

A multi-centre study of neurofilament assay reliability and inter-laboratory variability.

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Abstract

Objectives

Significantly elevated levels of neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) have been described in the blood and cerebrospinal fluid (CSF) of amyotrophic lateral sclerosis (ALS) patients. The aim of this study was to evaluate the analytical performance of different neurofilament assays in a round robin with 10 centres across Europe/U.S.

Methods

Serum, plasma and CSF samples from a group of five ALS and five neurological control patients were distributed across 10 international specialist neurochemical laboratories for analysis by a range of commercial and in-house neurofilament assays. The performance of all assays was evaluated for their ability to differentiate between the groups. The inter-assay coefficient of variation was calculated where appropriate from sample measurements performed across multiple laboratories using the same assay.

Results

All assays could differentiate ALS patients from controls in CSF. Inter-assay coefficient of variation of analytical platforms performed across multiple laboratories varied between 6.5% and 41.9%.

Conclusions

This study is encouraging for the growing momentum towards integration of neurofilament measurement into the specialized ALS clinic. It demonstrates the importance of 'round robin' studies necessary to ensure the analytical quality required for translation to the routine clinical setting. A standardized neurofilament probe is needed which can be used as international benchmark for analytical performance in ALS.

Introduction

Neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) are among the most promising candidate neurochemical biomarkers in amyotrophic lateral sclerosis (ALS). In recent years, there has been a surge in studies describing their performance in the diagnostic phase and their utility as prognostic biomarkers [1-8]. Although these studies have consistently demonstrated the elevation of pNfH and NfL in the cerebrospinal fluid (CSF) and blood (plasma or serum) of ALS patients, the concentrations among studies varies considerably.

Studies have identified pre-analytical factors that may influence neurofilament determination and demonstrated that efforts focussed on the optimisation and standardisation of biofluid collection and processing have been successful [9, 10]. In order to implement neurofilament assays into routine clinical practice, it is important to independently assess and standardize the performance of the currently available assays across multiple platforms and laboratories [11]. In this study, CSF, serum and/or plasma blood samples were sent from a single centre to 10 participating laboratories across Europe and the USA for the measurement of NfL and pNfH, investigating the consistency of differentiation between ALS and controls and inter-laboratory variation across assays.

Methods

Patients

Biofluid samples (five with ALS and five disease controls) were obtained from individuals attending the University of Ulm Department of Neurology Clinic (Ulm, Germany) with informed consent (ethical approval number 20/10). Patient characteristics are shown in Table 1. Disease controls had no neurodegenerative disease and CSF was collected to exclude a chronic neuroinflammatory process. Their final diagnoses were: myositis, adenoma of pituitary gland, polyneuropathy, subcortical vascular encephalopathy (SVE), vertigo.

Biofluid sample collection

CSF was obtained by lumbar puncture directly into polypropylene collection tubes. Venous blood was collected into serum separator tubes and EDTA tubes (for plasma extraction). Biofluid samples were centrifuged at 3000rpm for 10 minutes at 4°C within one hour of sampling and stored at -80°C. CSF, serum and plasma samples; one aliquot from each ALS (n=5) and control (n=5) patient were shipped on dry ice from the Department of Neurology, Ulm University Hospital to each participating centre: (Nuffield Department of Clinical Neuroscience, University of Oxford (UK); Department of Neurology, Ulm University Hospital (Germany); Clinical Neurochemistry Laboratory, University of Gothenburg (Sweden); Pitié-Salpêtrière Hospital, Paris (France); University of Basel (Switzerland); Queen Mary University of London UK; University of Leuven (Belgium); Amsterdam University Medical Centres, Amsterdam (Netherlands); University College London, Institute of Neurology (UK); Iron Horse Diagnostics, Inc., Scottsdale, AZ (USA). All samples were stored at -80°C until analysis.

Determination of pNfH and NfL in serum, plasma and CSF

The details of each assay platform used is outlined in Table 2. The concentration of NfL was measured using four different assays in CSF (Simoa NF-light® assay, In-house Simoa NFL assay [12], IBL International NF-light® ELISA and an in-house ELISA method [13]; three different assays in serum (Simoa NF-light® assay, In-house Simoa NFL assay [14] and an in-house NfL Meso Scale Discovery assay [15] and two different assays in plasma (Simoa NF-light® assay and an In-house Simoa

NFL [16]). pNfH was measured using five different assays in CSF (an in house MSD assay, Simoa pNF-Heavy, BioVendor, Euroimmun and an in-house pNfH Luminex assay [17]; four different assays in serum (Simoa, BioVendor, Euroimmun and an in house MSD assay) and four different assays in plasma (Simoa, BioVendor, Euroimmun and an in house MSD assay). sample was assayed twice to generate two replicates on each platform. Any samples for which neurofilament levels were below the limit of detection (LOD), the lowest standard value was used in statistical analysis.

Statistics

Statistical analysis was performed using GraphPad Prism 7. The ALS and disease control groups were compared by the Mann-Whitney test ($p < 0.05$ significant).

Results

Assay performance in CSF

The CSF concentration of NfL was significantly increased in ALS patients compared with neurological controls when measured by all four different analytical platforms in eight centres (Figure 1). The CSF concentration of pNfH was significantly increased in ALS patients compared with neurological controls as measured by all five different analytical platforms in eight centres (Figure 2). In CSF, the frequency of endogenous analyte detection for all NfL assay platforms tested was 100%. For the measurement of CSF pNfH levels, undetectable values were encountered in 20% of samples assayed using the BioVendor platform.

Assay performance in blood

The serum concentration of NfL was significantly increased in ALS patients compared with neurological controls when measured by the Simoa platform (commercial and in-house) in five centres (Figure 3a). The plasma concentration of NfL was significantly increased in ALS patients compared to neurological controls when measured by the Simoa platform (commercial and in-house) in four centres (Figure 3b). The serum and plasma concentration of pNfH was significantly increased in ALS patients compared with neurological controls when measured by the Simoa platform in one centre (Figure 3c, d). In blood, the frequency of endogenous analyte detection was 100% for both NfL and pNfH using the Simoa platform. Serum and plasma pNfH and serum NfL were quantified using a total of 3 other analytical platforms, whereby 90% of the sample concentrations were above the analytical sensitivity for each platform.

Inter-assay reliability of analytical platforms

Four different analytical platforms were used across multiple centres (Table 3). The coefficient of variation (CV%) of Simoa NfL measurements between laboratories was $18.3 \pm 4.2\%$ (serum, n=5), $7.2 \pm 3.4\%$ (plasma, n=3), $27.9 \pm 6.8\%$ (CSF, n=3). The coefficient of variation (CV%) of Euroimmun pNfH measurements between

laboratories was $17.6 \pm 9.5\%$ (serum, n=3), $18.5 \pm 12.2\%$ (plasma, n=2), $6.5 \pm 2.8\%$ (CSF, n=3). The coefficient of variation (CV%) of Uman NfL measurements between laboratories was $41.9 \pm 3.4\%$ (CSF, n=4). The coefficient of variation (CV%) of BioVendor pNfH measurements between laboratories was $11.5 \pm 6.1\%$ (CSF, n=3).

Discussion

This study sought to assess the performance of multiple neurofilament analytical platforms in a 'round-robin' exercise across 10 international experienced neurochemistry laboratories. [Previous studies \[10, 11\] examining the inter-laboratory variation of neurofilament measurements have determined that efforts to optimise and standardise biofluid collection have been successful and that neurofilament levels in ALS patient samples are largely comparable. In this study, we have demonstrated that all currently available neurofilament assay platforms have the required sensitivity for the measurement of NfL and pNfH in CSF.](#)

Our findings demonstrate that pNfH and NfL can be sensitively quantified by all of the analytical platforms applied to the CSF of ALS patients and neurological controls across the study. The CSF concentrations of both NfL and pNfH were all in the analytical range of these assays. In agreement with another study [18], the Simoa [platform](#) provided the most sensitive quantification of pNfH and NfL levels in blood with serum and plasma concentrations of all subjects detected across multiple laboratories.

For the assays that failed to differentiate ALS from disease controls in blood, 10% of serum and plasma pNfH and NfL concentrations using these assays were outside the analytical range in serum and EDTA plasma. These findings extend upon previous observations of increased pNfH and NfL levels in the blood and CSF of ALS patients and demonstrate that this increase is consistent and reliably detected across multiple analytical approaches. As this was seen with standard ELISA and also with the digital ELISA this seems to be independent of the platform. [The increased levels of pNfH and NfL reported in ALS biomarker studies and the robust correlations reported between CSF, serum and plasma \[1-6, 10\] suggest that both pNfH and NfL are](#)

reliable peripheral biomarkers of neuronal damage. The availability of easily accessible samples is of critical importance for integration into both clinic routine and therapeutic trials.

We consider the inter-assay variability across laboratories in this study performing the same assay of up to 20% to be acceptable. However, the measurement of NfL by two platforms exceeded this upper limit, which highlights that it is imperative that further round robins are performed to ensure the analytical quality for routine settings, especially to reduce the CV of assays below 10%. In another study a larger number of samples collected from a single centre were measured by only 3 assay platforms that are all present in each participating laboratory to complete further assessment of inter-laboratory reliability [18]. However this is quite often not feasible, as most labs have already performed an in-house validation and already made a decision for a specific assay. The aim of our round-robin is therefore to obtain an external quality assumption. In further studies a standardised NfL/pNfH probe that can be used as a gold standard to describe the analytical performance of each assay should be incorporated. However such material is difficult to obtain, if no mass spec data for standards is available. For future harmonisation of neurofilament measurements in the ALS field, all laboratories must begin using the optimal assay platform and engage in a quality control programme based on the principles highlighted by this study thereby ensuring alignment with all other users of the assay.

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Conflicts of interest

HZ has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg (all outside submitted work). KB has served as a consultant or at advisory boards for Abcam, Axon, Biogen, Lilly, MagQu, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. CT has a collaboration contract with ADx Neurosciences, performed contract research or received grants from Probiobdrug, AC Immune, Biogen-Esai, CogRx, Toyama, Janssen prevention center, Boehringer, AxonNeurosciences, Fujirebio, EIP farma, PeopleBio, Roche. [MRT independently received pNfH assay kits in kind \(Euroimmun UK\).](#)

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Figure Legends

Figure 1: Detection of neurofilament light chain (NfL) in the cerebrospinal fluid (CSF) of ALS patients and neurological controls. Scatter dot plots show neurofilament light chain concentrations in the CSF of patients with ALS and neurological controls. Filled circles, black - Lab 1 In-house Simoa assay; Filled circles, cyan – Lab 1 Simoa assay; Filled squares, cyan – Lab 2 Simoa assay; Filled inverted triangles, Lab 4 Simoa assay; Filled triangles, green – Lab 3 Uman ELISA; Empty circles, green – Lab 6 Uman ELISA; Empty triangles, green – Lab 8 Uman ELISA; Empty diamond, green – Lab 10 Uman ELISA; Filled hexagon, purple; Lab 7 – In-house ELISA. Median value and interquartile range are shown. Mann-Whitney U test was used for the comparisons. ** $p < 0.01$

Figure 2: Detection of phosphorylated neurofilament heavy chain (pNfH) in the cerebrospinal fluid (CSF) of ALS patients and neurological controls. Scatter dot plots show phosphorylated neurofilament heavy chain concentrations in the CSF of patients with ALS and neurological controls. Empty inverted triangles, blue – Lab 9 In-house MesoScale Discovery assay; Filled squares, cyan – Lab 2 Simoa assay; Filled squares, magenta – Lab 2 BioVendor ELISA; Filled triangles, magenta – Lab 3 BioVendor ELISA; Empty diamonds, magenta – Lab 10 BioVendor ELISA; Filled diamonds, yellow – Lab 5 Euroimmun ELISA; Empty inverted triangles, yellow – Lab 9 Euroimmun ELISA; Empty triangles, yellow – Lab 8 Euroimmun ELISA; Inverted triangles, red – Lab 4 Luminex Assay. Median value and interquartile range are shown. Mann-Whitney U test was used for the comparisons. ** $p < 0.01$

Figure 3: Detection of neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) by the Simoa platform in the serum (A) and plasma (B) of ALS patients and neurological controls. Scatter dot plots show **A** neurofilament light chain concentrations in the serum of patients with ALS and neurological controls; Filled circles, cyan; Simoa assay Lab 1, Filled circles, black; In-house Simoa assay Lab 1, Filled squares, cyan; Simoa assay Lab 2, Filled triangles, cyan; Simoa assay Lab 3, Filled inverted triangles, cyan; Simoa assay Lab 4; Empty circles, cyan; Simoa assay Lab, 6 **B** neurofilament light chain concentrations in the plasma of patients with ALS and neurological controls; Filled circles, cyan; Simoa

assay Lab 1, Filled circles, black; In-house Simoa assay Lab 1; Filled squares, cyan; Simoa assay Lab 2, Filled triangles, cyan; Simoa assay Lab 3, Filled circles, black; In-house Simoa assay Lab 7 **C** phosphorylated neurofilament heavy chain concentrations in the serum of patients with ALS and neurological controls; **D** phosphorylated neurofilament heavy chain concentrations in the plasma of patients with ALS and neurological controls. Median value and interquartile range are shown. Mann-Whitney U test was used for the comparisons. ** $p < 0.01$