Neurofilaments as a biomarker of axonal loss in multiple sclerosis

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Background Neurofilaments (NF-H) might be a potential biomarker of CNS tissue damage in MS, but their potential predictive value for the future disease course, disability, disease progression, and treatment response needs to be investigated.

Aim of the work The aim of this study is to evaluate the role of serum phosphorylated neurofilament H as a marker in multiple sclerosis and correlate it's level with clinical expanded disability status scale (EDSS) and magnetic resonance imaging findings.

Subjects and methods Study included 40 patients and 20 healthy control, 20 patients with relapsing remitting multiple sclerosis (RRMS) and 20 patients with secondary progressive multiple sclerosis (SPMS). All patients in this study were subjected to full history taking and clinical examination and scoring according to the EDSS scores, routine laboratory investigations, measuring of serum phosphorylated neurofilament-H (PNF-H) levels and neuroradiological assessment.

Results Patients with secondary progressive multiple sclerosis were more likely to have a high serum (PNF-H) titer compared to patients with relapsing remitting multiple sclerosis, and these levels were significantly correlated with EDSS scores in both patients groups.

Conclusion The increase in (PNF-H) titer during the progressive phase of the disease together with the correlation of (PNF-H) levels with all clinical scales and radiological lesions (e.g. gadolinium-enhanced lesions and black holes) suggests that cumulative axonal loss is responsible for sustained disability and that high (PNF-H) level is a poor prognostic sign.

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Introduction

Multiple sclerosis (MS) is a progressive inflammatory disease of the central nervous system (CNS). It is the most common cause of neurologic disability in young adult, with onset generally occurring between ages 15 and 40 years [1]. Current evidence suggests that axonal loss occurs at an early stage of MS, but because of CNS compensatory mechanisms, it remains clinically silent until a threshold level of axonal loss is achieved and the functional reserve capacity is exhausted [2]. As inflammation correlates poorly with disability whereas loss of neurons and axons may be subjected to biochemical monitoring, biochemical markers of neuroaxonal degeneration have gained increasing importance. Such biomarkers could provide tools for development and evaluation of new therapeutic strategies [3]. Neurofilaments (NFs) are cytoskeletal proteins that play a role in stabilizing axon, determining axon diameter, and participating in axonal transport [4]. As NFs are found exclusively within neurons, their detection in blood or cerebrospinal fluid (CSF) therefore reflects neuronal and axonal damage [5].

Patients and methods

Patients

A total of 40 Egyptian patients with MS were included in the study: 20 cases with relapsing-remitting MS (RRMS) and 20 cases with secondary progressive MS (SPMS). Patients were recruited from the neurology outpatient clinic of Al-Zahraa University Hospital and from Nasr-City Health Insurance Hospital during the period from April 2013 to March 2015. Moreover, 20 healthy individuals were included as a control group, who were age and sex matched with patients. Control individuals were volunteers selected from general population through personal communication.

Study design

This was a case–control comparative study.

Inclusion criteria

The following were the inclusion criteria:

(1) Definite MS according to the 2010 revised McDonald diagnostic criteria for MS [6].
(2) Relapsing, remitting and secondary progressive types.

Exclusion criteria

Patients having any other medical problems (neurological, immunological, cardiac, hepatic, and renal) were excluded from this study.

Methods

All patients in this study were subjected to the following:

(1) Full history taking and clinical examination:
  (a) Complete general and neurological examination.
  (b) Expanded Disability Status Scale (EDSS).
(2) Routine laboratory investigations.
(3) Serum phosporylated neurofilament-H (PNF-H) levels of both patients and control groups were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Human Phosphorylated Neurofilament-H; BioVendor Research and Diagnostic Product, Heidelberg, Germany), according to the manufacturer instructions.
(4) Neuroradiological assessment.

Conventional MRI

(1) T1-weighted axial images.
(2) T2-weighted axial images.
(3) Fluid-attenuated inversional recovery axial images.
(4) Gadolinium-enhanced T1-weighted axial and sagittal images.
(5) Conventional MRI was used as a part of the McDonald criteria. MRI criteria for definite MS was essential for inclusion in the study.

Statistical analysis

Data were analyzed using Statistical Program for Social Science, version 18.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data were expressed as mean±SD. Qualitative data were expressed as frequency and percentage.

The following tests were done:

(1) Independent samples *t*-test of significance was used when comparing between two means.
(2) One-way analysis of variance was used when comparing between more than two means.
(3) χ^2-test of significance was used to compare proportions between two qualitative parameters.
(4) Pearson's correlation coefficient (*r*) test was used for correlating data.
(5) Probability (P value)
  (a) *P* value less than 0.05 was considered significant.
  (b) *P* value less than 0.001 was considered as highly significant.
  (c) *P* value greater than 0.05 was considered insignificant.
(6) Receiver operating characteristic (ROC curve) analysis was used to find out the overall predictivity of parameters and to find out the best cutoff value with detection of sensitivity and specificity at this cutoff value.

Results

Characteristics of the study population

This study included 40 patients with MS and 20 age-matched and sex-matched healthy individuals.

The patient group comprised 20 patients with RRMS, five (25%) males and 15 (75%) females, and 20 patients with SPMS, five (25%) males and 15 (75%) females. The mean age of the patients with MS ranged from 17 to 42 years, with a mean of 31.3±6.7 years in RRMS and 31.6±7.3 years in SPMS. There was no statistically significant difference between patient subgroups regarding age (years) and sex (Table 1).

Results of EDSS

The disability scores among patients with MS in the present study were assessed and scored using the EDSS score. The EDSS scores in RRMS ranged from 1.5 to

Table 1 Comparison between relapsing-remitting group and secondary progressive group regarding age and sex

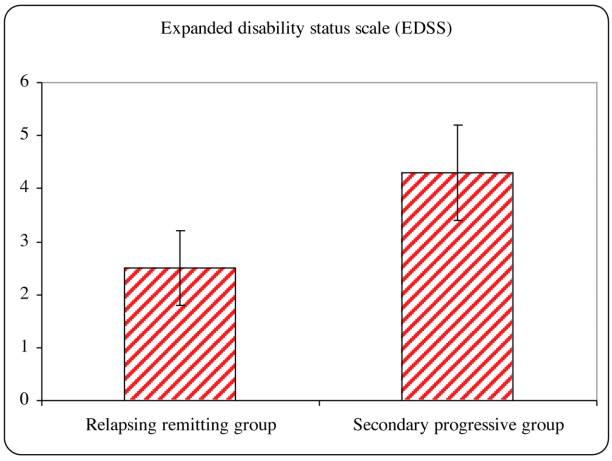
	Relapsing-remitting group	Secondary progressive group	t/χ^2	*P* value
Age (years)				
Mean±SD	31.3±6.7	31.6±7.3	0.013	0.911
Range	17.0–42.0	20.0–42.0		
Sex [*n* (%)]				
Male	5 (25)	5 (25)	0.000	1.000
Female	15 (75)	15 (75)		

3.5, with a mean of 2.5±0.7. The EDSS scores in SPMS ranged from 3 to 6 with a mean of 4.3±0.9. There is a high statistically significant difference between the two patient groups regarding EDSS, with disability being more in SPMS (Fig. 1).

Results of serum levels of phosphorylated neurofilament-H

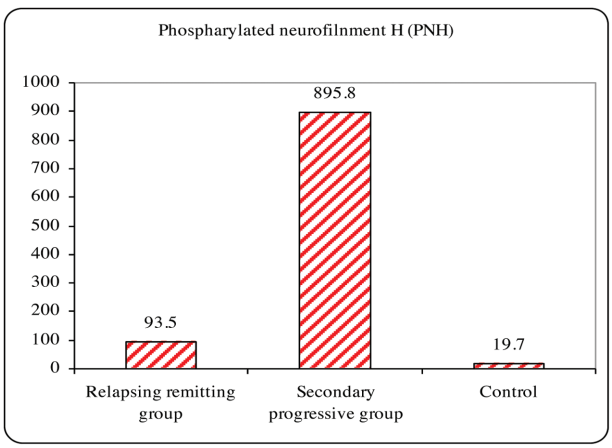
Analysis of PNF-H was performed for all patients and control groups. The mean level of neurofilament-H in RRMS group was 93.5±245.6, with a range of 6.2–1130.5, and the mean level of PNF-H in SPMS group was 895.8±1003.7, with a range of 46.1–4000. There was a highly statistically significant difference between the two groups. The mean level in the control group was 19.7±20.3, with a range 0–55.1, with statistically significant difference between patient subgroups and control group (Fig. 2).

Figure 1



Comparison between relapsing-remitting group and secondary progressive group regarding Expanded Disability Status Scale (EDSS).

Figure 2



Comparison between the groups regarding phosphorylated neurofilament-H (PNH) serum levels.

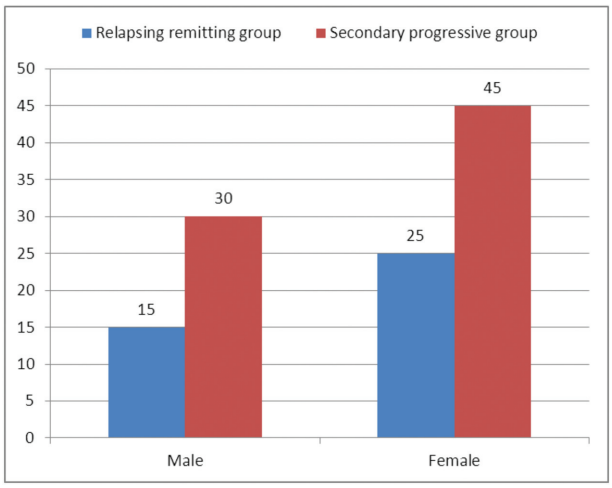
Results of MRI

The black holes were found in 23 patients: eight (40%) patients with RRMS and 15 (75%) patients with SPMS. So, there was a statistically significant difference between the two patient groups regarding T1-weighted black holes (Fig. 3).

Gadolinum-enhanced lesions were found in 22 patients: eight (40%) patients in RRMS groups and 14 (70%) patients in SPMS group. There was a statistically significant difference between the two patient groups regarding gadolinium-enhanced lesions (Fig. 4).

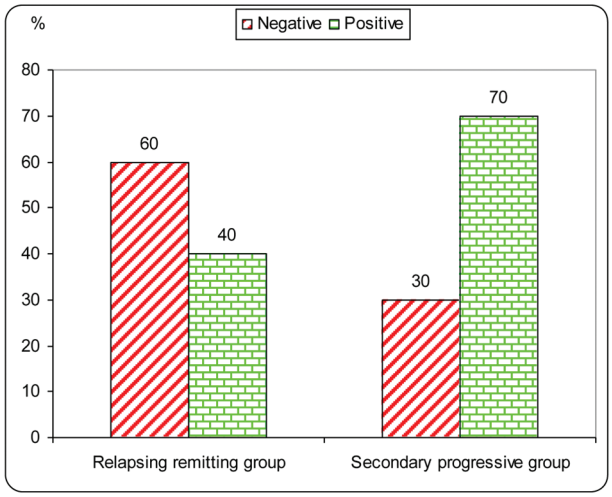
Regarding the correlation between PNF-H serum levels and the EDSS, black holes, and gadolinium-

Figure 3



Comparison between relapsing-remitting group and secondary progressive group regarding T_1-weighted black holes.

Figure 4



Comparison between relapsing-remitting group and secondary progressive group regarding gadolinium-enhanced lesions.

enhanced lesions, there was a positive significant correlation in both patient subgroups: RRMS (Table 2) and SPMS (Table 3).

Diagnostic performance of PNF-H in discrimination between relapsing-remitting and secondary progressive groups was done (Table 4).

ROC) curve was used to define the best cutoff value of PNF-H, which was less than 56.8, with sensitivity of 80%, specificity of 85%, positive predictive value of 84.2%, and negative predictive value of 81%, with diagnostic accuracy of 88.1% (Fig. 5).

ROC curve was used to define the best cutoff value of PNF-H, which was less than or equal to 56.8, with sensitivity of 80%, specificity of 85%, positive predictive value of 84.2%, and negative predictive value of 81%, with diagnostic accuracy of 88.1%.

Discussion

An ideal biomarker of axonal loss would have several properties: it should be expressed specifically in axons, it should be abundant enough so that it can be readily detectable after the significant dilution that occurs following release into fluid compartments such as CSF and blood, and it should be resistant to proteases so that it is not degraded before or following release.

Table 2 Correlation between phosphorylated neurofilament-H serum levels and the Expanded Disability Status Scale, black holes, and gadolinium-enhanced lesions in relapsing-remitting group using Pearson's correlation coefficient test

Relapsing-remitting groups	Phosphorylated neurofilament-H	
	r	*P* value
Expanded Disability Status Scale	0.312	0.039 (Significant)
Black holes	0.418	0.028 (Significant)
Gadolinum-enhanced lesions	0.456	0.015 (Significant)

r, Pearson's correlation coefficient.

Table 3 Correlation between phosphorylated neurofilament-H and the Expanded Disability Status Scale, black holes, and gadolinium-enhanced lesions in secondary progressive group using Pearson's correlation coefficient test

Secondary progressive group	Phosphorylated neurofilament-H	
	r	*P* value
Expanded Disability Status Scale	0.506	0.018 (Significant)
Black holes	0.474	0.003 (Significant)
Gadolinum enhancement lesions	−0.643	<0.001 (highly significant)

r, Pearson's correlation coefficient.

The phosphorylated subunits of neurofilaments, the major structural protein complexes of axon, meet these criteria.

Neurofilament-H is a major axonal cytoskeletal protein. After axonal injury, they are released into the extracellular compartment, and subsequently into the CSF and peripheral blood, thus analysis of CSF and serum neurofilaments levels may provide a valuable tool to estimate the extent of axonal damage in patients with MS [7].

Our study included 40 patients, 20 patients with relapsing-remitting MS and 20 patients with secondary progressive MS, along with 20 healthy controls.

This study included more females than males, with a female/male ratio of 3 : 1, suggesting that hormones may play a significant role in determining susceptibility to MS [8].

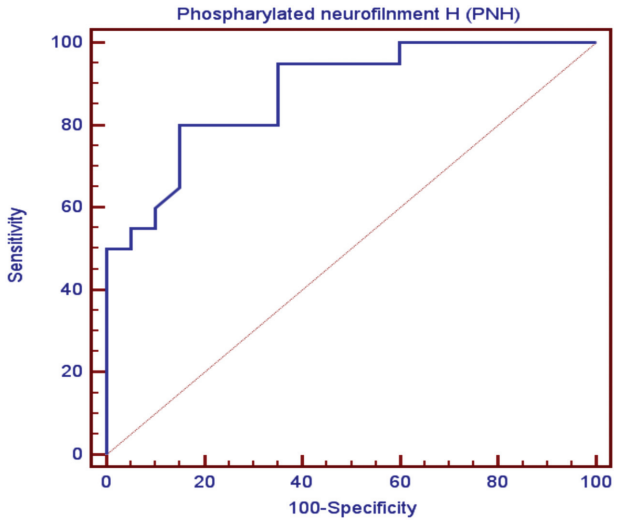
The EDSS scores show highly statistically significant difference between the RRMS and SPMS. Most patients with RRMS have a disability scores of less than 3, whereas most patients with SPMS have a disability scores greater than this. The disability

Table 4 Diagnostic performance of phosphorylated neurofilament-H in discrimination between relapsing-remitting and secondary progressive groups

Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy
≤56.8	80%	85%	84.2%	81%	88.1%

NPV, negative predictive value; PPV, positive predictive value.

Figure 5



Receiver operating characteristic curve shows sensitivity and specificity of phosphorylated neurofilament-H (PNH).

scores increase with disease progression owing to reduced relapses and irreversible disease progression [9].

In our study, there was a highly statistically significant difference in the levels of PNF-H in patients with MS when compared with control group. This finding was consistent with Petzold *et al.* [10], who found that the level of PNF-H was higher in patients with all subtypes of MS than control.

In the present study, there was a significant increase in the level of PNF-H in patients with SPMS more than patients with RRMS. This finding may indicate that the patients with SPMS have CNS axonal damage mediated by inflammatory mechanisms. Detection of neurofilament and its breakdown peptides within the serum can predict definitive axonal damage and disease progression.

This finding is consistant with Gresle *et al.* [11], who studied serum PNF-H by ELISA in cases with relapsing-remitting, secondary progressive, primary progressive, and first demyelinating event and found that a high serum PNF-H peptides titer was detected in 9% of relapsing-remitting and first demyelinating event cases, and 38.5% of SPMS cases.

Higher levels of neurofilament heavy chain in patients with more progressive disease would imply a continuous neurofilament release and thus reflect a severe irreversible neurodegenerative process in these patients [12].

Another study by Brettschneider and colleagues investigated neurofilament heavy chain as a marker for axonal damage in patients with clinically isolated syndrome.

They concluded that neurofilament heavy chain might increase sensitivity for predicting conversion to clinically definite MS during a follow-up time of 48 months. The relation of neurofilament heavy chain with disease progression suggests that high neurofilament heavy chain reflects late stages of neuronal damage [13].

Our result is consistent with Petzold and colleagues, where 34 patients with clinically definite MS were followed up after 3 years. The patients with MS were classified as having relapsing remitting and progressive disease. A significant increase in neurofilament heavy chain from baseline to follow-up was observed in a higher proportion of patients with SPMS/primary progressive MS when compared with patients with RRMS. The marked increase of neurofilament heavy chain levels suggests that axonal damage is a gradual cumulative process during the course of the disease and that loss of neurological function is a direct consequence of axonal injury [9].

Petzold found that phosphorylated neurofilament heavy chain were higher in patients with all subtypes of MS. These levels were on average 1.5-fold higher for progressive MS relative to RRMS cases. These studies suggest that the PNF-H may be more useful as a measure of ongoing neurodegenerative activity in patients with MS, which would make this protein a potential candidate for use as a surrogate marker for assessment of treatment aimed at reducing axonal injury [9].

Regarding the relation between PNF-H and expanded disability status scale, the present study showed that the level of PNF-H significantly increased with the progression of MS as measured by expanded disability status scale. This finding is consistent with Gresle and colleagues, who found that patients with SPMS were more likely to have a high serum PNF-H titer compared with patients with RRMS. As permanent disability in MS is thought to be largely attributable to CNS axonal injury as indicated by a high EDSS), the patients with SPMS had higher EDSS score than patients with RRMS [11].

These results agree with our result that PNF-H levels increase in both RRMS and SPMS cases but are higher in patients with SPMS, with more EDSS score. These findings are consistent with the premise that serum phosphorylated neurofilament titers, measured by ELISA, are likely to be reflecting CNS axonal injury, one of the main pathological mechanisms underlying accumulation of permanent disability in MS [14].

In another study done by Eikelenboom and colleagues, most of the patients with MS had progressed clinically on the EDSS. At follow-up, there was a difference between MS groups for the EDSS score. Disability progression appeared to be associated with higher serum levels for phosphorylated neurofilament [15].

In the present study, the serum levels of PNF-H was significantly increased in patients with gadolinium-enhanced lesions when compared with those without enhanced lesions. The gadolinium-enhanced lesions indicate active inflammation within the brain and the blood–brain barrier is disrupted. This was in consistent with the study done by Teunissen *et al.* [4], who found that neurofilament-H levels correlated with the

number of gadolinium-enhanced lesions in patients with progressive MS .

This supports the view that acute inflammation in MS results in axonal pathology and that the latter has a role in determining functional impairment.

This finding disagrees with Gresle *et al.* [11]; they were unable to show an association between serum phosphorylated neurofilament levels and the presence of cerebral gadolinium-contrast enhancing lesions. Their findings are potently concordant with the hypothesis that active lesions contribute less to the rate of axonal injury than macrophage-mediated injury at the edge of chronic active lesions and low-level inflammation in perilesional white matter, both occurring in the absence of ongoing blood–brain barrier breakdown [16].

There was a significant correlation between black holes and neurofilament-H level, which was significantly increased in patient with SPMS. 'Black holes' nonenhancing lesions that are visible on T_1-weighted sequences are suggestive of severe demyelination and axonal loss, and are most common in patients with long disease duration and progressive disease subtypes [17].

The transition from RRMS to SPMS occurs subtly and is difficult to define clinically. Identifying biomarkers that can distinguish between the different clinical phenotypes of MS is an important goal to ensure that the appropriate treatment regimens are adopted in a timely fashion [18].

Furthermore, such biomarkers may provide new insight into the pathologic basis and lead to the development of effective treatments for disability prevention [19].

In this study, ROC curve was used to define the best cutoff value of PNF-H with a good diagnostic value for PNF-H serum levels in discrimination between RRMS and SPMS.

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Conflicts of interest
There are no conflicts of interest.

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