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Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis

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Neuro-axonal injury is a key factor in the development of permanent disability in multiple sclerosis. Neurofilament light chain in peripheral blood has recently emerged as a biofluid marker reflecting neuro-axonal damage in this disease. We aimed at comparing serum neurofilament light chain levels in multiple sclerosis and healthy controls, to determine their association with measures of disease activity and their ability to predict future clinical worsening as well as brain and spinal cord volume loss. Neurofilament light chain was measured by single molecule array assay in 2183 serum samples collected as part of an ongoing cohort study from 259 patients with multiple sclerosis (189 relapsing and 70 progressive) and 259 healthy control subjects. Clinical assessment, serum sampling and MRI were done annually; median follow-up time was 6.5 years. Brain volumes were quantified by structural image evaluation using normalization of atrophy, and structural image evaluation using normalization of atrophy, cross-sectional, cervical spinal cord volumes using spinal cord image analyser (*cordial*). Results were analysed using ordinary linear regression models and generalized estimating equation modelling. Serum neurofilament light chain was higher in patients with a clinically isolated syndrome or relapsing remitting multiple sclerosis as well as in patients with secondary or primary progressive multiple sclerosis than in healthy controls (age adjusted $P < 0.001$ for both). Serum neurofilament light chain above the 90th percentile of healthy controls values was an independent predictor of Expanded Disability Status Scale worsening in the subsequent year ($P < 0.001$). The probability of Expanded Disability Status Scale worsening gradually increased by higher serum neurofilament light chain percentile category. Contrast enhancing and new/enlarging lesions were independently associated with increased serum neurofilament light chain (17.8% and 4.9% increase per lesion respectively; $P < 0.001$). The higher the serum neurofilament light chain percentile level, the more pronounced was future brain and cervical spinal volume loss: serum neurofilament light chain above the 97.5th percentile was associated with an additional average loss in brain volume of 1.5% ($P < 0.001$) and spinal cord volume of 2.5% over 5 years ($P = 0.009$). Serum neurofilament light chain correlated with concurrent and future clinical and MRI measures of disease activity and severity. High serum neurofilament light chain levels were associated with both brain and spinal cord volume loss. Neurofilament light chain levels are a real-time, easy to measure marker of neuro-axonal injury that is conceptually more comprehensive than brain MRI.

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Abbreviations: EDSS = Expanded Disability Status Scale; (s)NfL = (serum) neurofilament light chain

Introduction

Multiple sclerosis is a chronic immune-mediated disease of the CNS, leading to demyelination and neurodegeneration. Neuro-axonal injury represents the key morphological correlate for long-term disability progression (Compston and Coles, 2008). Biomarkers reflecting tissue damage, subclinical disease activity and therapeutic response are urgently needed both for clinical drug development and individual therapeutic decision-making for multiple sclerosis patients.

Neurofilament light chain (NfL) represents one of the main constituents of the neuronal cytoskeleton and plays an important role in axonal growth, stability and intracellular transport (Yabe *et al.*, 2001). Previous studies have shown that NfL levels in CSF are associated with the occurrence of MRI lesions, relapses, neurological disability and treatment status in multiple sclerosis (Teunissen *et al.*, 2009*a*; Gunnarsson *et al.*, 2011; Disanto *et al.*, 2017; Hakansson *et al.*, 2017; Novakova *et al.*, 2017). The advent of a more sensitive method, single molecule array technology (Simoa) now allows the reliable quantification of NfL in plasma or serum (Gisslén *et al.*, 2015; Kuhle *et al.*, 2016*a*). Several studies in multiple sclerosis and other diseases have recently demonstrated that serum and CSF NfL concentrations are highly correlated, suggesting that serum NfL (sNfL) could represent a reliable blood-derived biomarker of neuro-axonal injury (Gaiottino *et al.*, 2013; Lu *et al.*, 2015; Bacioglu *et al.*, 2016; Kuhle *et al.*, 2016*c*; Weydt *et al.*, 2016; Piehl *et al.*, 2017). In a recent study we have shown that sNfL levels: (i) can be reliably measured in multiple sclerosis patients; (ii) closely reflect CSF NfL levels; (iii) are positively associated with MRI lesion load; (iv) increase after the occurrence of clinical relapses; and (v) decrease after initiating a disease-modifying treatment (Disanto *et al.*, 2017). Preliminary findings also suggested that patients with higher sNfL levels have worse later clinical disease outcomes and more brain atrophy (Disanto *et al.*, 2017; Kuhle *et al.*, 2017*b*). However, in these studies, systematic long-term clinical and quantitative brain MRI follow-up was not available, and the correlation of sNfL with spinal cord atrophy was not investigated at all (Disanto *et al.*, 2017).

Here, we investigate a well-characterized cohort of 259 patients with multiple sclerosis with long-term MRI and clinical follow-up and 259 healthy control subjects aiming at: (i) replicating in a second independent cohort the association of sNfL with measures of concurrent and past clinical disease activity as well as its ability to predict future worsening on the Expanded Disability Status Scale (EDSS); (ii) investigating the cross-sectional association between sNfL and quantitative MRI measures of inflammation and degeneration; and (iii) investigating the potential of sNfL to predict short- and long-term brain and spinal cord volume changes.

Materials and methods

Patients

Patients and controls were recruited in Basel as part of a prospective multicentre study initiated in 2003 (Genome-Wide Association Study of Multiple Sclerosis, GeneMSA; continued as of 2011 as Serial Unified Multicenter Multiple Sclerosis Investigation, SUMMIT) (Baranzini *et al.*, 2009; Bove *et al.*, 2017). The study included patients with all clinical subtypes of the disease (Lublin *et al.*, 1996; McDonald *et al.*, 2001; Polman *et al.*, 2005). Secondary progressive multiple sclerosis was defined by six or more months of worsening neurological disability not explained by clinical relapses. Primary progressive multiple sclerosis was defined both by progressive clinical worsening for more than 12 months from symptom onset without any relapses, and by abnormal CSF as defined by the presence of two or more oligoclonal bands or an elevated IgG index. Our study included all 259 patients who were recruited at the Neurologic Clinic and Policlinic, University Hospital Basel (Switzerland) between June 2004 and October 2005 as part of this study. All data were collected between 2 July 2004 and 17 February 2015. All patients provided written informed consent and the study was approved by the local ethics committee.

Procedures

Follow-up visits were performed outside acute clinical relapses, i.e. in case patients experienced a relapse within the previous month, the follow-up visit was postponed by 30 days. The concomitant use of disease-modifying therapies for multiple sclerosis was permitted. Annual evaluations included standardized clinical assessments with functional system score and EDSS calculation by certified raters (http://www.neurostatus.

net/). The occurrence of relapses, disability worsening (as measured by the EDSS), disease-modifying treatment initiation or interruption and disease-modifying treatment-related adverse events were recorded at each visit. Brain MRI scans were performed within 1 week of clinical visits. An overview of available time points, including serum samples and MRI data is shown in Supplementary Table 1.

Image acquisition and data analysis

Brain MRI scans were performed on all patients at baseline and then yearly in a 1.5 T magnetic resonance scanner (Magnetom Avanto, Siemens Helthineers) equipped with a 12-element head matrix coil (see Supplementary material for description of cranial image acquisition and data analysis). Cervical spinal cord volume was analysed by cord image analyser (*cordial*) (Amann *et al.*, 2016). The segmentation was carried out over a 35-mm long spinal cord segment in the cranial T_1-weighted scan (MPRAGE, see Supplementary material), starting 27 mm below the cisterna pontis, which corresponds to the spinal cord volume between the foramen magnum and the C2/C3 intervertebral disc.

Serum sampling and serum neurofilament light chain measurements

Serum samples were collected on the same day as the clinical visit and stored at −80°C following standard procedures (Teunissen *et al.*, 2009*b*). SNfL in longitudinal serum samples was measured by Simoa assay as previously described (Disanto *et al.*, 2017). Inter-assay coefficients of variation for three native serum samples were 9%, 8%, and 6% for control samples with mean concentrations of 13.5, 25.8 and 269.5 pg/ml, respectively. The mean intra-assay coefficient of variation of duplicate determinations for concentration was 7.4%. Repeat measurements were done for few samples with intra-assay coefficient of variation above 20%. One sample from two patients and one healthy control showed an sNfL value below 1.3 pg/ml (i.e. the lower limit of quantification). These two patients and one healthy control were excluded from the analysis.

Healthy control subjects

Serum samples from 259 healthy controls were collected in the Neurologic Clinic and Policlinic, University Hospital Basel, as part of the Genome-Wide Association Study of Multiple Sclerosis between July 2004 and April 2006 (Baranzini *et al.*, 2009). A second serum sample 1 year after baseline was available for 226 of these healthy controls. Healthy controls were probands or genetically-unrelated friends and spouses of the patients. Inclusion criteria for healthy control included age 18–70 years, no diagnosis of multiple sclerosis and no known cases of multiple sclerosis in the family.

Statistics

Categorical variables are described by counts and percentages, continuous and ordinal variables by median and interquartile range (IQR). For all analyses with sNfL as dependent variable, sNfL levels were log-transformed. The distribution of sNfL in healthy controls and its association with age was modelled by means of Generalized Additive Models of Location, Scale and Shape (Rigby and Stasinopoulos, 2004) and age-dependent percentiles were derived as described recently (Disanto *et al.*, 2017). To obtain an age-independent measure of sNfL elevation, the patients' sNfL levels were finally dichotomized into levels above or below a given percentile category using five cut-offs (80%, 90%, 95%, 97.5% and 99%).

Several clinical and MRI parameters were tested for association with log sNfL using generalized estimating equation (GEE) models with an 'exchangeable' correlation structure. Estimates were backtransformed to the original scale and therefore represent multiplicative effects on the geometric mean of sNfL and are denoted by β_{mult} throughout this work. The Wald method was used to calculate confidence intervals [95% confidence interval (CI)]. Model quality was inspected visually using Q-Q plots.

EDSS worsening was modelled using binominal GEE models and odds ratios were estimated (denoted by β_{OR}). EDSS worsening was defined as an increase in EDSS to the subsequent visit of $\geqslant$1.5 points from an EDSS score of 0.0, $\geqslant$1.0 point from an EDSS score of 1.0–5.5 or $\geqslant$0.5 point from an EDSS score $\geqslant$6.0. Brain volume changes were measured using Structural Image Evaluation using Normalization of Atrophy (SIENA). Percentage brain volume change at 2 and 5 years of follow-up were modelled using ordinary linear regression models. The estimates represent additive effects and are denoted by β_{add}. The percentage change in spinal cord volume was calculated over all available 2- and 5-year follow-up intervals (i.e. baseline–Year 2, Year 1–Year 3 or baseline–Year 5, Year 1–Year 6, etc.) and modelled using GEE models.

All analyses were conducted using the statistical software R (version 3.4.1) (R Development Core Team 2016).

Results

Serum neurofilament light chain levels in healthy controls and reference percentile curves

The median (IQR) age in 258 healthy control subjects was 44.3 (36.3–52.4) years and 177 (68.6%) were females. The median sNfL level was 23.6 (18.4–31.3) pg/ml with similar values for males [23.0 (17.6–30.3) pg/ml] and females [24.5 (18.7–31.7) pg/ml, $P = 0.757$]. A significant increase in sNfL was observed with age, with a 2.2% increase in sNfL per year (estimated multiplicative effect $\beta_{mult} = 1.022$, 95% CI = 1.018–1.026, $P < 0.001$). Accordingly, the median sNfL level in the 226 healthy controls with a second serum sample after a median follow-up time of 368 (364–386) days was 24.6 (19.6–32.3) pg/ml [baseline: 24.4 (18.4–31.3) pg/ml]. The distribution of sNfL across different ages was modelled by using generalized additive models for location, scale and shape (GAMLSS) (Disanto *et al.*, 2017). The resulting 80th, 90th, 95th, 97.5th and 99th sNfL percentiles are shown in Supplementary Table 2 and Supplementary Fig. 1.

Association of serum neurofilament light chain with demographic and clinical variables in multiple sclerosis patients

The median follow-up in 257 patients with yearly serum samples was 6.5 (2.1–9.1) years. The study cohort included 29 samples from 11 (4.3%) patients with a clinically isolated syndrome, 1180 samples from 178 (69.3%) patients with relapsing remitting multiple sclerosis, 377 samples from 54 (21.0%) patients with secondary progressive multiple sclerosis and 98 samples from 14 (5.4%) patients with primary progressive multiple sclerosis summing up to 1684 samples. Baseline demographics and clinical characteristics are shown in Supplementary Table 3 and disease-modifying treatment received at baseline and during follow-up in Supplementary Fig. 2.

The median sNfL level in the multiple sclerosis patients was 32.9 (23.2–46.6) pg/ml. SNfL levels were significantly associated with age in both relapsing multiple sclerosis (β_{mult} = 1.016, 95% CI = 1.010–1.021, $P < 0.001$, n = 1209) and progressive multiple sclerosis (β_{mult} = 1.015, 95% CI = 1.007–1.023, $P < 0.001$, n = 475).

Both groups, relapsing multiple sclerosis and progressive multiple sclerosis patients had higher sNfL than healthy controls [relapsing multiple sclerosis: 29.7 (21.2–42.2) pg/ml and progressive multiple sclerosis: 41.9 (31.9–55.7) pg/ml; after age correction: β_{mult} = 1.263, 95% CI = 1.179–1.353 and β_{mult} = 1.423, 95% CI = 1.284–1.576, respectively, $P < 0.001$ for both comparisons] (Supplementary Fig. 3); SNfL levels were also higher in progressive multiple sclerosis as compared to relapsing multiple sclerosis (β_{mult} = 1.312, 95% CI = 1.198–1.436, $P < 0.001$, Supplementary Table 4; after age correction: β_{mult} = 1.154, 95% CI = 1.059–1.258, P = 0.001). Progressive multiple sclerosis patients with [51.4 (40.9–60.2) pg/ml] versus without [40.8 (30.6–52.5) pg/ml] contrast enhancing lesions had higher sNfL levels, but this did not reach statistical significance (β_{mult} = 1.121, 95% CI = 0.933–1.346, P = 0.223; after age correction: β_{mult} = 1.123, 95% CI = 0.932–1.352, P = 0.222). However, progressive multiple sclerosis patients without contrast enhancing lesions had higher sNfL levels than healthy controls (β_{mult} = 1.691, 95% CI = 1.526–1.874, $P < 0.001$; after age correction: β_{mult} = 1.406, 95% CI = 1.262–1.566, $P < 0.001$).

Univariable analyses showed significant positive associations of sNfL with EDSS (β_{mult} = 1.094, 95% CI = 1.070–1.120, $P < 0.001$) as well as with presence of a relapse within 120 days before sampling (β_{mult} = 1.118, 95% CI = 1.034–1.208, P = 0.005). In a multivariable model, the association of higher sNfL levels with higher age, EDSS and with a recent relapse were confirmed, whereas higher values of progressive versus relapsing multiple sclerosis were no longer statistically significant. There was a trend for a positive association between being under treatment at time of sampling and sNfL in both univariable and multivariable models, although this effect was not statistically significant (Supplementary Table 4).

Associations between serum neurofilament light chain and EDSS worsening in the following year

Next, we investigated the potential of sNfL to predict EDSS worsening. SNfL levels above the 90th percentile compared to levels below were associated with increased odds of EDSS worsening at the next visit (estimated odds ratio β_{OR} = 2.577, 95% CI = 1.553–4.278, $P < 0.001$, n = 677 observations) (Table 1). In the multivariable model sNfL above the 90th percentile (β_{OR} = 2.786, 95% CI = 1.609–4.826, $P < 0.001$, n = 677 observations) and T_2 lesion volume (β_{OR} = 1.061, 95% CI = 1.023–1.101, P = 0.001) were the only significant predictors of an EDSS worsening in the subsequent year (Table 1). Notably, the odds ratio and similarly the estimated average probability of EDSS worsening gradually increased with increasing sNfL percentile category (Fig.1 and Supplementary Table 5).

Association of serum neurofilament light chain with brain MRI measures of disease activity and normalized brain volume

In the univariable analysis (Table 2), sNfL was found to be associated with all established MRI measures and increased with increasing lesion load (see Supplementary Fig. 4 for contrast enhancing lesions and Supplementary Fig. 5 for new or enlarging T_2 hyperintense lesions).

In the multivariable model each contrast enhancing lesion was associated with a 17.8% increase in sNfL (β_{mult} = 1.178, 95% CI = 1.078–1.287, $P < 0.001$, n = 764), and each new or enlarging T_2 hyperintense lesion with an average increase in sNfL levels by 4.9% (β_{mult} = 1.049, 95% CI = 1.031–1.067, $P < 0.001$) (Table 2). A smaller normalized brain volume was associated with higher sNfL levels: sNfL was increased by 11.7% per 100 cm^3 reduction of normalized brain volume (β_{mult} = 0.883, 95% CI = 0.831–0.938, $P < 0.001$). Conversely, the relationship with T_2 lesion volume was no longer visible in the multivariable analysis (β_{mult} = 0.996, 95% CI = 0.987–1.006, P = 0.450).

Association of baseline serum neurofilament light chain with future brain volume changes

We tested whether baseline sNfL levels were associated with brain volume changes over the following years. In the univariable model sNfL levels at baseline were significantly associated with the percentage of brain volume change over 2 years: an increase in sNfL by 10 pg/ml was associated with an average additional reduction in brain

Table 1 Estimated odds ratios (β_{OR}) of EDSS worsening within the next year

Variables (677 observations)	Univariable			Multivariable		
	β_{OR}	95% CI	*P*	β_{OR}	95% CI	*P*
NfL > 90th percentile	2.577	1.553–4.278	<0.001	2.786	1.609–4.826	<0.001
Age at sampling (years)	1.003	0.978–1.029	0.816	1.005	0.977–1.034	0.714
Sex						
F (439)	–	–	–	–	–	–
M (238)	1.429	0.848–2.411	0.18	1.377	0.806–2.353	0.242
EDSS	1.080	0.924–1.263	0.332	0.926	0.762–1.125	0.440
Disease course						
RMS (502)	–	–	–	–	–	–
PMS (175)	1.655	0.934–2.933	0.084	1.297	0.647–2.600	0.464
Recent relapse (<120 days)						
No (611)	–	–	–	–	–	–
Yes (66)	1.847	0.888–3.841	0.1	1.993	0.892–4.454	0.093
DMT						
Untreated (181)	–	–	–	–	–	–
Treated (496)	0.630	0.363–1.092	0.099	0.596	0.320–1.108	0.102
T_2 lesion volume (per cm^3)	1.053	1.020–1.086	0.001	1.061	1.023–1.101	0.001
New/enlarging T_2	0.991	0.890–1.103	0.866	0.899	0.749–1.080	0.255
CEL	1.028	0.701–1.508	0.886	1.180	0.601–2.317	0.631
nBV (per 100 cm^3)	0.861	0.663–1.118	0.262	1.109	0.779–1.577	0.567

Univariable and multivariable GEE models testing associations between sNfL, clinical and MRI variables.
BL = baseline; CEL = contrast enhancing lesions; DMT = disease-modifying treatment; F = female; M = male; nBV = normalized brain volume; PMS = progressive multiple sclerosis; RMS = relapsing multiple sclerosis.

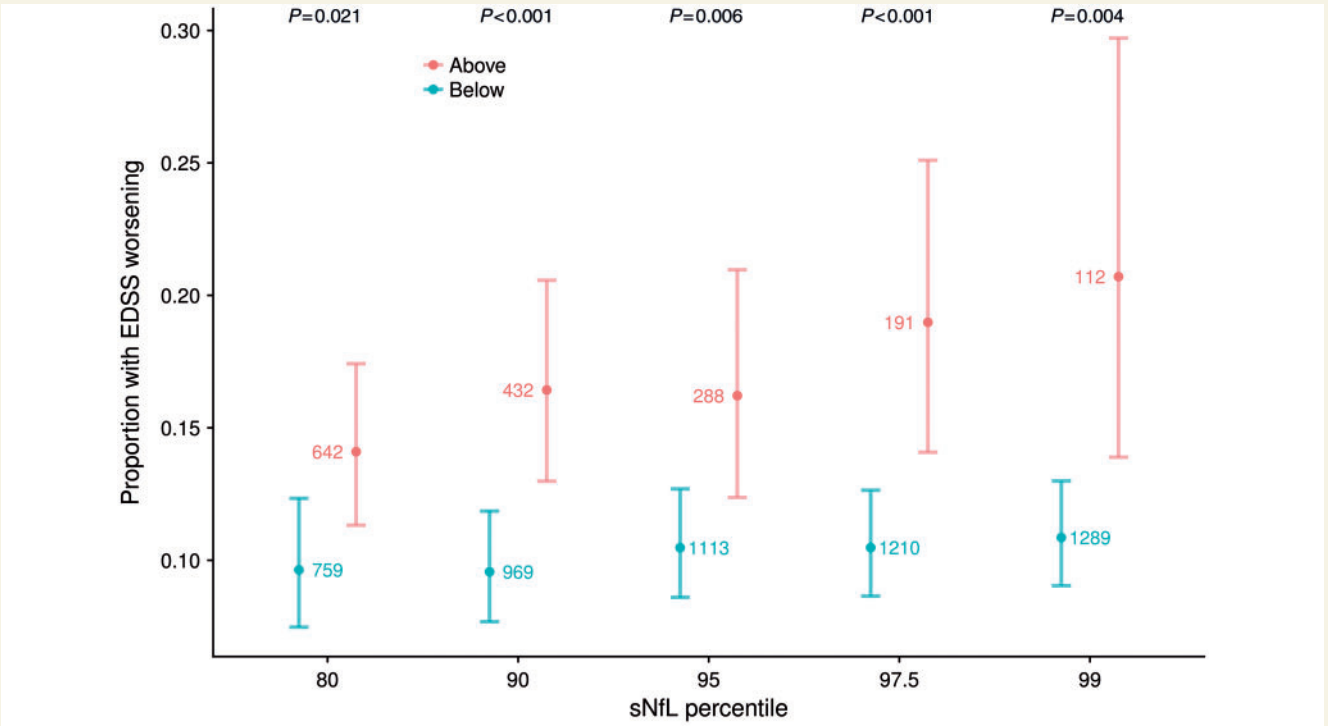

Figure 1 Estimated proportion of patients with EDSS worsening at the next visit against sNfL dichotomized based on age-corrected percentile curves from healthy controls. The estimated proportion of patients with EDSS worsening gradually increased with increasing sNfL percentile category based on healthy controls (above versus below 80th percentile: β_{OR} = 1.539, 95% CI = 1.067–2.219, *P* = 0.021; above versus below 99th percentile: β_{OR} = 2.143, 95% CI = 1.274–3.606, *P* = 0.004, *n* = 1401 observations). The percentiles were constructed based on healthy control samples. Numbers in the figure denote the number of samples above or below the respective percentiles of healthy controls.

Table 2 Estimates of univariable and multivariable GEE models testing associations between sNfL and lesional and brain volume MRI variables

Variables (764 observations)	Univariable			Multivariable		
	β_{mult}	95% CI	*P*	β_{mult}	95% CI	*P*
Age	1.015	1.010–1.021	<0.001	1.014	1.008–1.019	<0.001
Sex						
F (498)	–	–	–	–	–	–
M (266)	1.124	0.998–1.264	0.053	1.087	0.985–1.198	0.097
CEL	1.314	1.195–1.445	<0.001	1.178	1.078–1.287	<0.001
T_2 lesion volume (per cm^3)	1.012	1.004–1.020	0.003	0.996	0.987–1.006	0.450
New/enlarging T_2	1.057	1.037–1.077	<0.001	1.049	1.031–1.067	<0.001
nBV (per 100 cm^3)	0.856	0.812–0.903	<0.001	0.883	0.831–0.938	<0.001

The estimates represent multiplicative effects (β_{mult}) since the endpoint sNfL was log transformed.
CEL = contrast enhancing lesions; F = female; M = male; nbV = normalized brain volume.

volume of 0.17% after 2 years ($\beta_{add} = -0.171\%$, 95% CI = −0.226 to −0.116%, $P < 0.001$, $n = 197$ observations). Besides a weaker signal for baseline EDSS in the multivariable model ($\beta_{add} = -0.151$, 95% CI = −0.271 to −0.031, $P = 0.014$, $n = 197$ observations), sNfL remained the only strong predictor of brain volume change over 2 years ($\beta_{add} = -0.134\%$, 95% CI = −0.194 to −0.073%, $P < 0.001$; Supplementary Table 6), while this was not the case for acute and chronic lesional activity.

Repeating the same analysis for baseline to Year 5 percentage brain volume change showed similar results with sNfL (Fig. 2 and Table 3): confirming the 2-year results, baseline sNfL was a highly significant predictor of percentage brain volume change over 5 years of follow-up ($\beta_{add} = -0.287\%$, 95% CI = −0.432 to −0.142%, $P < 0.001$, $n = 132$) in a multivariable analysis that included EDSS ($\beta_{add} = -0.294\%$, 95% CI = −0.545 to −0.042%, $P = 0.023$), disease course and several MRI baseline variables (Table 3).

In addition, we compared sNfL measurements of multiple sclerosis patients against the age corrected percentile curves that were constructed based on healthy control samples. The mean percentage brain volume change in patients with sNfL above the respective percentiles gradually increased with increasing sNfL percentile category both over 2 (Fig. 3A) and over 5 years (Fig. 3B) of follow-up (Supplementary Table 7). We performed the same analysis in all progressive multiple sclerosis patients without contrast enhancing lesions over 2 and 5 years ($n = 45$ and 26 observations, respectively). Patients with sNfL levels above the 99th percentile showed increased brain volume loss versus those with values below the 99th percentile ($P < 0.001$ and $P = 0.003$ for 2 and 5 years, respectively; Supplementary Fig. 6A and B).

Association of serum neurofilament light chain with future spinal cord volume

We also investigated the association between sNfL and the change in spinal cord volume over 2 and 5 years of follow-up. A significant association between sNfL and the change in spinal cord volume over 2 and 5 years was present with an estimated additional average reduction in spinal cord volume of 0.19% over 2 years or 0.49% over 5 years per 10 pg/ml increase in sNfL ($\beta_{add} = -0.191\%$, 95% CI = −0.295 to −0.086%, $P < 0.001$, $n = 673$ observations and $\beta_{add} = -0.488\%$, 95% CI = −0.783 to −0.192%, $P = 0.001$, $n = 307$ observations, respectively; Fig. 4). The mean reduction in spinal cord volume over 2 and 5 years gradually increased with increasing sNfL percentile category (Fig. 3C, D, and Supplementary Table 8). For example, patients with sNfL above the 97.5th percentile as compared to those below the same percentile had on average a 1.7% and 2.5% lower spinal cord volume at 2 and 5 years of follow-up, respectively. Similarly, in progressive multiple sclerosis patients without contrast enhancing lesions over 2 and 5 years ($n = 161$ and 61 observations, respectively), the mean reduction in spinal cord volume with sNfL above versus below the 95th ($P = 0.012$ and 0.082, respectively), 97.5th ($P = 0.002$ and 0.02) and 99th ($P = 0.06$ and <0.001) percentile gradually increased with increasing sNfL levels (Supplementary Fig. 6C and D).

Discussion

There is an increasing interest in the potential use of sNfL as the first reliable blood-based marker of neuro-axonal damage in multiple sclerosis, as well as in other neurological conditions. Our study confirms in a large independent cohort our previous findings that sNfL levels in multiple sclerosis reflect the effect of ageing, recent relapses and concurrent disability (Disanto *et al.*, 2017). We also provide new evidence that sNfL levels are increased in the presence of focal active inflammation, as measured by the number of brain contrast enhancing lesions and new or enlarging T_2 lesions. We complemented our previous observation that the number of T_2 lesions is associated with sNfL by showing that T_2 lesion volume, a more comprehensive measure of brain lesion burden, is highly associated with sNfL. This finding further suggests that sNfL may be

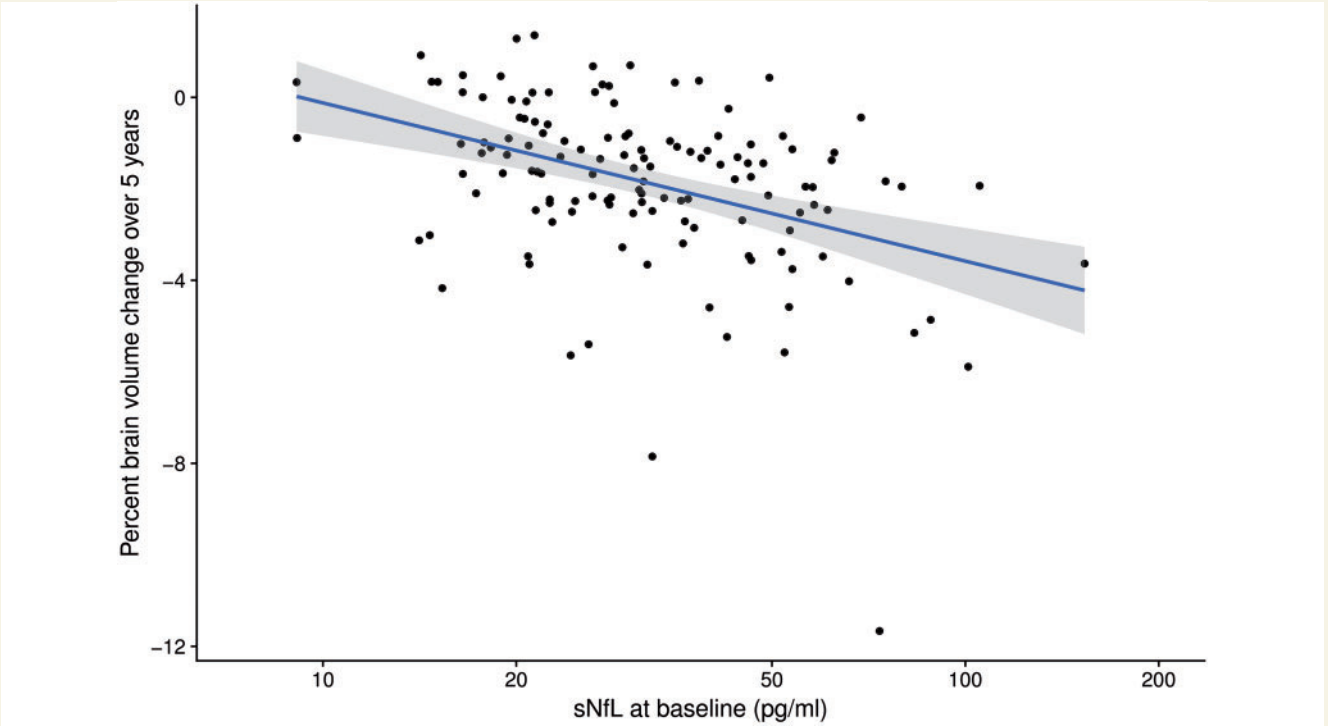

Figure 2 Association of sNfL at baseline with percentage change in brain volume over 5 years. In the univariable model sNfL levels at baseline were significantly associated with the percentage brain volume change over 5 years ($\beta_{add} = -0.352\%$, 95% CI = −0.490 to −0.214% per 10 pg/ml change in sNfL, $P < 0.001$, $n = 132$ observations), i.e. an estimated additional 0.35% reduction in brain volume over 5 years per 10 pg/ml increase in baseline sNfL.

Table 3 Estimated percentage brain volume change over 5 years (β_{add}) in univariable and multivariable linear models testing for associations with baseline variables

Baseline variables (132 observations)	Univariable			Multivariable		
	β_{add} (%)	95% CI (%)	*P*	β_{add} (%)	95% CI (%)	*P*
sNfL (per 10 pg/ml)	−0.352	−0.490 to −0.214	<0.001	−0.287	−0.432 to −0.142	<0.001
Age (years)	−0.025	−0.054 to 0.005	0.098	0.008	−0.025 to 0.040	0.642
Sex						
F (87)	–	–	–	–	–	–
M (45)	−0.394	−1.058 to 0.269	0.241	−0.229	−0.845 to 0.387	0.463
EDSS	−0.454	−0.654 to −0.255	<0.001	−0.294	−0.545 to −0.042	0.023
Disease course						
RMS (97)	–	–	–	–	–	–
PMS (35)	−0.839	−1.579 to −0.099	0.027	0.118	−0.734 to 0.971	0.784
T_2 lesion volume (per cm^3)	−0.064	−0.111 to −0.017	0.008	−0.028	−0.081 to 0.025	0.294
CEL	−0.259	−0.549 to 0.031	0.079	−0.055	−0.328 to 0.219	0.693
nBV (per 100 cm^3)	0.546	0.235 to 0.857	<0.001	0.167	−0.235 to 0.570	0.412

CEL = contrast enhancing lesions; F = female; M = male; nBV = normalized brain volume.

used to capture the extent of brain damage in individual patients. Importantly, we provide evidence that sNfL is also associated with the normalized brain volume at time of sampling. Taken together, these observations support that sNfL is a quantitative measure of the rate of neuronal loss within the CNS at the time of sampling.

More relevant for the utility of sNfL as biomarker for individual decision-making is its predictive power for the course of disease. We confirmed in this study that multiple sclerosis patients with higher sNfL levels are at higher risk of experiencing disability worsening in the following year (Disanto *et al.*, 2017). We had previously reported an association between sNfL and brain atrophy over 2 years, but this study was limited by the small sample size (42 multiple sclerosis patients) and by the limited sensitivity of the assay used at that time (18% of samples being not reliably

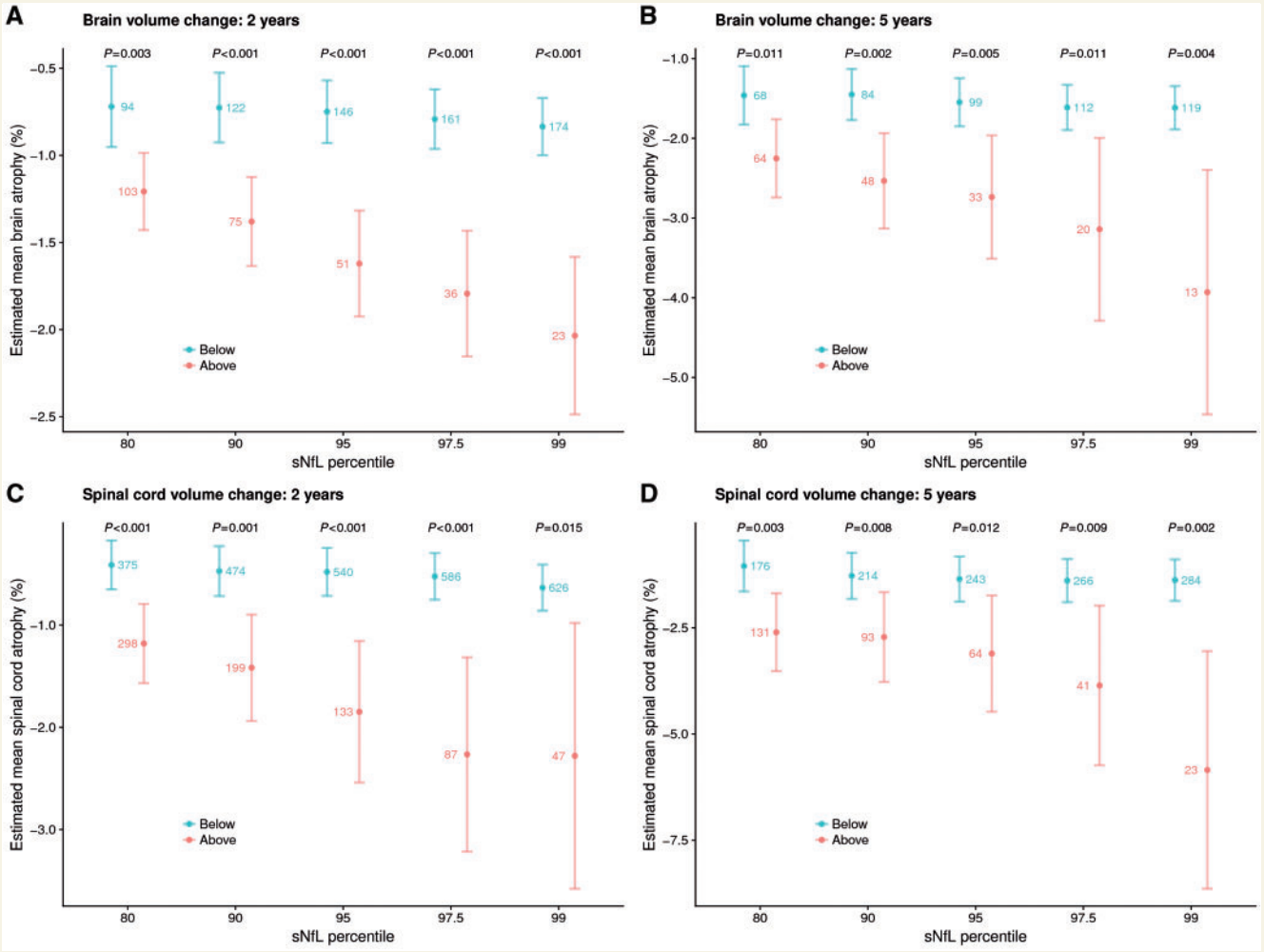

Figure 3 Estimated mean change in brain and spinal cord volume over 2 and 5 years against sNfL dichotomized based on age-corrected percentile curves from healthy controls. The estimated mean percentage of brain volume change in patients with sNfL above the respective age corrected percentiles gradually increased with increasing sNfL percentile category over 2 (**A**) and 5 years (**B**) of observation time. The mean reduction in spinal cord volume over 2 (**C**) and 5 years (**D**) gradually increased with increasing sNfL percentile category. For example, patients with sNfL above the 97.5th percentile had on average a 1.7% and 2.5% lower spinal cord volume at 2 (**C**) and 5 years (**D**) of follow-up as compared to those below the same percentile, respectively. Numbers in the figure denote the number of samples above or below the respective percentiles of healthy controls.

measurable) (Kuhle *et al.*, 2017*b*). We now provide for the first time, strong evidence that sNfL concentration is a predictor of brain atrophy in multiple sclerosis at 2 and 5 years. Notably, when included in a multivariable model, sNfL remained significantly associated with future brain volume loss while T_2 lesion volume, contrast enhancing lesions and baseline normalized brain volume did not. This suggests that sNfL can represent a more accurate indicator of ongoing neuro-axonal loss and a better predictor of brain atrophy than MRI measures of acute and chronic lesional activity.

This is further reinforced by the novel finding of an association between sNfL and spinal cord atrophy. Noteworthy, sNfL levels were still associated with spinal cord volume change after 5 years of follow-up. This observation is clinically relevant, given recent studies pointing at spinal cord pathology as a key driver of long term disability accumulation in multiple sclerosis (Schlaeger *et al.*, 2014; Zecca *et al.*, 2016; Hagstrom *et al.*, 2017). The fact that we were able to confirm the association of sNfL with spinal cord volume loss in the subgroup of progressive multiple sclerosis patients without detectable focal inflammatory MRI activity further underlines the independent contribution of sNfL as a prognostic marker of tissue damage.

Taken together, these observations show that multiple sclerosis patients with higher sNfL levels are at higher risk of experiencing accelerated brain and spinal cord volume loss and worsening of disability scores in the long term. A practical implication of our findings is that patients with highest sNfL levels might be candidates for an escalation to more active treatments, to better prevent the occurrence and accumulation of further neuronal damage.

Our study had some limitations. First, not all enrolled patients underwent MRI scans and atrophy measurements at 5 years. Although some patients were lost to follow-up

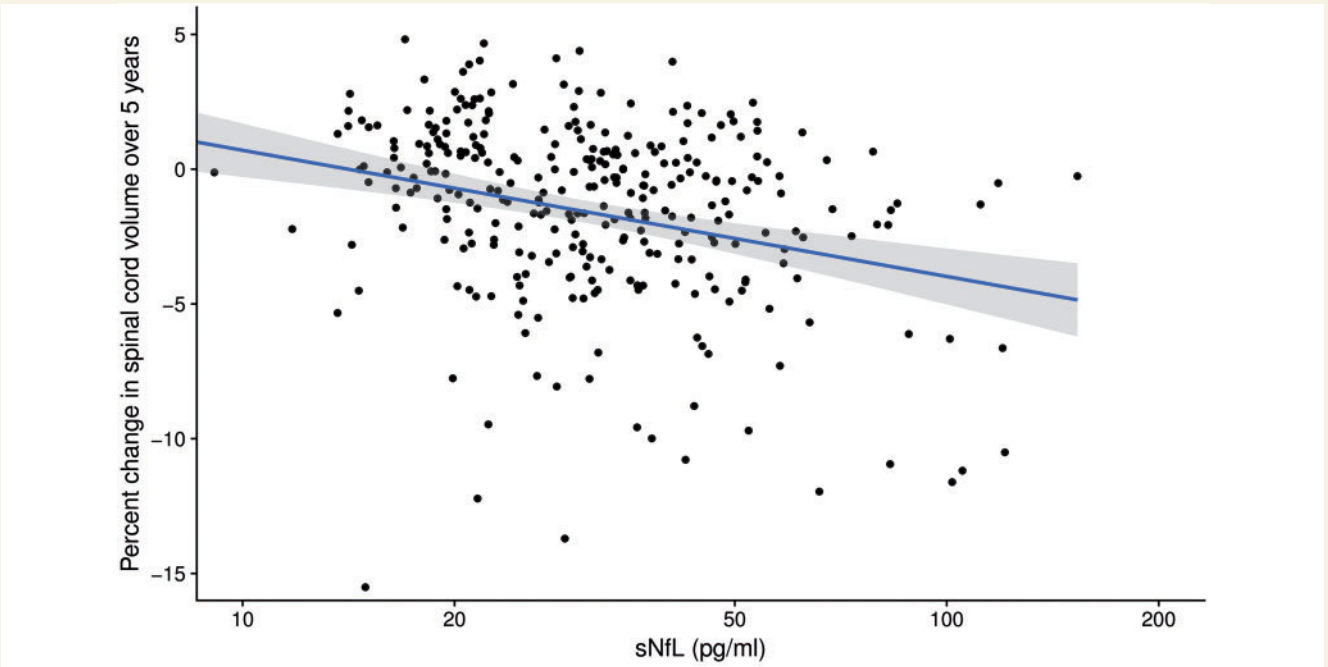

Figure 4 Association of sNfL and per cent change in spinal cord volume after 5 years of follow-up. A significant association between sNfL and the change in spinal cord volume over 5 years was found with an estimated additional reduction in spinal cord volume of 0.49% over 5 years per 10 pg/ml increase in sNfL (β_{add} = −0.488%, 95% CI = −0.783 to −0.192%, P = 0.001, n = 307 observations).

in the setting of our prospective observational study, the loss of more active or more disabled participants would rather have reduced the power of our study to show significant correlations than biased the associations described. As our study was not designed to investigate treatment effects, it is not surprising that in this setting we did not replicate the negative association between sNfL levels and immunomodulating treatments described in our previous study (Disanto *et al.*, 2017). Our failure to depict significant effects of treatment might be attributed to lower number of patients per treatment with variable follow-up and to the relative higher proportion of patients on first generation low efficacy drugs, most of these having started treatment already before inclusion in this study. For the study by Disanto *et al.* (2017), recruitment took place between 2009 and 2016 and was focused on active patients starting or switching disease-modifying therapies whereas patients in this study were recruited earlier (between 2004 and 2005), with limited access to the potentially more effective newer generation compounds. Ongoing or not yet fully published sNfL studies in the setting of randomized controlled trials are certainly much more appropriate to provide robust evidence that proves and quantifies potential treatment effects of currently available multiple sclerosis therapies on sNfL (Kuhle *et al.*, 2016*b*, 2017*a*).

The release of NfL into the peripheral blood represents a significant opportunity for monitoring disease activity and progression. A blood fluid biomarker has intrinsic characteristics such as providing a real-time signal covering the entirety of the CNS, lower cost and ability to measure repetitively in a non-invasive manner. The latter is also fundamental for the implementation of sNfL as an endpoint in clinical trials or routine clinical practice. The strong association with clinical relapses, disability scores, MRI measures of inflammation and with tissue damage in both brain and spinal cord, complemented by the intrinsic advantages of a peripheral body fluid biomarker, supports sNfL utility as a highly informative disease marker in multiple sclerosis.

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Supplementary material

Supplementary material is available at *Brain* online.

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