. Spinal Cord 2012;50:493-6.
9. Ahadi R, Khodagholi F, Daneshi A, Vafaee A, Mafi AA, Jorjani M. Diagnostic value of serum levels of GFAP, pNF-H, and NSE compared with clinical findings in severity assessment of human traumatic spinal cord injury. Spine (Phila Pa 1976) 2015;40:E823-30.
10. Takahashi H, Aoki Y, Nakajima A, Sonobe M, Terajima F, Saito M, et al. Phosphorylated neurofilament subunit NF-H becomes elevated in the cerebrospinal fluid of patients with acutely worsening symptoms of compression myelopathy. J Clin Neurosci 2014;21:2175-8.
11. Lewis SB, Wolper RA, Miriala L, Yang C, Shaw G. Detection of
phosphorylated NF-H in the cerebrospinal fluid and blood of aneurysmal subarachnoid hemorrhage patients. J Cereb Blood Flow Metab 2008;28:1261-71.

12. Ghonemi MO, Rabah AA, Saber HM, Radwan W. Role of phosphorylated neurofilament H as a diagnostic and prognostic marker in traumatic brain injury. Egypt J Crit Care Med 2013;1:139-44.

13. Lee JY, Vaccaro AR, Lim MR, Oner FC, Hulbert RJ, Hedlund R, et al. Thoracolumbar injury classification and severity score: A new paradigm for the treatment of thoracolumbar spine trauma. J Orthop Sci 2005;10:671-5.

14. Available from: http://www.asia-spinalinjury.org/elearning/ASIA_ISCOS_high.pdf. [Last assessed on 2016 Feb 25].

15. Shaw G, Yang C, Ellis R, Anderson K, Parker Mickle J, Scheff S, et al. Hyperphosphorylated neurofilament NF-H is a serum biomarker of axonal injury. Biochem Biophys Res Commun 2005;336:1268-77.

16. Iencean SM, Ungureanu D, Tascu A, Costachescu B, Iencean AS, Poeata I. CSF phosphorylated neurofilament subunit NF-H (pNF-H) levels are biomarkers of spinal cord injury. Rom Neurosurg 2013;20:148-50.

17. Ungureanu D, Iencean SM, Dimitriu C, Iencean AS, Tascu A. Determination of the phosphorylated neurofilament subunit NF-H (pNF-H) in cerebro-spinal fluid as biomarker in acute traumatic spinal cord injuries. Rev Rom Med Lab 2014;22:377-86.

18. Haqqani AS, Hutchinson JS, Ward R, Stanimirovic DB. Biomarkers and diagnosis; protein biomarkers in serum of pediatric patients with severe traumatic brain injury identified by ICAT-LC-MS/MS. J Neurotrauma 2007;24:54-74.

19. Gresle MM, Liu Y, Dagley LF, Haartsen J, Pearson F, Purcell AW, et al. Serum phosphorylated neurofilament-heavy chain levels in multiple sclerosis patients. J Neurol Neurosurg Psychiatry 2014;85:1209-13.