Phosphorylated Neurofilament H as a Diagnostic Marker in Acute Brain Insults

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Abstract

Background and Purpose: One of the main drawbacks in the management of patients with acute brain injuries is the absence of a widely available and rapid diagnostic test. The objective of our study was to assess whether Phosphorylated Neurofilament H (pNF-H) might provide useful diagnostic information and weather levels of the neurofilament correlated with different clinical variables.

Methods: A total of 90 patients presenting to the Critical Care Department of Cairo University were prospectively studied. Patients were stratified according to the presenting pathology into 3 main groups: Traumatic Brain Injury, Ischemic Stroke and Cerebral Hemorrhage. Blood samples for phosphorylated neurofilament H were assayed on admission and after 7 days. Neurofilament levels were correlated to Glasgow coma scale, CT findings and NIHSS on admission and after 7 days. Rankin score at 3 months was used to detect the degree of disability.

Results: Neurofilament H levels showed a negative correlation with GCS on admission and after 7 days in traumatic brain injury (r=0.66 & 0.78, respectively), ischemic stroke (r=0.3 & 0.5, respectively) and cerebral hemorrhage (r=0.56 & 0.65, respectively). In traumatic brain injury patients, there were a negative correlation between neurofilament levels and Marshal CT scores on admission and after 7 days (r=0.56 & 0.4, respectively) hence higher neurofilament levels correlated with worse CT findings. In ischemic CVS, there were a negative correlation between neurofilament levels and ASPECTS CT scores (r=0.64 & 0.89, respectively). In both ischemic CVS and cerebral hemorrhage, NIHSS showed positive correlations with neurofilament levels patients who died or had the greatest scores (Rankin 6 & 5) after 3 months had the highest levels of Neurofilament on admission and after 7 days. The cut off level of Neurofilament to detect death and disability was 35 pg/ml on admission (sensitivity 82%, specificity 78%) and was 1 pg/ml after 7 days (sensitivity 87%, specificity 92%).

Conclusion: Phosphorylated Neurofilament H can be used as a diagnostic and prognostic marker in patients with acute brain insults as seen by the presence of significant correlations between the marker levels and different clinical and radiological tools.

Key Words: Phosphorylated neurofilament H – Traumatic brain injury – Ischemic CVS.

Introduction

FOLLOWING CNS injury, certain proteins are released from neurons. A test that can quantify the levels of these released proteins might provide useful information about the level of injury, and would be particularly useful if such protein could be detected in blood. There has been a growing appreciation that many kinds of CNS injury and disease states are the result of axonal injury and degeneration [1,2]. Accordingly, a convenient method of detecting ongoing axonal loss might be particularly useful experimentally and clinically. The perfect marker to detect axonal injury should acquire several properties; it should be specific to axons, it should be profuse enough so that it can be readily detectable after the significant dilution that occurs following release into blood, and it should be resistant to proteases so that it is not broken down prior to or following release The Neurofilament H (NF-H) protein sequence contains unusual tandemly repeated 6-8-amino-acid sequences centered on the sequence lysine-serine-proline (LSP). The serine residues of the LSP are phosphorylated [3] and are axon specific [4]. This phosphorylated form of NF-H (here referred to as pNF-H) is known to be more resistant to calpain and other proteases [5,6]. Also, pNF-H is highly immunogenic, and the multiple repeated phosphorylated sites are an excellent target for antibody-based assays. Taken together, these facts suggest that pNF-H might be a good candidate for a biomarker of axonal injury.

Patients and Methods

Study design:

This is a randomized prospective included 90 patients presenting to the Critical Care Department
Cairo University Hospitals in the period from January 2010 to January 2011. Informed written consents had been obtained from the relatives and the study was approved by the Hospital’s Ethics Committee. Patients were stratified in 3 different groups according to the presenting pathology:

**Group A:** 30 Patients with isolated Traumatic Brain Injury (TBI) defined according to the Head Injury Interdisciplinary Special Interest Group of the American Congress of Rehabilitation Medicine [7] by having at least one of the following: Any period of loss of consciousness, any loss of memory for events immediately before or after the trauma, any alteration in mental state at the time of the accident focal neurologic deficits which may or may not be transient and abnormal CT scan findings.

**Group B:** 30 Patients with Ischemic Cerebrovascular Stroke defined as the presence neurological deficit with a clearly defined time of onset, and a baseline CT scan of the brain that shows no evidence of intracranial hemorrhage.

**Group C:** 30 Patients with Intracerebral Hemorrhage defined as the presence of neurological signs or deficit with a CT scan showing a typical homogeneous, hyperdense lesion with a sharp border with or without edema or mass effect within the brain.

This study excluded patients with one or more of the following criteria: Patients with any chronic Neurological Disease, seizure activity, renal impairment, patients with Multi-organ Failure Syndrome or Multiple trauma. Patients younger than 18 years old and older than 65 years old, Patients who were receiving or those candidates for thrombolytic therapy, five patients were excluded, because they had one or more reason for exclusion.

**Methods:**

Full history taking from the patients, relatives or witnesses with stress on the onset of neurological symptoms. Complete general and focused neurological examination. Blood samples were taken for routine labs and for pNF-H levels. Samples were taken within the first 24 hours (pNF-H1) and after 7 days (pNF-H2). For each patient, Non-contrast CT brain (NCCT), Glasgow coma scale was calculated on admission (GCS1) and repeated after 7 days (GCS2) Image review was independently performed on a workstation radiologists or neurologists.

- For patients with ischemic CVS and cerebral hemorrhage New York Institute of Health stroke scale (NIHSS) were used on admission (NIHSS 1) and after 7 days (NIHSS2). The level of stroke severity as measured by the NIH stroke scale scoring system: 0 = No stroke, 1-4 = Minor stroke, 5-15 = Moderate stroke, 15-20 = Moderate/severe stroke, 21-42 = Severe stroke.

- We used Marshal CT score and ASPECTS score to assess CT findings in traumatic brain injury patients and ischemic CVS respectively, on admission (MARSHAL 1 -ASPECTS 1) and after 7 days (MARSHAL2-ASPECTS2) (Table 1).

**The ASPECTS Score in Group B:**

The Alberta stroke program [9] early CT scores (ASPECTS) is a 10-point quantitative topographic CT scan score used in patients with ischemic stroke. Segmental assessment is made and 1 point is removed from the initial score of 10 if there is evidence of infarction in that region. The ASPECTS score was calculated on each of the CTs done to every patient in Group B on admission and after 7 days.

**Outcome analysis: Rankin score:**

Rankin score [10] was used to assess patients outcome after an interval of 3 months. Each patient was given a number from 1 to 6 according to level of disability (Table 2).

<table>
<thead>
<tr>
<th>Category</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>1: Diffuse injury I</td>
<td>No visible intra-cranial pathology seen on CT scan.</td>
</tr>
<tr>
<td>2: Diffuse injury II</td>
<td>Cisterns are present with midline shift &lt;5mm and/or lesion densities present. No high- or mixed-density lesion &gt;25ml, may include bone fragments and foreign bodies.</td>
</tr>
<tr>
<td>3: Diffuse injury III</td>
<td>Cisterns compressed or absent with mid-line shift 0-5mm No high- or mixed-density lesion &gt;25ml.</td>
</tr>
<tr>
<td>4: Diffuse injury IV</td>
<td>Mid-line shift &gt;5mm No high- or mixed-density lesion &gt;25ml.</td>
</tr>
<tr>
<td>5: Evacuated mass lesion</td>
<td>Any lesion surgically evacuated.</td>
</tr>
<tr>
<td>6: Non-evacuated mass lesion</td>
<td>High- or mixed-density lesion &gt;25ml, not surgically evacuated.</td>
</tr>
</tbody>
</table>

Each patient in Group A was given a number (1 -6) according to the Marshal CT.
Table (2): Rankin score.

<table>
<thead>
<tr>
<th>Rankin Score</th>
<th>Description</th>
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<tbody>
<tr>
<td>6</td>
<td>Death</td>
</tr>
<tr>
<td>5</td>
<td>Severe disability</td>
</tr>
<tr>
<td>4</td>
<td>Moderately severe disability</td>
</tr>
<tr>
<td>3</td>
<td>Moderate disability</td>
</tr>
<tr>
<td>2</td>
<td>Slight disability</td>
</tr>
<tr>
<td>1</td>
<td>No significant disability</td>
</tr>
</tbody>
</table>

Bedridden, incontinent, and requiring constant nursing care and attention.
Unable to walk without assistance, and unable to attend to own bodily needs without assistance.
Requiring some help, but able to walk without assistance.
Unable to carry out all previous activities but able to look after own affairs without assistance.
Able to carry out all usual duties and activities.

**Neurofilament H assay method:**

Blood samples were drawn from each patient on admission and after 7 days. The BioVendor Human Phosphorylated Neurofilament H ELISA, standards quality controls and samples were placed and left in microplate wells that contained with chicken polyclonal anti-pNF-H antibody. One hour later, detection rabbit polyclonal anti-pNF-H antibody was added and incubated with captured pNF-H for one hour. After another washing, HRP conjugated antibody against rabbit antibody was added. After one hour incubation and the last washing step, the remaining conjugate was allowed to react with the substrate solution. The reaction was stopped by addition of acidic solution and absorbance of the resulting yellow product was measured.

**Data analysis:**

Pearson correlation analysis of data was performed using SPSS statistical software. The association of subject characteristics to pNF-H levels was studied with multiple regressions. The pNF-H data were square root transformed to effect normality of distribution of residuals. Relationships between the square root of pNF-H were investigated within groups with Pearson correlation and the two-sample *t*-test. Analysis of correlation was used to assess the different relationships between pNF-H and other variables.

**Results**

A- Analysis of the whole study population:

1- Demographic data:

The study of demographic characteristics of the patients groups showed that the mean age of patients in the groups studied was 36.5 years in Group A, 49 years in Group B and 50.6 years in Group C with statistically significant difference *p* < 0.001 being youngest in Group A patients. There were a total of 54 (60%) male patients and 36 females (40%). The mean GCS on admission (GCS 1) was 6.6 in Group A, 7.1 in Group B and 7.8 in Group C. While the mean GCS after 7 days (GCS2) was 6.8, 8.1 and 8 respectively with non significant differences between groups. The mean Neurofilament level on admission (pNF-H1) and after 7 days (pNF-H2) were 40.9 pg/ml and 120.8 pg/ml respectively in the total 90 patients studied.

Group A patients had the lowest incidence of co morbidx illness compared to other groups. Group B patients had the highest incidence of all major risk factors than the other groups, except for hypertension which was more prevalent in Group C patients (Fig. 1).

2- Correlation of phosphorylated Neurofilament H with glasgow coma scale in the total study population:

The correlation between the Glasgow Coma Scale and the pNF-H level on admission was analyzed in the whole population studied there was a significant negative correlation (*p*<0.005) between
the GCS and pNF-H on admission and after 7 days of ($r=0.58$ & 0.43, respectively).

3- **Correlation between GCS and Neurofilament H levels in each group:**

As shown by the scatter diagram (Fig. 2), there was a negative correlation between the GCS and the mean neurofilament levels on admission and after 7 days in each group studied. In other words, higher neurofilament levels were associated with a lower GCS ($p<0.005$) this correlation was strongest in traumatic brain injury patients on admission and after 7 days in Group A ($r=0.66$ & 0.78, respectively) compared to the other groups.

4- **Analysis of outcome: Rankin score:**

Patients with higher levels of Neurofilament H on admission and after 7 days showed a greater Rankin score after 3 months and hence a greater disability ($p<0.005$). There were an apparent increase in mean neurofilament levels in patients with the greatest disability (Rankin 5, n=19) and those who died (Rankin 6, n=7). Patients with the least disability (Rankin 1 & 2) showed the lowest mean neurofilament levels on admission and after 7 days hence neurofilament H levels were significantly higher in patients with poor outcome (Table 3).

5- **Discriminative abilities of Neurofilament H for predicting outcome after 3 months:**

ROC curve showed cut-off point for pNF-H level at admission to predict severe disability or death was 35pg/ml with sensitivity 82.1% and specificity 78.4%, positive predictive value 74.4% and negative predictive value 14.9% with Area Under Curve (AUC 87.1%) hence patients with neurofilament H levels of 35pg/ml or more on admission had a worse Rankin score after 3 months (Fig. 3).

Table (3): Mean neurofilaments level pNF-H in each rankin group.

<table>
<thead>
<tr>
<th>Rankin: Outcome</th>
<th>Mean pNF-H1</th>
<th>Mean pNF-H2</th>
</tr>
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<tbody>
<tr>
<td>1: No significant disability</td>
<td>22</td>
<td>53</td>
</tr>
<tr>
<td>2: Slight disability</td>
<td>27.6</td>
<td>61</td>
</tr>
<tr>
<td>3: Moderate disability</td>
<td>32.91667</td>
<td>82.20833</td>
</tr>
<tr>
<td>4: Moderate severe disability</td>
<td>52.41667</td>
<td>140.3333</td>
</tr>
<tr>
<td>5: Severe disability</td>
<td>57.4</td>
<td>190.4</td>
</tr>
<tr>
<td>6: Death</td>
<td>67.85714</td>
<td>259.1429</td>
</tr>
</tbody>
</table>
Also ROC curve showed cut-off point for pNF-H level, one week later to predict severe disability or death was 111 pg/ml with sensitivity 87.2% and specificity 92.2%, positive predictive value 89.5% and negative predictive value 9.6% with Area Under Curve (AUC 95.7%). Hence patients with Neurofilament H levels of more than 111 pg/ml after 7 days had a worse Rankin score after 7 days (Fig. 3).

B- Patients subgroups analysis:

The mean Neurofilament Level on admission (pNF-H1) was 47±25.8 pg/ml in Group A 35.4±21 pg/ml in Group B and 40.3±20.4 in Group C and the mean Neurofilament H level after 7 days (pNF-H2) was 148±90.3 pg/ml, 89.3±54.7 and 125.1±65.4 in Group C hence the Neurofilament level was significantly higher in all groups after 7 days (p<0.005). Traumatic Brain injury patients (Group A) had the highest mean levels of Neurofilament H both on admission and after 7 days.

Group A: Traumatic brain injury group:

A strong negative correlation between the level of Neurofilament H and the Glasgow Coma Scale in patients with traumatic brain injury on admission (r=0.66 with a p<0.005) this applies also after 7 days (r=0.78, p<0.005). In other words, the higher the level of pNF-H levels correlated with lower GCS. The mean Marshal score in Group A was 2.1 on admission (Marshal1), and 3.7 after 7 days (Marshal2). Also there were a significant positive correlation r=0.56 between the mean Marshal score and the mean Neurofilament H level both on admission and after 7 days (r=0.56 and 0.4, respectively) p<0.005 In other words, the higher the level of Neurofilament the higher the Marshal score in TBI patients.

Group B: Ischemic CVS:

A negative correlation between the level of Neurofilament H and the Glasgow Coma Scale on admission and after 7 days in patients with ischemic CVS. This was a negative correlation of 0.374 with a p-value of <0.005 on admission and a negative correlation of 0.51 with a p-value of <0.005 after 7 days. CT assessment showed mean ASPECTS score of 8.1 on admission and 6.7 after 7 days. The difference was statistically significant, lower after 7 days hence most patients with ischemic CVS showed an increase in infarct size after 7 days (p<0.005). There was a negative correlation between the level of Neurofilament detected and the ASPECTS scores on admission and after 7 days of r=0.64 and 0.89 respectively (p<0.005). In other words, the higher the level of pNF-H the lower the ASPECTS score (Fig. 4).
Also there were a positive correlation between the Neurofilament level and NIHSS in Group B patients. This was a positive correlation \( r=0.55 \) on admission and 0.8 after 7 day \( (p<0.005) \).

**Group C: Hemorrhagic CVS group:**

A negative correlation between the level of Neurofilament H and the Glasgow Coma Scale on admission and after 7 days in patients with hemorrhagic CVS. This was a negative correlation of 0.56 and 0.65 respectively, with a \( p \)-value of \( <0.005 \). In Group C, most patients had intracerebral hemorrhage 53.3% while 36.6% had intraventricular hemorrhage and 10% had thalamic hemorrhage. A significant positive correlation between the Neurofilament level (pNF-H1) and the NIHSS on admission and after 7 days. In other words, the higher the level of pNF-H the higher the NIHSS \( (r=0.8 & 0.5, \text{respectively}) \).

**Discussion**

In this study, we have demonstrated that pNF-H can be detected in the blood of patients with different forms of acute brain injuries. The presence of neurofilaments in blood indicated damage of neurons after such injuries. The importance of pNF-H lies in the fact that we were able to detect levels of it in the blood of patients, which makes such marker a convenient and simple marker of brain damage, on contrast to other markers measured in the CSF. Blood collection is more practical and safer than CSF sampling; this suggests that analysis of blood for pNF-H could be a useful clinical tool to conveniently assess axonal damage. Recent data have emphasized that the morphology of vertebrate neurons renders the axons sensitive to mechanical and metabolic damage and a major portion of the pathophysiology following brain injury is due to axonal damage \([1]\). Therefore, a convenient blood assay of axonal loss could be of great utility.

In the 3 groups of patients studied, we found a negative correlation between the GCS and pNF-H levels both on admission and after 7 days. This correlation was strongest in traumatic brain injury patients, followed by patients with cerebral hemorrhage. The correlation was weakest in ischemic CVS group. This might be explained fact that leak of proteins may be slower in the early phases of stroke. pNF-H levels also correlated with CT findings. In traumatic brain injury patients, levels showed an inverse relationship with MARSHAL CT scores, and similarly in ischemic CVS there was a negative correlation with ASPECTS and pNF-H. NIHSS showed positive correlations in both ischemic CVS and cerebral hemorrhage with pNF-H, hence higher levels of the marker correlated with higher NIHSS hence more disability.

Rankin score was used to assess outcome after 3 months. Patients with higher levels of Neurofilament H on admission and after 7 days showed a greater Rankin score after 3 months and hence a greater disability \( (p<0.005) \). There was an apparent increase in mean Neurofilament levels in patients with the greatest disability (Rankin 5, \( n=19 \)) and those who died (Rankin 6, \( n=7 \)). Patients with the least disability (Rankin 1 & 2) showed the lowest mean Neurofilament levels on admission and after 7 days. Hence Neurofilament H levels were significantly higher in patients with poor outcome. ROC curve showed cut-off point for NFH level at admission to predict severe disability or death was 3.5 pg/ml and 1.1 pg/ml after 7 days.

Several other proteins have been proposed as potential biomarkers in TBI \([11]\). For example, the small Ca2+-binding modulator protein S-100\( \beta \) has been extensively studied in both animal models of TBI and human TBI victims \([11]\). While S-100\( \beta \) has been shown to be elevated following TBI in humans, its specificity to brain injury was debated since it appears to be elevated in humans with other forms of trauma not related to the brain. In addition, S-100\( \beta \) levels have been shown to be elevated following femoral fractures without any accompanying brain injury \([12]\) and is elevated in long distance runners \([13]\). Although pNF-H was elevated post traumatic brain injury in our study and correlated with the level of consciousness and CT findings, we still do not have any data about other causes that will increase neurofilaments in blood. And weather our marker-pNF-H-is more specific to axonal damage than other markers. However, patients included in our study suffered only trauma to the head, and patients with multiple trauma or those with multiorgan dysfunction were excluded. Thus further studies may be needed to assess the degree of specificity of pNF-H.

Previous studies have also examined serum levels of c-tau as a marker of TBI in both humans and animal models. However, the level of c-tau in the serum of injured humans and animals is significantly less than pNF-H. Furthermore, tau is not neuronal specific; it is expressed in in non-neuronal tissues such as heart, skeletal muscle, lung, kidney, and testis \([7,14]\). Again further studies will be required before we understand if similar problems will occur with the use of pNF-H protein as a biomarker and if it will be elevated in other non-neurological conditions.
Another issue is, as mentioned above, if pNF-H is located solely in axons, axonal loss is a major problem in many kinds of human neurological damage and disease states, such as TBI, multiple sclerosis, and amyotrophic lateral sclerosis [1]. This can be problematic in patients with chronic CNS disease. However, in our study, we excluded patients with any neurological disorders other than traumatic brain injury thus we can say that increases levels of pNF-H in our population was solely due to acute brain injury and not due to chronic CNS diseases.

Johann Sellner and Colleagues [15] studied serum biomarkers in patients presenting with acute stroke. They studied 18 patients (15 ischemic, 3 hemorrhagic) and analyzed 3 biomarkers: Neurofilaments, S-100β and GFAP. Serial blood samples were collected, starting within the first 6 hours and daily up to 6 days. There data showed a significant increase in neurofilament levels within the first day and at all other times compared to the S-100β, and GFAP was not detected at any time in all patients. Although there study shows at increase in the level of neurofilament in ischemic stroke patients, they did not correlate their findings with the clinical parameters as GCS or NIHSS or with radiological findings.

Singh and Yan [16], measured levels of pNF-H in stroke and correlated these levels with measures of stroke severity. Blood samples were collected from 54 ischemic stroke patients at day 1, week 1 (days 7-10) and weeks 3-6, and an ELISA was used to measure pNF-H levels in each patient at each time-point. Serum pNF-H levels were significantly elevated in stroke patients compared to healthy controls. Blood pNF-H levels that reflect the severity of ischemic stroke correlated with outcome and rise during the weeks after stroke. Similarly in our study, the pNF-H levels correlated with the severity of the stroke as reflected by the GCS, ASCPECTS and NIHSS.

Conclusion:

We can conclude that blood levels of pNF-H were quantifiable in patients with traumatic brain injury on admission to the ICU and after 7 days. The blood levels were significantly higher levels in patients with poor outcome as compared with those with good outcome in the first 3 months following TBI. Phosphorylated Neurofilaments levels showed significant correlations with the level of consciousness and CT findings in such patients. In patients with acute ischemic CVS pNF-H can be detected in the plasma. Levels of pNF-H correspond to the severity of injury as shown by the presence of significant correlations between Neurofilament levels and GCS, NIHSS and the radiological findings. Thus pNF-H seems to be a promising marker for the diagnosis and prognosis of patients with ischemic CVS and for the short term follow-up of such patients. Further studies will be needed to complement our results in larger patients’ samples and more importantly to elucidate other non cranial causes of increased pNF-H level in serum.

References

12- PELINKA L.E., SZALAY L., JAFARMADAR M., et al.: Circulating S-100β is increased after bilateral femur


