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Correlations between serum and CSF pNfH levels in ALS, FTD and controls: a comparison of three analytical approaches

https://doi.org/10.1515/cclm-2019-0015
Received January 5, 2019; accepted April 5, 2019

Abstract

Background: Phosphorylated neurofilament heavy (pNfH), a neuronal cytoskeleton protein, might provide a promising blood biomarker of neuronal damage in neurodegenerative diseases (NDDs). The best analytical approaches to measure pNfH levels and whether serum levels correlate with cerebrospinal fluid (CSF) levels in NDDs remain to be determined.
Methods: We here compared analytical sensitivity and reliability of three novel analytical approaches (homebrew Simoa, commercial Simoa and ELISA) for quantifying pNfH in both CSF and serum in samples of amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and control subjects.
Results: While all three assays showed highly correlated CSF measurements, Simoa assays also yielded high between-assay correlations for serum measurements ($\varrho = 0.95$). Serum levels also correlated strongly with CSF levels for Simoa-based measurements (both $\varrho = 0.62$). All three assays allowed distinguishing ALS from controls by increased CSF pNfH levels, and Simoa assays also by increased serum pNfH levels. pNfH levels were also increased in FTD.
Conclusions: pNfH concentrations in CSF and, if measured by Simoa assays, in blood might provide a sensitive and reliable biomarker of neuronal damage, with good between-assay correlations. Serum pNfH levels measured by Simoa assays closely reflect CSF levels, rendering serum pNfH an easily accessible blood biomarker of neuronal damage in NDDs.

Keywords: amyotrophic lateral sclerosis (ALS); cerebrospinal fluid (CSF); frontotemporal dementia (FTD); phosphorylated neurofilament heavy chain (pNfH); serum; single molecule array (Simoa).

Introduction

Fluid biomarkers which capture and quantify neuronal damage in a sensitive and reliable manner and are easily accessible in peripheral blood are urgently warranted to monitor disease progression and response to molecular treatments which are now on the horizon for neurodegenerative diseases. Neurofilament light (NfL) shows

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great promise as a diagnostic and possibly also treatment outcome biomarker in neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), Alzheimer's disease and Huntington's disease, both in cerebrospinal fluid (CSF) and blood [1–5]. However, optimal assessment and correlation of CSF levels with blood levels are less clear for phosphorylated neurofilament heavy (pNfH). Analytical approaches for pNfH quantification by ELISA differ largely between laboratories [6–9] and promising novel single molecule array (Simoa) approaches have not yet been compared in a standardised manner. Moreover, associations of pNfH concentrations in CSF with disease progression and survival do not yet consistently reproduce in blood [6, 10, 11]. In view of these challenges, we compared three novel analytical approaches to measuring pNfH levels, comprising homebrew Simoa, commercial Simoa and ELISA approaches, analysing matched pairs of CSF and serum samples of ALS, FTD and control subjects. We hypothesised that pNfH quantification in CSF and serum is sensitive and reliable with all three assays, expecting high correlations across analytical approaches as well as high correlations between CSF and serum pNfH levels. We further predicted all three approaches to detect clinically relevant pNfH differences between neurodegenerative patients and controls, possibly even in blood.

Materials and methods

Subjects

We recruited 34 subjects from the Department of Neurodegenerative Disorders, Hertie Institute for Clinical Brain Research, Tübingen, comprising 10 ALS, 10 FTD and 14 control subjects. Patients fulfilled established diagnostic criteria for ALS and FTD, respectively. The controls did not have any history or clinical signs of neurodegenerative disease, as ascertained by neurologists with special expertise in neurodegenerative diseases. The university's Ethics Committee approved the study. All subjects gave written informed consent prior to participation.

Biomaterial

Paired CSF and blood samples were taken within a time interval of less than 20 min. CSF was centrifuged at 2000 × *g* for 10 min at room temperature, serum at 2000 × *g* for 10 min at 4 °C (after clotting for 30 min at room temperature). Samples were stored at −80 °C within 60 min after collection in the local biobank and analysed without any previous thaw-freeze cycle.

pNfH measurements

The same 34 CSF and serum sample pairs were measured by three approaches in parallel: by homebrew Simoa, by commercially available Simoa and by ELISA, each time in duplicates (for a methodological overview, see Table 1). Technicians were blinded to the clinical diagnosis. In an explorative add-on analysis, pNfH levels were also correlated with NfL levels available for 22 matched CSF-serum pairs, determined by a previously established electrochemiluminescence immunoassay [2] (Supplementary materials 3 and 4).

Homebrew Simoa

The Simoa pNfH assay was developed in-house (University Hospital Basel) using a Homebrew kit (Quanterix Corp, Lexington, MA, USA). pNfH was captured by an anti-human-pNfH mouse monoclonal antibody (Iron Horse Diagnostics, Phoenix, AZ, USA; for ordering the antibody, the manufacturer should be contacted directly with reference to this article) and detected by anti-human-pNfH chicken polyclonal antibody (Iron Horse Diagnostics, Phoenix, AZ, USA). The antibodies recognise the KSP site of pNfH, with the signal

Table 1: Methodological comparison of three analytical approaches to pNfH quantification.

	Homebrew Simoa	Commercial Simoa	ELISA
Capture antibody	Monoclonal, mouse, *Iron Horse Diagnostics*	Monoclonal, mouse (IgG purified), *Quanterix*	Monoclonal, mouse (NF-01) *Abcam* (catalogue: ab7795)
Detection antibody	Polyclonal, chicken, *Iron Horse Diagnostics*	Polyclonal, chicken (affinity purified), *Quanterix*	Monoclonal, mouse (NF-05) *Abcam* (catalogue: ab118812)
Standard origin	Porcine spinal cord, purified	Bovine spinal cord, purified	Human recombinant protein expressed in HEK293T cells, *OriGene* (cat.: TP313487)
Buffer pH	Calibrator diluent: pH 7.0 Sample diluent: pH 7.0 Bead diluent: pH 7.0 Detector diluent: pH 7.0	Calibrator diluent: pH 7.5 Sample diluent: pH 7.5 Bead diluent: pH 7.5 Detector diluent: pH 7.5	Coating: pH 9.6 Sample dilution: pH 8.0 Blocking: pH 7.16 Washing: pH 7.16 Detection: pH 7.16

The analytical approaches to pNfH quantification are described in detail in the Methods section. Simoa, single molecule array.

disappearing on Western blot after alkaline phosphatase treatment. Before measurements, the capture was coupled to paramagnetic beads (Quanterix) and the detector was biotinylated.

Coupling of the capture: Seventy-eight micrograms of antibody were buffer exchanged with Beads Conjugation Buffer (2-(N-morpholino) ethane sulfonic acid [MES] buffer, Quanterix) using spin filtration (Amicon Ultra-2, 50 kDa, Sigma) and concentrated to 0.3 mg/mL. Paramagnetic beads (1.4×10^6 beads per 1 µL of recovered antibody) were buffer exchanged to Beads Conjugation Buffer on a magnetic separator (Ambion, #AM10055), and underwent a 30 min incubation on a rotator (HulaMixer, Thermofisher Scientific) with 5% EDC 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, Thermofisher Scientific). The coupling reaction was started by adding the washed 0.3 mg/mL capture antibody to the activated beads followed by a 2 h incubation on the rotator. The coupled beads were then washed with Bead Wash Buffer (phosphate buffer with detergent and ProClin 300, Quanterix) and blocked by the addition of Bead Blocking Buffer (phosphate buffer with 1% bovine serum albumin and ProClin 300, Quanterix) followed by 30 min incubation on the rotator.

Biotinylation of the detector: One hundred and thirty micrograms of polyclonal anti-pNfH were buffer exchanged to Biotinylation Reaction Buffer (100 mM phosphate buffered saline, Quanterix) and diluted to 1 mg/mL. N-hydroxysuccinimide ester-polyethylene glycol 4-Biotin (NHS-PEG4-Biotin, Thermofisher Scientific) was then added with a ratio of 40:1, followed by 30 min incubation. Finally, the biotinylated detector was purified using spin filtration and the concentration was measured. Coupled beads and biotinylated detector were respectively stored in bead diluent (50 mM Tris pH 7.8, 50 mM NaCl, 10 mM EDTA, 1% bovine serum albumin, 0.1% Tween 20) and biotinylation reaction buffers at 4 °C until analysis.

Purified native pNfH for calibration was obtained from Iron Horse Diagnostics (porcine spinal cord). Calibrators ranged from 0 to 1000 pg/mL (diluent: tris base saline [TBS] pH 7.0; 0.1% Tween 20; 1% milk powder; 300 µg/mL Heteroblock [Omega Biologicals Inc., Bozeman, MT, USA]). Batch prepared calibrators were stored at −80 °C. Calibrators (neat) and samples (1:4 dilution; diluent: TBS pH 7.0, 0.1% Tween 20, 1% milk powder, 400 µg/mL Heteroblock [Omega Biologicals]) were measured in duplicates from a single well. Reagents were prepared as follows: pNfH beads and Helper Beads (Quanterix) were diluted to 1×10^4 beads/µL; in a second tube the detector concentration was adjusted to 0.1 µg/mL (beads and detector diluent: TBS pH 7.0, 0.1% Tween 20, 1% milk powder, 300 µg/mL Heteroblock [Omega Biologicals]). The assay was run on a Simoa HD-1 instrument (Quanterix) using a 2-step Assay Neat 2.0 protocol: 100 µL of calibrator/sample, 25 µL conjugated beads, and 20 µL of detector were incubated for 47 cadences (1 cadence = 45 s). After a first washing, 100 µL of streptavidin conjugated β-galactosidase (150 pM; SBG, Quanterix) was added, followed by a 7-cadence incubation and a second wash. Finally, after addition of 25 µL Resorufin β-D-galactopyranoside (RGP, Quanterix) pNfH was quantified. CSF and serum samples were diluted by factor 4.

Commercial Simoa

A commercially available pNfH Simoa assay (pNfH discovery kit) was run on the Simoa HD-1 Analyzer (Quanterix, Lexington, MA, USA) [12]. The calibrator curve was constructed using the standard provided by the manufacturer. CSF samples were diluted by a factor of 26, serum samples were measured without dilution. The assay used a monoclonal mouse antibody (IgG purified) for capture and a polyclonal chicken antibody (affinity purified) for detection. The antibodies specifically recognise the phosphorylated KSP sequences of NfH, with the signal disappearing on western blot after alkaline phosphatase treatment. The provided calibrator is native pNfH isolated from bovine spinal cord.

ELISA

The pNfH ELISA, described previously [13] was used with minor modifications (University of Gothenburg). Briefly, the antibodies (monoclonal anti-human mouse antibodies) used for the in-house sandwich ELISA were NF-01 (Abcam, ab7795) for coating and NF-05 (Abcam, ab118812) as a biotinylated detector. The blocking of the plate was performed with 1% bovine serum albumin (BSA) in phosphate-buffered saline with Tween (0.05%) (PBS-T). The sample buffer used to dilute the NfH calibrants (OriGene, TP313487; human recombinant protein expressed in HEK293T cells) and samples was 5mM urea in PBS-T. The buffers used were carbonate buffer (pH 9.6) for coating, PBS-Tween (0.05%) with 1% BSA (pH 7.16) for blocking and detection, 5 mM urea in PBS-T (pH 8.0) for sample dilution, and PBS-Tween (0.05%) (pH 7.16) for washing. CSF samples were diluted by a factor of 4, serum samples by a factor of 2. The substrate used was SuperSignal ELISA Femto Substrate and the plates were read with a Viktor X4 2030 Multilabel Reader after being shaken for 2 min. Four washes with PBS-T were performed in between all steps. The phosphorylation specificity was assessed by incubating the recombinant protein used as calibrant with alkaline phosphatase from *Escherichia coli* and measured using the assay previously described, a control with no alkaline phosphatase was also included [13].

Analysis

Analytical sensitivity of each assay was defined as the pNfH concentration of the calibrator with the lowest pNfH concentration fulfilling established acceptance criteria (i.e. coefficient of variation [CV] of duplicate determination ≤20% and accuracy within the range of 80%–120%) [14]. Within-run precision and between-run precision [15] were derived from four consecutive runs. For CSF and serum, sample CVs were based on duplicate measurements of all 34 samples. Data were reported as median and interquartile range, unless stated otherwise. We used non-parametric procedures to analyse correlations of pNfH concentrations between and within analytical approaches (Spearman's correlation) and group effects on pNfH levels (Mann-Whitney tests). *Post-hoc* tests were Bonferroni-corrected for multiple comparisons. The effect size of group effects was reported as Pearson's r. For comparing pNfH levels across groups, we analysed an age-matched subset (n = 10) of our controls (Supplementary material 1) to control for potential age effects on pNfH levels [10].

As prior data on correlations of pNfH measurements across different analytical approaches were not available, sample size calculation was not possible. We therefore chose a total sample size (n = 34) in the range of comparative studies of analytical platforms for neurofilament light [16].

Results

Sensitivity and reliability of the three analytical approaches

Analytical sensitivity [16] was 0.3 pg/mL for the homebrew Simoa, 8.6 pg/mL for the commercial Simoa and 192.0 pg/mL for the ELISA (Table 2). For CSF, concentrations of all samples were above these levels. For serum, 97.1% (33/34) of sample concentrations were above the respective analytical sensitivity of the homebrew Simoa, 100% for the commercial Simoa and 50% (17/34) for the ELISA. Within- and between-run precision and sample coefficients of variation (CVs) of duplicate measurements in CSF and serum are summarised in Table 2. Overall, sample CVs were lower in CSF than in serum.

pNfH correlations between different analytical approaches

CSF pNfH measurements by all three analytical approaches were highly correlated (homebrew Simoa vs. commercial Simoa: $\varrho = 0.91$, $p < 0.001$; homebrew Simoa vs. ELISA: $\varrho = 0.99$, $p < 0.001$; commercial Simoa vs. ELISA: $\varrho = 0.94$, $p < 0.001$; Figure 1A–C), yet with varying absolute pNfH values (for detailed results and normalised analytical sensitivity, see Supplementary material 2).

For serum measurements, pNfH levels were also highly correlated between both Simoa approaches ($\varrho = 0.95$, $p < 0.001$), yet correlations were weaker between ELISA and each of the Simoa approaches (homebrew Simoa: $\varrho = 0.55$, $p = 0.001$; commercial Simoa: $\varrho = 0.37$, $p = 0.035$) (Figure 1D–F). The corresponding Bland-Altman plots (Figure 2) indicate proportional differences between the three approaches to pNfH quantification, which increase together with the absolute concentrations. This suggests that additional normalisation of analytical approaches is necessary for improved comparability.

pNfH correlations between CSF and peripheral blood

Correlations between paired CSF and serum samples were strong for homebrew Simoa ($\varrho = 0.62$, $p < 0.001$) and commercial Simoa ($\varrho = 0.62$, $p < 0.001$) measurements, but not significant for the ELISA approach ($\varrho = 0.19$, $p = 0.292$) (Figure 1G–I).

pNfH levels across diagnostic groups

In CSF, all three approaches yielded significantly higher pNfH levels in ALS than in both FTD ($p < 0.001$, $r = 0.85$ for all three assays) and control subjects ($p < 0.001$, $r = 0.85$

Table 2: Assay performance of three analytical approaches to pNfH quantification in CSF and serum.

	Homebrew Simoa	Commercial Simoa	ELISA
Analytical sensitivity	0.3 pg/mL	8.6 pg/mL	192.0 pg/mL
Normalised analytical sensitivity	0.3 pg/mL	4.9 pg/mL	11.9 pg/mL
Within-run precision	5.4% (19.7 pg/mL)	5.2% (14.5 pg/mL)	6.6% (1802 pg/mL)
	5.4% (31.9 pg/mL)	6.3% (106.4 pg/mL)	3.9% (13,174 pg/mL)
	3.7% (39.1 pg/mL)	2.7% (1211 pg/mL)	7.1% (18,593 pg/mL)
Between-run precision	7.3% (19.7 pg/mL)	41.3% (14.5 pg/mL)	16.9% (1802 pg/mL)
	5.7% (31.9 pg/mL)	17.5% (106.4 pg/mL)	9.4% (13,174 pg/mL)
	7.3% (39.1 pg/mL)	11.5% (1211 pg/mL)	20.5% (18,593 pg/mL)
Sample CV, CSF	2.1% (0.8–3.8)	5.4% (3.0–9.5)	1.9% (0.7–3.6)
Sample CV, serum	3.9% (1.8–7.5)	6.5% (2.7–11.2)	2.7% (2.1–6.0)

Analytical sensitivity [pg/mL] of each analytical approach was defined as the pNfH concentration of the calibrator with the lowest pNfH concentration fulfilling established acceptance criteria (i.e. CV of duplicate determination ≤20% and recovery within the range of 80%–120%) [16]. While measurements of pNfH concentrations in CSF were highly correlated across the three analytical approaches (Figure 1), absolute measurement values differed considerably and consistently across assays (Figure 2), necessitating normalisation of analytical sensitivities for better comparability. The normalised analytical sensitivity reports the analytical sensitivity of each approach normalised to the scale of the homebrew Simoa. For instance, measurement of a sample with a concentration corresponding to the analytical sensitivity of the commercial Simoa approach would result in a concentration of 8.6 pg/mL on the scale of the commercial Simoa and 4.9 pg/mL on the scale of the homebrew Simoa assay (Supplementary material 2). Within-run precision and between-run precision were derived from four consecutive runs of each assay [16]. For CSF and serum, sample CVs were based on duplicate measurements of all 34 samples.

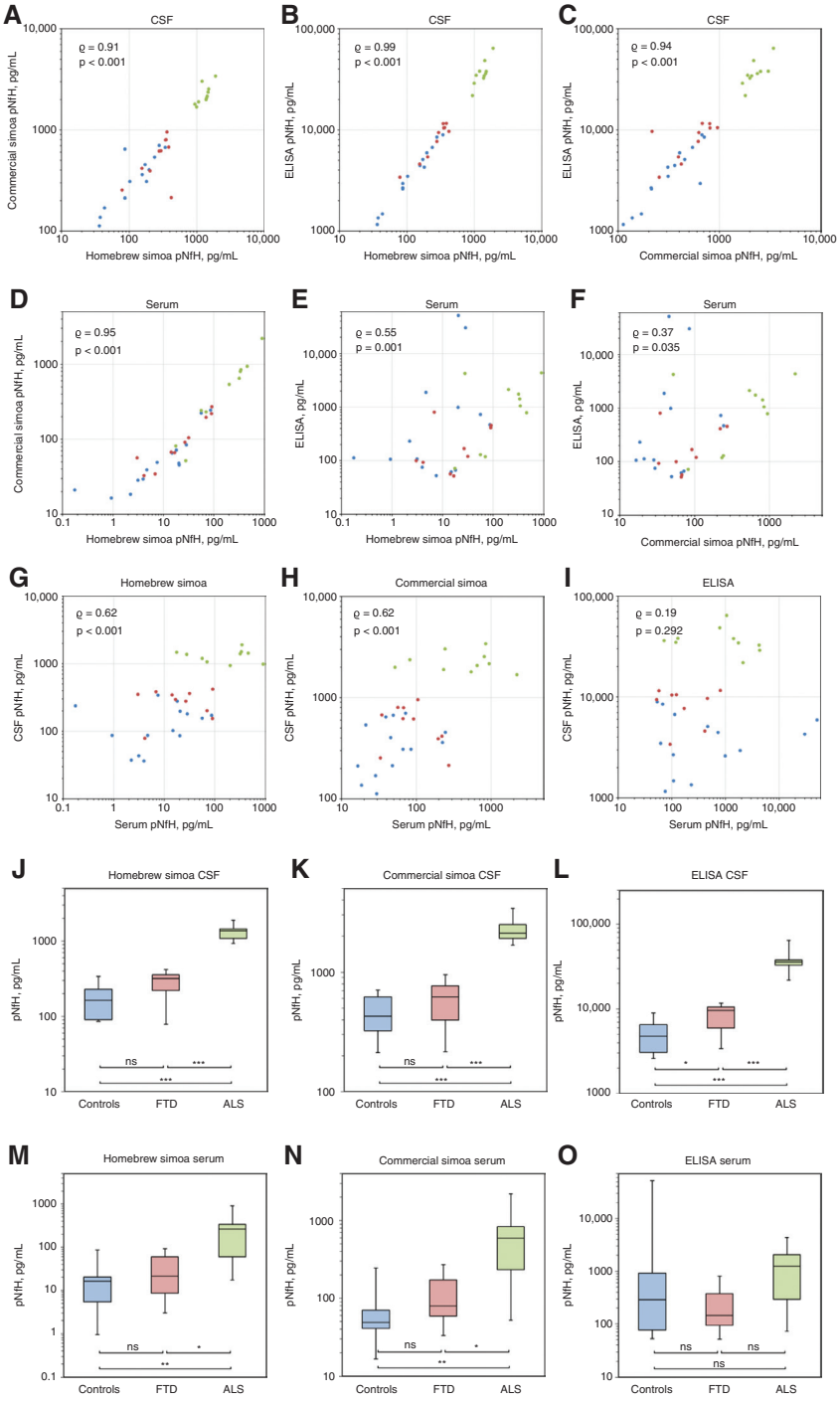

Figure 1: Associations between pNfH measurements across analytical approaches in CSF (A–C) and serum (D–F), correlations between CSF and serum pNfH measurements within analytical approaches (G–I), pNfH concentrations in FTD, ALS and control subjects measured with different approaches in CSF (J–L) and serum (M–O).
CSF pNfH measurements were highly correlated across all three analytical approaches (A–C). Dot colour indicates diagnosis (blue: controls, red: FTD, green: ALS). For serum measurements, pNfH levels were highly correlated between both Simoa approaches, while the correlations between ELISA and both Simoa approaches were considerably weaker (E–F). Correlation between paired CSF and serum samples was strong for both the homebrew Simoa and the commercial Simoa approach, but not significant for the ELISA measurements (G–I). In CSF, all three approaches yielded significantly higher pNfH levels in ALS than in both FTD and age-matched control subjects (J–L), CSF pNfH levels of FTD subjects were significantly higher than those of controls if measured by ELISA (L) (p-values Bonferroni-corrected for multiple comparisons, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; ns, not significant). In serum, significantly higher pNfH levels in ALS than in FTD and control subjects were only found by the Simoa approaches (M–O). Central horizontal lines indicate median values, boxes illustrate the ranges between lower and upper quartiles, and error bars represent the full ranges of data. Please note the logarithmic scale of the x- and y-axis. The corresponding Bland-Altman plots are depicted in Figure 2.

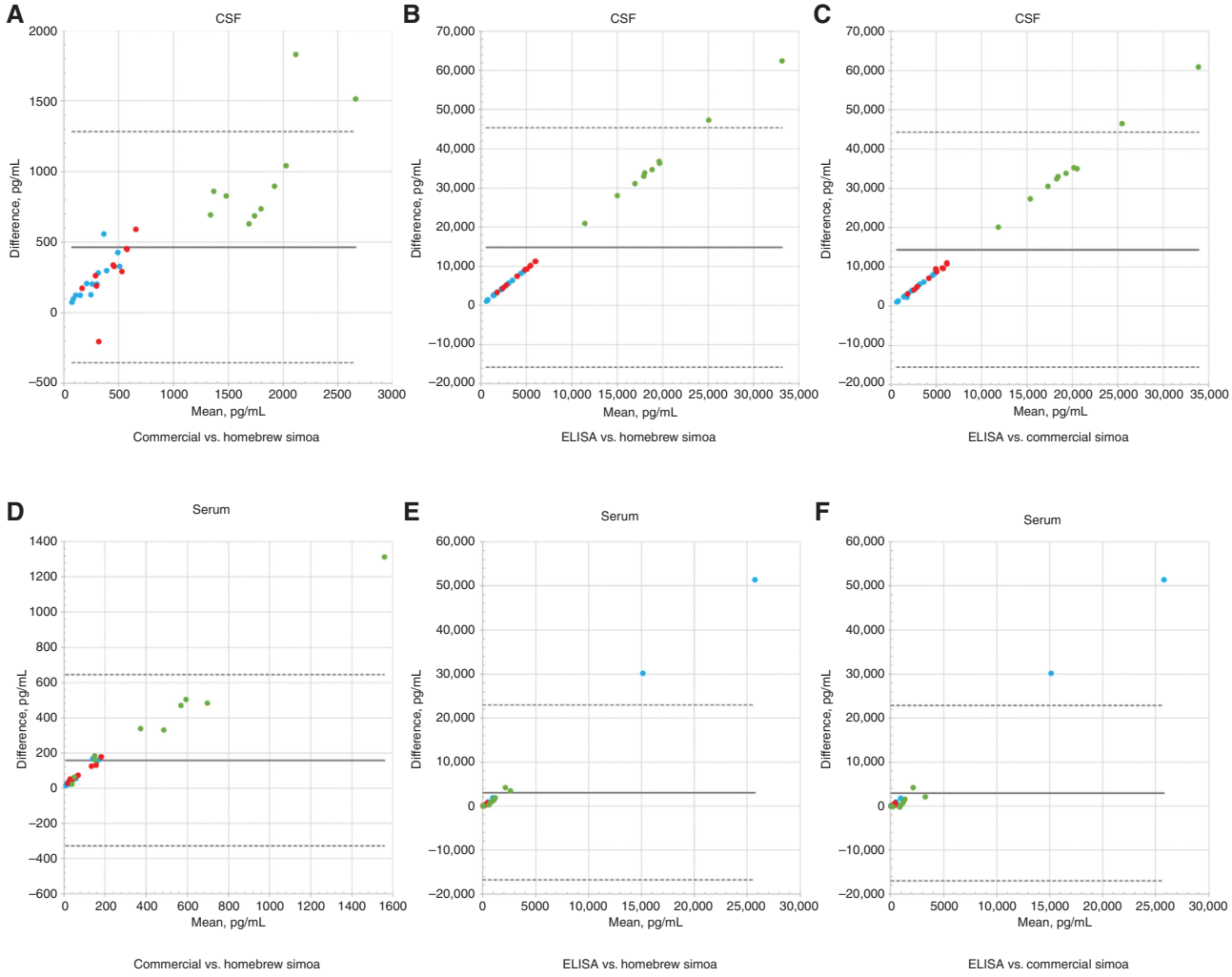

Figure 2: Bland-Altman plots for comparison of three analytical approaches to pNfH quantification in CSF (A–C) and serum (D–F). The Bland-Altman plots for CSF (A–C) demonstrate that there are proportional differences between the three approaches to pNfH quantification, i.e. errors increasing with increasing absolute concentrations. Proportional errors were also observed for Simoa-based measurements in serum (D). For the ELISA measurements in serum, the plots furthermore support the notion that the ELISA measurements in serum are affected by the occurrence of unexpectedly high pNfH ELISA levels in serum samples of healthy controls which yielded low concentrations in Simoa measurements (E–F). Mean (solid line) and 95% confidence interval (dotted line) were derived from log-transformed data. Dot colour indicates diagnosis (blue: controls, red: FTD, green: ALS).

for all three assays) (Figure 1J–L, Supplementary material 1). Moreover, CSF pNfH levels of FTD subjects were significantly higher than those of controls (Figure 1L) if measured by ELISA ($p = 0.015$, $r = 0.54$), but not if measured by Simoa approaches.

In serum, significantly higher pNfH levels in ALS than in FTD (homebrew Simoa: $p = 0.005$, $r = 0.61$; commercial Simoa: $p = 0.007$, $r = 0.59$) and control subjects (homebrew Simoa: $p = 0.001$, $r = 0.69$; commercial Simoa: $p = 0.001$, $r = 0.71$) were found by the two Simoa approaches (Figure 1M–O), but not by the ELISA. Though cohorts were not sufficiently powered to yield statistical significance, serum pNfH levels were also quantitatively higher in FTD than control subjects across all three approaches.

Explorative analysis of the relation of pNfH to NfL levels

This explorative analysis indicated that pNfH concentrations measured by all three approaches yielded significant correlations with concentrations of NfL in CSF (Supplementary materials 3 and 4). If measured by Simoa approaches, the correlation of pNfH levels with NfL levels was maintained to a lesser extent also in serum (Supplementary material 3 for visualisation of the pNfH-NfL correlations, Supplementary material 4 for Bland-Altman plots), suggesting that pNfH and NfL in peripheral blood might potentially be subject to different release and/or clearance dynamics.

Discussion

Sensitive and reliable fluid biomarkers reflecting neuronal damage, ideally accessible even in blood, are highly warranted in neurodegenerative diseases. Our findings demonstrate that pNfH levels can be sensitively and reliably quantified by all three novel analytical approaches in CSF: the homebrew Simoa, the commercially available Simoa and the ELISA approach, with stable proportional differences between the three approaches to pNfH quantification. Given the high analytical sensitivity, the CSF concentrations of pNfH in our neurodegenerative subjects were all in the analytical range of the three assays. Importantly, the Simoa assays also provided sensitive and reliable pNfH measurements in serum, with serum concentrations of 97%–100% of subjects being well quantifiable by these two assays. For the ELISA, 50% of serum concentrations were outside the analytical range.

In CSF, pNfH concentrations were strongly correlated between the three analytical approaches, as required to allow comparability across centres and studies in future multicentre trials. While assay specificity for the phosphorylated form of neurofilament heavy has been demonstrated only for the pNfH ELISA [13], the high correlation of CSF pNfH levels between the three approaches suggests that also the two Simoa assays indeed capture phosphorylated NfH.

In serum, pNfH concentrations also showed strong correlations between the two Simoa assays, but not between Simoa assays and ELISA. This low between-assay correlation of the ELISA in serum might be due to its lower analytical sensitivity, the high share of pNfH serum concentrations outside its analytical range and the occurrence of unexpectedly high pNfH ELISA levels in serum samples which yielded low concentrations in Simoa measurements [16] (Figure 2). These factors might, at least partially, be related to a relatively higher susceptibility of the ELISA to disturbances by matrix effects [17] (e.g. heterophilic antibodies) and/or more robust measurements by the Simoa technique which decreases non-specific interactions by confining the detection reaction to small-volume compartments.

Serum pNfH levels correlated highly with CSF levels, if measured by any of the two Simoa approaches. The finding of a robust correlation between CSF and serum pNfH values in both ALS and FTD extends previous findings of increased pNfH levels in ALS [6–10, 18]. Given this strong CSF-serum association, pNfH levels in peripheral blood might present a valid peripheral biomarker for neuronal damage in the central nervous system in neurodegenerative diseases. For the ELISA measurements, the absence of a significant correlation between CSF and serum pNfH levels might result from its limitations in serum measurements. The performance differences between the three analytical approaches might relate to further factors which were not directly controlled for in our measurements, including differences of the antibodies, the calibration with different protein standards and concentrations, the buffer composition and pH values, and local laboratory conditions (including differently calibrated pipettes). To improve direct comparability across analytical approaches, the standard curves of the three assays could be run on each of the three assays. Moreover, pNfH quantification might have been affected by the use of phosphate buffered solutions in the immunoassays as the phosphate binding capacity of neurofilament heavy is such that NfH phosphorylation might occur during the application [5, 11].

All three pNfH assays indeed seem suited to reveal relevant findings in neurodegenerative disease cohorts. All three of them allowed distinguishing ALS subjects from control and FTD subjects by CSF pNfH concentrations, with the Simoa assays also allowing this distinction by serum concentrations (homebrew Simoa: ALS-controls 0.91 [0.78–1.00], ALS-FTD AUC = 0.86 [0.70–1.00]; commercial Simoa: ALS-controls AUC = 0.92 [0.80–1.00], ALS-FTD AUC = 0.85 [0.67–1.00]). These findings support the validity of the assays and extends previous findings of increased pNfH levels in ALS [6–10, 18] by demonstrating that this increase is consistent and reliable across different analytical approaches. The diagnostic value of CSF pNfH levels for differentiating ALS subjects from controls was high throughout all three analytical approaches (Table 3), with a total allowable

Table 3: Diagnostic value of pNfH concentrations for ALS across analytical approaches.

Matrix	Approach	Significance	AUC (95% CI)
CSF	Homebrew	$p < 0.001$	1.00 (1.00–1.00)
CSF	ELISA	$p < 0.001$	1.00 (1.00–1.00)
CSF	Commercial	$p < 0.001$	1.00 (1.00–1.00)
Serum	Homebrew	$p = 0.001$	0.92 (0.81–1.00)
Serum	ELISA	$p = 0.128$	0.69 (0.47–0.91)
Serum	Commercial	$p < 0.001$	0.93 (0.83–1.00)

Receiver operating characteristics (ROC) for the discrimination of ALS subjects from controls show that all three analytical approaches discriminated ALS subjects from healthy controls with 100% diagnostic accuracy in CSF, corresponding to an area under the curve (AUC) of 1.0 for each approach. Diagnostic accuracy was lower in serum. Supplementary material 5 depicts the diagnostic accuracy of each analytical approach as a function of the pNfH cut-off level.

Table 4: Reliability of pNfH concentrations in CSF for distinguishing ALS subjects from controls.

Approach	Upper limit of reference range for controls	Lower limit of reference range for ALS subjects	Total error allowable
Homebrew	493 pg/mL	848 pg/mL	72%
ELISA	12,923 pg/mL	20,725 pg/mL	60%
Commercial	1041 pg/mL	1436 pg/mL	38%

We calculated the total allowable error (TEa) at the upper reference range limit of healthy controls which still allowed to maintain the 100% discrimination of ALS patients and controls by calculating the difference between the upper reference range limit of controls and the lower reference range limit of ALS subjects. Both reference ranges were derived from log-transformed data as 95% confidence intervals. We expressed the TEa in percent of the upper reference range limit of controls. TEa was ≥38% for all three analytical approaches in CSF, indicating high agreement of all three analytical approaches in their diagnostic reliability. The limits of the reference ranges refer to the original, non-normalised measurement values.

error (TEa) ≥38% (Table 4). Thus, pNfH concentrations in CSF, as measured by any of the three approaches, might reliably support the diagnosis of ALS.

Quantitatively, pNfH levels were also increased in FTD, yet without statistical significance given the small patient cohort. Sufficiently powered studies on both serum and CSF pNfH in FTD are warranted to confirm the hypothesis that pNfH levels in FTD might be increased not only in CSF, but also in serum.

In sum, our results indicate that pNfH concentrations in CSF and, if measured by Simoa assays, also in blood might provide a sensitive and reliable biomarker of neuronal damage, assessment of which is valid across different analytical approaches. The increases of pNfH levels in several neurodegenerative disorders and the correlations between CSF and serum values suggest that pNfH, like NfL, might provide a promising peripheral fluid biomarker of neuronal damage in neurodegeneration [5]. Such easily accessible blood biomarkers with high sensitivity and reliability are important to promote acceptance by patients, longitudinal sampling and their broad use in both research and clinical routine [5]. Previous research suggested that pNfH measurement might be more robust than NfL measurements [18] and more stable against preanalytical variables [19], and our findings indicate that blood pNfH and NfL levels might potentially be subject to different release and/or clearance dynamics (Supplementary materials 3 and 4). Yet both pNfH and NfL appear valuable candidates, possibly used together in composite neurodegenerative biomarker panels.

Acknowledgments: Biosamples were obtained from the Neuro-Biobank of the University of Tübingen, Germany, which is supported by the University of Tübingen, the Hertie Institute for Clinical Brain Research (HIH) and the German Center for Neurodegenerative Diseases (DZNE). CW was supported by the Wilhelm Vaillant Stiftung. HZ is a Wallenberg Academy Fellow and is supported by grants from the Swedish Research Council and the European Research Council. JK is supported by the Swiss National Research Foundation (320030_160221) and the University of Basel. FP is funded by the MIROCALS project from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 633413. This study was further supported by the National Institutes of Health (NCATS, NINDS) through collaborative funding for the CReATe consortium (U54NS092091) which is part of the Rare Diseases Clinical Research Network (RDCRN) (pilot grant to RS and MS) and by funding from the HSP Research Foundation (grant to RS). KB holds the Torsten Söderberg professorship in Medicine and is supported by grants from the Swedish Research Council, the Swedish Alzheimer Foundation, and the Swedish Brain Foundation.

Author contributions: CW: design and conceptualisation of the study, acquisition of data, analysis of the data, drafting and revision of the manuscript. FP: acquisition of data, analysis of the data, revision of the manuscript. CB: acquisition of data, analysis of the data, revision of the manuscript. ES: acquisition of data, revision of the manuscript. KB: revision of the manuscript. ZM: acquisition of data, revision of the manuscript. CD: acquisition of data, revision of the manuscript. AJ: provision of antibodies, analysis of the data, revision of the manuscript. HZ: design and conceptualisation of the study, revision of the manuscript. RS: design and conceptualisation of the study, revision of the manuscript. KH: design and conceptualisation of the study, analysis of the data, revision of the manuscript. JK: design and conceptualisation of the study, analysis of the data, revision of the manuscript. MS: design and conceptualisation of the study, analysis of the data, drafting and revision of the manuscript. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Competing financial interests: JK's institution (University Hospital Basel) received in the last 3 years and used exclusively for research support: consulting fees from Novartis, Protagen AG; speaker fees from the Swiss MS Society, Biogen, Novartis, Roche, Genzyme; travel expenses from Merck Serono, Novartis; grants from ECTRIMS Research Fellowship Programme, University of Basel, Swiss MS Society, Swiss National Research Foundation, Bayer (Switzerland) AG, Genzyme, Merck, Novartis. MS received speaker's honoraria and research support from Actelion Pharmaceuticals, unrelated to the current project and manuscript. AJ is an advisor Quanterix Corporation, Lexington, Massachusetts. HZ has served at scientific advisory boards for Eli Lilly, Roche Diagnostics, CogRx, Samumed and Wave, has received travel support from Teva and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. KB has served as a consultant or at scientific advisory boards for IBL International, Fujirebio Europe, Roche Diagnostics, BioArctic, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. The other authors declare no competing financial interests.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Data availability: The datasets analysed in the current study are available from the corresponding author on reasonable request.

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/cclm-2019-0015).