Cerebrospinal fluid neurofilament light chain as a biomarker in neuroaxonal damage: Comparing and analysing quantification with two analytical assays

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Abstract

**Introduction:** Neurofilament light chain (NfL) is a potential biomarker in cerebrospinal fluid (CSF) and blood for neuroaxonal degeneration. Many studies have examined its concentration in various neurodegenerative diseases but its turnover in CSF has not been established. To prepare for stable isotope labelling kinetics (SILK) experiments, with which turnover can be examined, a Mass spectrometry-based quantification method for NfL would be needed.

**Aim:** To confirm quantification of NfL concentration in CSF and serum using two different assays and as a substudy, to develop a targeted proteomic assay to detect NFL in CSF using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**Methods:** The study population contains 36 paired samples in CSF and serum collected from patients under investigation for neurodegenerative diseases, at Sahlgrenska University Hospital, Gothenburg. In Gothenburg, CSF NFL was measured using an in-house ELISA. In London, CSF and serum concentrations of NfL were analysed using the Single Molecular Array (Simoa) technique.

In a sub study, pilot experiments to develop an LC-MS/MS-based method for NfL were performed. The assay was developed to detect tryptic peptides of NfL, which are likely to be detected and will be appropriate for Stable Isotope Labeling Kinetics (SILK) in future clinical UCL-SILK studies.

**Result:** CSF NfL measured at UCL in London strongly correlated with CSF NfL previously measured in Gothenburg (Spearman Rho=0.86, p< .001). In addition, correlation between serum NfL and CSF NfL was highly significant (Spearman Rho =0.76, p < 0.001). Development of a method to detect NfL in CSF by LC-MS/MS was started and optimisation is on-going.
Conclusions: In conclusion, this master thesis showed correlations in CSF concentrations of NFL detected by two different laboratories and using two different assays. Furthermore, quantifying NFL in serum and CSF as an indicator of potential biomarker for neuroaxonal damage gave similar results that were highly correlated. NfL in different populations will be a useful tool to develop targeted screening tools and monitor disease activity in the future.

There are still unanswered questions concerning the turnover and function of the protein. However, potential development of a LC-MS/MS platform for using SILK as a tool for investigate the turnover in the protein NfL in vivo will be an important step forward for the field.

Keywords: Neurofilament light chain, Neuroaxonal diseases, immunoassay, Liquid Chromatography-tandem Mass Spectrometry
Abbreviations

NFL – Neurofilament Light chain

CSF – Cerebrospinal fluid

SIMOA- Single Molecular Array

LC-MS/MS – Liquid Chromatography-tandem Mass Spectrometry

ELISA - Enzyme-Linked Immunosorbent assay

AD – Alzheimer Disease

MS – Multiple Sclerosis

BBB – Blood Brain Barrier

SILK – Stable Isotope Labelling Kinetics

TBI – Traumatic Brain Injury
Background

Neurofilament light chain (NfL) is a promising proteomic biomarker for neurodegenerative axonal damage (1). NfL exists partially in the cytoskeleton and can be found in axonal myelinated axon in nerve cells. Neurological disorders are often associated with neuronal damage or loss and these factors have a pathological role in dementia, neuroinflammatory diseases, stroke, white matter changes and traumatic brain injuries (TBI) (2-4).

Neuroaxonal damage leads to protein leakage into the cerebrospinal fluid (CSF) and it is possible to collect CSF and detect biomarkers by a lumbar puncture (LP) procedure. The connection between the CSF and blood through the blood brain barrier (BBB) is strictly regulated; yet, in pathological and normal conditions NfL concentration is measureable in the bloodstream using sensitive assays (5). Studies have reported detection of NfL in plasma as a possible screening tool for axonal degeneration. With new high sensitivity assays to quantify normal concentrations of NfL in controls, this biomarker has potential to detect drug efficacy and indicate neuronal damage activity of disease in clinical trials. (4-6) NfL measurements in CSF have been strongly correlated between different immunoassay platforms (7). Furthermore, there is interest in confirming detection and correlations in CSF NFL as a protein that marks axonal damage, which is part of the pathogenesis in neurodegenerative diseases.

Definition of biomarkers

Biomarker abnormality may indicate and reflect the underlying pathogenic events of a disease. Recently, there has been renewed interest in finding biomarkers of neurodegenerative
diseases (1). The Jack model (Figure 1) demonstrates the importance of biomarker discovery and quantification, where abnormal changes in biomarkers can be detected before neuronal cell loss and clinical onset of disease (8). Alzheimer’s disease (AD) is a multifaceted disease, caused by different pathologies along different pathways. Biomarkers may highlight these pathologies and are potential factors to indicate disease stage and severity (9). NfL is increased in patients with AD and changes over time. The degeneration of axons in AD is a part of the pathophysiology, however it is not independent of any other pathological path known in AD.

Figure 1. The Jack model. A pathological cascade model for Alzheimer’s disease (AD), as described by Jack CR Jr et al. (8). This model presents the biomarkers in dementia and explains the correlation between time and abnormality of biomarkers, together with diagnostic factors. On that basis, the importance of biomarker detection and correlation to time of symptoms is demonstrated. In this figure, following biomarkers are presented; CSF Amyloid β42 and CSF Tau. Also, diagnostic imaging of the brain with Amyloid-PET, MRI; magnetic resonance imaging, and cognitive impairment are showed.

AD is a challenge to healthcare systems worldwide, affecting around 50 million people globally and expected to double before 2030 (10). Currently, there is no cure and absolute clinical diagnosis remains challenging and only definite post-mortem when pathology can be
confirmed. Present biomarkers include proteins found in CSF, as well as brain imaging, which are used alongside cognitive testing in the clinic to aid diagnosis. A benefit for blood-based biomarkers in the clinic would be to screen a population with the possibility to detect and follow the alterations over time (11).

**Neurofilament light chain**

Rosengren *et al.* confirmed higher levels of CSF NfL patients with various neurodegenerative diseases compared with healthy individuals and was the first study to identify NfL as a potential biomarker of neurodegeneration (12). The neurofilament family is divided into; light-, medium- and heavy-chain microfilaments, as well as α-internexin, which are all incorporated into the cytoskeleton of myelinated axons. The NfL microfilament diameter is approximately 10 nm with a mass of 61.5 kDa (13).

![Figure 2. The structure of neurofilament light (NfL). The protein consists of three domains with an N-terminus (head), mid-domain (rod) and a C-terminus (tail). Phosphorylation sites are located on the head domain.](image)

The full protein sequence consists of 543 amino acids and in the tail-domain partly lysine and serine are involved. The whole protein consists of more negatively charged amino acids and the rod in particular is more hydrophobic in character. Studies have shown that mutations in NfL can cause aggregation of the protein, leading to neurodegeneration in various
neuroaxonal disorders (14). It thus seems proper structure of NfL is crucial for neuronal health and normal function (1).

When axon degeneration appears in neurons, NfL is released into the extracellular fluid and can leak into the blood stream. Gisslén et al. were the first group to publish the correlation between concentrations of NfL in plasma and in CSF (15) and a later study by Mattsson et al. found an association of plasma NfL concentration with AD (16). A number of studies suggest that NfL may be a marker of disease activity in multiple sclerosis (MS). MS is a disease where axonal injury is ongoing with an inflammatory factor. These patients had a higher concentration of NfL in serum with positive correlation to disease intensity and activity in magnetic resonance imaging (MRI) (2).

NfL has an unknown kinetic profile in biofluids. However, increased levels of NfL are reported after traumatic brain injury (TBI) but this increase is not measurable immediately in CSF. The highest increased concentration of NfL in serum was reported with 144h after concussion (17). Also, higher concentrations of NFL were detected 36 weeks post-trauma in knocked-out boxers (18). Additional studies have also suggested that NfL is a marker of activity and intensity of the degenerative process in amyotrophic lateral sclerosis (ALS), with one study finding a correlation between the magnitude of increased NfL concentration and time to death (19, 20). All these findings currently imply that NfL is a promising and emerging biomarker reflecting neurodegenerative mechanisms in the human central nervous system (CNS).
Assays to quantify NfL

Methods to quantify NfL in biofluids have been developed quickly in recent years. Immunoassays are commonly used to detect NfL in CSF, based on monoclonal specific antibodies against the target. A well-known example of an immunoassay is the enzyme-linked immunosorbent assay (ELISA). Quantification of NfL in CSF has been used diagnostically in neurological diseases but more recently, improved analytical sensitivity has made it possible to quantify NfL in blood. Latterly, Kuhle et al. (7) compared different assays for detection of NfL and confirmed that the most sensitive assay was the Single molecule array (Simoa) -based method.

Simoa

This ultrasensitive instrument uses an antibody conjugated to magnetic beads to capture NfL in the sample and a detection antibody labelled with a fluorogenic enzyme to quantify it. The capture antibodies interacts with the target protein and the detection limit reaches fg/mL (21). With this high sensitivity assay, it is possible to quantify NfL in CSF and serum samples from healthy individuals where NfL levels are expected to be lower (21, 22).

Liquid Chromatography - tandem Mass Spectrometry

Liquid chromatography- mass spectrometry (LC-MS) is an analytical platform that can be used to discover, characterize and quantify metabolites, proteins and lipids based on their mass to charge ratios (m/z). The sensitivity and specificity for the detection and quantitation of target biomarkers is drastically increased by using a LC-MS/MS method (Figure 3). For larger proteins such as NfL that do not fall within the mass range of a mass spectrometer,
design and development of a method require the protein to be digested by a protease such as trypsin into smaller, more detectable fragments. The peptide fragments selected must be ‘proteotypic’ or unique in sequence to only your target protein. Liquid chromatography (LC) is used to separate peptides based on their biochemical properties, before ionisation, fragmentation and subsequent detection by the mass spectrometer. Development of a method requires the identification of the peptide elution time off an LC column (termed retention time) and the selection of the peptides precursor ions and product ions, the latter are produced after fragmentation of the precursor ion using a high-energy collision gas (23-25).

Optimization of a LC-MS/MS method requires sample preparation, multiple LC and multiple MS parameters to be optimized at each stage for each analyte (24). Clinically, some analyte multiple reaction monitoring (MRM) methods may be run together, thus LC-MS/MS multiplex assays can be used to measure multiple biomarkers at once. For this study, an LC-MS/MS assay was designed and developed for detection of NfL in CSF.

**Figure 3. Explanation of the Mass Spectrometry.** Before running the LC-MS/MS for the target protein, a method must be designed, developed and optimized. Protein within the CSF is digested using trypsin or another protease. The peptides are separated by liquid chromatography (LC), ionized and introduced into the mass spectrometer (MS). Thereafter, mass-to-charge (m/z) ratios are generated and the fragment pattern can be used to identify the protein.

A research group in Washington, Bateman et al. (26), have been able to measure the turnover of the biomarker, amyloid β42, in CSF and blood in patients with AD, where synthesis and
clearance rates were measured using stable isotope labeling kinetics (SILK). Isotopically labeled $^{13}$C$_6$-leucine was administrated intravenously into the patients and incorporated into newly synthesized amyloid β-protein, which makes it possible to differentiate newly produced Aβ42 from older, unlabeled protein in a patient sample. Ratios of labelled to unlabelled Aβ42 across various time points following administration of the labeled leucine may then be calculated and used to determine production and clearance rates of the protein (27). Detection of label incorporation relies heavily on analysis of the samples by mass spectrometry, the only platform able to distinguish between stable isotope labeled peptides and endogenous peptides. Targeted LC-MS/MS assays can be set up to measure peptide fragments of the protein of interest and leucine incorporation over time. In this study, we are trying to develop a method for detecting NfL in CSF using LC-MS/MS. By understanding the kinetics of NfL we could gain more insight into what this biomarker truly represents in neurodegeneration and therefore how best to interpret the delay and changes in CSF levels over time and across neurodegenerative diseases.

**Aim**

The aim of this study is to confirm quantification of NfL concentration in CSF and serum using two different assays. We hypothesize that there is a correlation of NfL concentrations between the first method, in-house ELISA and the second, Simoa assay. To prepare for SILK studies of NfL turnover, we also performed preliminary experiments to develop an LC-MS/MS method for detection of NfL.
Material and Methods

Study population

Samples were obtained from the Biobank Sweden and de-identified. All participants were under investigation and suspected of having a neurodegenerative disorder. De-identified paired CSF and serum samples were collected at Sahlgrenska University Hospital, Gothenburg. The biomarkers CSF t-TAU, CSF p-TAU and CSF Ab42 were measured in clinical laboratory practice before de-identification with INNOTEST ELISAs (Fujirebio, Ghent, Belgium). CSF NfL was measured in Gothenburg with an in-House ELISA technique previously described (Gaetani et al (28)). These samples where centrifuged (1300 x g, 10 min), thereafter aliquoted in polypropylene tubes and stored in aliquots at -80°C pending analysis. The Gothenburg analyses were made before the samples arrived to DRI UCL, London.

NfL Analyses - Simoa

In London, we measured NFL levels in CSF using immunoassays on an ultra-sensitive Single molecule array (Simoa) platform (the HD-1 Analyzer, Quanterix, Lexington, MA). These analyses were performed in March 2019. A commercially available kit was used for detection of the protein NfL in CSF and serum (Quanterix, Lexington, MA). 10 uL of CSF and 200 uL of serum were spun for 5 minutes at 21C 1000xg. We diluted samples of CSF 1:100 (4 uL of CSF in 396 uL of sample diluent). Serum was diluted 4-fold on board the machine. The plate was prepared with 350 uL aliquot of CSF or 150 uL serum in each well. The resorufin-β-D-galactopyranoside (RGP) Agent was shaken at 800rpm for 2h before loaded to the assay. All steps were performed in accordance with the kit insert. The paramagnetic particles coupled with antibodies (antibody-bead) were vortexed (VORTEX) 30 seconds at speed 10 before
being loaded. We constructed the calibration curve from premade Quanterix calibrators, and the acceptance levels for sample concentrations were set to the lowest and highest calibrator, respectively. All samples were replicated twice in the run (21).

Figure 4. Assay principle for Simoa. The target protein is bound by the capture antibody attached to a magnetic bead. The detection antibody with a biotin tag binds to another part of the target analyte. A magnet pulls down beads into micro wells (one bead per micro well) and immunocomplexes are quantified using an enzymatic reaction that produces light (22).

NfL Analyses - Liquid chromatography tandem mass spectral assay

Designing peptides for NfL

We tried to develop a method of measuring NfL using targeted LC-MS/MS techniques (Waters Xevo TQ-S) to detect the protein of interest – in this case NfL. We tried to develop this assay based on tryptically digested peptide sequences that are likely to be detected/fly well. The full amino acid of human NfL (Figure 5) was downloaded from the UniProt database (www.uniprot.org).

Peptide fragments produced after in-solution digestion of NfL with Trypsin were generated in Skyline Software (MacCoss Labs) and all sequences transferred into Excel (Microsoft office). Peptides that would be appropriate for future SILK studies at UCL were selected, these
sequences had to include one leucine, and the sequences were run through the UniProt Basic Local Alignment Tool (BLAST) to ensure the peptide was unique to human NFL only. Three peptide standards were ordered from GenScript (Netherlands) and stored at -20°C until use. For each peptide ordered, all theoretical transitions were generated in Skyline software and exported as a method compatible with the Water Xevo TQ-S instrument and MassLynx software.

MSSFSYEPYSSYKRRYVETPRVHISVSGYSTARAYSSYAP VSSLSSVRRSSSSSSGRLMPSLENLDSLQVAAISNDIKRTQOEKA QLDLNDRFASFIERVHELEQONKVLEAEALLVLRLQKHSEPSFRAL LYEQEIRDLRALLADATNEKQALQGERGEEITLRNLQARYEEEV LSREDDEGRLMEARKGAEAAARTNLRKEDISQFFLVKHC EEEAEALQAIQIQAYQISVEMDVTQPDLSAAKDIARQYKEKLAAN MQNAEEWFKSRTVLTESSAASKNTDAAKDEVSESRRLLKAKT LEIEACGMNEALEQKQLEDQANADISAMQTDINKLELERT TSEMARYLKEYQDLLNVXMADEIAAYRKLEGEEETRLFSTSVG SITSGYSSQSFGRSAVYGLQTSSYLMSTRSFPSYTYVQEEQJ EVEETIAAAKAEAKDEPPSEGAEAEEDKKKEAEEEEEAEAEAAAAA KEESEEKQEPEEGGESEETKEAEEEKKKVEGAGEEQAAKKDK

Figure 5. AA-sequence of the peptide NfL with 543 amino acids.

AA: Amino acids. NfL: Neurofilament light

Figure 6. Flow chart for targeted method design and development for detection of human NfL by LC-MS/MS.

LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry
Peptide tuning

Peptide standards were reconstituted in ultra-pure water (18MΩ Milli-Q, Millipore) to stock concentrations of 2mg/mL and stored at -20°C until use. For peptide tuning, all peptides were spiked into ultra-pure water at 1 pmol/µl and a total volume of 2mL. Peptides were then directly injected into the IntelliStart fluidics system of the Waters Xevo TQ-S triple quadrupole mass spectrometer. Samples were injected at a flow rate of 15µl/min and the mass spectrometer was operated in MS mode and data acquired over 0.5 min to identify the most abundant precursor ions after ionisation. To identify the most abundant product ions for each precursor ion, samples were infused at 25µl/min and the mass spectrometer operated in MS/MS mode (with the selected precursor ion m/z set for MS1). Various collision energies were tested in MS/MS mode to determine the optimal collision energy (in eV) for the most efficient collision-induced dissociation (CID) of the precursor ion. The top 3 most intense transitions for each product ion of each peptide were identified and compared to the m/z values predicted by skyline. These transitions and the optimal collision energy for fragmentation were taken through to the final developed MRM for each NfL peptide.

Determining Peptide Retention Times

Peptide standards were prepared by spiking into ultra-pure water (18MΩ Milli-Q, Millipore) at three different concentrations (0.5 pMol, 1pmol, 5pMol). Samples were injected (1µl injection per sample) onto a CORTECS™ UPLC C18 column (90 Å, 1.6 µm, 2.1 mm x 50 mm) attached to a CORTECS™ UPLC C18+ VanGuard pre-column at total column loads of 0.5, 1 and 5 pmol. Standards were run on an Acquity UPLC system (Waters) coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer, which was operated in positive electrospray ionisation (ESI+) and MS/MS mode using the optimised NfL LC-MS/MS
method. The previously published 10-minute peptide UPLC gradient from the UCL group was used (29). Retention times for each peptide were determined, by checking all expected transitions of each peptide also eluted at the same time.

Target proteomics: Running CSF

We prepared a CSF pool from 13 different samples. From this pool two CSF standards (to ensure expected retention times of each peptide were the same for the peptides in a CSF matrix), and an endogenous non-spiked sample were run. We also ran one NfL CSF sample with a known detectable high concentration of NfL by Simoa measurements and chosen to maximise our chances of detection. All together 4 samples (Figure 7) prepared to run into the Waters LC-MS/MS.

Figure 7. Sample preparation for testing detection of NfL in patient CSF using the developed targeted LC-MS/MS method.

NfL: Neurofilament light. LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry.

Aliquots (500uL of 13 samples) of CSF were freeze-dried overnight (≥ 15hr) at -40°C. Protein pellets were then resuspended in digest buffer (100mM Tris, 6M Urea, 2M Thiourea, 2%
amidosulfobetaine-14, pH 7.8) and allowed to shake (1500rpm) for 1hr at room temperature. 3 µl of dithioerythritol (DTE) was added to each sample and allowed to shake at room temperature for 1hr, before 6 µl of 5M iodoacetamide (IAA) was added and allowed to incubate in the dark for 50 minutes. Ultra-pure water was added to each sample (331 µl) and 20 µl of trypsin gold (Mass Spectrometry Grade, Promega, USA) added and allowed to digest overnight at 37˚C (Heywood et.al(30)). Digested samples then underwent a C18 clean up (Isolute™ column, 100mg/1ml, Biotage). For samples to be used as CSF standards, peptides were spiked into the samples immediately after C18 clean up. All samples were then dried down under vacuum at room temperature in a SpeedVac for 5 hours. Samples were then reconstituted to desired concentrations using 3% ACN 0.1% TFA ready for injection.

Proteomic Screening of Abundant Digested NfL Peptides

A recombinant human NfL protein (R &D Systems, Bio-techne, UK) was ordered in to determine the most abundant and detectable peptides produced after digestion with trypsin. Recombinant NfL was spiked into ultrapure water at a concentration on 0.1pmol/µl and a final volume of 500 µl. The sample was then digested and cleaned using a C18 column as described above. After drying, the sample was reconstituted to a final concentration of 0.1pmol/µl. The sample was then analysed by the Translation Mass Spectrometry Group at the Institute of Child Health, UCL, London using a nanoAcquity UPLC system coupled to a Waters Quadrupole Time of Flight (QToF) mass spectrometer, where data was acquired over a 60 minute run. The data was then analysed and peptide ions identified in Progenesis QI for proteomics software (v4.1 nonlinear dynamics, Waters), which preformed searches and identified peptides using the UniProt Human database.
**Statistical methods**

SPSS statistics 25 for PC (IBM Corp. 2015) were used for all statistical analyses. Tests of normality was made with the Shapiro-Wilk test. For data which was not normally distributed the spearman coefficient was used for correlation calculations. Differences between the assays for detection of NFL, London (Simoa) compared to Gothenburg (in-house Elisa) was assessed by the Mann Whitney U test. Grubb’s test was used for calculating significant outliers in the correlation plots and a Bland-Altman plot with data from both platforms were used to evaluates the detectable differences.

**Ethics**

In this study all CSF - and serum-samples were de-identified when collected from the Biobank. These samples are therefore no longer covered by the Swedish ethical legislation and Swedish Biobank law (Biobanks in Medical Care Act). Further, it is not possible to go back for further journal information about the patients when the samples are de-identified.

**Result**

**NFL quantification and correlations**

See Table 1 for additional demographics of the 36 samples. The age range were widely spread, and the population were strongly equal in sex. Non -parametric statistical tests were used due to not normally distributed data (D(36)=0.566, p=<0.01) calculated with the Shapiro Wilks test (Figure 8).
Table 1. Study population. Detectable concentrations of NFL CSF Gothenburg analysed with the In-house ELISA, Gaetani et. al. (28). NFL CSF London was analysed with the Simoa assay.

<table>
<thead>
<tr>
<th></th>
<th>N=36</th>
<th>MEAN</th>
<th>MEDIAN</th>
<th>STD.DEVIATION</th>
<th>MINIMUM</th>
<th>MAXIMUM</th>
</tr>
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<tbody>
<tr>
<td>Sex (F/M=N)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>56</td>
<td>56</td>
<td>21</td>
<td>5</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>NFL CSF GOTHENBURG (pg/mL)</td>
<td>2258</td>
<td>1030</td>
<td>3601</td>
<td>50</td>
<td>16400</td>
<td></td>
</tr>
<tr>
<td>NFL CSF LONDON (pg/mL)</td>
<td>2300</td>
<td>1085</td>
<td>3888</td>
<td>45</td>
<td>20735</td>
<td></td>
</tr>
<tr>
<td>NFL SERUM LONDON (pg/mL)</td>
<td>28</td>
<td>18</td>
<td>36</td>
<td>3</td>
<td>216</td>
<td></td>
</tr>
</tbody>
</table>

Figure 8. Histogram showing skewed distribution of the data.

The detected concentrations of NfL CSF with the Simoa assay in London, significantly correlated with CSF NfL levels previously measured by an in-house ELISA in Gothenburg.

Further correlation between NfL CSF Gothenburg vs NfL CSF London showed strong correlation (Spearman Rho = 0.86, p< .001) (Figure 9A). Grubbs test was screened for outliers and one sample was detected. No other variables had outlier that were found to skew results. A graph representing higher correlation between the methods (n=35, Spearman Rho = 0.98, p<0.01) is showed in Figure 9B. A Mann-Whitney U test was used to indicated that CSF NfL
concentrations measured in Gothenburg and then in London were not significantly different (n=36, U=669, p=0.813). Further, the serum concentrations of NfL were significant correlated positive to concentration of NFL in CSF with, London assay (Spearman Rho =0.76, p< .001) (Figure 9C).

Figure 9. (A-C) Correlation plots of NfL (neurofilament light) measured in 36 paired serum and CSF samples using assays as indicated in the graphs. A. CSF vs CSF strongly correlated. B. Plot presented without the detectable outlier (n=35). C. Serum vs CSF measurements with London assay positively correlated.

None of the 36 samples fell under the sensitivity limits of the NFL Simoa assay, as all concentrations measured in CSF and serum were between the lowest and highest calibrators. All sample fell below the CV-cut off of $\leq 20\%$ for duplications thus all 36 samples were used in the analysis. A Bland Altman plot (Figure 10) was used to determine between the in-house ELISA in Gothenburg compared to the Simoa array in London. This analysis showed that there is little difference between the techniques.

Figure 10. A Bland-Altman plot shows the difference between CSF NfL concentrations measured using the Gothenburg and London assays including (A) and without including the detected outlier (B). NfL: Neurofilament light. CSF: Cerebrospinal fluid.
A calculated bias; -41 represents the difference of absolute measured concentrations of NfL. 33 of 36 samples were within limits of agreement (-1.96/+1.96 of mean) (Figure 10A). In Figure 10B, a plot without the outlier sample is shown; bias was calculated to -428. However, the graph shows a slightly falling trend towards the right down corner, which indicates a considerably higher difference at higher concentrations.

**NfL CSF – detection with tandem mass spectral assay**

**Peptide tuning – Development for NfL detection**

In this sub-study, LC-MS/MS methods were developed with the aim to detect NfL in CSF. The figure below (Figure 11), demonstrates results from the peptide-tuning for one peptide; LAAEDNATEK. The precursor was set to 531.313 with an optimal collision energy of 5v and identified product ions for the chosen precursor. Following transitions were adapted; 562.165, 677.186, 806.222, 877.203. The most abundant precursor: 531.313 was inconsistently from the suggested precursor in Skyline. These precursor and product ions were chosen and adapted to the method to detect NfL in CSF.

![Figure 11. Peptide tuning. Selection of precursor and product ions for the peptide: LAAEDNATEK is showed in the chromatograms.](image-url)
Of the peptides ordered, two peptides in particular were found to fly the best and were the most detectable at biological NFL concentrations: ALYEQEIR, LAAEDNATEK. These peptides were spiked into water at increasing concentrations in order to identify retention times for each peptide.

Figure 12. The manufactured peptide sequences spiked into water at different concentrations: ALYEQEIR and LAAEDNATEK in three different concentrations A: 0.5 pmol/mL, B: 1 pmol/mL, C: 5 pmol/mL.
The intensity of all transitions increased accordingly with standard concentration. Spiked peptide standards of ALYEQEIR, LAAEDNATEK into CSF showed that retention time of the peptide in a CSF sample matrix remained the same as seen in an ultra-pure water matrix (Figure 12). However, there was no detection of endogenous NfL in the non-spiked CSF pool sample or in the CSF sample where NfL was previously detected at high concentration (2070 pg/ml) using the Simoa assay.

Proteomic Screening of Abundant Digested NfL Peptides

The data from the digested recombinant NfL screening showed that the 3rd most abundant peptide after digestion was the ALYEQEIR as presented below in Table 2. The other peptides were not detected.

Table 2. Screening of most detectable peptides in digested Neurofilament light (NfL). The 3rd most abundant peptide is the ALYEQEIR-sequence, as presented in the table below.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Peptide Mass</th>
<th>Charge</th>
<th>Drift time (ms)</th>
<th>Average Normalised Abundances</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 SAYSSYSAPVSSLSVRR</td>
<td>1902.9656</td>
<td>+3</td>
<td>3.67</td>
<td>2.22e+005</td>
</tr>
<tr>
<td>2 SSFSYEPYYSTSYK</td>
<td>1707.7530</td>
<td>+2</td>
<td>4.64</td>
<td>2.17e+005</td>
</tr>
<tr>
<td>3 ALYEQEIR</td>
<td>1020.5406</td>
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<td>3.39</td>
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</tr>
</tbody>
</table>
Discussion

In this study, we successfully measured NfL in samples of CSF and confirmed correlations between two different immunoassays and laboratories. There was tight agreement within the Bland Altman plot which supports the idea of a perfect correlation between ELISA-based and Simoa-based measurements in NfL. Additionally, our hypothesis regarding no significant difference between the London and Gothenburg assay was confirmed with the Mann-Whitney U test.

NfL - Comparison of analytical assays

In general, the concentrations of NfL in CSF, measured with the in-house ELISA technique in Gothenburg (28) were lower than what was quantified in London by Simoa. These results are in agreement with a previous study (7). Also, that study confirms the analyses of the trend in the Bland-Altman graph, where concentrations of NfL higher than 5000 pg/mL have considerable differences between the two methods. The antibodies in the Simoa kit are specifically targeted to the mid-domain of NfL, which is very similar to where the antibodies in the Gothenburg assay binds (22, 28). The lowest concentration of serum NFL in this study was measured to 3.4 pg/mL with the London assay. This range is not a detected concentration with the in-house ELISA (28) method in Gothenburg and highlight the difference in sensitivity of these two methods. Further research should be undertaken to investigate if these two methods correlate the same with detecting NfL in serum.

It remains important to adapt comparability of concentrations in labs and hospitals worldwide, before committing to clinical trials or diagnostic lab work. Today, globally, research groups have started up collaborations to standardise CSF biomarkers in AD and validate different
assays between labs. This community improves the analytical sensitivity and robustness of assays to be used in upcoming clinical trails (31).

Secondly, we investigated how well serum and CSF concentrations of NfL correlated, particularly as a blood test is easier to implement in clinical practice as CSF sampling requires a lumbar puncture. Also, a blood test does not require a specialist to take a sample therefore making it easier to screen and test much larger populations of patients. The correlation of serum and CSF NfL was shown in the study to be significantly strong which was keeping with previously published research by Kuhle et al. (7), who correlated samples using the same analytical platform. A strong relationship between increased NfL concentrations and pathophysiological mechanisms has been reported in the literature (32). Mild cognitive impairment (MCI) is a pre-stage for dementia, and Mattsson et al. (16) confirms increased concentrations of NfL in patients within this diagnostic group. A recently published paper suggested prediction of clinical AD 16 years before estimated time of onset in patients who carry mutations that cause familial AD when examining rate of change of serum NfL concentrations. These studies suggest serum NfL as a useful screening tool in pre-symptomatic AD (33, 34).

Conversely, in other diseases, NfL may be used in a slightly different manner. According to Tsagkas et al. (35), NfL concentration is more useful as a biomarker to estimate disease intensity after symptoms accrue in the neuroinflammatory disease MS. One study suggested increased concentrations of NfL with a delay of 144 h after onset of symptoms in hockey players with traumatic brain injuries (TBI) (17). However, there is a lack of studies on temporal changes of NfL concentrations in acute as well as chronic CNS disorders, which would be important to know when interpreting NfL concentrations in biofluids.
Further development of LC-MS/MS assay

Bateman et al. (26) has detected and measured the turnover of Aβ42 in blood for the first time in AD with LC-MS/MS and isotopically labeled $^{13}$C$_6$-leucine (27). At UCL, London, a new study started in the spring of 2019 in which the SILK method was implemented for use in study participants. SILK is a technique in which proteins are labelled in vivo using an isotope-labelled amino acid that is incorporated in newly synthesized proteins. Through this approach, clearance and production rates of individual proteins can be measured. This method can improve our knowledge on biomarker kinetics in patients and further explain some of the changes observed in underlying pathogenesis. Dementias and other diseases that involve the CNS can be investigated and new therapies can be examined in terms of efficacy and target engagement in vivo.

To allow for SILK experiments on NfL, a sensitive and specific LC-MS/MS assay for NfL in CSF has to be developed. There is so far, to the best of our knowledge, no other report detecting NFL CSF with a targeted LC-MS/MS method. Preliminary experiments have shown the method developed can detect the selected NfL peptides at biological concentrations, however detection of NfL in CSF prepared without immunoprecipitation enrichment remains challenging and requires further optimisation. There are several possible explanations for not detecting NfL in CSF. Firstly, biofluids in particular contain a complex mixture of lipids, metabolites, peptides and large proteins. These molecules can cause ion suppression of the analyte of interest; however, this problem can be avoided with different types of pre-fractionation. In this study, we only did a post-digest C18 clean-up of the samples. Also, if any molecule co-elutes at the same retention time as the target analyte, there may be interference.
To get around the issue of ion suppression as a result of matrix effects, studies detecting turnover in other proteins using SILK, most often have had to use antibody-based enrichment of the target protein to de-complex the sample. Future experiments for the detection of NfL will most likely have to use this preparation strategy (36). Secondly, a possible explanation could be that the NfL is simply present at too low concentrations to allow for detection in the mass spectrometer, however we used samples in this study known to have high concentrations of NfL, therefore further supporting the idea this is in fact a matrix-effect related issue for detection. Thirdly, post translations modifications (PTMs) of NfL could cause the peptides in biofluids to acquire a higher mass than the synthetic peptide standards, however this unlikely to be the case as the peptides selected during the design of the assay do not lie within areas of NfL known to be effected by PTMs (37). Further investigation and method optimisation, particularly sample preparation strategies to address the issues of matrix effects, are required before NfL is likely to be detected by the developed LC-MS/MS assay in biofluids.

**Limitations and strengths**

In this study there are limitations, especially the small sample size. The 36 deidentified samples randomly chosen from leftover samples for method development in the Clinical Neurochemistry Laboratory at Sahlgrenska had no clinical information, which makes it impossible to draw any conclusions regarding the pathophysiology that may underlie the NfL concentrations measured. However, in this type of method comparison and method development study this is not needed; we simply wanted to see how well we could characterise CSF and blood samples using two different immunoassays in preparation for the development of a novel MS-based quantification method to allow for SILK experiments on
NFL turnover. Regarding the development of an LC-MS/MS-based quantification method for NFL, this turned out to be very challenging. Especially, one of the most difficult parts was detecting NFL in the more complex biofluid matrices, a well-known and documented problem in LC-MS/MS based research. Currently, there are no other published reports using a targeted LC-MS/MS method of NFL in biofluids as all methods rely on immunoprecipitation and detection by proteomic screening-based methods. In this study, the fact peptide standards were detectable, and at low biological concentrations, but not in CSF known to have a high concentration of NFL suggest matrix-based effects remains the key problem. Further work is needed to determine the extent of the matrix effect and LC-based parameters adjustments as well as sample preparation strategies that can counteract this common issue.

Conclusions and implications

In conclusion, this master thesis showed correlations in CSF concentrations of NFL detected with two different assays in two different laboratories. Furthermore, we managed to quantify NFL concentrations in both serum and CSF samples to corroborate the high correlation between NFL in these two sample types. The samples with known NFL concentrations will be used to examine a future LC-MS/MS method for NFL. Before that has been achieved, we can conclude that the high correlation of serum with CSF NFL suggests that a lumbar puncture may not be needed if the only biomarker of interest is NFL.
Populärvetenskaplig sammanfattning

TVÅ METODER ATT KVANTIFIERA KONCENTRATION AV BIOMARKÖREN NEUROFILAMENT LIGHT PROTEIN VID NEURODEGENERATIVA TILLSTÅND


I den här studien har vi tittat på hur vi kan mäta koncentrationen av biomarkören; NfL. Vi använde 36 prover av ryggmärgsvätska och blod från patienter under utredning för neurodegenerativa sjukdomar. Dessa prover analyserades med två högkänsliga metoder som använder specifika antikroppar mot NfL; In-House ELISA samt en ytterligare känslig metod, Simoa. Vi mätte den absoluta koncentrationen NfL och tittade därefter på om det förekom någon korrelation mellan de uppmätta koncentrationerna i ryggmärgsvätska och blod. För att
vi ska kunna mäta proteinet i patienter över hela världen och i olika laboratorier testas olika
detektions-metoder för att få gränsvärden och förväntansfullt använda biomarkören i klinik.

En del av studien var inriktad på ett pilotprojekt som innebär att bygga upp en ny
detektionsmetod av NfL som bygger på masspektrometri. NfLs halveringstid och
produktionshastighet är okänt, men med ett nytt forskningsprojekt där en isotop injiceras i en
människa med ett neurodegenerativt tillstånd kan eventuellt frågan besvaras. Isotopen flaggar
för proteiner som bildas på nytt och genom kontinuerlig provtagning och analys av flaggade
proteiner jämfört med icke flaggade kan en produktionshastighet uppmätas.

I den här studien kom vi fram till att båda metoderna för analys av NfL i ryggmärgsvätska
korrelerade starkt och kunde identifiera låga koncentrationer NfL i proverna. Vårt arbete
styrker även användningen av koncentration NfL i blod som korrelerar med koncentrationer i
ryggmärgsvätska. Dock kommer förståelse för proteinet att öka i och med att nya studier är på
framfart och har potential att förklara grundläggande egenskaper hos proteinet. Det blir
därmed en skjuts framåt att potentiellt använda NfL i kliniken som biomarkör i en rad olika
tillstånd, som t. ex screening av sjukdomsaktivitet eller hur effektivt ett läkemedel är mot en
nervcellsnedbrytande sjukdom.
Acknowledgement

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