A comparative study of CSF neurofilament light and heavy chain protein in MS

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Abstract

Background: There is a lack of reliable biomarkers of axonal degeneration. Neurofilaments are promising candidates to fulfil this task. We compared two highly sensitive assays to measure two subunits of the neurofilament protein (neurofilament light (NfL) and neurofilament heavy chain (NfH)).

Methods: We evaluated the analytical and clinical performance of the UmanDiagnostics NF-light® enzyme-linked immunosorbent assay (ELISA) in the cerebrospinal fluid (CSF) of a group of 148 patients with clinically isolated syndrome (CIS) or multiple sclerosis (MS), and 72 controls. We compared our results with referring levels of our previously-developed CSF NfH-SMI35 assay.

Results: Exposure to room temperature (up to 8 days) or repetitive thawing (up to 4 thaws) did not influence measurement of NfL concentrations. Values of NfL were higher in all disease stages of CIS/MS, in comparison to controls (p ≤ 0.001). NfL levels correlated with the Expanded Disability Status Scale (EDSS) score in patients with relapsing disease (r = 0.31; p = 0.002), spinal cord relapses and with CSF markers of acute inflammation. The ability of NfL to distinguish patients from controls was greater than that of NfH-SMI35 in both CIS patients (p = 0.001) and all MS stages grouped together (p = 0.035).

Conclusions: NfL proved to be a stable protein, an important prerequisite for a reliable biomarker, and the NF-light® ELISA performed better in discriminating patients from controls, compared with the ECL-NfH-SMI35 immunoassay. We confirmed and expanded upon previous findings regarding neurofilaments as quantitative markers of neurodegeneration. Our results further support the role of neurofilaments as a potential surrogate measure for neuroprotective treatment in MS studies.

Keywords
Cerebrospinal fluid, multiple sclerosis, clinically isolated syndrome, neurodegeneration, neurofilament, neurofilament heavy chain, neurofilament light chain, relapse, disability, immunoassay, biomarker, study design

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Introduction

Axonal injury is increasingly being recognised as the cause of permanent disability in multiple sclerosis (MS).1,2 Neurofilaments (NFs) are major structural elements of neurons that are specifically expressed in axons and dendrites. They are heteropolymers composed of four subunits: the triplet of the NF light (NFL), medium (NFM) and heavy (NFH) chain, and either α-interneuron in the central or peripherin in the peripheral nervous system. NFL and NFH have emerged as promising biomarkers for neurodegeneration in a range of neurological disorders.3–5

NFH is the most extensively phosphorylated protein of the human brain, with regulatory influences on cell structure homeostasis and axonal transport;5–8 while NFL is the most...
Patients and methods

Patients and CSF samples

CSF samples were collected in the Department of Neurology of the University Hospital Basel, in the course of routine diagnostic measures, as indicated by the treating physicians and after patient informed consent. The sample collection procedure, clinical measurement methodology and immunomodulatory treatment were previously described in detail.14,16 We included 86 patients with definite MS and 62 patients with CIS. MS patients were sub-classified by a trained neurologist as having clinically definite relapsing-remitting MS (RRMS; n = 38), secondary progressive MS (SPMS; n = 25), or primary progressive MS (PPMS; n = 23).14,17

The control group consisted of 72 patients who, based on extensive diagnostic evaluation, had no objective clinical nor paraclinical signs of a neurological disease. Due to a lack of enough CSF sample, we were unable to assay one CIS, one RRMS and one control patient’s sample that was used in NFHSM35 testing, for NFH (Table 1, available only online).14

The investigators who conducted the NF measurements had no access to the clinical data.

UmanDiagnostics NF-light® ELISA and ECL-NF-HSM35 assay

The UmanDiagnostics NF-light® enzyme-linked immunosorbent assay (ELISA) was performed according to the ELISA kit instructions. Our ECL-NF-HSM35 assay and basic CSF analysis is described in detail elsewhere.13

Precision of the NF-light® ELISA and stability of NFH

We evaluated the reproducibility (intra-assay variability) and repeatability (inter-assay variability) of the NF-light® ELISA, and the stability of NFH at room temperature (RT), 4°C and during freeze-thawing cycles, as described previously.13 We conducted the study according to the current regulations on research with patient samples and on documentation of disease-related information of the University Hospital Basel. Approval was granted by the Common Institutional Review Board of the Cantons of Basel.

Statistical evaluation

Continuous variables are described by their median and interquartile range (IQR), and categorical variables by numbers and percentages. Comparison of basic quantitative CSF parameters across groups was performed using the Kruskal-Wallis test, and pair-wise post-hoc comparisons using the Mann-Whitney U test. Comparisons of categorical variables were done using the chi-square test. CSF levels of NFH, Q_ab and other basic CSF parameters were log-transformed to achieve a normal distribution for subsequent analyses. Yet, for simplicity of notation, we used the original terms of CSF parameters when reporting and discussing results. To control for age as a potential confounding factor, we performed an analysis of covariance with age as a covariate and disease stage group as a fixed factor. Group-specific levels of NFH and other biomarkers were expressed as geometric means with 95% confidence intervals (CIs). For log-normal variables, the geometric mean equals the median. Partial correlations adjusted for age were computed by first regressing the two variables on age, and then determining the Spearman rank correlation coefficient (r) of the respective residuals.

Receiver operating characteristic (ROC) curves were derived from logistic regression (with age as a covariate), to compare the discriminatory power of NFH and NFHSM35 between CIS or different stages of MS, and healthy controls. We calculated the area under the curve (AUC) for NFH and NFHSM35 and compared them using the method of DeLong et al.18 A 2-sided p-value < 0.05 was considered significant. We adjusted the p-values of post hoc comparisons using a Bonferroni correction. We prepared all statistical analyses and graphs with SPSS (Version 15.0 SPSS, Chicago, IL) and Graph Pad Prism 5.02 for Windows (GraphPad Software, San Diego, CA).

Results

Analytical performance of the NFH assay and stability of the analyte. The mean coefficients of variation (CV) of duplicates within given assays were 5.6% (5680 pg/ml), 3.1% (564 pg/ml), 5.5% (242 pg/ml) and 3.6% (156 pg/ml). In-between-assay variation was 8.9% (5680 pg/ml), 7.3% (564 pg/ml), 11.3% (242 pg/ml) and 13.5% (156 pg/ml). There was no significant change in the measured concentration in CSF samples that were stored at room temperature (RT) nor at 4°C for up to 8 days (RT, day 8: 1.04 ± 0.053;
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a mean normalised ratio between day 0 and day 8 ± SD; \( p = 1.0 \) and 4°C, day 8: 1.05 ± 0.051; \( p = 0.5 \)) (Figure 1). Similarly, there was no significant effect of freeze-thawing the CSF up to 4 times on the measured concentrations of NfL in three samples (4 freeze-thawing cycles: 1.03 ± 0.026; \( p = 0.25 \)) (Figure 2).

**NfL levels in CSF as a function of disease features and age**. The CSF NfL levels were increased (\( F_{4, 215} = 26.89; \ p < 0.0001 \)) in all forms and stages of MS, as compared to controls (\( p < 0.0001 \) for all comparisons). A strong correlation with age was seen for NfL in the controls (\( r = 0.61; \ p < 0.0001 \)), while this association was absent in CIS (\( r = 0.06; \ p = 0.778 \)), RRMS (\( r = 0.11; \ p = 0.417 \)), SPMS (\( r = 0.13; \ p = 0.444 \)) and PPMS (\( r = -0.08; \ p = 0.694 \)).

Subsequent analysis with age as a covariate confirmed the previous highly significant group differences of CIS and all stages of MS, in comparison to the controls (\( F_{4, 214} = 26.05; \ p < 0.0001 \); \( p = 0.001 \) for SPMS; \( p < 0.0001 \) for CIS, RRMS and PPMS). Moreover, this analysis also revealed a difference between RRMS and SPMS (\( p = 0.025 \)) (Figure 3).

**Correlations of NfL with CSF markers of inflammation**. In CIS and RRMS, the levels of NfL correlated with the CSF cell count (\( r_s = 0.27; \ p = 0.016 \) and \( r_s = 0.43; \ p = 0.01 \), respectively) and with qAlb in CIS, RRMS and SPMS (\( r_s = 0.28; \ p = 0.025 \); \( r_s = 0.49; \ p = 0.002 \); and \( r_s = 0.67; \ p < 0.0001 \), respectively). Patients with CIS and RRMS with a CSF cell count > 5 cells/mm\(^3\) had almost twice higher NfL concentrations, as compared to patients with normal CSF cytosis (> 5 cells/mm\(^3\); \( n = 47: 1252 \) pg/ml (899 – 1744), versus ≤ 5 cells/mm\(^3\); \( n = 53: 684 \) pg/ml (544 – 859); \( p = 0.0078 \)). In contrast, no such correlation could be observed in patients with progressive MS.

**Correlations of NfL with disability and disease activity**. Age-corrected NfL levels correlated with the Expanded Disability Status Scale (EDSS) score in patients with relapsing disease (CIS and RRMS: \( r_s = 0.31; \ p = 0.002 \)), but not in progressive stages of MS (SPMS and PPMS: \( r_s = -0.18; \ p = 0.218 \)).

Patients with a relapse at the time of their lumbar puncture tended to have higher NfL values (\( n = 61: 1070 \) pg/ml (818 – 1401)) than those in remission (\( n = 64: 734 \) pg/ml (598 – 900)); \( p = 0.054 \)). Similarly, the levels of NfL in patients with relapses due to spinal cord pathology was nearly double the levels seen in patients with relapses due to cerebral lesions (\( n = 16: 1728 \) pg/ml (924 – 3229) versus \( n = 45: 906 \) pg/ml (674 – 1217); \( p = 0.037 \)).

**Discriminatory power of NfL and NfH**

**Figure 1.** Stability of the NfL protein in CSF samples kept at RT (left) and 4°C (right) prior to testing. Three CSF samples (S. 1 – 3) were thawed on day 0 (0 hours, the reference); 3 hours in advance of measurement; and 1, 4 and 8 days before the experiment; and then stored at either RT or 4°C until testing. There was no significant change in the measured concentration of NfL in samples that were stored at RT and at 4°C, for up to 8 days (RT, day 8: 1.04 ± 0.053 (mean normalised ratio between day 0 and day 8 ± SD), \( p = 1.0 \); and 4°C, day 8: 1.05 ± 0.051, \( p = 0.5 \)). Mean calculated pg/ml of duplicates (SD) are displayed.

CSF: cerebrospinal fluid; NfL: neurofilament protein type light; RT: room temperature; S: sample; SD: standard deviation.
NiF<sub>SMI35</sub> in CIS patients (Figure 4, left) and RRMS, SPMS and PPMS together (Figure 4, right) (CIS: AUC 0.83 versus 0.67; p = 0.001 and all MS patients (RRMS, SPMS, PPMS): AUC 0.91 versus 0.85, p = 0.035).

There was a highly significant correlation of NiF<sub>SMI35</sub> and NiF in controls (r = 0.40; p < 0.0001), CIS (r = 0.44; p < 0.0001), RRMS (r = 0.57; p < 0.0001) but not SPMS (r = 0.23; p = 0.163) or PPMS (r = 0.40; p = 0.061). After age correction, this relationship was no longer observed in controls (r<sub>s</sub> = 0.058; p = 0.627); conversely, in CIS (r<sub>s</sub> = 0.46; p < 0.0001) and RRMS (r<sub>s</sub> = 0.56; p < 0.0001) it remained strong, whereas in SPMS (r<sub>s</sub> = 0.31; p = 0.128) and PPMS it was absent (r<sub>s</sub> = 0.41; p = 0.054).

**Discussion**

The primary findings of this comparative study were that NiF proved to be a stable analyte, and the assay system used here for NiF is more sensitive than that for NiF<sub>SMI35</sub>. NiF is considered to represent the most abundant and also the most soluble subunit, but there have been concerns about its susceptibility to proteases, especially in the protease-rich CSF or blood. Several groups, including ours, have therefore previously concentrated on NiF as a biomarker for axonal damage, as its phosphorylated state is assumed to be more stable.

In the present study, CSF NiF levels were demonstrated to be stable up to 8 days at RT and for up to four freeze-thaw cycles. We concluded that there is no basis to prefer NiF over NiF as a biomarker of axonal damage due to concerns of sample stability.

Persistent neurological deficits in MS likely emerge as a consequence of the accumulating nerve injury, starting in the very early phases of the disease. Interestingly, differences between CIS patients and controls were more pronounced for NiF than for NiF<sub>SMI35</sub>, while differences in the other disease stages were more similar and strong (Figure 3). The higher abundance of NiF and/or better performance of the two monoclonal antibodies included in the UmanDiagnostics NF-light<sup>®</sup> assay seem to outweigh the known high sensitivity and higher dynamic range of the ECL technology used in the NiF<sub>SMI35</sub> assay.

In our previous study, NiF<sub>SMI35</sub> showed a strong correlation with age in controls and in patients with a CIS; the correlation was weaker in RRMS and absent in SPMS and PPMS. In the present results, for NiF this correlation was even more pronounced in the controls (r<sub>s</sub> = 0.61; p < 0.0001); conversely, it was absent in all the disease stages. Both these NiF<sub>SMI35</sub> and NiF findings are well in line with a recent report on CSF NiH and NiF levels and age correlation in CIS patients.

More pronounced as compared to NiF<sub>SMI35</sub>, the disease-related neurodegenerative processes paired with higher assay sensitivity seem to outweigh physiologic, age-related changes of NiF clearance, even in the earliest disease stages of MS.

Similarly to the above-mentioned study, our results also suggested that CSF NiF levels do not only reflect chronic
neurodegenerative processes.\textsuperscript{25} In CIS, RRMS and SPMS, the levels of NfL correlated with the extent of blood-CSF barrier damage, relapses, and for CIS and RRMS, with inflammatory cell counts in the CSF. This further supported the concept that in MS, neurofilament release reflects two parallel neurodegenerative processes: first as a result of chronic brain-diffuse neuroinflammation and second due to acute focal inflammatory activity in the course of plaque formation.

Replicating our findings on NfH\textsubscript{SMI35}, the NfL levels correlated with disability in earlier (CIS and RRMS), but not in progressive (SPMS and PPMS) stages.\textsuperscript{14} Likewise, NfL and NfH\textsubscript{SMI35} concentrations in CIS and RRMS (but not in controls) showed a robust correlation, whereas it was not seen in progressive disease. It remains speculative whether the relatively small sample number of progressive MS stages, the difficulty in quantifying neurological deficits by the EDSS, or a dissociation of the liberation of different Nf subunits in progressive MS contributed to these findings.

NfL levels in CSF were reported to be higher in different stages of MS, compared to healthy controls and in relapses versus remissions.\textsuperscript{26,27} Previous studies also report relatively weak correlations of NfL levels with the EDSS\textsuperscript{28-30} and, in fewer studies, with age in the controls.\textsuperscript{31} In a study by Teunissen and colleagues, NfL is determined by the Uman-Diagnostics NF-light\textsuperscript{®} ELISA and NfH by a modified conventional ELISA assay.\textsuperscript{21,32} In comparison to our findings, their differences for NfH using this assay system were less pronounced, information towards correlation of NfL and NfH and/or age in controls and separate stages of MS are not given and performance of the NfL versus the NfH assay is not described.\textsuperscript{14,32}

Taken together, we confirmed and expanded on previous findings describing Nf as quantitative markers of neurodegeneration in CSF. We found NfL and NfH\textsubscript{SMI35} are both stable proteins, an important prerequisite for biomarkers. It is important to note that based on our findings, we could not conclude that NfL is a superior analyte over NfH, in general. Some of our results are likely to reflect the properties of the assays used, and not wholly the properties of these proteins. Rather, in comparison to the ECL-NfH\textsubscript{SMI35} assay, we found that the NF-light\textsuperscript{®} ELISA differentiated better between health and disease, especially in the CIS stage. All our analyses were performed in CSF; conversely, serum Nf measurements would be the most relevant for clinical practice, an aim so far reached more frequently by analyses of NfH\textsubscript{SMI35} as compared to NfL.\textsuperscript{37} Based on this study, further development of a NfL assay that would include the benefits of ECL technology seemed a promising approach in aiming towards NfL measurement in serum/plasma samples. Our findings support the role of Nf as a useful measure of neurodegeneration and their potential usefulness as a surrogate measure in MS treatment studies.

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**Conflict of interest**

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Serono and Novartis Pharma that were used exclusively for research. His work is supported by an ECTRIMS Research Fellowship Programme and by the Forschungsfonds of the University of Basel, Switzerland. RL Lindberg received research support from the Swiss MS Society, Swiss National Science Foundation, European FP6 and IMI JU programs, Roche Postdoc Fellowship Program, and unrestricted research grants from Novartis and Biogen. N Norgren is employed by UmanDiagnos AB, Sweden; D Leppert is employed by Hoffmann-La Roche AG, Basel, Switzerland. G Giovannoni serves on scientific advisory boards for Merck Serono, Biogen Idec and Vertex Pharmaceuticals; served on the editorial board of Multiple Sclerosis; received speaker honoraria from Bayer Schering Pharma, Merck Serono, Biogen Idec, Pfizer, Teva Pharmaceutical Industries, Sanofi-Aventis, Vertex Pharmaceuticals, Genzyme Corporation, Ironwood and Novartis Pharma; served as a consultant for Bayer Schering Pharma, Biogen Idec, GlaxoSmithKline, Merck Serono, Protein Discovery Laboratories, Teva Pharmaceutical Industries, Sanofi-Aventis, UCB, Vertex Pharmaceuticals, GW Pharma, Novartis Pharma and FivePrime; serves on the speakers bureau for Merck Serono; and received research support from Bayer Schering Pharma, Biogen Idec, Merck Serono, Novartis Pharma, UCB, Merz Pharmaceuticals, Teva Pharmaceutical Industries, Sanofi-Aventis, GW Pharma and Ironwood. L Kappos participated in the last 24 months as a principal investigator, member or chair of planning and steering committees or advisory boards in corporate-sponsored clinical trials in MS and other neurological diseases, sponsored by: Abbott, Actelion, Advancell, Allozyne, BaroFold, Bayer Health Care Pharmaceuticals, Bayer Schering Pharma, Bayhill, Biogen Idec, BioMarin, CSL Behring, Elan, Gennab, GeNeuro SA, Genmark, GlaxoSmithKline, Lilly, Merck Serono, Novartis Pharma, Novonordisk, Peptimmune, Sanofi-Aventis, Santhera, Roche, Teva, UCB and Wyeth. He also lectured at medical conferences or in public on various aspects of the diagnosis and management of MS: in many cases, these talks were sponsored by non-restricted educational grants to his institution from the above companies. Honoraria and other payments were used exclusively to fund departmental research. Both research and clinical operations (nursing, patient care services) of the MS Centre in Basel were supported by non-restricted grants from one or more of these companies.

References


