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Original article

Ultrasensitive immunoassay allows measurement of serum neurofilament heavy in multiple sclerosis

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ABSTRACT

Background: Neurofilament heavy (NfH) is a promising biomarker for neuro-axonal damage in Multiple Sclerosis (MS). We compared the performance of high-sensitivity serum-NfH immunoassays, with as aim to investigate the value of serum-NfH as biomarker for MS.
Methods: We measured serum-NfH in 76 MS patients with Simoa (one commercial, one in-house) or Luminex assays. Serum-NfH measured by the immunoassay with greatest sensitivity was related to clinical and radiological outcomes with age and sex-adjusted linear regression analysis, and to biological outcomes cerebrospinal fluid (CSF)-NfH, serum neurofilament light (NfL) and CSF-NfL with Spearman's correlation analysis.
Results: With the commercial Simoa assay, we obtained 100% serum-NfH detectability (in-house Simoa: 70%, Luminex: 61%), with lowest coefficient of variation (CV) between duplicates of 11%CV (in-house Simoa: 22%CV, Luminex: 30%CV). Serum-NfH quantified with the commercial Simoa assay was associated with disease duration (standardized beta (sβ) = 0.28, $p = 0.034$), T2 lesion volume (sβ = 0.23, $p = 0.041$), and tended to associate with black hole count (sβ = 0.21, $p = 0.084$) but not with Expanded Disease Disability Score (EDSS) or normalized brain volume (all: $p>0.10$). Furthermore, serum-NfH showed correlations with CSF-NfH (rho = 0.27, $p = 0.018$) and serum-NfL (rho=0.44, $p < 0.001$), but not with CSF-NfL.
Conclusions: Serum-NfH can be quantified with high-sensitivity technology. Cross-sectionally, we observed some weak correlations of serum-NfH with MS disease burden parameters, suggesting there might be some utility for serum-NfH as biomarker for MS disease burden.

1. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory and degenerative disease of the central nervous system (Thompson et al., 2018). Converging evidence indicates that neuronal damage occurs from MS disease onset and most likely underlies the progressive, irreversible accumulation of disability. A number of treatment options are available to reduce relapse rate in relapsing-remitting MS (RRMS) (Thompson et al., 2018; Ziemssen et al., 2015). However, those drugs have little, if any, efficacy in preventing disability progression during secondary progressive MS (SPMS). Clinical trials targeting prevention of disability progression require tools to dynamically quantify axonal damage over a relatively short time period. Such tools would be useful in the clinical setting as well, to monitor disease progression and to assist therapeutic decisions. Neurofilaments are the major components of the axonal cytoskeleton (Khalil et al., 2018; Petzold, 2005), and are promising biomarker candidates to fulfill these roles.

Neurofilaments are heteropolymers composed of the light (NfL), medium and phosphorylated heavy (NfH) subunits (Khalil et al., 2018). NfL and NfH levels are elevated in the CSF of MS patients compared to controls (Bridel et al., 2019; Khalil et al., 2018; Kuhle et al., 2011, 2013; Trentini et al., 2014), and levels correlate with focal inflammatory

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disease activity measures such as relapse rate and new T2 lesions (Sellebjerg et al., 2018; Disanto et al., 2017; Barro et al., 2018; Damasceno et al., 2019; Kuhle et al., 2019; Dalla Costa et al., 2019; Varhaug et al., 2018; Kuhle et al., 2013). Neurofilament levels may also capture progressive neuronal damage in MS, which is thought to underlie disability progression. Indeed, CSF-NfH levels were reported to correlate with clinical disease severity measures (Khalil et al., 2013; Kuhle et al., 2011; Petzold et al., 2016) and to relate to neurodegeneration measures on MRI (Khalil et al., 2013; Petzold et al., 2016).

The development of an ultrasensitive NfL immunoassay on Simoa (Quanterix) (Rissin et al., 2010), has allowed robust measurement of serum NfL (Kuhle et al., 2016). Consequently, it became possible to demonstrate that serum-NfL has clear relationships with measures of acute disease activity and decreases upon effective treatment (Khalil et al., 2018; Barro et al., 2018; Kuhle et al., 2019; Novakova et al., 2017). The assessment of NfH in blood has not yet led to reproducible results in MS (Fialova et al., 2013; Ljubisavljevic et al., 2016; Gnanapavan et al., 2013; Gresle et al., 2014; Kuhle et al., 2017). So far, studies reporting NfH values in blood in MS applied traditional immunoassays (Fialova et al., 2013; Ljubisavljevic et al., 2016; Gnanapavan et al., 2013; Gresle et al., 2014; Kuhle et al., 2017). Conflicting results are likely due to poor assay sensitivity hampering reliable sample measurement. In this study, we first aimed to compare the analytical sensitivity of three bead-based immunoassays developed on high-sensitive analytical platforms, comprising two Simoa assays (commercially available and in-house developed) and one Luminex assay (in-house developed). Subsequently, we selected the best performing assay to explore the potential of serum-NfH as a biomarker for inflammatory disease burden, neuronal damage and clinical disease severity in MS. As a comparative analysis, we investigated the relationships of CSF-NfH, serum-NfL and CSF-NfL with these severity measures as well.

2. Materials and methods

2.1. Subjects

We included 76 MS patients, who visited the Amsterdam UMC MS center between 2000 and 2004. Patients were diagnosed with MS according to McDonald criteria (McDonald et al., 2001) and subtyped according to Lublin and Reingold criteria (Lublin and Reingold, 1996) ($n = 64$ relapse-onset MS ($n = 38$ RRMS; $n = 26$ SPMS) and $n = 12$ primary progressive MS (PPMS)). Clinical disease severity was assessed by neurological examination and quantified with the Expanded Disability Status Scale (EDSS). Serum was collected by vena puncture and CSF ($n = 75$) was collected by lumbar puncture within three weeks of the clinical visit. Two patients experienced a relapse in the month preceding body fluid collection. Body fluids were biobanked until use according to consensus guidelines (Teunissen et al., 2009). The local medical ethical committee consented with this research and all patients gave written informed consent for use of biomaterials and medical data. The study was conducted in accordance with the ethical principles of the Helsinki Declaration of 1975.

2.2. MRI

MRI was performed in 73 (96%) patients using a 1.0 Tesla scanner (Siemens Magnetom Impact, Erlangen, Germany), mostly within three weeks of the serum and CSF collection ($n = 72$, 99%; one patient had a MRI scan 362 days prior to blood and CSF collection). Both T1-weighted axial pre- and post-contrast images (repetition time (TR) = 700 ms, echo time (TE = 15 ms) and T2-weighted (TR = 2700 ms, TE = 90 ms) were acquired with 5.0 mm slice thickness and 0.5 mm inter-slice gap. Using in-house developed semi-automated seed growing software, gadolinium enhanced (Gd+) lesions, black hole lesions (T1 hypo-intense lesions) and T2 lesions were quantified based on a local thresholding technique. Normalized brain volume was assessed as a measure of brain atrophy from the pre-contrast T1-weighted images using the 'Structural Image Evaluation, using Normalization, of Atrophy Cross-sectional' (SIENAX) technique (Smith et al., 2002).

2.3. Serum neurofilaments analysis

Prior to serum analysis, samples were thawed at room temperature and centrifuged at 14,000xg for 10 min to remove any debris from the samples. All samples were measured in duplicates.

2.3.1. In-house Luminex serum-NfH assay

We measured serum-NfH with an in-house developed SinglePlex xMAP® assay (Luminex), following the procedure described previously (Koel-Simmelink et al., 2014). Samples were diluted 40-fold.

2.3.2. In-house Simoa serum-NfH assay

An in-house developed Simoa (Quanterix) serum-NfH assay was developed by transferring the Luminex NfH assay set-up (Koel-Simmelink et al., 2014) onto the Simoa HD-1 platform, using the homebrew assay development kit (Quanterix). We measured serum-NfH with this automated assay with onboard 4-fold sample dilution. In short, paramagnetic carboxylated beads (Quanterix) were activated using 0.3 mg/mL EDC (ThermoScientific) and coated with 0.3 mg/mL anti-NfH monoclonal 9C9 antibody (shared by Carsten Korth; Schmitz et al., 2014). Detector antibody N4142 (Sigma Aldrich) was biotinylated using NHS-PEG4-Biotin (Thermo Scientific) in antibody to biotin challenge ratio of 40x. Reagent and sample diluent consisted of TBS + 6 mM EDTA + 1% BSA + 0.1% Tween + 10 μg/mL Rabbit IgG (DAKO, Agilent Pathology Solutions). In the first step, 25μl of 250 K assay beads with 250 K Simoa dye-encoded helper beads (Quanterix) were incubated for 30 min with 20μl 0.3 μg/mL biotinylated detector antibody and 100μl of 4-fold diluted sample or calibrator (bovine NfH protein; Neurofilament 200 kD; Progen). After a wash cycle, in step two, 100 μl 100pM enzyme streptavidin β-galactosidase (Quanterix) was added and incubated for 5 min 15 sec. After a last wash cycle, 25 μl of Resorufin β-D-galactopyranoside (Quanterix) was added and beads were pulled directly onto the imaging disk for time-lapsed fluorescent imaging.

2.3.3. Commercial Simoa serum-NfH assay

We measured serum-NfH with the commercially available Simoa™ pNF-Heavy Discovery kit (Quanterix) on the Simoa HD-1 analyzer according to manufacturer's instructions, with onboard automated 4-fold dilution.

2.3.4. Simoa serum-NfL

We measured serum-NfL with an in-house assay on the Simoa HD-1 analyzer, with onboard automated 4-fold dilution. The Uman Diagnostics antibodies (capture: mAb 47:3, detector: biotinylated mAb 2:1) were transferred onto the Simoa HD-1 platform using the homebrew assay development kit (Quanterix), as described previously (Kuhle et al., 2016).

2.4. CSF-NfH and CSF-NfL measurements

CSF-NfH was measured with the in-house NfH Luminex assay (Koel-Simmelink et al., 2014) and CSF-NfL was measured with the commercially available NF-light® ELISA assay (Uman Diagnostics).

2.4.1. Assay performance comparison and statistical analyses

We used SPSS for windows (version 24.0) for data analysis, and R (version 3.4.2) to construct graphs. $p<0.05$ was considered statistically significant, and $p<0.10$ a statistical trend. When duplicate serum-NfH values were at or below blank reading (i.e. non-detectable), we assigned the concentration 0. When at least one of the duplicate measurements gave a detectable serum-NfH concentration, we counted the

measurement as detectable and used the monoplo concentration for statistical analysis. Coefficients of variation (CV; standard deviation divided by the mean) of the detectable duplicate serum-NfH measurements were calculated. We plotted %CV against the detected concentrations to visualize precision for each assay. A local regression line (LOESS) was fitted to the precision plots, and lower limit of quantification (LLOQ) was extracted at the point where the fitted line crossed 20%CV. We did not exclude serum-NfH values below LLOQ or values with a duplicate %CV above 20% from statistical analysis. We conducted age and sex-adjusted linear regression analyses between natural log-transformed neurofilament levels and clinical or MRI measures Additionally, we conducted Spearman's correlation analysis between neurofilament levels. Analyses were performed both on the total cohort and restricted to the relapse-onset subset (i.e. RRMS + SPMS).

3. Results

3.1. Comparison of the analytical performance of the three serum-NfH assays

The commercially available Simoa assay could detect serum-NfH in all samples, of which serum-NfH level was below LLOQ for 10/76 (13%) samples. The in-house developed Simoa assay could detect serum-NfH in 53/76 (70%) samples, of which 31 were below the LLOQ. The Luminex assay could detect serum-NfH in 46/76 (61%) samples, of which 21 were below the LLOQ. Average intra-assay precision of detectable duplicate values was 11%CV for the commercial Simoa assay (calculated on 74/76 samples; 11/74 samples had%CV>20%), 22%CV for the in-house Simoa assay (calculated on 30/76 samples; 9/30 samples had%CV>20%) and 30%CV for the Luminex assay (calculated on 46/76 samples; 21/46 samples had%CV>20%). Precision profiles (supplemental figure 1) show a notably steeper increase in %CV at lower serum-NfH values for the Luminex (supplemental figure 1A) and in-house Simoa (supplemental figure 1C) assays, as compared to the commercial Simoa assay (supplemental figure 1E).

Correlations between the serum-NfH levels generated by the three assays showed a strong correlation of Spearman's rho = 0.90 ($p < 0.001$) between both Simoa serum-NfH measurements, whereas the serum-NfH Luminex measurement correlated with rho = 0.44 ($p < 0.001$) and rho = 0.48 ($p < 0.001$) with the commercial Simoa and in-house Simoa assays respectively (supplemental figure 2).

Because of its highest sensitivity (i.e. maximal detectability) and highest robustness (i.e. lowest mean duplicate %CV) among the three assays investigated, we selected the commercial Simoa assay for further clinical validation.

3.2. Neurofilament levels in relation to MS clinical features and MRI measures

The MS patients had a median (interquartile range (IQR)) age of 46 (38 – 53) years and median disease duration prior to serum sampling of 11 (IQR: 4 – 20) years (Table 1). Measured by the commercially available Simoa assay, the cohort's median serum-NfH level was 48 pg/mL (IQR: 26 – 111). In RRMS, median serum-NfH was 37 pg/mL (IQR: 23 – 78), in SPMS this was 67 pg/mL (IQR: 29 – 163) and in PPMS this was 72 pg/mL (IQR: 32 – 111).

Relationships between serum-NfH measured by the commercially available Simoa assay and MRI and clinical outcome measures are presented in Table 2 and Fig. 1. Adjusted for age and sex, in the total study cohort we observed positive moderate associations between serum-NfH and T2 lesion volume (standardized (s)β = 0.23, $p = 0.041$) but not T2 lesion count, a trend for a positive association with black hole count (sβ = 0.21, $p = 0.084$) but not black hole volume and a positive association with disease duration (standardized beta (sβ) = 0.28, $p = 0.034$). When focusing on the relapse-onset subset, serum-NfH tended to positively associate with T2 lesion volume (sβ = 0.24, $p = 0.053$) and remained

Table 1
Demographics and clinical characteristics of the MS cohort.

	Total cohort	Relapse-onset subset
	$n = 76$	$n = 64$
Characteristics		
Age, years	46 (38 – 53)	46 (36 – 53)
Female (%)	36 (47%)	32 (50%)
MS subtype: RRMS/SPMS/PPMS	38 / 26 / 12	38 / 26 / 0
Disease duration, years	11 (4 – 20) (	11 (4 – 20)
EDSS	4 (3 – 6)	4 (3 – 6)
Interferon-beta used (%)	24 (32%)	23 (36%)
MRI measures		
Normalized brain volume, cm^3	-	1197 (1134 – 1281)
Black hole count	3 (2 – 14)	3 (2 – 12)
Black hole volume, mm^3	226 (58 – 1189)	188 (55 – 1188)
T2 lesion count	25 (11 – 41)	25 (11– 44)
T2 lesion volume, mm^3	3736 (939 – 9639)	4011 (1100 – 9981)
Gd+ lesions present	10 (13%)	9 (14%)
Serum measures		
NfH, pg/mL, Commercial Simoa	48 (26 – 111)	46 (25 – 111)
NfH, pg/mL, In-house Simoa	1.7 (0 – 18)	1.8 (0 – 20)
NfH, pg/mL, Luminex	18 (0 – 61)	21 (0 – 61)
NfL, pg/mL	13 (9 – 19)	13 (9 – 18)
CSF measures		
NfH, pg/mL,	605 (453 – 796)	574 (453 – 790)
NfL, pg/mL,	665 (480 – 925)	665 (480 – 925)

Demographic features of the total cohort (RRMS, SPMS, PPMS), and for the relapse-onset subset (RRMS and SPMS). Features are presented as median (interquartile range: 25th – 75th percentile) or n(%). Normalized brain volume was unavailable for the PPMS group. Serum-NfL was available for 71 patients. CSF-NfH and CSF-NfL were available for 75 patients. When the immunoassay could not detect serum-NfH, we assigned the value 0 pg/mL. RRMS=relapsing-remitting multiple sclerosis, SPMS=secondary progressive multiple sclerosis, PPMS=primary progressive multiple sclerosis, EDSS=Expanded Disability Status Scale, Gd+=Gadolineum enhanced, NfH=Neurofilament heavy, Simoa=Single Molecule Array, NfL=Neurofilament light, CSF=Cerebrospinal fluid.

Table 2
Neurofilaments levels in relation to MS clinical features and MRI measures.

		Serum-NfH sβ	CSF-NfH sβ	Serum-NfL sβ	CSF-NfL sβ
Normalized brain volume	Total cohort	–	–	–	–
	Relapse-onset subset	−0.19	−0.42 **	0.00	−0.05
T2 lesion count	Total cohort	0.08	0.22	0.23	0.20
	Relapse-onset subset	0.05	0.28 *	0.21	0.26 *
T2 lesion volume	Total cohort	0.23 *	0.17	0.17	0.07
	Relapse-onset subset	0.24	0.17	0.16	0.09
Black hole count	Total cohort	0.24	0.12	0.15	0.12
	Relapse-onset subset	0.22	0.10	0.15	0.07
Black hole volume	Total cohort	0.12	0.13	0.06	0.09
	Relapse-onset subset	0.12	0.12	0.07	0.07
EDSS	Total cohort	0.13	0.33 **	0.12	−0.02
	Relapse-onset subset	0.20	0.29 *	0.15	−0.06
Disease duration	Total cohort	0.28 *	0.06	0.15	−0.07
	Relapse-onset subset	0.41 **	−0.03	0.20	−0.10

Results are presented as standardized betas, obtained from linear regression analysis adjusted for relevant confounders age and sex, with natural log-transformed neurofilament data as independent and disease activity and MRI parameters as dependent variables. Normalized brain volume was available only for the relapse-onset subset. Analysis was performed both on the total dataset and on the relapse-onset subset (RRMS and SPMS cases) * $p<0.05$ ** $p<0.01$.

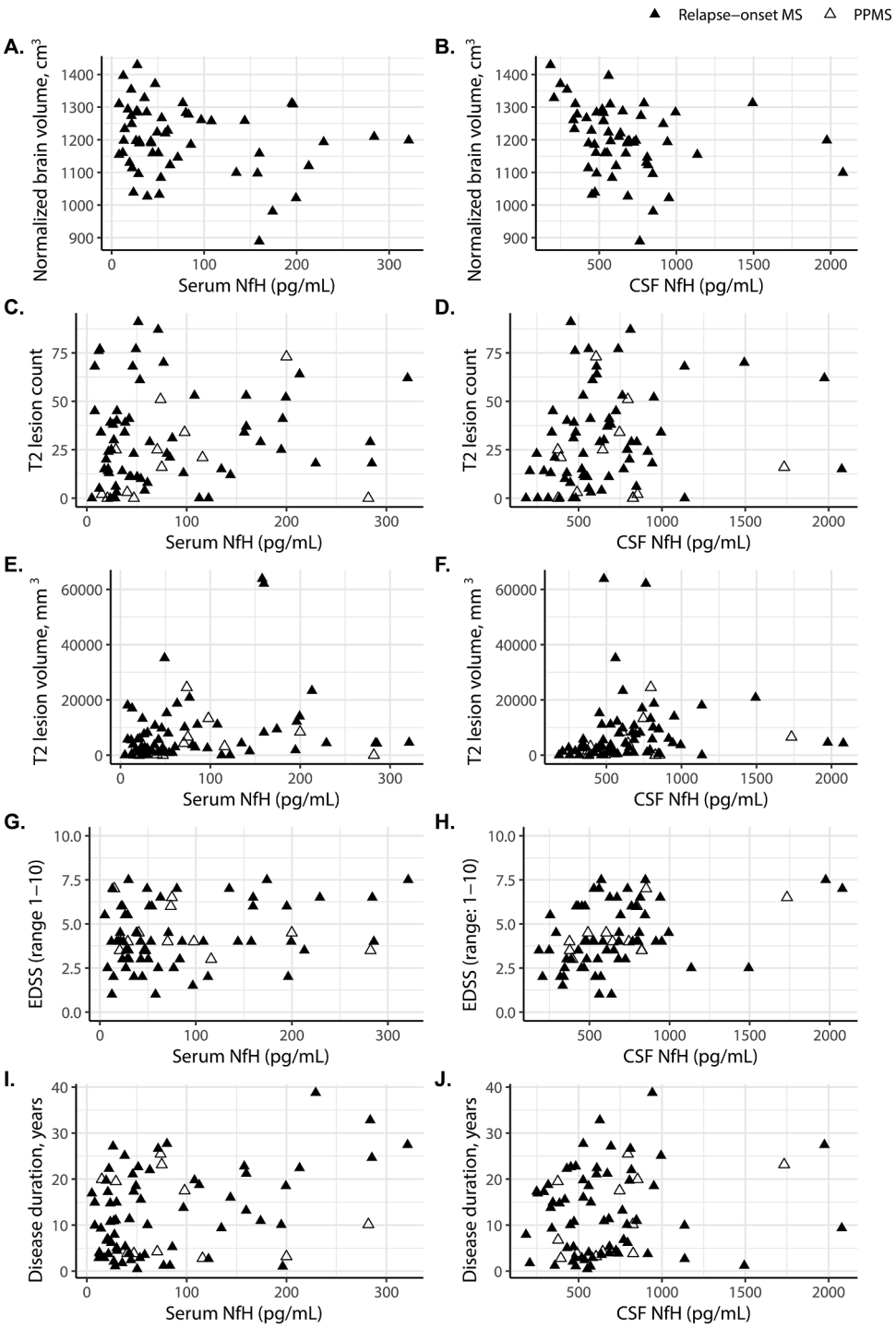

Fig. 1. Correlation of serum-NfH and CSF-NfH with MS clinical features and MRI measures
Scatterplots for the total study cohort ($n = 76$). Closed triangles represent the relapse-onset subset (RRMS and SPMS cases), open triangles are the PPMS cases. Serum NfH was measured by the commercial Simoa assay. NfH=Neurofilament heavy, CSF=Cerebrospinal fluid, EDSS=Expanded Disability Status Scale.

positively associated with disease duration ($s\beta = 0.41$, $p = 0.006$). No relationship of serum-NfH with normalized brain volume or EDSS was observed.

As comparative analysis, we investigated relationships of CSF-NfH, serum-NfL and CSF-NfL with clinical features and MRI outcome measures (Table 2, Fig. 2). In contrast to serum-NfH, in the total study cohort CSF-NfH tended to positively associate with T2 lesion count ($s\beta = 0.22$, $p = 0.053$) and positively associated with EDSS ($s\beta = 0.33$, $p = 0.007$) (Table 2, Fig. 2). In the relapse-onset subset, CSF-NfH showed a positive association with T2 lesion count ($s\beta$=0.28, $p = 0.024$), a negative association with normalized brain volume ($s\beta$=−0.42, $p = 0.002$), and a positive association with EDSS ($s\beta = 0.29$, $p = 0.033$). Serum-NfL and CSF-NfL showed a trend to a moderate positive association with T2 lesion count in the total study cohort (serum-NfL: $s\beta$=0.23, $p = 0.064$; CSF-NfL: $s\beta = 0.20$, $p = 0.085$), but were not associated with any of the other MRI or clinical measures. In the relapse-onset subset, this relationship with T2 lesion count disappeared for serum-NfL ($s\beta = 0.21$, $p = 0.113$) whereas it became significant for CSF-NfL ($s\beta = 0.26$, $p = 0.041$).

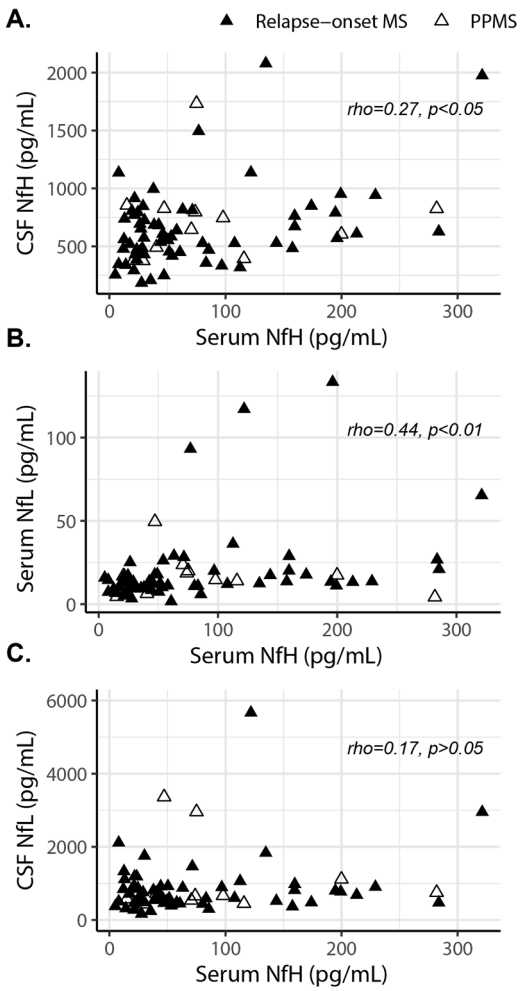

Fig. 2. Correlation of serum-NfH (commercial Simoa assay) with CSF-NfH, serum-NfL and CSF-NfL

Scatterplots including spearman's rho correlation coefficients for the total study cohort ($n = 76$). Closed triangles represent the relapse-onset subset (RRMS and SPMS cases). Serum-NfH was assessed using the commercial Simoa assay. CSF neurofilament data was missing for one subject. NfH=Neurofilament heavy, CSF=Cerebrospinal fluid, NfL=Neurofilament light.

3.3. Correlations of serum-NfH with CSF-NfH, serum-NfL and CSF-NfL

Serum-NfH assessed by the commercial Simoa assay correlated with CSF-NfH (Table 3, Fig. 2A), with comparable Spearman's correlation coefficients in the total study cohort (rho = 0.27, $p = 0.018$) and in the relapse-onset subset (rho = 0.26, $p = 0.036$). Serum-NfH correlated with serum-NfL as well (Table 3, Fig. 2B), again with comparable correlation coefficients in the total study cohort (rho = 0.44, $p < 0.001$) and the relapse-onset subset (rho = 0.49, $p < 0.001$). No correlation between serum-NfH and CSF-NfL levels was observed (Table 3, Fig. 2C).

Table 3

Spearman's correlation of serum-NfH (commercial Simoa assay) in relation to CSF-NfH, serum-NfL and CSF-NfL.

Total cohort	CSF-NfH	Serum-NfL	CSF-NfL
Serum-NfH	0.27 *	0.44 **	0.17
Relapse-onset subset			
Serum-NfH	0.26 *	0.49 **	0.11

Spearman's rho correlation coefficients for the total study cohort ($n = 76$) and for the relapse-onset subset only ($n = 64$; RRMS and SPMS), between serum-NfH measured by the commercially available Simoa assay and CSF-NfH, serum-NfL and CSF-NfL. CSF neurofilament data was missing for one subject. * $p<0.05$, ** $p<0.01$. NfH=Neurofilament heavy, NfL=Neurofilament light, CSF=Cerebrospinal fluid.

4. Discussion

We found that NfH levels in serum of MS patients can be detected with an ultrasensitive immunoassay. With this assay, serum-NfH levels were associated with T2 lesion volume and disease duration, and there was a tendency for an association with black hole count. These findings suggest that serum-NfH might have some utility as biomarker of disease burden in MS.

Analytical sensitivity was an issue in four out of five previously published studies on blood-based NfH as biomarker for MS (Fialova et al., 2013; Ljubisavljevic et al., 2016; Gnanapavan et al., 2013; Gresle et al., 2014; Kuhle et al., 2017). These studies all applied conventional ELISA immunoassays from different vendors. We reasoned that high-sensitive technology is needed for reliable serum-NfH measurement. We first performed a comparison of three different phosphorylated serum-NfH immunoassays, on two different platforms: a Luminex assay, an in-house developed Simoa assay using the same antibodies and comparable buffers as the Luminex assay, and a commercially available Simoa assay. All three assays were bead-based, thus capture-antibodies are coupled to paramagnetic beads instead of coated on the surface of 96-well plates. In bead-based assays, incubation steps are more effective compared to ELISAs, because the capture beads are in suspension with samples and as such can easier encounter antigens. Additionally, the washing steps in bead-based assays are more effective compared to ELISA, by using the magnetic properties of the beads resulting in reduced background signals thus increased signal to noise ratios. Simoa has an additional advantage over Luminex, because in Simoa the beads are pulled into femtoliter-sized reaction chambers that are sized to fit no more than one bead (Rissin et al., 2010). Due to this extremely low reaction volume a minimal increase in fluorescent signal can be detected, whereas in Luminex this minimal increase in signal would diffuse in the larger reaction volume and consequently remain below detection limit. In our assay comparison, we found that the correlation of serum-NfH levels between the Luminex assay and both Simoa assays was substantially weaker (rho<0.48), as compared to the between-Simoa assays correlation (rho = 0.90), even though the Simoa assays employed different antibodies. This weaker correlation suggested that the Luminex results were more hampered by lack of measurement reliability compared to Simoa, which fits with the technological benefits Simoa has over Luminex (Rissin et al., 2010). Still, only the commercial Simoa assay yielded 100% serum-NfH detectability in the samples, indicating higher analyte-affinity of the antibodies as compared to the in-house Simoa assay. It is to note that still not all samples were measured above LLOQ and with a reliable%CV of duplicate measurements however, showing that serum-NfH measurement remains challenging. A recent study applied a similar design as our study, and compared serum-NfH assay performance in patients with amyotrophic lateral sclerosis and frontotemporal dementia (Wilke et al., 2019). Serum-NfH levels are generally higher in those patients as compared to MS patients as included in our study. Yet, their conclusion that serum-NfH can be most reliably measured by a Simoa assay and not by lower-sensitivity alternatives supports our conclusion.

Upon exploration of the possible clinical value of serum-NfH in MS, we observed positive relationships of serum-NfH with T2 lesion volume, and a trend for a positive relationship with black hole count. The correlations were moderate, which could be due to the fact that the T2 lesions and black holes were not aged precisely, and a proportion of these lesions may be old and inactive. We hypothesized that serum-NfH reflects neuronal damage, which increases with MS disease duration. Similarly, T2 and black hole lesion load increases with disease duration. The positive relationships we observed between serum-NfH and T2 lesions and black holes support this hypothesis. We did not find significant

associations of serum-NfH with normalized brain volume and EDSS, although the direction of the effect sizes were in line with expectations. Since previous studies on serum-NfH in MS were hampered by analytical sensitivity (Fialova et al., 2013; Ljubisavljevic et al., 2016; Gnanapavan et al., 2013; Gresle et al., 2014; Kuhle et al., 2017), and there were notable cohort differences between our study and those previous studies (e.g. inclusion of only SPMS patients (Gnanapavan et al., 2013), additional inclusion of unaffected controls (Gresle et al., 2014; Ljubisavljevic et al., 2016) or patients with one clinically isolated event of MS (Ljubisavljevic et al., 2016) and/or larger sample sizes (Gresle et al., 2014)), we are limited in comparing our findings against findings of previous studies. We however did a comparative analysis with other neurofilament measures in both CSF and serum in this cohort. We made seemingly contrasting observations, with serum-NfH being associated with disease duration, T2 lesion volume and black hole count (trend), and CSF-NfH being associated with EDSS, T2 lesion count and normalized brain volume. Also, we only observed a moderate correlation between serum-NfH and CSF-NfH levels (rho = 0.27). Our results for the relation of CSF-NfH with clinical parameters are in line with several previous studies (Trentini et al., 2014; Kuhle et al., 2011; Khalil et al., 2013; Fialova et al., 2013). Relations between CSF-NfH and MRI parameters have been less studied. The one study that explored such relationships investigated clinically isolated syndrome (CIS) patients who have lower amounts of axonal loss compared to clinically definite MS, and in contrast to our study they did not observe any relationships between CSF-NfH and T2 lesions or normalized brain volume (Khalil et al., 2013). Not all patients with CIS will develop definite MS and according to two previous studies, CSF-NfH levels were not elevated yet in CIS patients that eventually convert to MS (Khalil et al., 2013; Arrambide et al., 2016), which could potentially explain our discrepancy in findings.

Based on previous literature on NfL, we expected that we would observe relationships between increased NfL and worse MS disease severity scores (Barro et al., 2018; Dalla Costa et al., 2019; Disanto et al., 2017; Kuhle et al., 2013; Sellebjerg et al., 2018) or MRI outcomes (Disanto et al., 2017; Barro et al., 2018; Damasceno et al., 2019; Kuhle et al., 2019; Dalla Costa et al., 2019; Varhaug et al., 2018). However, we only observed a trend for a relationship of serum-NfL and CSF-NfL with T2 lesion count, while for serum-NfH and CSF-NfH various relationships were shown. Taken together, these data suggest that NfH measurement in MS, in addition to NfL, could be of added clinical value.

The strength of our study lies in the selection of several high sensitive serum-NfH assays that were compared head-to-head in a relevant cohort of MS patients with paired serum and CSF samples available. With the emergence of more treatment possibilities for MS with potential beneficial effect on disability progression, the need for easy-to-use dynamic tools to monitor focal inflammatory activity and neurodegeneration becomes stronger, making the current study timely. Among the limitations is that we conducted the assay comparison only for the serum-NfH measurement. As a consequence, different assays and platforms were used for the CSF and the serum measurements, which might have influenced our comparisons of the observed relationships of the various neurofilament measures with the clinical and MRI measures. Although we could detect serum-NfH in all samples with the commercial Simoa assay, still some samples were measured below LLOW or with a duplicate %CV higher than 20%. Since the selected cohort was not very large, statistical analyses for clinical validity were regarded as exploratory and therefore not corrected for multiple testing. Furthermore, we did not include healthy controls, since it was our aim to investigate associations with MS disease burden and not investigate diagnostic utility for differentiating MS patients and controls. It is recommended to repeat the comparative serum (and CSF) NfH and NfL analysis in independent cohorts, preferably with data on relapses and medication as well as longitudinal serum samples and longitudinal clinical data. Longitidudinal analysis is recommended, because likely, neurofilament biomarkers NfH and NfL are most useful in therapeutic effectiveness monitoring (i.e. having increased or decreased levelslevels compared to previous visits).

To conclude, serum-NfH can be assessed with a high-sensitivity assay and showed some potential as biomarker for neuro-axonal disease burden in MS.

5. Author's contributions

IV conceptualized the study, curated and formally analysed the data and wrote the original draft. CT and CB conceptualized the study, and reviewed and edited the text. MK, HT, HV, CK and JK curated the data and reviewed and edited the text. All authors read and approved the final manuscript.

Declaration of Competing Interest

IV, MK, HT, CK, CB have nothing to disclose. HV has received research grants from Pfizer, MerckSerono, Novartis and Teva, speaker honoraria from Novartis, and consulting fees from MerckSerono; all funds were paid directly to his institution. JK has accepted speaker and consulting fees from Merck, Biogen, TEVA, Sanofi, Genzyme, Roche and Novartis. CT has a collaboration contract with ADx Neurosciences, performed contract research or received grants from Probiodrug, AC Immune, Biogen-Esai, CogRx, Toyama, Janssen prevention center, Boehringer, AxonNeurosciences, Fujirebio, EIP farma, PeopleBio and Roche.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2021.102840.

References

Thompson, A.J., Baranzini, S.E., Geurts, J., Hemmer, B., Ciccarelli, O., 2018. Multiple sclerosis. Lancet 391 (10130), 1622–1636.
Ziemssen, T., De Stefano, N., Sormani, M.P., Van Wijmeersch, B., Wiendl, H., Kieseier, B. C., 2015. Optimizing therapy early in multiple sclerosis: an evidence-based view. Mult. Scler Relat. Disord. 4 (5), 460–469.
Khalil, M., Teunissen, C.E., Otto, M., Piehl, F., Sormani, M.P., Gattringer, T., et al., 2018. Neurofilaments as biomarkers in neurological disorders. Nat. Rev. Neurol. 14 (10), 577–589.
Petzold, A., 2005. Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. J. Neurol. Sci. 233 (1–2), 183–198.
Kuhle, J., Plattner, K., Bestwick, J.P., Lindberg, R.L., Ramagopalan, S.V., Norgren, N., et al., 2013. A comparative study of CSF neurofilament light and heavy chain protein in MS. Mult. Scler. 19 (12), 1597–1603.
Trentini, A., Comabella, M., Tintore, M., Koel-Simmelink, M.J., Killestein, J., Roos, B., et al., 2014. N-acetylaspartate and neurofilaments as biomarkers of axonal damage in patients with progressive forms of multiple sclerosis. J. Neurol. 261 (12), 2338–2343.
Kuhle, J., Leppert, D., Petzold, A., Regeniter, A., Schindler, C., Mehling, M., et al., 2011. Neurofilament heavy chain in CSF correlates with relapses and disability in multiple sclerosis. Neurology 76 (14), 1206–1213.
Bridel, C., van Wieringen, W.N., Zetterberg, H., Tijms, B.M., Teunissen, C.E., the, N.F.L. G., et al., 2019. Diagnostic value of cerebrospinal fluid neurofilament light protein in neurology: a systematic review and meta-analysis. JAMA Neurol.
Sellebjerg, F., Royen, L., Soelberg Sorensen, P., Oturai, A.B., Jensen, P.E.H, 2018. Prognostic value of cerebrospinal fluid neurofilament light chain and chitinase-3-like-1 in newly diagnosed patients with multiple sclerosis. Mult. Scler. 25 (11), 1352458518794308.
Disanto, G., Barro, C., Benkert, P., Naegelin, Y., Schadelin, S., Giardiello, A., et al., 2017. Serum Neurofilament light: a biomarker of neuronal damage in multiple sclerosis. Ann. Neurol. 81 (6), 857–870.

Barro, C., Benkert, P., Disanto, G., Tsagkas, C., Amann, M., Naegelin, Y., et al., 2018. Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis. Brain 141 (8), 2382–2391.
Damasceno, A., Dias-Carneiro, R.P.C., Moraes, A.S., Boldrini, V.O., Quintiliano, R.P.S., da Silva, V., et al., 2019. Clinical and MRI correlates of CSF neurofilament light chain levels in relapsing and progressive MS. Mult Scler Relat. Disord. 30, 149–153.
Kuhle, J., Kropshofer, H., Haering, D.A., Kundu, U., Meinert, R., Barro, C., et al., 2019. Blood neurofilament light chain as a biomarker of MS disease activity and treatment response. Neurology 92 (10) e1007-e15.
Dalla Costa, G., Martinelli, V., Sangalli, F., Moiola, L., Colombo, B., Radaelli, M., et al., 2019. Prognostic value of serum neurofilaments in patients with clinically isolated syndromes. Neurology 92 (7) e733-e41.
Varhaug, K.N., Barro, C., Bjornevik, K., Myhr, K.M., Torkildsen, O., Wergeland, S., et al., 2018. Neurofilament light chain predicts disease activity in relapsing-remitting MS. Neurol. Neuroimmunol. Neuroinflamm. 5 (1), e422.
Kuhle, J., Malmestrom, C., Axelsson, M., Plattner, K., Yaldizli, O., Derfuss, T., et al., 2013. Neurofilament light and heavy subunits compared as therapeutic biomarkers in multiple sclerosis. Acta Neurol. Scand. 128 (6), e33–e36.
Khalil, M., Enzinger, C., Langkammer, C., Ropele, S., Mader, A., Trentini, A., et al., 2013. CSF neurofilament and N-acetylaspartate related brain changes in clinically isolated syndrome. Mult. Scler. 19 (4), 436–442.
Petzold, A., Steenwijk, M.D., Eikelenboom, J.M., Wattjes, M.P., Uitdehaag, B.M., 2016. Elevated CSF neurofilament proteins predict brain atrophy: a 15-year follow-up study. Mult. Scler. 22 (9), 1154–1162.
Rissin, D.M., Kan, C.W., Campbell, T.G., Howes, S.C., Fournier, D.R., Song, L., et al., 2010. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat. Biotechnol. 28 (6), 595–599.
Kuhle, J., Barro, C., Andreasson, U., Derfuss, T., Lindberg, R., Sandelius, A., et al., 2016. Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. Clin. Chem. Lab. Med. 54 (10), 1655–1661.
Novakova, L., Zetterberg, H., Sundstrom, P., Axelsson, M., Khademi, M., Gunnarsson, M., et al., 2017. Monitoring disease activity in multiple sclerosis using serum neurofilament light protein. Neurology 89 (22), 2230–2237.
Fialova, L., Bartos, A., Svarcova, J., Zimova, D., Kotoucova, J., 2013. Serum and cerebrospinal fluid heavy neurofilaments and antibodies against them in early multiple sclerosis. J. Neuroimmunol. 259 (1–2), 81–87.
Ljubisavljevic, S., Stojanovic, I., Basic, J., Pavlovic, D.A., 2016. The Validation study of neurofilament heavy chain and 8-hydroxy-2′-deoxyguanosine as Plasma Biomarkers of Clinical/paraclinical activity in first and relapsing-remitting demyelination acute attacks. Neurotox. Res. 30 (3), 530–538.
Gnanapavan, S., Grant, D., Morant, S., Furby, J., Hayton, T., Teunissen, C.E., et al., 2013. Biomarker report from the phase II lamotrigine trial in secondary progressive MS - neurofilament as a surrogate of disease progression. PLoS ONE 8 (8) e70019.
Gresle, M.M., Liu, Y., Dagley, L.F., Haartsen, J., Pearson, F., Purcell, A.W., et al., 2014. Serum phosphorylated neurofilament-heavy chain levels in multiple sclerosis patients. J. Neurol. Neurosurg. Psychiatry 85 (11), 1209–1213.
Kuhle, J., Nourbakhsh, B., Grant, D., Morant, S., Barro, C., Yaldizli, O., et al., 2017. Serum neurofilament is associated with progression of brain atrophy and disability in early MS. Neurology 88 (9), 826–831.
McDonald, W.I., Compston, A., Edan, G., Goodkin, D., Hartung, H.P., Lublin, F.D., et al., 2001. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann. Neurol. 50 (1), 121–127.
Lublin, F.D., Reingold, S.C., 1996. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. Neurology 46 (4), 907–911.
Teunissen, C.E., Petzold, A., Bennett, J.L., Berven, F.S., Brundin, L., Comabella, M., et al., 2009. A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. Neurology 73 (22), 1914–1922.
Smith, S.M., Zhang, Y., Jenkinson, M., Chen, J., Matthews, P.M., Federico, A., et al., 2002. Accurate, robust, and automated longitudinal and cross-sectional brain change analysis. Neuroimage 17 (1), 479–489.
Koel-Simmelink, M.J., Vennegoor, A., Killestein, J., Blankenstein, M.A., Norgren, N., Korth, C., et al., 2014. The impact of pre-analytical variables on the stability of neurofilament proteins in CSF, determined by a novel validated SinglePlex Luminex assay and ELISA. J. Immunol. Methods 402 (1–2), 43–49.
Schmitz, M., Greis, C., Ottis, P., Silva, C.J., Schulz-Schaeffer, W.J., Wrede, A., et al., 2014. Loss of prion protein leads to age-dependent behavioral abnormalities and changes in cytoskeletal protein expression. Mol. Neurobiol. 50 (3), 923–936.
Wilke, C., Pujol-Calderon, F., Barro, C., Stransky, E., Blennow, K., Michalak, Z., et al., 2019. Correlations between serum and CSF pNfH levels in ALS, FTD and controls: a comparison of three analytical approaches. Clin. Chem. Lab. Med. 57 (10), 1556–1564.
Arrambide, G., Espejo, C., Eixarch, H., Villar, L.M., Alvarez-Cermeno, J.C., Picon, C., et al., 2016. Neurofilament light chain level is a weak risk factor for the development of MS. Neurology 87 (11), 1076–1084.