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Title Page

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Cerebrospinal fluid neurofilament light chain predicts disease activity after the first demyelinating event suggestive of multiple sclerosis

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Abstract

Introduction. The prediction of disease activity in patients with a first demyelinating event suggestive of multiple sclerosis (MS) is of high clinical relevance. Cerebrospinal fluid (CSF) neurofilament light chain (NfL) has shown to have prognostic value in MS patients.

Objective. In this work, we measured CSF NfL in patients at the first demyelinating event in order to find a cut-off value able to discriminate patients who will have disease activity from those who will remain stable during the follow-up.

Patients and methods. We included CSF samples collected from 32 patients followed-up for 3.8 ± 2.5 years. CSF NfL was measured with a newly developed in-house ELISA.

Results. Patients with subsequent disease activity had significantly higher baseline CSF NfL values as compared to clinically and radiologically stable patients (median 812.5 pg/mL, range 205-2359 pg/mL vs 329.5 pg/mL, range 156-3492 pg/mL, $p = 0.002$). A CSF NfL cut-off value of 500 pg/mL significantly discriminated these two groups of patients with 90% sensitivity and 83% specificity.

Conclusions. Our results confirm that CSF NfL is a prognostic marker in the very early phases of MS and suggest that clinically relevant cut-off values can be established. The validation of a cut-off value of 500 pg/mL could provide clinicians with a dichotomous variable that can simplify the prognostic assessment of patients at the first demyelinating event.

Introduction

In 85% of cases, multiple sclerosis (MS) presents at onset with an isolated central nervous system (CNS) demyelinating event, the clinically isolated syndrome (CIS) [1]. CIS patients have an intrinsic high risk to further develop MS since about 2/3 of them will experience a second clinical relapse or additional changes in follow-up magnetic resonance images (MRI) [2]. Therefore, CIS is now included among the clinical phenotypes of MS [3]. The most recent revisions of the diagnostic criteria for MS allow defining both dissemination in space and in time of demyelinating lesions soon after the first manifestation of the disease, thus facilitating an earlier diagnosis of MS [4, 5]. In the management of patients at the first demyelinating event, there is a compelling need for reliable tools that could help to identify those patients who will show disease activity. Indeed, these patients could benefit from an early and more effective treatment [6]. Several studies, performed on large multicenter CIS populations, have shown that a combination of demographical, clinical, laboratory and MRI features is able to stratify the risk of subsequent conversion into MS [7–9]. Among baseline characteristics of CIS patients, the presence of cerebrospinal fluid (CSF) oligoclonal bands (OCBs) is one of the factors associated with a higher risk of MS development [7].

Importantly, in the most recent update of the diagnostic criteria for MS, CSF-specific OCBs have been re-entered as a diagnostic tool [5], which gives the opportunity to analyze additional biomarkers reflecting other early pathophysiological mechanisms underlying MS, such as axonal damage. Neurofilament light chain (NfL) is a structural protein in the axonal cytoplasm. Its expression is particularly high in large-calibre myelinated axons within the CNS [10]. NfL levels increase in the CSF as a consequence of axonal damage and their changes reflect ongoing disease activity in MS [11]. So far, studies on CSF NfL have been performed using the same commercially available enzyme-linked immunosorbent assay (ELISA) [12]. In the present study, we measured CSF NfL in a cohort of patients at the first demyelinating event by means of a newly developed ELISA [13]. We retrospectively

investigated the prognostic value of CSF NfL in defining the risk of subsequent disease activity, and we looked for a possible cut-off value able to discriminate patients who presented clinical and/or radiological signs of disease activity from those who were stable during the follow-up.

Patients and methods

CSF sample collection and storage. We analyzed 32 CSF samples stored in the Biobank of the Section of Neurology, Department of Medicine, University of Perugia (Perugia, Italy). CSF was collected over a 10-year period (from 2006 to 2016), via lumbar puncture, in the same Institution, with the same standard operating procedures, and stored according to a protocol following international guidelines [14]. Specifically, in all patients, lumbar punctures were performed in the morning between 8:00 and 11:00 and CSF was collected in sterile polypropylene tubes, centrifuged for 10 minutes at 2000 × g, divided into 0.5 mL aliquots and immediately frozen at −80 °C, pending analysis.

CSF sample selection. We selected for this study CSF samples from patients satisfying, at the time of CSF collection, the following inclusion criteria: (i) a diagnosis of a first demyelinating event suggestive of MS, including both CIS and possible MS (i.e. CIS with MRI evidence of dissemination in space but not in time or vice-versa) according to the 2010 revision of the McDonald criteria [4]; (ii) a follow-up time of at least 1 year; and (iii) age > 18 years.

Patients clinical and MRI assessment. At baseline, main demographic, clinical and neuroradiological characteristics of the patients were recorded. Patients were followed-up clinically and radiologically according to the routine monitoring program and none of them underwent disease-modifying treatments before the appearance of subsequent clinical and/or MRI signs of disease activity. Disease activity was defined by the appearance, at any time during the follow-up, of one or more of the

following: (i) a clinical relapse, (ii) new T2 lesions on a follow-up MRI scan, (iii) enlarging T2 lesions on a follow-up MRI scan, and/or (iv) gadolinium-enhancing lesions on a follow-up MRI scan. Experienced neurologists diagnosed a relapse in the presence of new neurological symptoms and signs suggestive of an acute inflammatory demyelinating event in the CNS with a duration of at least 24 hours, in the absence of fever or infection [15]. All the patients underwent brain and spinal cord MRI at baseline and follow-up as part of the usual workup (3, 6 and 12 months after the onset, then yearly if asymptomatic). All images were acquired with a 1.5 Tesla magnet according to published guidelines [16].

CSF NfL assessment. CSF NfL concentration was measured at the Institute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, the Sahlgrenska Academy at the University of Gothenburg (Mölndal, Sweden), through a newly developed in-house ELISA, as previously described in detail [13].

Standard protocol approvals, registrations and patient consents. Patients gave informed consent to participate in the study. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Local Ethics Committee.

Statistical analysis. Continuous variables are reported as mean ± standard deviations (median; range). For continuous variables, normal distribution was tested with the Shapiro-Wilk test. Group differences were assessed with an unpaired T-test for normally distributed variables or Mann-Whitney test for variables with a skewed distribution. Categorical variables are reported as numbers and percentages. Fisher exact test was performed to test for group differences. The accuracy of the diagnostic value of NfL was assessed by calculating the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. The cut-off value of CSF NfL was calculated as the concentration that gave the maximum

Youden's index (J = sensitivity + specificity – 1). All tests were 2-sided and significance was set at $p < 0.05$. Statistical analyses were performed using R software, version 3.3.1.

Results

Characteristics of the patients. The CSF samples were from 32 consecutive patients (mean age 38.3 ± 11.5 years, F/M = 1.7), who were followed-up for a mean time of 3.8 ± 2.5 years. In all patients, CSF samples were collected within 30 days of the onset of the first demyelinating event. The details of demographic, clinical and neuroradiological characteristics of the patients are reported in Table 1. During follow-up, 20 patients (62.5%) presented disease activity, with a mean time to the appearance of clinical/MRI signs of disease activity of 1.4 ± 1.1 years. The remaining 12 patients (37.5%) were stable during the follow-up period. The two groups were homogeneous concerning the main clinical and MRI features (Table 1).

CSF NfL. The median CSF NfL concentration was 631 pg/mL (range 156-3492) in the entire cohort. Patients with subsequent disease activity had significantly higher CSF NfL concentrations (median 812.5 pg/mL, range 205-2359) as compared to stable patients (median 329.5 pg/mL, range 156-3492) ($p = 0.002$) (Figure 1A). In the ROC analysis, NfL was found to have a high diagnostic accuracy to differentiate these two groups of patients, with a specificity of 91% and a sensitivity of 90% (AUC = 0.89, 95% CI 0.76-1.00) (Figure 1B). A CSF NfL cut-off value of 500 pg/mL was found to discriminate the two groups of patients at the first demyelinating event with a sensitivity of 90% and a specificity of 83.3%.

Discussion

The main finding of our study is that CSF NfL, as measured with a new ELISA soon after the first demyelinating event, was able to predict future inflammatory disease activity. Indeed, patients with subsequent relapses and/or new/enhancing MRI lesions at the follow-up had significantly higher baseline concentrations of CSF NfL than clinically and MRI stable patients. CSF NfL was able to discriminate these two groups of patients with high accuracy. Previously, CSF NfL has been examined in CIS patients as a predictor of conversion to MS with conflicting results. Indeed, while some authors have found that higher CSF NfL concentrations are associated with a higher risk of subsequent MS development [11, 17–19], others have not replicated these results [20–22]. Our findings are in line with studies showing a prognostic value of CSF NfL in the earliest phases of MS and strengthen the evidence on NfL as a disease-severity marker by confirming previous results with a different assay.

In our cohort, when setting a cut-off value of 500 pg/mL, CSF NfL was able to identify patients with subsequent disease activity with high sensitivity (90%) and specificity (83.3%). Two other studies have previously tested a cut-off value for CSF NfL in early MS patients [18, 23]. In these studies, cut-off values were set at 450 pg/mL (with a sensitivity of 93% and a specificity of 62%) by Håkansson and colleagues [23], and at 900 pg/mL (in this case, sensitivity and specificity were not reported) by Arrambide and colleagues [18]. The cut-off value we found is close to the one identified by Håkansson and colleagues but has slightly lower sensitivity and higher specificity. The differences in cut-off values between studies could be explained by differences in assay formats and/or kit lots. There may also be differences in the clinical characteristics of the cohorts that could result in different cut-offs; for example, in the paper by Håkansson, disease activity could also be defined by the presence of sustained disability worsening, a parameter that was not included in our study.

Our results suggest that CSF NfL, as a measure of ongoing axonal damage, can accurately identify early MS patients who will show further evidence of disease activity. As a biomarker for axonal injury, CSF NfL measures the final downstream effect of different pathophysiological processes that take place during MS. It could thus be considered a biomarker able to summarize the ongoing pathology in MS. Of interest, CSF NfL has been shown to correlate with T2 lesion load and gadolinium-enhancing lesions, thus suggesting that the prognostic value of this biomarker could reflect other well-defined prognostic factors [11, 13]. One limitation of our study is the relatively small sample size, which did not allow us considering multivariable analyses. Therefore, we did not test whether or not CSF NfL correlates with other prognostic markers and if it could be considered an independent prognostic factor. Nevertheless, the measurement of CSF NfL could represent a reliable and easily-quantifiable measure of the individual risk of early MS patients to have subsequent disease activity.

The identification of a reproducible CSF NfL cut-off value could provide clinicians with a dichotomous variable able to simplify the management of patients after a first demyelinating event. Further studies performed on different and larger populations should retest a CSF NfL cut-off value of around 450-500 pg/mL in order to confirm and refine our findings. Given the promising results for this marker, a reference material should be produced for external calibration of NfL assays to allow for the establishment of assay-independent cut-off values.

Figure legends

Figure 1. A: CSF NfL values (pg/mL) at the first demyelinating event in clinically/MRI stable patients and in patients with subsequent clinical/MRI disease activity; p = 0.002. **B:** Diagnostic value of CSF NfL in discriminating between the two groups of patients (AUC = 0.89, 95% CI 0.76-1.00).

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Declaration of Conflicting Interests

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References

[1] Miller DH, Chard DT, Ciccarelli O. Clinically isolated syndromes. *The Lancet Neurology* 2012; 11: 157–169.

[2] Fisniku LK, Brex PA, Altmann DR, et al. Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. *Brain* 2008; 131: 808–

17.

[3] Lublin FD, Reingold SC, Cohen JA, et al. Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology* 2014; 83: 278–86.

[4] Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. *Ann Neurol* 2011; 69: 292–302.

[5] Thompson AJ, Banwell BL, Barkhof F, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol* 2018; 17: 162–173.

[6] Comi G, Radaelli M, Soelberg Sørensen P. Evolving concepts in the treatment of relapsing multiple sclerosis. *Lancet (London, England)* 2017; 389: 1347–1356.

[7] Tintore M, Rovira À, Río J, et al. Defining high, medium and low impact prognostic factors for developing multiple sclerosis. *Brain* 2015; 138: 1863–1874.

[8] Spelman T, Meyniel C, Rojas JI, et al. Quantifying risk of early relapse in patients with first demyelinating events: Prediction in clinical practice. *Mult Scler J* 2016; 23: 135245851667989.

[9] Kuhle J, Disanto G, Dobson R, et al. Conversion from clinically isolated syndrome to multiple sclerosis: A large multicentre study. *Mult Scler* 2015; 21: 1013–24.

[10] Zetterberg H. Neurofilament Light: A Dynamic Cross-Disease Fluid Biomarker for Neurodegeneration. *Neuron* 2016; 91: 1–3.

[11] Teunissen CE, Iacobaeus E, Khademi M, et al. Combination of CSF N-acetylaspartate and neurofilaments in multiple sclerosis. *Neurology* 2009; 72: 1322–1329.

[12] Norgren N, Karlsson J-E, Rosengren L, et al. Monoclonal antibodies selective for low molecular weight neurofilaments. *Hybrid Hybridomics* 2002; 21: 53–9.

[13] Gaetani L, Höglund K, Parnetti L, et al. A new enzyme-linked immunosorbent assay for neurofilament light in cerebrospinal fluid: analytical validation and clinical evaluation. *Alzheimers Res Ther* 2018; 10: 8.

[14] Teunissen CE, Petzold A, Bennett JL, et al. A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. *Neurology* 2009; 73: 1914–1922.

[15] Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. *Ann Neurol* 2011; 69: 292–302.

[16] Filippi M, Rocca MA, Bastianello S, et al. Guidelines from the Italian Neurological and Neuroradiological Societies for the use of magnetic resonance imaging in daily life clinical practice of multiple sclerosis patients. *Neurological Sciences* 2013; 34: 2085–2093.

[17] Modvig S, Degn M, Sander B, et al. Cerebrospinal fluid neurofilament light chain levels predict visual outcome after optic neuritis. *Mult Scler* 2016; 22: 590–598.

[18] Arrambide G, Espejo C, Eixarch H, et al. Neurofilament light chain level is a weak risk factor for the development of MS. *Neurology* 2016; 87: 1076–84.

[19] Martínez MAM, Olsson B, Bau L, et al. Glial and neuronal markers in cerebrospinal fluid predict progression in multiple sclerosis. *Mult Scler* 2015; 21: 550–61.

[20] Avsar T, Korkmaz D, Tütüncü M, et al. Protein biomarkers for multiple sclerosis: Semi-quantitative analysis of cerebrospinal fluid candidate protein biomarkers in different forms of multiple sclerosis. *Mult Scler J* 2012; 18: 1081–1091.

[21] Khalil M, Enzinger C, Langkammer C, et al. CSF neurofilament and N-acetylaspartate related brain changes in clinically isolated syndrome. *Mult Scler J* 2013; 19: 436–442.

[22] Fialová L, Bartos A, Švarcová J, et al. Serum and cerebrospinal fluid light neurofilaments and antibodies against them in clinically isolated syndrome and multiple sclerosis. *J Neuroimmunol* 2013; 262: 113–120.

[23] Hakansson I, Tisell A, Cassel P, et al. Neurofilament light chain in cerebrospinal fluid and prediction of disease activity in clinically isolated syndrome and relapsing

remitting multiple sclerosis. *Eur J Neurol* 2017; 24: 703–712.