Neurofilament light oligomers in neurodegenerative diseases: quantification by homogeneous immunoassay in cerebrospinal fluid


ABSTRACT
Background Neurofilament light (NFL) is a widely used biomarker for neurodegeneration. NFL is prone to oligomerisation, but available assays do not reveal the exact molecular nature of the protein variant measured. The objective of this study was to develop a homogeneous ELISA capable of quantifying oligomeric NFL (oNFL) in cerebrospinal fluid (CSF).

Methods A homogeneous ELISA, based on the same capture and detection antibody (NFL21), was developed and used to quantify oNFL in samples from patients with behavioural variant frontotemporal dementia (bvFTD, n=28), non-fluent variant primary progressive aphasia (nfvPPA, n=23), Alzheimer’s disease (AD, n=20) and healthy controls (n=20). The nature of NFL in CSF, and the recombinant protein calibrator, was also characterised by size exclusion chromatography (SEC).

Results CSF concentration of oNFL was significantly higher in nfvPPA (p<0.0001) and svPPA patients (p<0.05) compared with controls. CSF oNFL concentration was also significantly higher in nfvPPA compared with bvFTD (p<0.001) and AD (p<0.01) patients. SEC data showed a peak fraction compatible with a full-length dimer (~135 kDa) in the in-house calibrator. For CSF, the peak was found in a fraction of lower molecular weight (~53 kDa), suggesting dimerisation of NFL fragments.

Conclusions The homogeneous ELISA and SEC data suggest that most of the NFL in both the calibrator and human CSF is present as a dimer. In CSF, the dimer appears to be truncated. Further studies are needed to determine its precise molecular composition.

INTRODUCTION
Neurofilament light (NFL) is a 68kDa type IV intermediate filament with structural functions in the neuronal cytoskeleton, mainly found in axons. Under physiological conditions, there is a basal release of NFL into the cerebrospinal fluid (CSF), which increases with age. However, after axonal damage in neurodegenerative conditions and other acute neurological conditions such as traumatic brain injury or stroke, its release greatly increases. Previous studies indicate NFL as a general marker of neurodegeneration, with its concentration in CSF and blood being increased in several neurological diseases including Alzheimer’s disease (AD), multiple sclerosis (MS), amyotrophic lateral sclerosis, Huntington’s disease and frontotemporal dementia (FTD). NFL is a well-established biomarker for neurodegeneration and several assays, either immunoassay or mass spectrometry (MS) based, have previously been developed intended to quantify it in body fluids. In a recent study, Budelier et al showed that, in CSF, no full-length NFL was found. Instead, it was detected by immuno-precipitation-MS analysis as various truncated species, shedding some light on the actual molecular state of this protein in CSF.

The hypothesis in this study is that NFL could be present in CSF as an oligomer. Our goal was to develop a homogeneous ELISA, using the same capture and detection antibody, to test this hypothesis and to quantify these oligomers in CSF. We also set out to examine the apparent size of NFL by size exclusion chromatography (SEC) in CSF and the calibrator used in the ELISA.

METHODS
Participants This study included FTD patients (n=61) from different variants, including behavioural variant FTD (bvFTD, n=28), non-fluent variant primary progressive aphasia (nfvPPA, n=23) and semantic variant primary progressive aphasia (svPPA, n=10). Among the FTD patients, 17 were mutation carriers affecting...
The homogeneous ELISA for oNFL is based on a previously developed sandwich assay\textsuperscript{13} in which in-house anti-NFL antibodies, NfL21 and NfL23 (raised against the rod domain of NFL) were used to quantify CSF NFL. Here, we verified that either antibody could be used in a homogeneous set-up to detect an NFL signal in CSF, indistinguishable from that obtained using the regular ELISA. For the oNFL assay, we chose NfL21+NfL21 (but NfL23+NfL23 worked as well, online supplemental figure 1). In brief, 96-well microtiter plates were coated with capture NfL21 antibody at a final concentration of 0.5\,µg/mL (100\,µL/well) in bicarbonate buffer (50\,mM NaHCO\textsubscript{3}, pH 9.6), overnight at +4°C. Washes were performed between every step with 4×350\,µL PBS with 0.05\% Tween20, and all incubations at room temperature. Unspecific binding was blocked with 1\% bovine serum albumin (BSA) in PBS (0.01 M phosphate buffer, 0.14 M NaCl, pH 7.4; 250\,µL/well) for 1 hour. The assay buffer used for dilutions was optimised to PBS-Tween20 0.01\% with 1\% BSA. After blocking, eight-step serial twofold dilutions of calibrator, prepared from bovine brain tissue (range 1562–2000000\,pg/mL), blank, quality controls (QCs) and samples were added to the plate (100\,µL/well). Samples and QCs were diluted 1:1 in assay buffer.

A 3-hour incubation period preceded the addition of biotinylated NfL21 detection antibody (1\,µg/mL in assay buffer) with a 1-hour incubation. After that, enhanced streptavidin horseradish peroxidase (Enhanced Streptavidin-HRP, 4740\,n, Kem En Tech) diluted 1:20000 in assay buffer was added (100\,µL/well) and incubated for 30\,min. 3,3′,5,5′-Tetramethylbenzidine (One Solution, 4380A, Kem En Tech; 100\,µL/well) was used as substrate. Finally, after 30\,min incubation in the dark, the reaction was stopped with 0.2 \,M H\textsubscript{2}SO\textsubscript{4} (100\,µL/well), and the absorbance measured at 450\,nm (with 650\,nm reference) using an ELISA plate reader (Sunrise, Tecan Trading AG).

To assess any differences in oNfL concentrations, samples representing several neurodegenerative diseases were included in a pilot study. All samples were randomised and analysed blinded, under the same conditions. Results showed significant increases in oNfL concentrations in nfvPPA (2.90e+4 ± 4.66e+3 pg/mL, p<0.0001) and svPPA (2.36e+4 ± 3.24e+3 pg/mL, p<0.05) compared with controls (8.50e+3 ± 8.22e+2 pg/mL). nfvPPA also displayed increased oNfL compared with bvFTD (1.35e+4 ± 1.72e+3 pg/mL, p<0.001) and AD (1.30e+4 ± 2.74e+3 pg/mL, p<0.01). However, neither bvFTD nor AD had any significant differences compared with controls (figure 1A).

**DISCUSSION**

This study shows that most NfL in CSF and the assay calibrator is a multimer (likely a dimer). None of the currently used NfL immunoassays give any information regarding the multimeric state of the protein. In the newly developed homogeneous assay, where the capture and detector antibodies are the same, the binding of the capture antibody to the target protein blocks the epitope for the detector antibody, thus nullifying the output signal if the captured protein is in monomeric form. However, if the captured protein is in oligomerised form, there will still be epitopes available for detector antibody-binding which consequently results in measurable absorbances.

CSF NfL concentration, measured using a standard ELISA, is increased in several neurodegenerative diseases, FTD in particular. CSF NfL even distinguishes FTD variants; in a recent meta-analysis, higher NfL levels were noted in semantic PPA patients when compared with bvFTD and non-fluent PPA patients. Here, we replicate most of these findings using an immunoassay selective for oNfL. In fact, our data suggest that most of the NfL signal in earlier studies may have been derived from oNfL rather than NfL monomers.

**Size exclusion chromatography**

SEC analysis of in-house calibrator showed a peak fraction (~135kDa) that was consistent with full-length NfL dimers (figure 1B). Still, in CSF, the peak was found in fractions corresponding to lower molecular weight (~53kDa), suggesting that oligomers in CSF are formed of truncated NfL (figure 1C).

Figure 1  Oligomeric NfL (oNfL) concentration (pg/mL) measured with the homogeneous assay in different neurodegenerative diseases and healthy controls (A) and after SEC on in-house calibrator (B) or CSF (C). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, by one-way ANOVA test; bars represent 95% CIs; green dotted line, measured concentrations in collected fractions and dashed black line, absorbance at 280 nm. AD, Alzheimer’s disease; ANOVA, analysis of variance; bvFTD, behavioural variant frontotemporal dementia; CSF, cerebrospinal fluid; nfvPPA, non-fluent variant primary progressive aphasia; SEC, size exclusion chromatography; svPPA, semantic variant primary progressive aphasia.
It is also important to emphasise that our assay may not detect all forms of oNfL, as oligomerisation might mask NfL21 epitopes in certain oligomeric species. However, from earlier studies, we are positive that this type of assay only detects oligomers of the target protein,\(^1\) and hence our results highlight this novel aspect of NfL as a biomarker. To examine the relative amount of oligomeric vs monomeric forms of NfL, as currently available assays cannot distinguish the two, an assay specific to monomeric NfL needs to be developed.

According to Budeliers et al, NfL is present in the CSF as several different fragments, of which rod domain 2B fragments were identified and found to be correlating with results from the most widely used NfL ELISA (UmanDiagnosics).\(^9\) Our SEC results are in line with these findings, as in CSF, the peak fraction cannot correspond to full-length NfL, but rather oligomers of truncated NfL.\(^9\) Furthermore, we also observed that our calibrator eluted as a full-length dimer, confirming the oligomiserate state of the protein. Previous studies, which also support our findings, state that the central part human NfL (detected by NfL21) has a strong propensity for self-assembly.\(^17\)

One question that remains to be answered is whether the oligomerisation occurs prior to or after release of the protein from the axon, and if oligomerisation is a part of the neurodegenerative process. We hypothesise that the protein is released as a dimer in both physiological and pathological conditions, given the strong propensity for oligomerisation of the coiled coil domain of NfL.\(^17\) To address this hypothesis, further research, including cell-based experiments and/or studies on acute axonal injury, is needed.

Nevertheless, the novel homogeneous ELISA described here, along with the SEC data, provide strong evidence of the presence of oNfL in CSF. In fact, the data suggest that most of NfL in calibrators and human CSF is a dimer; an important result if we are to standardise NfL assays by reference measurement procedures for which full characterisation of the target analyte is a prerequisite.

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Contributors FJM and UA contributed to the study design, acquisition and interpretation of data, and drafted the manuscript. HK and HZ contributed to the study design, supervision, interpretation of data and drafted the manuscript. KK, IJS, AS-E, JDR, AD, IS and SK provided the samples and revised the manuscript. BB and KB contributed to the study design and revised the manuscript.

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Competing interests HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, ALZPath, Annexon, Apollis, Apollis Therapeutics, AZTherapies, Cogex, Denali, Eisai, Neurgen, Novo Nordisk, Passage Bio, Prion Therapeutics, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Thrombopep, Triplight Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectrictron, Fujirebio, Aluceze, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KB has served as a consultant, at advisory boards, or at data monitoring committees for Abbcam, Axon, BioArctica, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, Pharmatrophix, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. SK has served at scientific advisory boards and/or as a consultant for Geras Solutions and Biogen.

Patient consent for publication Not applicable.

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Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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