Towards quantifying axonal injury in blood samples of patients affected by multiple sclerosis

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List of publications as part of the thesis main text

Asterisks (*) denote equal contributions.

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Abbreviations

ARR	Annualized relapse rate
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DMT	Disease modifying treatment
ECL	Electrochemiluminescence
EDSS	Expanded Disability Status Scale
ELISA	Enzyme-linked immunosorbent assay
GeneMSA	Genome-Wide Association Study of Multiple Sclerosis
HC	Healthy control
IRR	Incidence rate ratio
MIAC	Medical Image Analysis Center
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NEDA	No Evidence of Disease Activity
NEPAD	No Evidence of Progression or Active Disease
Nf	Neurofilaments
NfH	Neurofilament heavy chain
NfL	Neurofilament light chain
NfM	Neurofilament medium chain
PBVC	Percentage brain volume change
RRMS	Relapsing remitting multiple sclerosis
SIMOA	SIngle MOlecule Array
SMSC	Swiss Multiple Sclerosis Cohort
sNfL	serum neurofilament light chain

Abstract

Background. Neuro-axonal injury is a hallmark of the underlying pathological processes in neurodegenerative disorders. Reliable quantification and longitudinal follow-up of such damage via a biofluid marker would be a highly relevant adjunctive tool in the treatment workup for patients with multiple sclerosis (MS). The neurofilament proteins have emerged as the first biomarker bearing promise for a clinical application beyond a research tool. For the first time a biomarker specifically indicative of neuronal damage can be quantified in an easily accessible fluid source, i.e. in serum or plasma. Second (Enzyme-linked immunosorbent assays (ELISA)) and third generation (electrochemiluminescence based (ECL) assays) measuring systems lacked sufficient sensitivity to reliably measure neurofilaments throughout the range of concentrations found in blood samples, and specifically failed to define normal levels. The single molecule array system (SIMOA) marks a qualitative technological advancement as it provides the sensitivity to quantify physiologic neurofilament levels. This has paved the way to investigate neurofilaments in a range of neurological disorders, and specifically in diseases with smoldering course of neurodegeneration.

Objective. We aimed to develop and validate a highly sensitive SIMOA assay for the neurofilament light chain (NfL). Using this assay, we investigated blood-based neurofilament light chain (NfL) as fluid biomarker of disease activity, treatment response, and as a predictor of the long-term course of disability and morphological features of neurodegeneration in MS. Further, we are evaluating in a third work stream the validity of NfL as a tool to detect suboptimal treatment with current standard MS therapies.

Methods. In the first study, we quantified serum NfL (sNfL) in two independent MS patient cohorts: (i) in a cross-sectional cohort (142 patients) NfL in serum and CSF was correlated with magnetic resonance imaging (MRI) data, ii) in a longitudinal cohort (246 patients) from the Swiss MS Cohort study (SMSC) NfL levels in two samples post-switch to a new disease modifying treatment were compared to pre-switch levels and with those from 254 healthy controls from the Genome-Wide Association Study of Multiple Sclerosis (GeneMSA).

In the second study, we quantified yearly serum sNfL in 259 MS patients followed up in the GeneMSA study for up to 10 years and 259 healthy controls who had also a one year follow up blood sampling.

Results and interpretation. NfL levels in CSF and blood were highly correlated, thus supporting the concept that serum is a valid biofluid source to determine accurately neuronal damage within the central nervous system compartment. sNfL levels were higher in relapsing and progressive forms of MS, compared to healthy controls and were associated with current clinical and MRI disease activity. Finally, sNfL levels independently predicted future disability worsening, and cranial and spinal cord volume loss.

Conclusion. Our data demonstrate that NfL can be reliably quantified in peripheral blood and CSF. Levels are associated with a) concurrent clinical and MRI measures of acute and chronic disease activity, b) response to DMT and c) long-term course of disability. This supports the potential of sNfL to become the first precision medicine tool to monitor subclinical disease activity and suboptimal treatment response.

Zusammenfassung

Hintergrund. Neuroaxonale Schädigung spielt bei verschiedensten neurologischen und neurodegenerativen Erkrankungen eine wichtige Rolle. Die Multiple Sklerose (MS) ist eine sehr heterogen verlaufende Erkrankung, so dass gerade hier eine verlässliche und longitudinale Messung dieser Schädigungen zur Beurteilung der Krankheitsaktivität, Überwachung der Behandlungswirksamkeit und prognostischen Einschätzung sehr wichtig wäre. Neurofilament Proteine, die unabhängig von ursächlichen Mechanismen neuroaxonale Schädigung anzeigen sind hier erstmals vielversprechende Kandidaten: im Zusammenhang mit neuroaxonaler Schädigung kommt es zu deren Anstiegen im Liquor, aber darüber hinaus auch im Blut. Enzyme-linked immunosorbent assays (ELISAs) und Elekktrochemilumineszenz basierte Nachweisverfahren sind von begrenzter Sensitivität. Im Gegensatz dazu erlauben neue „single molecule array“ (SIMOA) Detektionsverfahren die zuverlässige Messung von Neurofilamenten auch in Blutproben, insbesondere auch von gesunden Kontrollpersonen. Diese wichtige technologische Weiterentwicklung erlaubt nun die longitudinale Messung von Neurofilamenten bei einer Reihe von vor allem auch chronischen neuroinflammatorischen oder neurodegenerativen Erkrankungen.

Ziele. Ein wichtiges Ziel dieser Arbeit war es ein hochsensitives SIMOA basiertes Nachweisverfahren für die leichte Kette der Neurofilamente (NfL) zu entwickeln, zu optimieren und zu validieren. Weitere Ziele waren es dann mit Hilfe dieses entwickelten Testsystems die Wertigkeit der Konzentrationen von NfL in Serumproben (sNfL) als Mass der Krankheitsaktivität, des Therapieansprechens, der Prognose der Behinderungsentwicklung und morphologischer Veränderungen im MRI zu untersuchen.

Methoden. In der ersten Studie hatten wir Zugang zu Serumproben zur Bestimmung von NfL von zwei unabhängigen Studienkollektiven: i) von 142 MS Patienten mit gepaarten Serum/Liquorproben und Magnetresonanztomographie (MRI) Daten, ii) von 246 MS Patienten der Schweizerischen MS Kohorten Studie (SMSC) mit jeweils zwei Proben nach Umstellung bzw. Beginn einer immunmodulierenden Therapie und einer Probe vor der Umstellung und 254 gesunde Kontrollpersonen aus der Genome-Wide Association Study of Multiple Sclerosis (GeneMSA). In Rahmen der zweiten Studie haben wir jährlich sNfL bei 259 MS-Patienten, die im Rahmen der GeneMSA-Studie bis zu 10 Jahre nachuntersucht wurden, und 259 gesunden Kontrollen quantifiziert.

Resultate. Es zeigte sich ein starker Zusammenhang zwischen den gemessenen Liquor und Serum NfL Konzentrationen. Diese Korrelation unterstützt die Wertigkeit von NfL Messungen im Blut zur Quantifizierung neuroaxonaler Schädigung innerhalb des zentralen Nervensystems. Sowohl schubförmige als auch progrediente MS Patienten wiesen im Vergleich zu gesunden Kontrollen höhere sNfL Spiegel auf, und höhere sNfL Konzentrationen waren mit aktueller klinischer und bildgebender Krankheitsaktivität assoziiert. Zusätzlich waren erhöhte sNfL Spiegel signifikant und unabhängig mit zukünftiger Behinderungszunahme und Hirn- und Rückenmarksatrophie assoziiert.

Schlussfolgerungen. Zusammenfassend zeigen unsere Daten, dass NfL im peripheren Blut und Liquor zuverlässig quantifiziert werden kann. Erhöhte Werte sind mit a) akuten und chronischen klinischen und bildgebenden Massen der MS Krankheitsaktivität, b) der Therapieantwort und c) der Behinderungsentwicklung assoziiert. Unsere Daten unterstützen den möglichen Nutzen von individuellen sNfL Bestimmungen zur Detektion subklinischer Krankheitsaktivität und suboptimalen Therapieansprechens.

Chapter 1: Introduction

1.1 A biomarker approach to MS

A. The need for a reliable blood based biomarker in multiple sclerosis

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disease of the central nervous system (CNS) of unknown aetiology. Response to therapy and short, but especially long-term course are not predictable as current disease measures are not sufficiently precise and accurate to predict the course of disease in individual patients. The inability to accurately quantify acute and chronic clinical worsening may be an important reason for a series of failures in the development of neuroprotective treatments for MS. Biofluid markers bear the advantage of measuring ongoing pathologic changes real-time and being specific for molecular mechanisms of disease. Such a biomarker would be helpful in monitoring ongoing damage and potential treatment across all forms and stages of MS.

Currently, oligoclonal bands and to a lesser extent the IgG-index are the only biofluid markers that play a role in the diagnostic workup for MS[1]. However, these measures are relatively insensitive to change over time and there is no monitoring biomarker established for MS where change reflects disease activity and treatment response. Monitoring biomarkers imply a longitudinal assessment, which could be implemented on the basis of cerebrospinal fluid (CSF) analysis for neu NfL[2]. Due to their invasiveness, sequential lumbar punctures are however impractical outside of research settings. Hence, the profile of a biomarker for use in routine clinical practice requires a) easy accessibility of fluid source for sequential measurements, i.e. blood or urine, b) more dynamic change over time in function of disease activity than current clinical and magnetic resonance imaging (MRI) measures, and c) reliable quantification vis-à-vis its physiological levels. This PhD thesis focuses on the development of a high sensitivity assay for NfL and its validation as the first biomarker that may fulfill all these three premises.

1.2 Neurofilaments

There is increasing evidence that neuronal degeneration is the key factor in the pathogenesis of sustained neurological disability in MS and hence may be the main driver for what we call 'disease progression' (disability worsening independent of relapse)[3]. Neuronal degeneration is seen in acute and chronic MS lesions, as well as in extralesional gray and white matter[4].

Neurofilaments (Nf) are a family proteins that are present exclusively in neuro-axonal structures[5]. Their main role is to stabilise axon caliber of myelinated axons and consequently their conduction velocity[6]. Nf belong to the class IV intermediate filaments comprising in the nervous system: α-internexin, peripherin, neurofilament light (NfL; 60–70 kDa), medium (NfM, 130–170 kDa), and heavy chain (NfH; 180–200 kDa), **Figure 1**. NF are obligate heteropolymers composed of the NFL, NFM and NFH subunits with a subunit stoichiometry of 4:2:1[5]. This ratio varies during neuronal development[7] and likely in neurodegenerative disorders like amyotrophic lateral sclerosis[8]. NfL is the most abundant Nf protein and acts as the backbone to which other Nf chains bind to. Because Nf are exclusive products of neuronal cells, their key advantage over other biomarkers is their specificity in terms of cellular source, reflection of pathomechanism and hence signal interpretation, i.e. they are highly specific for neuronal cell damage and eventual neuronal cell death.

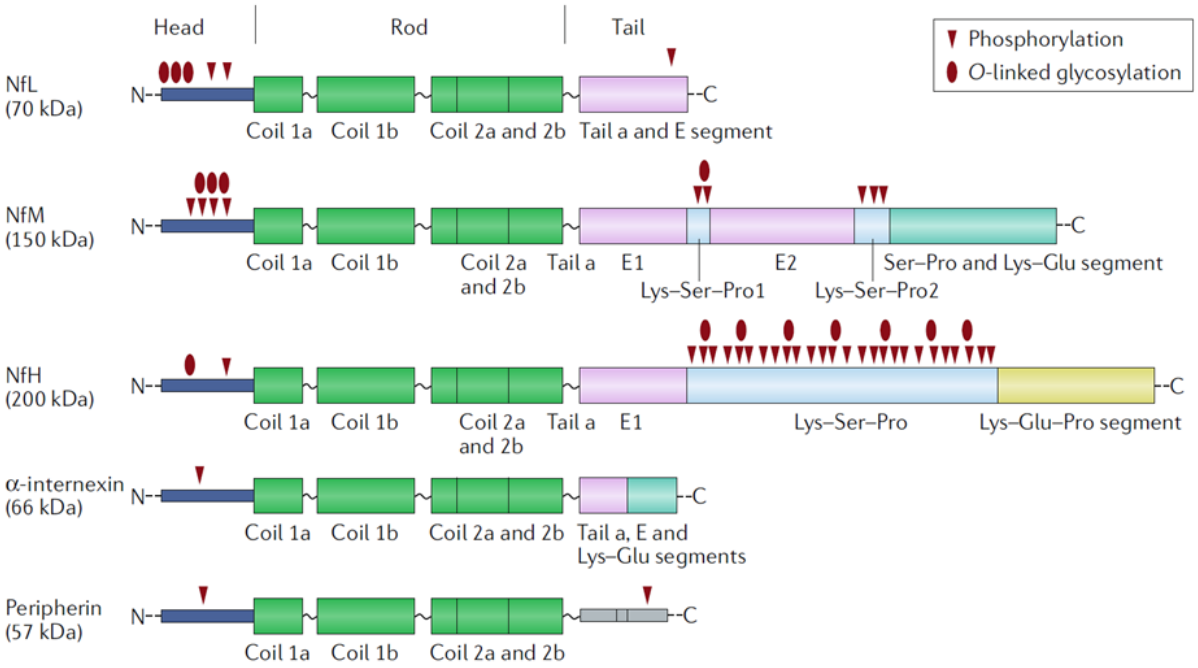

Figure 1. Structure of Neurofilaments. Domain structure and post-translational modifications of neurofilament subunits. Neurofilament light chain (NfL), neurofilament medium chain (NfM), neurofilament heavy chain (NfH), α-internexin and peripherin are the subunits of neurofilaments in the mature nervous system. All neurofilament subunits include a conserved α-helical rod domain that comprises several coiled coils, and variable amino-terminal globular head regions and carboxy-terminal tail domains. NfM and NfH subunits are unique among the intermediate filament proteins in that they have long carboxy-terminal domains with multiple

Lys–Ser–Pro repeats that are heavily phosphorylated. Phosphorylation and *O*-linked glycosylation sites on neurofilament subunits are shown[2].

1.3 Neurofilament light chain as biomarker in multiple sclerosis

A. Evidence from NfL measurements in cerebrospinal fluid

The advent of the first Enzyme-linked immunosorbent assay (ELISA) detecting NfL, allowed its reliable quantification in CSF. Several studies showed a positive association between CSF NfL levels and degree of disability, clinical and MRI disease activity[9-15]. Further studies highlighted the predictive value of CSF NfL in patients with a clinically isolated syndrome for conversion to clinically definite MS[10,12]. An early indication of the role of NfL as treatment response biomarker came from a longitudinal study showing a decrease of NfL levels over 6-12 months in 92 MS patients treated with natalizumab[16]. Similar findings could be reproduced in observational as well as in placebo-control settings for relapsing remitting MS (RRMS) treated with fingolimod[13,14,17] (**Figure 2**) and progressive MS patients treated with natalizumab[18], mitoxantrone and rituximab[19].

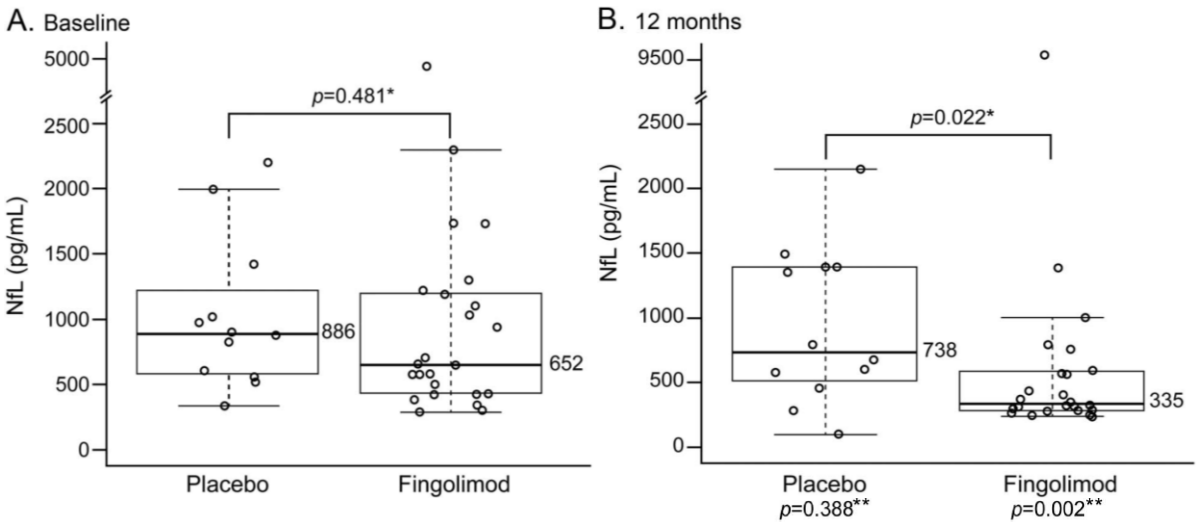

Figure 2. Neurofilament light chain levels at baseline and after 12 months. *NfL levels at baseline (A); pooled fingolimod0.5/1.25mg: 652pg/ml; placebo: 886pg/ml, p=0.481. At 12 months (B); NfL levels pooled fingolimod group: 335pg/ml; placebo: 738pg/ml, p=0.022. *Mann-Whitney test.**Sign test: baseline vs month 12. Dots represent individual samples. Box and whiskers plotted according to theTukey method*[17].

B. Previous evidence from NfL measurements in serum and plasma

The need for an easily accessible biomarker led to further efforts towards a quantification of NfL in serum or plasma samples and the consequent development of more sensitive immunoassays. In 2013 my group developed an electrochemiluminescence (ECL) based immunoassay[20]. Despite a suboptimal sensitivity to quantify serum NfL (sNfL, 18%[21] and 27%[22] of samples were below detection limit), we detected that NfL levels in serum were highly correlated to levels in corresponding CSF samples ($r = 0.62$, $p = 0.0002$). Also, concentrations in serum were higher in MS patients than in healthy controls and levels correlated with white matter lesion volume ($r = 0.68$, $p < 0.0001$), mean T1 ($r = 0.40$, $p = 0.034$) and T2* relaxation time ($r = 0.49$, $p = 0.007$) and with magnetization transfer ratio in normal appearing white matter ($r = -0.41$, $p = 0.029$)[22]. In a follow-up study again using the ECL NfL assay, changes in sNfL were correlated with Expanded Disability Status Scale (EDSS) change ($p = 0.009$), and brain volume decreased more rapidly in patients with high baseline sNfL values ($p = 0.05$ at 12 months and $p = 0.008$ at 24 months), while higher and increasing sNfL predicted the occurrence of higher numbers of gadolinium-enhancing lesions ($p < 0.001$ for both)[21].

Based on these findings we initiated the development and validation of a more sensitive NfL assay on the so called single molecule array (SIMOA) platform[23]. The SIMOA technology is based on the simultaneous counting of a large number (n=500 000 per sample)[23] of single capture microbeads in very small reaction volumes (40 femtoliters)[23,24]. In contrast to conventional ELISA where the enzyme-substrate reaction is conducted in relatively large volumes (50–100 μL), SIMOA restricts the diffusion of the fluorescent molecules by femtoliter-sized wells that can be counted with a camera simultaneously in thousands of microwells. The counting of active and inactive wells constitutes a digital signal corresponding to the presence or absence of single enzyme molecules. The resulting gain in sensitivity permits the use of low quantities of labeling reagent, which lowers nonspecific interactions and increases signal to background ratios[23,25]. In collaboration with colleagues in Gothenburg and applying the SIMOA NfL assay this group had developed,[26] we found the SIMOA platform to be 126- and 25-fold more sensitive than ELISA and our ECL assay, respectively, to quantify NfL[25]. Correlations between paired CSF and serum samples were strongest for SIMOA ($r=0.88$, $p<0.001$) and the ECL assay ($r=0.78$, $p<0.001$), but only moderate for the ELISA measurements ($r=0.38$, $p=0.030$), **Figure 3**[25]. SIMOA allowed the reliable detection of NfL in all serum samples. In contrast, more than 50% of the samples were not reliably quantifiable by the ECL

assay and ELISA. Serum levels of NfL are 50-100-fold lower in serum compared to CSF. Note that serum levels in **Figure 3 A, B, G, H, I** are assigned to a calculated lower limit of quantification, as the actual levels cannot be quantified. This leads to an artificial vertical or horizontal line for serum values below this limit. Only with the SIMOA technology lower range NfL values can quantified (**Figure 3 C, F, I**), allowing a continuation of the correlation of serum and CSF values in these low concentrations.

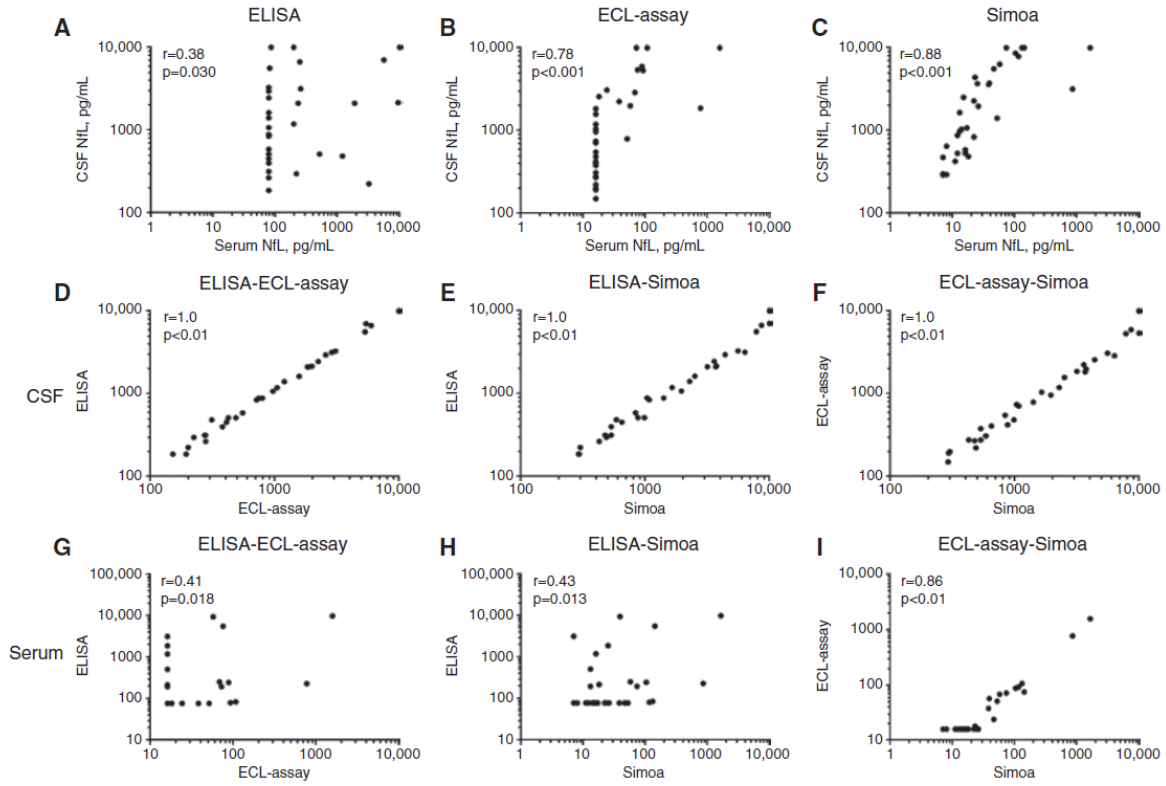

Figure 3. CSF and serum NfL correlation in different assays (A–C), associations between NfL measurements on different platforms in CSF (D–F) and serum (G–I). *Correlations between paired CSF and serum samples were strongest for SIMOA (r = 0.88, p < 0.001, Figure 1C), and the ECL assay (r = 0.78,p < 0.001, Figure 1B). This was less clear for the ELISA measurements (r = 0.38, p = 0.030, Figure 1A). CSF NfL measurements on the three different platforms were highly correlated: ELISA-ECL: r = 1.0, p < 0.001, Figure 1D; ELISA-SIMOA assay: r = 1.0, p < 0.001, Figure 1E; and ECL assay -SIMOA: r = 1.0, p < 0.001, Figure 1F. For serum measurements, NfL levels were highly correlated between ECL assay and SIMOA (r = 0.86,p < 0.001, Figure 1I), whereas this relation was weaker for ELISA-ECL assay (r = 0.41, p = 0.018, Figure 1G) and ELISA-SIMOA (r = 0.43, p = 0.013, Figure 1H)*[25].

Chapter 2: Research objectives

The urgent need for a biomarker able to monitor neuro-axonal injury in a disease with very variable course like MS motivated us in pursuing the development, analytical and clinical validation of NfL measurements in peripheral blood samples. Important prerequisites were our group's previous experience in assay development and the access to a wide collection of CSF, serum and plasma samples from our department's CSF bank and high quality observational studies including longitudinal biosampling.

We aimed to develop an immunoassay on the SIMOA platform with high sensitivity, and proven parallelism, spiking and dilution linearity for the detection of NfL in blood samples.

Using this assay we investigated:

a) the association between blood and CSF NfL levels (chapter 3.1);

b) sNfL levels in a large collection of healthy controls and explore their association with demographical characteristics like sex and age (chapters 3.1, 3.2)

c) sNfL's association with current clinical and MRI measures of disease activity in MS (chapters 3.1, 3.2)

d) if sNfL could predict future clinical disease activity (chapters 3.1, 3.2)

e) if sNfL levels predict future brain and spinal cord volume changes (chapter 3.2)

f) the effect of disease modifying treatment (DMT) on sNfL levels (chapters 3.1)

Chapter 3: Publications

3.1 Serum Neurofilament Light: A Biomarker of Neuronal Damage in Multiple Sclerosis

Note: This publication was awarded with the Neurowind prize 2017 and the Franco Regli prize 2017.

Serum neurofilament light: a biomarker of neuronal damage in multiple sclerosis

Running head: Serum NfL as a biomarker in multiple sclerosis

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ABSTRACT

Objective: Neurofilament light chains (NfL) are unique to neuronal cells, are shed to the CSF and are detectable at low concentrations in peripheral blood. Various diseases causing neuronal damage have resulted in elevated CSF concentrations. We explored the value of an ultrasensitive single-molecule array (Simoa) serum NfL (sNfL) assay in multiple sclerosis (MS).

Methods: sNfL levels were measured in healthy controls (HC, n=254) and two independent MS cohorts: (1) cross-sectional with paired serum and CSF samples (n=142), and (2) longitudinal with repeated serum sampling (n=246, median (IQR) follow-up 3.1 (2.0-4.0) years). We assessed their relation to concurrent clinical, imaging and treatment parameters and to future clinical outcomes.

Results: sNfL levels were higher in both MS cohorts than in HC (p<0.001). We found a strong association between CSF NfL and sNfL (β=0.589, p<0.001). Patients with either brain or spinal (43.4 (25.2-65.3) pg/ml) or both brain and spinal gadolinium enhancing lesions (62.5 (42.7-71.4) pg/ml) had higher sNfL than those without (29.6 (20.9-41.8) pg/ml; β=1.461, p=0.005 and β=1.902, p=0.002 respectively). sNfL was independently associated with EDSS assessments (β=1.105, p<0.001) and presence of relapses (β=1.430, p<0.001). sNfL levels were lower under disease modifying treatment (β=0.818, p=0.003). Patients with sNfL levels above the 80th, 90th, 95th, 97.5th and 99th HC based percentiles had higher risk of relapses (97.5th percentile: IRR=1.94, 95%CI=1.21-3.10, p=0.006) and EDSS worsening (97.5th percentile: OR=2.41, 95%CI=1.07-5.42, p=0.034).

Interpretation: These results support the value of sNfL as a sensitive and clinically meaningful blood biomarker to monitor tissue damage and the effects of therapies in MS.

INTRODUCTION

The clinical course of multiple sclerosis (MS) is highly variable, ranging from rapidly reversible episodes of impairment to severe disability within months after disease onset. Focal inflammation, chronic diffuse neuronal damage and failure of repair or compensation, all contribute to the development of permanent disability.[1] Biomarkers reflecting tissue damage and allowing to monitor subclinical disease activity are highly desirable for assessment of therapeutic response and prediction of disability in both clinical studies and management of individual patients.[2]

Together with the medium and heavy subunits, neurofilament light chain (NfL) represents one of the scaffolding proteins of the neuronal cytoskeleton and is released in the extracellular space following axonal damage.[3] NfL levels are increased in the cerebrospinal fluid (CSF) of MS patients as well as in degenerative and traumatic neurological diseases (e.g. dementia, amyotrophic lateral sclerosis and spinal cord injury).[4-9] CSF NfL levels are further increased during relapses and are positively associated with MRI lesion load and disability scores in MS.[10-12] Noteworthy, CSF NfL levels have also been shown to be a marker of treatment response in this disease.[13-17] However, lumbar punctures are relatively invasive procedures, limiting the value of CSF NfL in routine clinical settings.

A commercially available ELISA (UmanDiagnostics) can be used to measure CSF NfL, but is not recommended for blood measurements. Using an electrochemiluminescence (ECL) based assay we have found increased serum NfL (sNfL) concentrations in clinically isolated syndrome (CIS) and MS patients.[11,12,18,19] However, these studies were limited by the still relatively low sensitivity of the assay.[20] A novel single-molecule array (Simoa) assay has shown 126- and 25-fold higher sensitivity than the ELISA and ECL assays respectively.[20,21] This high sensitivity allows a more accurate quantification of the low sNfL concentrations expected in healthy controls and can help to better differentiate abnormal from normal values. Recent studies using this assay have shown that sNfL levels are increased in patients suffering from acute brain damage or chronic neurodegenerative disorders.[22-24]

This study had several aims: I) to obtain a pilot estimate of the distribution of sNfL concentrations in healthy controls (HC) and to investigate the potential influence of age and gender; II) to compare paired sNfL and CSF NfL levels in MS patients; III) to investigate the association between sNfL and number of T2 and contrast enhancing lesions in brain and spinal cord; IV) to investigate the association between sNfL and clinical features including occurrence of relapses, worsening of disability and treatment status; V) to test whether elevated sNfL levels can predict later disease activity and disability worsening.

SUBJECTS AND METHODS

Clinical settings, patient selection and sample collection

Lugano cohort

A cross-sectional cohort (n=142) was recruited between 2004 and 2015 at the Neurocentre of Southern Switzerland (Lugano, Switzerland), where paired serum and CSF samples are prospectively collected and stored as part of the diagnostic workup.[25] Inclusion criteria were: I) a diagnosis of CIS, relapsing remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS) or radiologically isolated syndrome (RIS)[26]; II) availability of serum and preferentially also paired CSF samples at time of diagnosis; III) availability of demographic and clinical data at time of diagnosis; IV) availability of brain and preferentially also spinal cord MR images acquired as part of the diagnostic workup at time of diagnosis. All brain and spinal MRI included in the analysis were performed with a standardized protocol and using 1.5T and 3T scanners (Siemens Sonata and Siemens Skyra, Erlangen, Germany).[27]

Swiss MS Cohort Study cohort

A longitudinal cohort (n=246) was recruited between 2009 and 2016 at the Neurologic Clinic and Policlinic, University Hospital Basel (Switzerland) as part of the Swiss Multiple Sclerosis Cohort Study (SMSC), a prospective observational study in which demographic, neuroimaging and clinical data as well as serum samples are collected every 6 or 12 months. Standardized clinical assessments with functional system score and Expanded Disability Status Scale (EDSS) calculation are performed by certified raters (http://www.neurostatus.net/).[28,29] All samples are collected within 8 days from the clinical visit and stored at -80°C following standardized procedures.[25] Criteria for inclusion in this study were: I) a diagnosis of CIS, RRMS, PPMS or SPMS; II) at least 2 but preferentially 3 available serum samples collected at baseline and at follow-up (FU) visits 1 and 2; III) start of disease modifying treatment (DMT) or switch to a different DMT shortly after baseline sample and before first FU sample (this only for CIS and RRMS patients); IV) availability of demographic and clinical data at time of sample collection including information on relapses and disability scores as measured by standardised assessment of the EDSS.

Healthy controls

Serum samples from 254 HC were collected between 2004 and 2007 in the Neurologic clinic and Policlinic, University Hospital Basel, as part of the international cohort study "GeneMSA" (Genetic MS Associations).[30] A one year FU serum sample was available for 87 HC. Inclusion criteria were age 18-70 years and no diagnosis of MS as well as no known cases of MS in the family.

Standard Protocol Approvals, Registrations, and Patient Consents

The study received ethical approval by independent ethics committees of the participating centres; all patients provided written, informed consent. The SMSC is registered with ClinicalTrials.gov (NCT02433028).

CSF and sNfL Measurements

We developed and validated a Simoa NfL assay using the capture monoclonal antibody (mAB) 47:3, and the biotinylated detector mAB 2:1 from UmanDiagnostics (UmanDiagnostics, Umeå, Sweden)[31], transferred onto the Simoa platform. mAB 47:3 was buffer exchanged and diluted to 0.3 mg/ml. $4x10^6$ paramagnetic beads (Quanterix) were buffer exchanged and activated using 0.5 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Quanterix), followed by a 30 minutes incubation at room temperature (RT, HulaMixer®, Thermofisher, USA). During a 2 hours incubation at RT (HulaMixer®) the diluted capture mAB was conjugated with the washed and activated beads. Subsequently the beads were washed and blocked. After three washes, the conjugated beads were suspended and stored at 4 °C. Biotinylated mAB 2:1 was obtained from UmanDiagnostics and stored at 4°C pending analysis.

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 (diluent: tris buffered saline (TBS); 0.1% Tween 20; 1% milk powder; 400 µg/ml Heteroblock (Omega Biologicals Inc., Bozeman, USA)), 25 µL conjugated beads (diluent: TBS; 0.1% Tween 20; 1% milk powder; 300 µg/ml Heteroblock), and 20 µL of mAB 2:1 (0.1 µg/ml; diluent: TBS; 0.1% Tween 20; 1% milk powder; 300 µg/ml Heteroblock) were incubated for 47 cadences (1 cadence=45 s). After washing, 100 µL of streptavidin conjugated β-galactosidase (150 pM; Quanterix) was added, followed by a 7-cadence incubation and a wash. Prior to reading, 25 µL Resorufin β-D-galactopyranoside (Quanterix) was added. Calibrators (neat) and samples (serum: 1:4 dilution; CSF: 1:10 dilution) were measured in duplicates. Bovine lyophilized NfL was obtained from UmanDiagnostics. Calibrators ranged from 0 to 2,000 pg/ml for serum and 0 to 10,000 pg/ml for CSF measurements. Batch prepared calibrators were stored at -80°C.

Intra- and inter-assay variability of the assay was evaluated with 3 native serum and 3 native CSF samples in 22/12 consecutive runs on independent days, respectively. For serum the mean coefficients of variation (CVs) of duplicate determinations for concentration were 5.6% (13.3 pg/ml, sample 1), 6.9% (22.5 pg/ml, sample 2) and 5.3% (236.5 pg/ml, sample 3). In CSF the mean intra-assay CVs were 2.5% (572.6 pg/ml, sample 1), 0.7% (1601.8 pg/ml, sample 2) and 3.8% (6110.2 pg/ml, sample

3). Inter-assay CVs for serum were 11.3% (sample 1), 9.3% (sample 2) and 6.4% (sample 3). In CSF inter-assay CVs were 10.1% (sample 1), 6.2% (sample 2) and 15.5% (sample 3). We used the concentration of the lowest calibrator fulfilling acceptance criteria [accuracy: 80%–120%, CV of duplicate determination ≤ 20%] as an estimate of the analytical sensitivity.[32] The analytical sensitivity was 0.32 pg/ml. All samples produced signals above the analytical sensitivity of the assay. Few samples with intra-assay CVs above 20% were repeat measured. Recovery rates ((Concentration spiked sample-concentration native sample)/Spiked concentration*100) were tested in 4 serum and 4 CSF samples from healthy controls spiked with 5, 50 and 200 pg/ml and 500, 2000 pg/ml of NfL, respectively. The mean recovery for serum after spiking was 107% and for CSF 121%. Parallelism and linearity of the assay for serum and CSF were confirmed by serial dilution experiments. [32]

Statistics

Categorical variables were described by counts and percentages, continuous and ordinal variables by median and interquartile ranges (IQR). For all analyses NfL levels were log-transformed to meet the normal assumption. The distribution of sNfL in HC and its association with age was modelled by means of Generalized Additive Models for Location, Scale and Shape (GAMLSS) using a Box-Cox t distribution according to Rigby & Stasinopoulos [33] and cubic splines and percentile curves were obtained. To quantify the variability bootstrapping was applied by drawing 100 random samples from the HCs. From each sample the percentile curves were estimated and the final reference percentiles across different ages represent averages over the 100 replicates together with the bootstrap confidence intervals.

In the cross-sectional Lugano cohort, linear regression models were used to investigate the associations with log sNfL. Linear generalized estimating equation (GEE) models were similarly used to investigate associations with log sNfL in the SMSC cohort with repeated measurements. In all linear models with log sNfL as the dependent variable, regression coefficients (denoted with "ß"

throughout this work) were back-transformed to the original scale and therefore reflect multiplicative effects (i.e. an estimate of 1.05 means an increase of approximately 5% in sNfL).

In GEE models, different correlation structures were investigated and model selection was performed based on QIC (quasi-likelihood under the independence model criterion).[34] Based on expert input and signals observed in the graphical analysis, several interaction terms were investigated and the final model was selected based on the QIC. To investigate the course of sNfL after treatment initiation, a linear GEE was used with time under treatment and baseline sNfL as additional covariates in the multivariable model thereby excluding treatment status. This analysis was performed on all samples after treatment start.

Patients' sNfL levels were finally categorized based on the percentiles derived from the HC samples. Clinically meaningful events (relapses, annualized relapse rate (ARR) or EDSS worsening, both before and after sample collection) were then tested for association with sNfL levels above vs below various percentile cut-offs using GEE models. These analyses were performed for the percentiles curves from each of the 100 bootstrap replicates. The 100 results were integrated into a final result using Rubin's rule. Therefore the final results not only incorporate the standard errors of the GEE models but also take into account the uncertainty of the reference percentile curves. EDSS worsening was defined as an increase in EDSS since previous SMSC visit of ≥1.5 points from an EDSS score of 0.0, ≥1.0 point from an EDSS score of 1.0–5.5 or ≥0.5 point from an EDSS score ≥6.0 (median duration between visits=6.4 (5.2 - 11.7) months). GEEs using a Poisson distribution were used to compare the incidence of relapses between percentile categories and calculate incidence rate ratios (IRR) with 95% confidence intervals (95%CI). The models were tested for overdispersion [35] and the null hypothesis of equidispersion was not violated in any model. As a sensitivity analysis negative binomial mixed effect models were used. However, these models tended to not converge further supporting the use of a Poisson distribution. GEE models were similarly used to model binary outcomes (e.g. presence vs absence of relapses and presence vs absence of EDSS increase) and estimate odds ratios (OR) with 95%CI. For all models, model-predicted means (marginal means) and

95%CI were calculated using the lsmeans-package [36] and predicted odds were converted to probabilities (p=odds/(1+odds)). All analyses in which NfL was used to predict past and future clinical events were performed on a subset of the data excluding samples within 30 days after a relapse. As a sensitivity analysis, all analyses were repeated using all samples (i.e. without removing samples shortly after a relapse) and using only the last sample at which patients were under similar conditions using generalized linear models (data not shown). The quality of all models was investigated by visually inspecting residuals and quantile-quantile plots. All analyses were conducted using the statistical software R.[37]

RESULTS

sNfL levels in HC

Age, gender and temporal variation

Most HC were females (n=173, 68.1%) and the median age was 44.3 (36.4-52.4) years. The median sNfL concentration was 22.9 (16.8-31.4) pg/ml, with no statistically significant difference between males and females (23.4 (17.1-32.1) vs 22.8 (16.6-30.3) pg/ml; β=1.032, 95%CI=0.910-1.171, p=0.622). A positive association was instead observed between sNfL and age, with a 2.2% increase in sNfL for each additional year (β=1.022, 95%CI=1.018-1.026, p<0.001). Accordingly, median serum NfL slightly increased (by 1.8%) in the 87 HC with a second serum sample after a median follow-up time of 367 (364-385) days (baseline: 27.3 (20.3-35.2) pg/ml; FU: 27.8 (22.1-36.3) pg/ml). There was no association between sNfL and storage time (β=0.959, 95%CI=0.906-1.016, p=0.157 after age correction).

Reference percentile curves

The distribution of sNfL across different ages was modelled by using GAMLSS (see methods). The resulting 80th, 90th, 95th, 97.5th and 99th sNfL percentiles are presented in table 1.

Table 1: Estimated sNfL percentiles including bootstrap confidence intervals across different ages calculated based on sNfL from HC samples.

Age (years)	sNfL percentiles (pg/ml)				
	80th	**90th**	**95th**	**97.5th**	**99th**
30	20.9 (19.3-22.4)	24.3 (22.3-26.3)	27.9 (25.1-30.4)	31.6 (27.6-35.7)	37.2 (30.9-44.4)
35	23.3 (21.9-24.9)	27.1 (25.3-29.2)	31.1 (28.6-34.0)	35.2 (31.7-39.6)	41.5 (35.8-49.4)
40	26.0 (24.7-27.5)	30.3 (28.6-32.3)	34.7 (31.9-37.8)	39.3 (35.4-44.0)	46.3 (40.1-54.9)
45	29.1 (27.7-30.7)	33.9 (32.2-35.9)	38.9 (36.1-41.9)	44.1 (39.8-49.2)	51.9 (44.8-61.5)
50	32.7 (31.1-34.8)	38.1 (35.9-40.3)	43.6 (40.7-47.0)	49.5 (44.7-55.4)	58.3 (50.3-69.4)
55	36.5 (34.2-39.2)	42.5 (39.7-45.4)	48.7 (45.4-52.5)	55.2 (50.4-61.6)	65.0 (56.2-77.3)
60	40.5 (37.7-44.0)	47.2 (43.6-51.0)	54.0 (49.6-58.8)	61.3 (55.4-68.1)	72.1 (62.3-85.1)
65	44.6 (41.0-49.1)	52.0 (47.3-57.1)	59.5 (53.4-65.8)	67.5 (60.0-75.9)	79.5 (68.2-93.4)
70	48.8 (44.2-54.3)	56.9 (51.1-63.4)	65.1 (57.2-73.2)	73.9 (64.3-84.0)	87.0 (73.8-102.7)

sNfL: serum neurofilament light chain; HC: healthy control.

sNfL levels in the Lugano Cohort

Demographic and clinical variables

Serum and paired CSF samples were available in 142 and 132 patients. The median age was 37.9 (29.8-47.8) years and 92 (64.8%) were female. There were 48 (33.8%) CIS, 62 (43.7%) RRMS, 16 (11.3%) PPMS, 3 (2.1%) SPMS and 13 (9.1%) RIS patients. Brain and spinal cord MRI data were available at time of sample collection for 142 and 124 individuals. The median time between sample collection and the acquisition of brain and spinal cord MRI images was 5.0 (1.0-19.5) and 13.0 (4.0-30.0) days.

Serum and CSF NfL

Median NfL in serum (35.9 (22.1-61.7) pg/ml) was 42-fold lower than that in CSF (1521.1 (814.1-2888.1) pg/ml). There was a strong positive association between CSF NfL and sNfL levels, with a 10% increase in CSF leading to a 5.9% higher sNfL ($\log_{10}(sNfL) = 0.0509 + 0.589*\log_{10}(NfL_{CSF})$, $p<0.001$; Pearson's r=0.77, 95%CI=0.69-0.83, $p<0.001$; figure 1A).

sNfL in patients and controls and associations with MRI

As in HC samples, sNfL was positively associated with age (β=1.015, 95%CI=1.006-1.025, $p=0.002$), but not with gender (β=1.165, 95%CI=0.911-1.489, $p=0.226$). There was no association between sNfL and storage time (β=1.030, 95%CI=0.977-1.086, $p=0.274$, after age correction). All remaining analyses were corrected by including age as a covariate in the regression models. Patients had higher sNfL levels than HC (β=1.914, 95%CI=1.717-2.135, $p<0.001$). In addition, sNfL progressively increased with increasing number of T2 and gadolinium enhancing (GE) lesions in both brain and spinal cord (table 2 and figures 1B, 1C). Median sNfL levels progressively increased from 29.6 (20.9-41.8) pg/ml in patients with GE lesions in neither brain nor spinal cord, to 43.4 (25.2-65.3) pg/ml in those with GE lesions in either brain or spinal cord, to 62.5 (42.7-71.4) pg/ml in those with GE lesions in both brain and spinal cord (either vs neither: β=1.461, 95%CI=1.128-1.892, $p=0.005$; both vs neither: β=1.902, 95%CI=1.278-2.830, $p=0.002$; both vs either: β=1.302, 95%CI=0.861-1.969, $p=0.213$; table 2 and figure 1D).

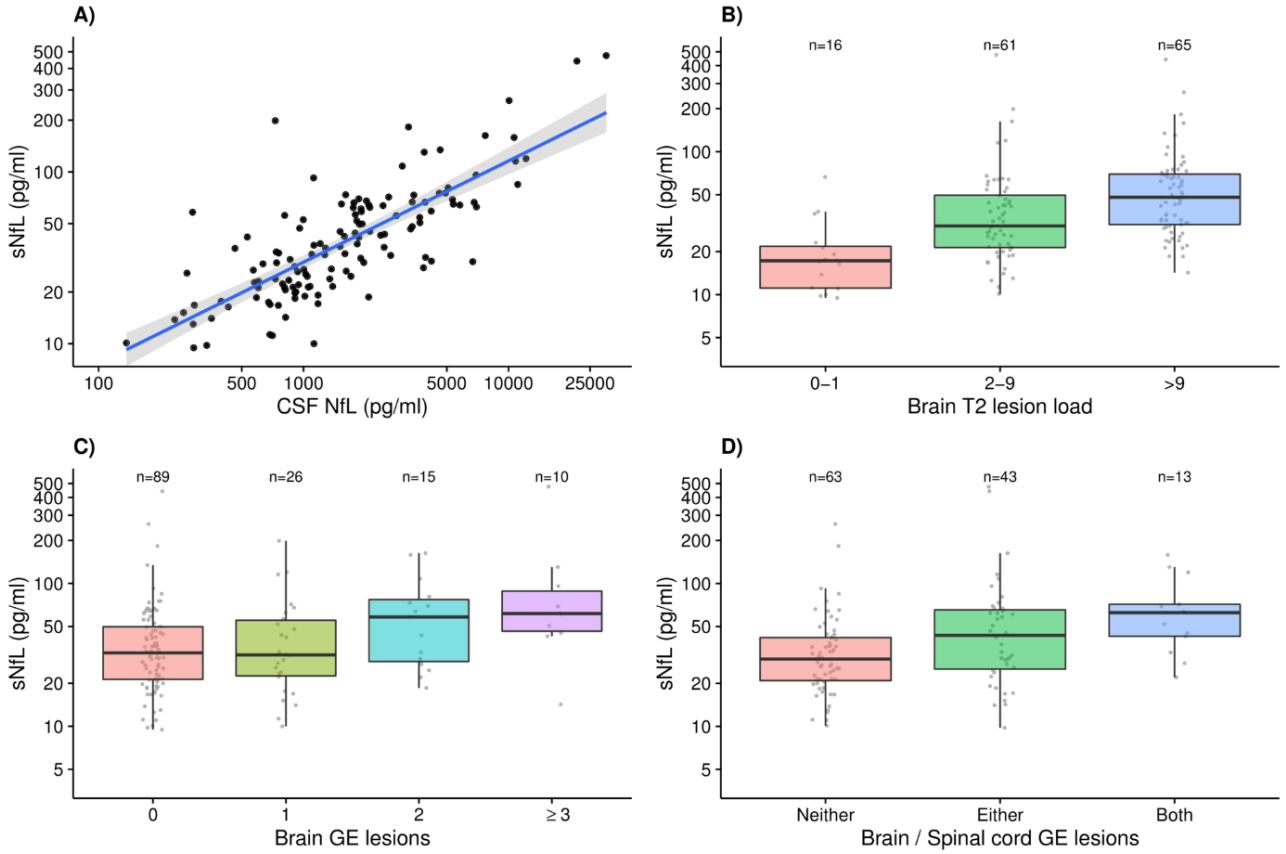

Figure 1: A) Association between CSF NfL and sNfL levels in the Lugano cohort. A 10% increase in CSF NfL corresponds to an increase of approximately 5.9% in sNfL (β=0.589, p<0.001). Grey band: 95% confidence interval. B) Association between brain T2 lesion load and sNfL levels in the Lugano cohort (2-9 vs 0-1: β=1.849, p=0.001; >9 vs 0-1: β=2.524, p<0.001). C) Association between number of brain GE lesions and sNfL levels in the Lugano cohort (1 vs 0: β=1.077, p=0.630; 2 vs 0: β=1.551, p=0.024; ≥3 vs 0: β=2.138, p=0.001). D) Association between brain and spinal cord GE lesions and sNfL levels in the Lugano cohort (either brain or spinal vs neither: β=1.461, p=0.005; both brain and spinal vs neither: β=1.902, p=0.002).

Table 2: sNfL concentration and associations with different clinical and MRI variables in the Lugano cohort.

Variables		median (IQR) / n (%)	sNfL (median (IQR)) pg/ml	β	95%CI	*p*
Age (years)		37.9 (29.8-47.8)	-	1.015	1.006-1.025	0.002
Gender	F	92 (64.8)	33.0 (21.5-55.3)	-	-	-

	M	50 (35.2)	44.2 (25.7-62.4)	1.165	0.911-1.489	0.226
Oligoclonal bands	Negative	13 (9.1)	26.8 (16.8-49.6)	-	-	-
	Positive	129 (90.9)	36.2 (22.7-61.9)	1.114	0.740-1.676	0.606
Brain T2 lesion number	0-1	16 (11.3)	17.3 (11.1-21.8)	-	-	-
	2-9	61 (43.0)	30.2 (21.4-49.6)	1.849	1.283-2.666	0.001
	>9	65 (45.7)	48.0 (30.9-69.7)	2.524	1.744-3.653	<0.001
Brain GE lesions	0	89 (63.6)	32.7 (21.3-49.7)	-	-	-
	1	26 (18.6)	31.6 (22.6-55.3)	1.077	0.797-1.456	0.630
	2	15 (10.7)	58.3 (28.4-77.0)	1.551	1.064-2.259	0.024
	≥3	10 (7.1)	61.6 (46.4-89.1)	2.138	1.362-3.355	0.001
Spinal T2 lesion number	0	31 (25.0)	26.4 (17.2-42.8)	-	-	-
	1	26 (21.0)	25.4 (18.5-42.5)	0.819	0.574-1.167	0.271
	≥2	67 (54.0)	44.0 (29.6-64.6)	1.332	0.992-1.788	0.059
Spinal GE lesions	0	95 (78.5)	32.4 (21.5-53.5)	-	-	-
	1	26 (21.5)	49.2 (30.9-66.0)	1.467	1.091-1.974	0.013
Brain/Spinal GE lesions	Neither	63 (52.9)	29.6 (20.9-41.8)	-	-	-
	Either	43 (36.1)	43.4 (25.2-65.3)	1.461	1.128-1.892	0.005
	Both	13 (10.9)	62.5 (42.7-71.4)	1.902	1.278-2.830	0.002

sNfL: serum neurofilament light chain; IQR: interquartile range; CI: confidence interval; F: female; M: male; GE lesions: gadolinium enhancing lesions. Age was included as additional variable in all models.

sNfL Levels in the SMSC

Demographic, clinical variables and treatment switches

Three and two serum samples were available for 227 and 19 patients, respectively (i.e. total number of samples=719). Most patients started or switched to a new DMT shortly after baseline sample ("starters", n=212, 86.2%), while 34 (13.8%) were patients with progressive MS who were either untreated or on continuous DMT ("non-starters"). The median time between baseline sampling and DMT initiation in the starters group was 41 (5.0-93.8) days. Demographic and clinical characteristics are shown in table 3.

Table 3: Descriptive statistics of demographic and clinical variables of the SMSC patients at baseline (median (IQR) or counts (percentages)).

Variables		SMSC (n=246)	SMSC starters (n=212)	SMSC non-starters (n=34)
Age (years)		42.2 (33.6-51.4)	40.6 (32.8-48.8)	54.5 (49.2-60.9)
Gender	F	162 (65.9)	151 (71.2)	11 (32.4)
	M	84 (34.1)	61 (28.8)	23 (67.6)
Diagnosis (at baseline)	CIS	14 (5.7)	14 (6.6)	0 (0.0)
	RRMS	185 (75.2)	185 (87.3)	0 (0.0)
	SPMS	27 (11.0)	11 (5.2)	16 (47.1)
	PPMS	20 (8.1)	2 (0.9)	18 (52.9)
Disease duration (years)		7.4 (1.8-15.3)	6.6 (1.6-14.3)	15.3 (7.9-23.7)
EDSS		3.0 (1.5-4.0)	2.5 (1.5-3.5)	4.8 (3.6-6.0)
DMT at baseline	Injectable DMTs	77 (31.3)	73 (34.4)	4 (11.8)
	Natalizumab	22 (8.9)	22 (10.4)	0 (0.0)
	Fingolimod	9 (3.7)	9 (4.2)	0 (0.0)
	Azathioprine	4 (1.6)	4 (1.9)	0 (0.0)
	Mitoxantrone	6 (2.4)	3 (1.4)	3 (8.8)
	Dimethyl fumarate	2 (0.8)	2 (0.9)	0 (0.0)
	Rituximab	1 (0.4)	1 (0.5)	0 (0.0)
	Other	4 (1.6)	0 (0.0)	4 (11.8)
	Untreated	121 (49.2)	98 (46.2)	23 (67.6)
Switch after baseline to	Fingolimod	-	136 (64.2)	-
	Injectable DMTs	-	39 (18.4)	-
	Natalizumab	-	21 (9.9)	-
	Rituximab	-	16 (7.5)	-
Baseline to first follow-up (days)		224.0 (188.0-368.0)	217.0 (183.5-365.0)	363.5 (335.2-377.2)
Baseline to second follow-up (days)		540.0 (386.0-725.5)	511.0 (383.5-700.8)	731.0 (664.5-753.0)
Baseline to new DMT start (days)		-	41.0 (5.0-93.8)	-

SMSC starters: patients starting or switching to a new disease modifying treatment (DMT) after baseline sampling. SMSC non-starters: progressive MS patients who were either untreated or had not changed DMT. F: females; M: males; CIS: clinically isolated syndrome; RRMS: relapsing remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS; EDSS: Expanded Disability Status Scale; DMT: Disease modifying treatment.

Associations between sNfL and demographic and clinical variables

The median sNfL level in the SMSC cohort was 29.4 (20.1-45.2) pg/ml. Several variables were tested for association with sNfL in all patients (n=246) (table 4). As in the HC and Lugano cohorts, sNfL levels were positively associated with age (β=1.018, 95%CI=1.012-1.024, p<0.001) and no gender association was detected (table 4). Storage time was not significantly associated with sNfL (β=1.048, 95%CI=0.999-1.099, p=0.057 after age correction). Disease duration was also significantly associated with sNfL (β=1.011, 95%CI=1.003-1.018, p=0.004). However, this association disappeared when correcting for age (β=1.001, 95%CI=0.993-1.010, p=0.755), while the age

association was unchanged (β=1.016, 95%CI=1.008-1.023, p<0.001). This implies disease duration as a proxy for age and only the latter was therefore considered in following analyses. The age association was present and of similar strength in both CIS/RRMS and PPMS/SPMS patients (β=1.015, 95%CI=1.007-1.023, p<0.001 and β=1.015, 95%CI=1.003-1.028, p=0.016; figure 2A). Both groups had higher sNfL than HC, even after correcting for age (CIS/RRMS: 27.2 (19.2-57.2) pg/ml, β=1.418, 95%CI=1.288-1.560, p<0.001; PPMS/SPMS: 41.4 (32.1-57.2) pg/ml, β=1.620, 95%CI=1.417-1.851, p<0.001; figure 2B). sNfL concentrations were higher in PPMS/SPMS as compared to CIS/RRMS (β=1.450, 95%CI=1.245-1.688, p<0.001; after correcting for age: β=1.205, 95%CI=1.106-1.418, p=0.029). Positive associations were also found in univariable analyses between sNfL and EDSS (β=1.141, 95%CI=1.106-1.178, p<0.001; figure 2C), presence of a relapse within 60 days before sampling (β=1.563, 95%CI=1.303-1.874, p<0.001) and recent EDSS worsening (β=1.294, 95%CI=1.090-1.536, p=0.003). Noteworthy, sNfL levels were lower in DMT treated versus untreated patients (β=0.717, 95%CI=0.634-0.810, p<0.001).

All following variables were then included in the same multivariable model: age, gender (F vs M), EDSS, disease course (CIS/RRMS vs PPMS/SPMS), presence of relapses within 60 days before sampling (yes vs no), recent EDSS worsening (yes vs no) and DMT treatment status (treated vs untreated). sNfL levels remained significantly associated with age, EDSS, presence of relapses within 60 days before sampling and DMT treatment status (table 4). Disease course (CIS/RRMS versus PPMS/SPMS) did not survive as an independent factor. We tested potential interactions between variables of interest and observed that the increase in sNfL per EDSS unit increase was lower in PPMS/SPMS than in CIS/RRMS patients (β=1.024, 95%CI=0.952-1.101 vs β=1.133, 95%CI=1.081-1.187, respectively; interaction p=0.021; figure 2D, supplementary table 1).

Table 4: Univariable and multivariable models testing associations between age, gender, EDSS, disease course, recent relapses, recent EDSS worsening and DMT status and sNfL in the SMSC cohort.

Variables (samples n)		sNfL pg/ml	Univariable			Multivariable		
			β	95%CI	*p*	β	95%CI	*p*
Age (719)		-	1.018	1.012-1.024	<0.001	1.012	1.005-1.019	<0.001
Gender	F (474)	29.1 (20.1-44.3)	-	-	-	-	-	-
	M (245)	30.9 (20.2-48.0)	1.054	0.902-1.232	0.505	0.991	0.858-1.145	0.905
EDSS (719)		-	1.141	1.106-1.178	<0.001	1.105	1.063-1.149	<0.001
Disease course	CIS/RRMS (581)	27.2 (19.2-57.2)	-	-	-	-	-	-
	PPMS/SPMS (138)	41.4 (32.1-57.2)	1.450	1.245-1.688	<0.001	0.924	0.742-1.151	0.483
Recent relapse (<60 days)	No (643)	28.9 (20.0-43.8)	-	-	-	-	-	-
	Yes (76)	39.3 (25.9-60.2)	1.563	1.303-1.874	<0.001	1.430	1.156-1.768	<0.001
Recent EDSS worsening	No (615)	29.0 (20.1-43.9)	-	-	-	-	-	-
	Yes (51)	38.5 (27.8-64.0)	1.294	1.090-1.536	0.003	1.119	0.962-1.303	0.146
DMT	Untreated (162)	38.0 (23.8-56.7)	-	-	-	-	-	-
	DMT treated (557)	27.0 (20.1-45.2)	0.717	0.634-0.810	<0.001	0.818	0.716-0.934	0.003

sNfL: serum neurofilament light chain; CI: confidence interval; F: female; M: male; CIS: clinically isolated syndrome; RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; SPMS: secondary progressive multiple sclerosis; EDSS: Expanded Disability Status Scale; DMT: disease modifying treatment. The number of samples for each variable is indicated within brackets (e.g. number of samples collected in patients being under treatment at time of sampling = 557, number of samples collected in patients being untreated at time of sampling = 162). Information on age, gender, EDSS, disease course, recent relapses, and DMT treatment was available for 719 (100%) sampling time points. No data were available for preceding EDSS scores at 53 (7.4%) sampling time points.

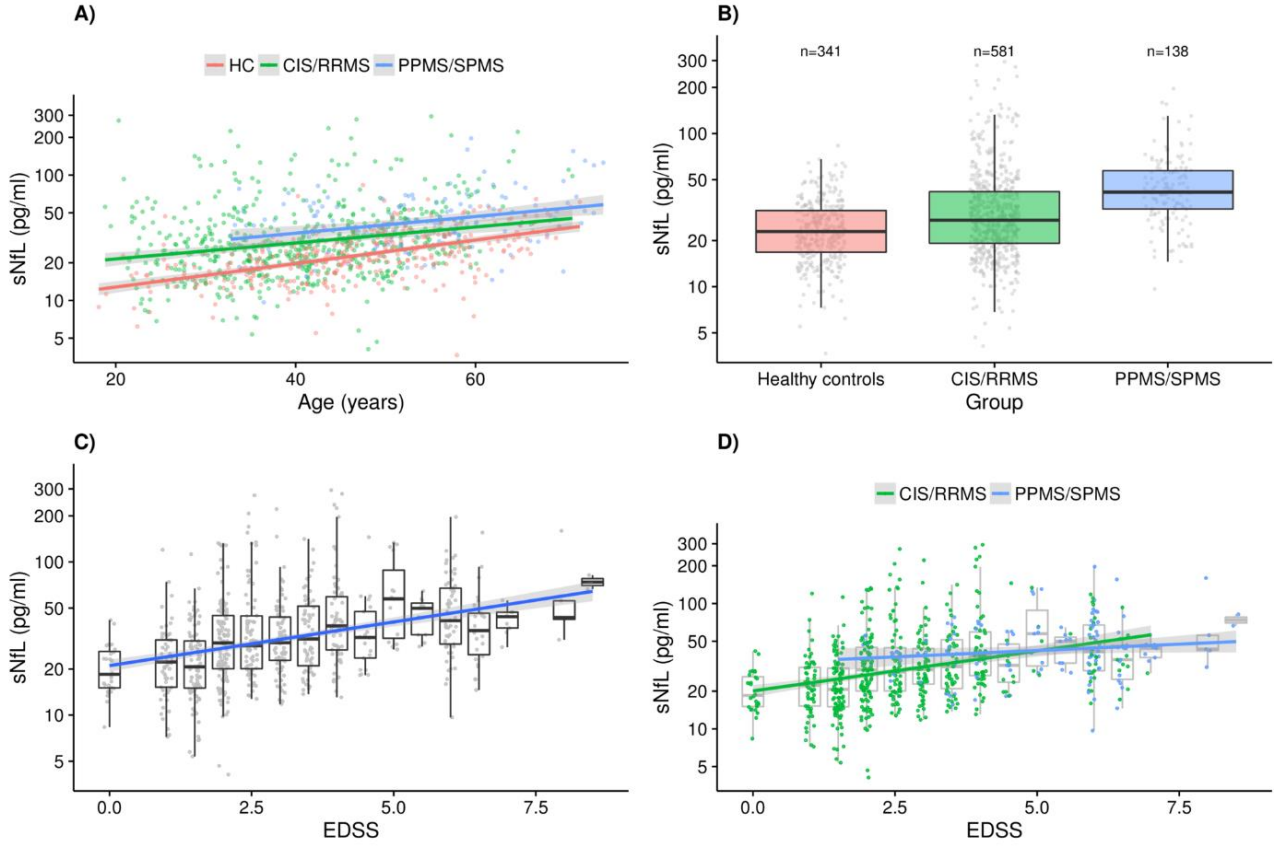

Figure 2: A) Association between age and sNfL levels in HC, CIS/RRMS and PPMS/SPMS patients from the SMSC cohort. An increase of 1 year in age corresponds to an increase of approximately 2.2%, 1.5% and 1.5% in sNfL in the three groups, respectively. Grey band: 95% confidence interval. B) Serum NfL in healthy controls versus CIS/RRMS and SPMS/PPMS from the SMSC cohort. C) Association between EDSS and sNfL levels in the SMSC cohort. A one point EDSS increase corresponds to a sNfL increase of approximately 14.1%. Grey band: 95% confidence interval. D) Significant interaction between EDSS and disease course (CIS/RRMS vs PPMS/SPMS) in the association with sNfL in the SMSC (interaction β=0.904, interaction *p*=0.021). Grey shading: 95% confidence interval.

Associations between sNfL and time under new treatment

Baseline sNfL levels were higher in patients starting natalizumab (50.8 (20.8-77.0) pg/ml) and rituximab (51.0 (29.1-71.4, pg/ml) than those initiating fingolimod (29.8 (20.7-46.4) pg/ml) and injectable DMTs (28.1 (18.0-43.2) pg/ml). sNfL levels at baseline were higher in all patient groups as compared to HC ($p<0.001$ for all, figure 3). We explored the association between time under treatment and sNfL during FU while correcting for baseline sNfL and other covariates. After adjustment, time since start of new treatment in years was negatively associated with FU sNfL (β=0.900, 95%CI=0.830-0.976, $p=0.011$, figure 3, supplementary table 2). The decrease in sNfL with time since start of new treatment appeared similar across different DMTs, but numbers were too low to investigate differences further.

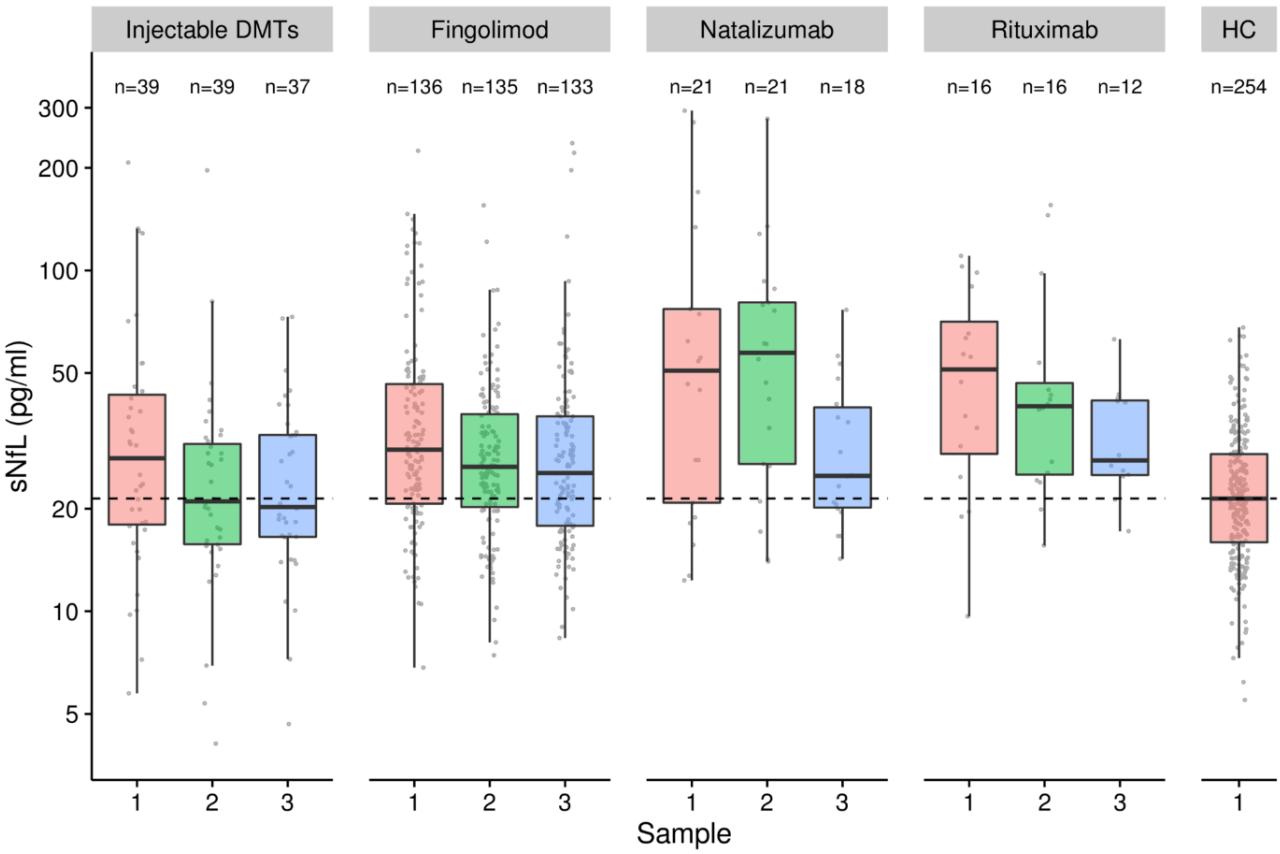

Figure 3: Baseline sNfL was higher in patients starting natalizumab (50.8 pg/ml) and rituximab (51.0 pg/ml) than in those initiating fingolimod (29.8 pg/ml) and injectable DMTs (28.1 pg/ml). sNfL levels decreased in patients starting injectable DMTs, fingolimod, natalizumab or rituximab over time.

Serum NfL and previous and future disease activity

We finally investigated whether high sNfL levels were associated with past and future clinical disease activity (relapses and EDSS worsening). To this purpose we compared sNfL measurements from the SMSC against the age corrected percentile curves that were constructed based on HC samples. In order to have a more homogeneous population, this analysis was only performed in CIS/RRMS patients. Out of a total of 581 samples, 287 (49.4%) samples had sNfL values above the 80th percentile, 228 (39.2%) above the 90% percentile, 171 (29.4%) above the 95th percentile, 135 (23.2%) above the 97.5th percentile and 105 (18.1%) above the 99th percentile. The median FU time after sample collection was 3.1 (2.0-4.0) years.

Previous clinical disease activity (relapses, annualized relapse rate and EDSS worsening)

The probability of having experienced a relapse within 60 days before sampling was increased for sNfL measurements above versus below the 80th, 90th, 95th, 97.5th and 99th percentiles (figure 4A, supplementary table 3). Patients with sNfL above the 97.5th percentile were at approximately 4.0 fold odds of having experienced a relapse in the previous 60 days (OR=3.89, 95%CI=2.30-6.58, p<0.001). The mean ARR during 1 and 2 years before sample collection was higher in patients with sNfL levels above these percentiles (figure 4A, supplementary table 3). The incidence of relapses 1 and 2 years before sample collection was approximately 1.5-2.0 times higher with sNfL levels above the 97.5th percentile (IRR=2.08, 95%CI=1.64-2.63, p<0.001 and IRR=1.39, 95%CI=1.18-1.64, p<0.001 respectively).

The probability of having experienced worsening of the EDSS within 6-12 months before sampling was higher in patients with sNfL values above vs below the 90th, 95th, 97.5th and 99th percentiles (figure 4A, supplementary table 3). Patients with sNfL above the 97.5th percentile were at more than 4.0 fold odds of having experienced EDSS worsening in the previous 6-12 months (OR=4.36, 95%CI=2.09-9.09, p<0.001). Notably, there was a strikingly progressive probability of having experienced past relapses or EDSS worsening with increasing percentile categories.

Future clinical disease activity (ARR and EDSS worsening)

The mean ARR was increased during 1 and 2 years after the collection of samples with sNfL levels above the 80th, 90th, 95th, 97.5th and 99th percentiles (figure 4B, supplementary table 4). The incidence of relapses was approximately 2.0 times higher both 1 and 2 years after the collection of samples with sNfL levels above the 97.5th percentile (IRR=1.94, 95%CI=1.21-3.10, p=0.006 and IRR=1.96, 95%CI=1.22-3.15, p=0.005). The proportion of patients experiencing EDSS worsening within 12 months after sampling gradually increased with increasing sNfL percentile category (from 6.7% for samples <80th percentile to approximately 15% for samples >97.5th percentile (OR=2.41, 95%CI=1.07-5.42, p=0.034)) (figure 4B, supplementary table 4).

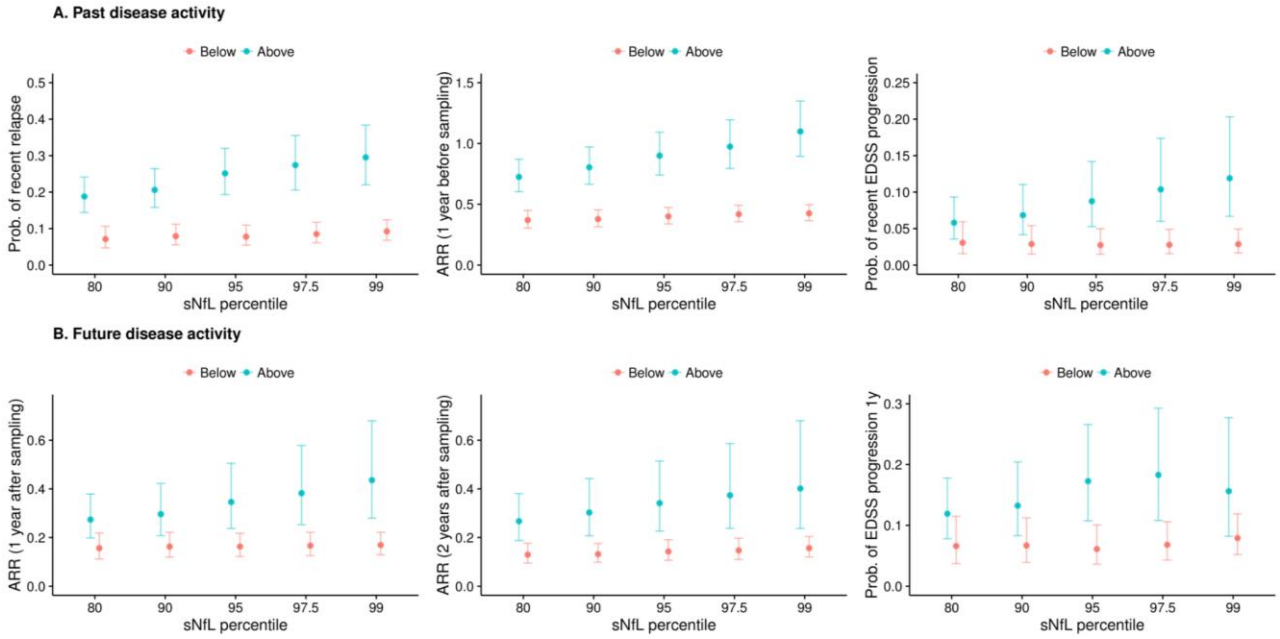

Figure 4: Model-predicted means (marginal means) and model estimates including 95% confidence intervals from GEE models: A) Probability of a recent relapse (within 60 days before sampling), ARR in the one year before sampling and probability of EDSS worsening since 6-12 months before sampling according to sNfL percentiles. B) ARR in the one year after sampling, ARR in the two years after sampling and probability of EDSS worsening within one year after sampling according to sNfL percentiles. 287 samples (49.4%) had sNfL values above the 80th percentile, 228 samples (39.2%) above the 90% percentile, 171 samples (29.4%) above the 95th percentile, 135 samples (23.2%) above the 97.5th percentile and 105 (18.1%) above the 99th percentile.

DISCUSSION

Several candidate biomarkers have been proposed in MS [2], but their clinical relevance remains uncertain and none is currently accepted as a sensitive and reliable measure to monitor disease course in clinical practice. In two independent cohorts of patients we provide evidence that measurement of sNfL has several features necessary to qualify as an urgently needed laboratory marker of neuronal damage in MS. sNfL levels are not only significantly higher in MS patients versus controls, they correlate with focal lesion presence and activity in both the brain and the spinal cord, as depicted by MRI but also with relevant static and dynamic clinical outcomes, i.e. previous, concurrent and future relapses and disability worsening.

Our results confirm and expand on previous studies [20,21] showing that NfL can be reliably measured in serum using the Simoa technology, even at very low concentrations (down to a few pg/ml). The observed increase of NfL levels in serum with age seen in both HC and patient cohorts mirrors the age association described for CSF NfL levels,[38] and it is best explained by ongoing age related neuronal degeneration. We did not observe a difference in sNfL between genders. The tight positive association between CSF and sNfL levels, highlights that serum levels closely reflect NfL release within the CNS as already indicated by previous studies.[19,39,40]

Both patient cohorts included in this study had higher sNfL concentration than healthy individuals. This confirms what has been observed in CSF NfL studies [4,7,10-12,41-44] and the results of a single previous investigation of CIS patients in which sNfL levels were measured using a less sensitive ECL assay.[18] sNfL levels were also slightly higher in the Lugano than in the SMSC samples, likely because the former were collected as part of the diagnostic work-up, which is frequently performed shortly after relapses. The close association of increased blood NfL levels with neuronal damage has been suggested in other neurological conditions including ALS, neurodegenerative disorders, and acute

brain and spinal cord injury.[8,22-24,39,40] In conjunction with findings in other neurological diseases, our results in MS strongly suggest that increased sNfL levels reflect ongoing neuronal damage irrespective of the underlying pathogenic mechanism.

The relation between neuronal damage and NfL concentration is also supported by the clear positive association between sNfL and focal inflammatory MRI lesions in both brain and spinal cord. We found gradually increased sNfL levels in patients with higher brain T2 and GE lesion counts. A similar significant association was found between sNfL and presence of spinal GE lesions and was most pronounced when GE lesions were present in both brain and spinal cord. Several studies have shown associations between CSF NfL and brain T2 and GE lesions. [7,12,45] We have also previously shown weak associations between sNfL (as measured by the ECL assay) and brain T2 and GE lesions in CIS patients [18] and in a small cohort of RRMS patients (n=29).[19] Our current results confirm and expand these findings in a larger cohort of patients and suggest that also spinal cord damage contributes to increased NfL concentrations in serum. This appears relevant since spinal cord pathology is a key factor in the development of disability in MS.[27,46]

We made use of the longitudinal SMSC cohort with repeated measurements to simultaneously analyse the association between several clinical variables and sNfL. In addition to age, both presence of a recent relapse and disability as measured by the EDSS were positively and independently associated with sNfL. This suggests sNfL levels may be related to both acute inflammatory damage and chronic diffuse neuronal loss leading to disability progression in its proper sense. Interestingly, the EDSS association was more evident in CIS/RRMS than in PPMS/SPMS patients, perhaps resembling the slower and gradual disability accumulation characterizing progressive MS. It may also be an indication that disease progression in this later stage of disease is reflecting both direct tissue damage and reduced/exhausted compensation capacity.

Of particular interest in the search for biomarkers reflecting therapeutic effects is that sNfL levels were significantly lower in DMT treated as compared to untreated patients, independently of all other variables. In CIS/RRMS patients the decrease in sNfL levels correlated inversely with longer time since start of DMTs independent of recent relapses. Notably, treatment effects on CSF NfL levels have already been shown for fingolimod, natalizumab and rituximab in MS patients.[13-17,47] Although this study was not primarily designed to investigate treatment effects, our results suggest that DMTs reduce sNfL levels, supporting their value for monitoring treatment response.

Patients with sNfL levels above different HC based percentiles had considerably higher risk of having experienced a recent relapse or EDSS worsening. sNfL measurements could therefore be used to indicate recent neuronal damage and this could be particularly useful in case of 'clinically silent disease' or when clinical changes are difficult to interpret. Moreover, high sNfL levels were also associated with a higher risk of future clinical relapses and EDSS worsening. This confirms findings from two relatively small studies suggesting that patients with higher CSF neurofilament levels have a worse long-term disease outcome.[48,49] Taken together, these results support the potential use of sNfL as a prognostic marker of clinical disease course.

Our study has some limitations. Only one single standardized high resolution MRI scan was available as part of the clinical diagnostic workup of the Lugano cohort and no lesion volume measurements were available in addition to the T2 lesion counts to test for association with sNfL. Second, the follow-up in the SMSC cohort was relatively short and did not allow an estimate of sNfL association with long term disease worsening or progression. The observational study design does not allow to separate potential treatment effects from regression to the mean phenomena in this relatively active cohort of patients. The percentile curves are currently based on a limited number of HC samples (n=254) and we did not include information on comorbidities and vascular risk factors. This will need to be assessed in the future as we move to application of this measure in individual patients. Finally,

samples were stored in different facilities and for different storage periods, but collection procedures were standardized [25] and we did not observe an association between storage time and sNfL in either patient or control cohorts.

Based on the investigation of healthy controls and two large independent samples of MS patients with the recently developed ultrasensitive sNfL assay, this study provides a number of important findings that further our understanding and support the value of sNfL levels as a biomarker of tissue damage in MS: I) sNfL levels can be reliably and reproducibly measured in serum samples from MS patients; II) in independent HC and patient cohorts sNfL levels are positively associated with age but not gender; III) sNfL levels closely reflect NfL concentration in the CSF of MS patients; IV) sNfL levels are increased in MS patients as compared to HC and positively associated with T2 and GE lesions in both brain and spinal cord; V) sNfL levels are increased in patients with recent relapses or worsening of disability, are higher with increasing EDSS scores, and decrease with increasing duration of disease modifying treatment; VI) sNfL levels are associated with an increased risk of future relapses and EDSS worsening. These findings indicate that sNfL may have a role in assessing disease severity and worsening, as well as in monitoring the effect of disease –modifying therapy. Before being implemented in clinical practice, more data and research will be needed to establish reference ranges in the general population and sensitivity and specificity of NfL based predictions, by using larger cohorts of controls, and taking into account relevant comorbidities and treatment effects. Assay protocols will need to be standardized and validity of the assay tested across different centres.[50] Ongoing investigations of samples obtained in the setting of prospective controlled clinical trials will help to further elucidate the utility of sNfL measurements in monitoring treatment effects.[51]

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AUTHOR CONTRIBUTIONS

Significant contribution to: conception and design of the study (GD, CB, YN, CZ, CG, JK); acquisition and analysis of data (GD, CB, PB, SS, KB, HZ, AG, JK); participation in drafting a significant portion of the manuscript or figures (GD, DL, LK, CG, JK). All authors approved the final version of the manuscript.

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POTENTIAL CONFLICTS OF INTEREST

There are no potential conflicts of interest related to this work.

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Supplementary Material

Supplementary table 1

Multivariable GEE model testing associations between several demographic and clinical variables including EDSS*Disease course interaction and sNfL in the SMSC cohort.

Variables / Interactions (*)		Multivariable		
		β	**95%CI**	***p***
Age (years)		1.011	1.004-1.018	0.001
EDSS		1.133	1.081-1.187	<0.001
Disease course	CIS/RRMS	-	-	-
	PPMS/SPMS	1.459	0.948-2.244	0.086
Recent relapse (<60 days)	No	-	-	-
	Yes	1.408	1.140-1.740	0.002
Recent EDSS worsening	No	-	-	-
	Yes	1.131	0.970-1.317	0.115
DMT	Untreated	-	-	-
	Treated	0.813	0.711-0.929	0.002
EDSS * Disease course		0.904	0.829-0.985	0.021

GEE: Generalized Estimated Equations model. sNfL: serum NfL; EDSS: Expanded Disability Status Scale; CIS: clinically isolated syndrome; RRMS: relapsing remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS; DMT: Disease modifying treatment.

Supplementary table 2
Multivariable GEE model testing associations between several demographic and clinical variables and sNfL in the SMSC cohort *after* start of new DMT.

Variables		Multivariable		
		β	**95%CI**	***p***
Time under DMT (years)		0.900	0.830-0.976	0.011
sNfL at baseline		1.003	1.001-1.004	0.001
Age (years)		1.015	1.008-1.021	<0.001
Gender	F	-	-	-
	M	0.952	0.832-1.090	0.478
EDSS		1.096	1.054-1.139	<0.001
Disease course	CIS/RRMS	-	-	-
	PPMS/SPMS	0.980	0.764-1.257	0.875
Recent relapse (<60 days)	No	-	-	-
	Yes	1.422	1.024-1.973	0.035
Recent EDSS worsening	No	-	-	-
	Yes	1.203	0.964-1.501	0.102

GEE: Generalized Estimated Equations model. sNfL: serum NfL; DMT: Disease modifying treatment; F: female; M: male; EDSS: Expanded Disability Status Scale; CIS: clinically isolated syndrome; RRMS: relapsing remitting MS; SPMS: secondary progressive MS; PPMS: primary progressive MS.

Supplementary table 3

Model-predicted means (marginal means) and model estimates including 95% confidence intervals from GEE models: Probability of a relapse within 60 days before sampling, ARR one and two years before sampling and probability of EDSS worsening before sampling according to different sNfL percentile categories.

Percentiles	samples n (%) with sNfL > percentile	Relapses <60 days before sampling					ARR 1 year before sampling*				
		Above (%)	Below (%)	OR	95%CI	*p*	Above	Below	IRR	95%CI	*p*
80th	287 (49.4)	18.7 (14.2-24.1)	7.1 (4.6-10.6)	3.02	1.72-5.28	<0.001	0.77 (0.66-0.90)	0.41 (0.34-0.50)	1.86	1.46-2.38	<0.001
90th	228 (39.2)	21.0 (16.0-27.0)	7.5 (5.2-10.8)	3.26	1.98-5.38	<0.001	0.85 (0.72-1.01)	0.42 (0.35-0.50	2.03	1.60-2.59	<0.001
95th	171 (29.4)	23.8 (17.9-30.9)	8.1 (5.7-11.4)	3.56	2.16-5.88	<0.001	0.92 (0.76-1.10)	0.44 (0.38-0.52)	2.06	1.62-2.62	<0.001
97.5th	135 (23.2)	26.6 (19.5-35.1)	8.5 (6.1-11.8)	3.89	2.30-6.58	<0.001	0.97 (0.80-1.18)	0.47 (0.40-0.55)	2.08	1.64-2.63	<0.001
99th	105 (18.1)	29.8 (21.2-40.2)	9.0 (6.6-12.3)	4.28	2.46-7.44	<0.001	1.06 (0.84-1.33)	0.48 (0.42-0.56)	2.19	1.70-2.81	<0.001
Percentiles	**samples n (%) with sNfL > percentile**	**ARR 2 years before sampling***					**EDSS worsening since visit before sampling***				
		Above	**Below**	**IRR**	**95%CI**	***p***	**Above (%)**	**Below (%)**	**OR**	**95%CI**	***p***
80th	287 (49.4)	0.64 (0.57-0.73)	0.53 (0.46-0.60)	1.23	1.06-1.42	0.007	9.1 (6.1-13.4)	3.6 (1.9-6.6)	2.72	1.23-6.02	0.014
90th	228 (39.2)	0.68 (0.59-0.77)	0.53 (0.46-0.60)	1.28	1.11-1.49	0.001	10.7 (7.0-15.9)	3.6 (2.0-6.4)	3.23	1.48-7.04	0.003
95th	171 (29.4)	0.70 (0.61-0.80)	0.53 (0.47-0.61)	1.31	1.13-1.51	<0.001	12.9 (8.4-19.2)	3.6 (2.1-6.3)	3.93	1.85-8.33	<0.001
97.5th	135 (23.2)	0.74 (0.64-0.87)	0.53 (0.47-0.60)	1.39	1.18-1.64	<0.001	14.8 (9.4-22.5)	3.8 (2.3-6.3)	4.36	2.09-9.09	<0.001
99th	105 (18.1)	0.78 (0.66-0.92)	0.54 (0.48-0.61)	1.45	1.22-1.73	<0.001	17.0 (10.4-26.5)	4.0 (2.5-6.5)	4.86	2.30-10.25	<0.001

OR: odds ratios; ARR: annualized relapse rate; IRR: incidence rate ratios; 95%CI=95% confidence intervals. * Samples from patients with a relapse within 30 days excluded.

Supplementary table 4

Model-predicted means (marginal means) and model estimates including 95% confidence intervals from GEE models: ARR one and two years after sampling and EDSS worsening within 1 year after sampling according to different sNfL percentiles.

Percentiles	samples n (%) with sNfL > percentile	ARR 1 year after sampling*					ARR 2 years after sampling*				
		Above	Below	IRR	95%CI	*p*	Above	Below	IRR	95%CI	*p*
80th	287 (49.4)	0.28 (0.21-0.37)	0.17 (0.12-0.24)	1.62	1.08-2.44	0.021	0.24 (0.18-0.32)	0.14 (0.10-0.19)	1.69	1.19-2.39	0.003
90th	228 (39.2)	0.30 (0.22-0.41)	0.18 (0.13-0.24)	1.71	1.12-2.64	0.014	0.26 (0.19-0.35)	0.14 (0.11-0.19)	1.80	1.23-2.65	0.003
95th	171 (29.4)	0.32 (0.22-0.46)	0.18 (0.14-0.24)	1.75	1.10-2.76	0.017	0.27 (0.18-0.39)	0.16 (0.12-0.21)	1.69	1.03-2.79	0.038
97.5th	135 (23.2)	0.36 (0.24-0.52)	0.18 (0.14-0.24)	1.94	1.21-3.10	0.006	0.30 (0.21-0.45)	0.15 (0.12-0.21)	1.96	1.22-3.15	0.005
99th	105 (18.1)	0.40 (0.26-0.62)	0.19 (0.14-0.24)	2.15	1.29-3.60	0.003	0.33 (0.21-0.50)	0.16 (0.12-0.21)	2.03	1.26-3.27	0.004

Percentiles	samples n (%) with sNfL > percentile	EDSS worsening within 1 year after sampling*				
		Above (%)	Below (%)	OR	95%CI	*p*
80th	287 (49.4)	11.0 (7.3-16.3)	6.7 (3.8-11.5)	1.72	0.84-3.50	0.135
90th	228 (39.2)	12.7 (8.1-19.3)	6.5 (3.9-10.6)	2.10	1.03-4.29	0.042
95th	171 (29.4)	15.1 (9.4-23.4)	6.3 (3.8-10.3)	2.63	1.25-5.54	0.011
97.5th	135 (23.2)	15.3 (8.8-25.4)	7.0 (4.4-10.9)	2.41	1.07-5.42	0.034
99th	105 (18.1)	14.6 (7.5-26.4)	7.7 (5.0-11.6)	2.06	0.85-4.98	0.110

OR: odds ratios; ARR: annualized relapse rate; IRR: incidence rate ratios; 95%CI=95% confidence intervals. *Samples from patients with a relapse within 30 days excluded.

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

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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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Running title: Neurofilament light chain as a predictor of disease severity in MS

Abstract:

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 (sNfL) 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 multiple sclerosis patients (189 relapsing and 70 progressive) and 259 healthy controls. Clinical assessment, serum sampling and magnetic resonance imaging 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 analyzer (*cordial*). Results were analysed using ordinary linear regression models and generalized estimating equation modeling.

sNfL 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). sNfL 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 sNfL percentile category. Contrast enhancing and new/enlarging lesions were independently associated with increased sNfL (17.8% and 4.9% increase per lesion respectively; $p<0.001$). The higher the sNfL percentile level, the more pronounced was future brain and cervical spinal volume loss: sNfL above the 97.5^{th} 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 five years ($p=0.009$).

sNfL correlated with concurrent and future clinical and MRI measures of disease activity and severity. High sNfL 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 magnetic resonance imaging.

Keywords: neurofilament light chain, biomarker, atrophy, multiple sclerosis

Abbreviations: EDSS: Expanded Disability Status Scale; HC: healthy controls; sNfL: 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 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 Neurofilament light chain levels in CSF are associated with the occurrence of MRI lesions, relapses, neurological disability and treatment status in multiple sclerosis (Teunissen *et al.* 2009a;Hakansson *et al.* 2017;Gunnarsson *et al.* 2011;Novakova *et al.* 2017;Disanto *et al.* 2017). The advent of a more sensitive method, the Single Molecule Array technology (Simoa) now allows the reliable quantification of Neurofilament light chain in plasma or serum (Kuhle *et al.* 2016a;Gisslen *et al.* 2016). Several studies in multiple sclerosis and other diseases have recently demonstrated that serum and CSF Neurofilament light chain concentrations are highly correlated, suggesting that sNfL could represent a reliable blood derived biomarker of neuro-axonal injury (Kuhle *et al.* 2016b;Gaiottino *et al.* 2013;Bacioglu *et al.* 2016;Weydt *et al.* 2016;Lu *et al.* 2015;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 Neurofilament light chain 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 (Kuhle *et al.* 2017a;Disanto *et al.* 2017). 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 multiple sclerosis patients with long-term MRI and clinical follow-up and 259 HC aiming at: 1) 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 in EDSS; 2) investigating the cross-sectional association between sNfL and quantitative MRI measures of inflammation and degeneration;

3) 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 multicenter study initiated in 2003 (Genome-Wide Association Study of Multiple Sclerosis, GeneMSA; continued as of 2011 as Serial Unified Multicenter Multiple Sclerosis Investigation, SUMMIT) (Bove *et al.* 2017;Baranzini *et al.* 2009). The study included patients with all clinical subtypes of the disease (McDonald *et al.* 2001;Polman *et al.* 2005; Lublin *et al.*, 1996). Secondary progressive multiple sclerosis was defined by 6 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 cerebrospinal fluid as defined by the presence of ≥2 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 July 2, 2004 and February 17, 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 one 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 Tesla MR scanner (Magnetom Avanto, Siemens Helthineers, Germany) 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 analyzed by cord image analyzer (*cordial*) (Amann

et al. 2016). The segmentation was carried out over a 35 mm long spinal cord segment in the cranial T1-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 sNfL measurements

Serum samples were collected on the same day as the clinical visit and stored at -80°C following standard procedures (Teunissen *et al.* 2009b). 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 pg/ml, 25.8 pg/ml, 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 HC showed a sNfL value below 1.3 pg/ml (i.e. the lower limit of quantification). These two patients and one HC were excluded from the analysis.

Healthy controls

Serum samples from 259 HC 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 one year after baseline was available for 226 of these HC. HC were probands or genetically unrelated friends and spouses of the patients. Inclusion criteria for HC included age 18-70 years and no diagnosis of multiple sclerosis as well as 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. For all analyses with sNfL as dependent variable, sNfL levels were log-transformed. The distribution of sNfL in HC 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 cutoffs (80%, 90%, 95%, 97.5% and 99%).

Several clinical and MRI parameters were tested for association with log sNfL using Generalized Estimating Equation 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%CI). Model quality was inspected visually using Q-Q-plots.

EDSS worsening was modelled using binominal Generalized Estimating Equation models and odds ratios were estimated (denoted by β_{OR}). EDSS worsening was defined as an increase in EDSS to the subsequent visit of ≥1.5 points from an EDSS score of 0.0, ≥1.0 point from an EDSS score of 1.0–5.5 or ≥0.5 point from an EDSS score ≥6.0. Brain volume changes were measured using Structural Image Evaluation using Normalization of Atrophy. 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 Generalized Estimating Equation models.

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

Results

1. sNfL levels in healthy controls and reference percentile curves

The median age in 258 HC 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 β_{mult}=1.022, 95%CI=1.018-1.026, p<0.001). Accordingly, the median sNfL level in the 226 HC 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 (Disanto *et al.* 2017). The resulting 80th, 90th, 95th, 97.5th and 99th sNfL percentiles are shown in Supplementary Table 2 and Supplementary Figure 1.

2. Association of sNfL 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 1,684 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 Figure 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 HC (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 Figure 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 HC (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).

3. Associations between sNfL 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 T2 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 (Figure 1, Supplementary Table 5).

Table 1. Estimated odds ratios (β_{OR}) of EDSS worsening within the next year (univariable and multivariable GEE models testing associations between serum NfL, clinical and MRI variables).

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
T2 lesion vol. (per cm3)		1.053	1.020-1.086	0.001	1.061	1.023-1.101	0.001
New/enlarging T2		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 100cm^3)		0.861	0.663-1.118	0.262	1.109	0.779-1.577	0.567

BL: baseline; CEL: contrast enhancing lesions; CI: confidence interval; DMT: disease modifying treatment; EDSS: Expanded Disability Status Scale; F: female; IQR: interquartile range; M: male; nBV: normalized brain volume; NfL: serum neurofilament light chain; PMS: progressive multiple sclerosis; RMS: relapsing multiple sclerosis.

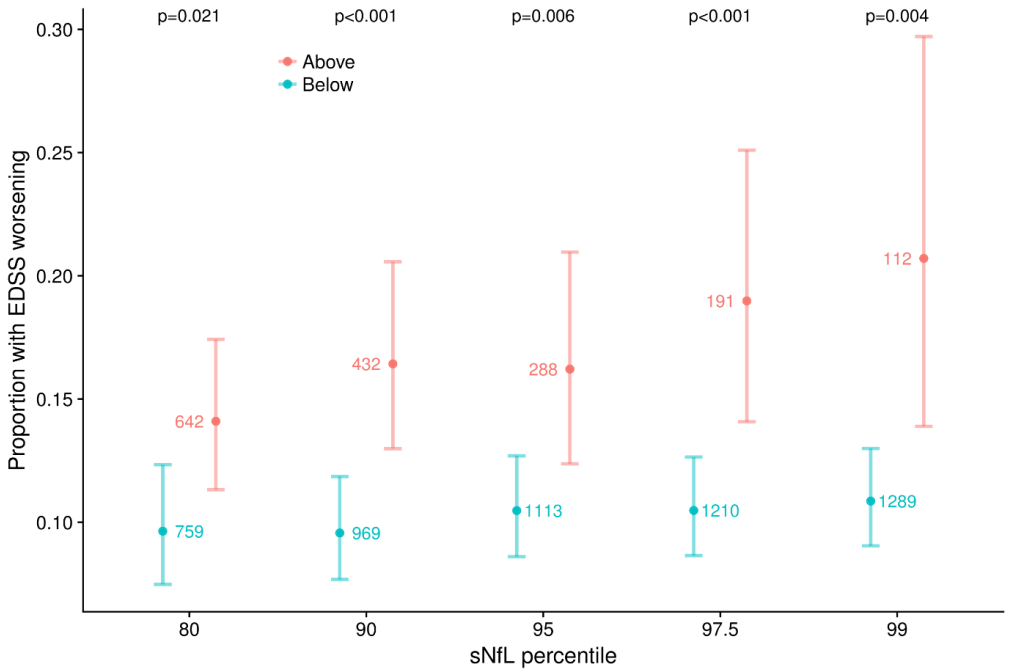

Figure 1. The estimated proportion of patients with EDSS worsening gradually increased with increasing sNfL percentile category based on healthy controls (above vs. below 80th percentile: β_{OR}=1.539, 95%CI=1.067-2.219, p=0.021; above vs. below 99th percentile: β_{OR}=2.143, 95%CI=1.274-3.606, p=0.004, n=1401 observations). The percentiles were constructed based on HC samples. Numbers in the figure denote the number of samples above or below the respective percentiles of healthy controls.

4. Association of sNfL 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 Figure 4 for contrast enhancing lesions and Supplementary Figure 5 for new or enlarging T2 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 T2 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 100cm^3 reduction of normalized brain volume (β_{mult}=0.883, 95%CI=0.831-0.938, p<0.001). Conversely, the relationship with T2 lesion volume was no longer visible in the multivariable analysis (β_{mult}=0.996, 95%CI=0.987-1.006, p=0.450).

Table 2. Estimates of univariable and multivariable GEE models testing associations between sNfL and lesional and brain volume MRI variables. The estimates represent multiplicative effects (β_{mult}) since the endpoint sNfL was log transformed.

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
T2 lesion vol. (per cm^3)		1.012	1.004-1.020	0.003	0.996	0.987-1.006	0.450
New/enlarging T2		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

CEL: contrast enhancing lesions; CI: confidence interval; F: female; M: male; nbV: normalized brain volume; NfL: serum neurofilament light chain; IQR: interquartile range.

5. Association of baseline sNfL 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 volume of 0.17% after 2 years (β_{add}=-0.171%, 95%CI=-0.226--0.116%, p<0.001, n=197 observations). Besides a weaker signal for baseline EDSS in the multivariable model (β_{add}=-0.151, 95%CI=-0.271--0.031, p=0.014, n=197 observations), sNfL remained the only strong predictor of brain volume change over two years (β_{add}=-0.134%, 95%CI=-0.194--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 (Figure 2, 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 (β_{add}=-0.287%, 95%CI=-0.432--0.142%, p<0.001, n=132) in a multivariable analysis that included EDSS (β_{add}=-0.294%, 95%CI=-0.545--0.042%, p=0.023), disease course and several MRI baseline variables (Table 3).

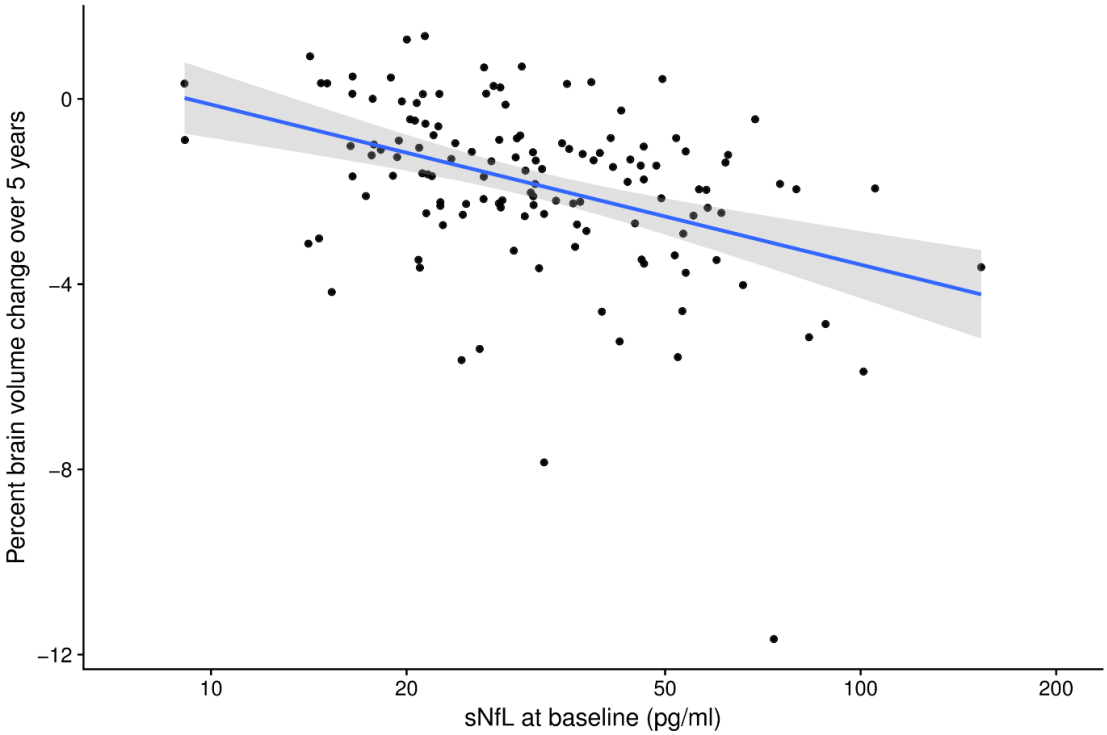

Figure 2. In the univariable model sNfL levels at baseline were significantly associated with the percentage brain volume change over 5 years (β_{add}=-0.352%, 95%CI=-0.490--0.214% per 10 pg/ml change in sNfL, p<0.001, n=132 observations), i.e. an estimated additional 0.35% reduction in brain volume 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 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--0.214	<0.001	-0.287	-0.432--0.142	<0.001
Age (years)		-0.025	-0.054-0.005	0.098	0.008	-0.025-0.040	0.642
Sex	F (87)	-	-	-	-	-	-
	M (45)	-0.394	-1.058-0.269	0.241	-0.229	-0.845-0.387	0.463
EDSS		-0.454	-0.654--0.255	<0.001	-0.294	-0.545--0.042	0.023
Disease course	RMS (97)	-	-	-	-	-	-
	PMS (35)	-0.839	-1.579--0.099	0.027	0.118	-0.734-0.971	0.784
T2 lesion vol. (per cm^3)		-0.064	-0.111--0.017	0.008	-0.028	-0.081-0.025	0.294
CEL		-0.259	-0.549-0.031	0.079	-0.055	-0.328-0.219	0.693
nBV (per 100 cm^3)		0.546	0.235-0.857	<0.001	0.167	-0.235-0.570	0.412

CEL: contrast enhancing lesions; CI: confidence interval; EDSS: Expanded Disability Status Scale; F: female; IQR: interquartile range; M: male; sNfL: serum neurofilament light chain.

In addition, we compared sNfL measurements of multiple sclerosis patients against the age corrected percentile curves that were constructed based on HC 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 (Figure 3A) and over 5 years (Figure 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 Figure 6A and B).

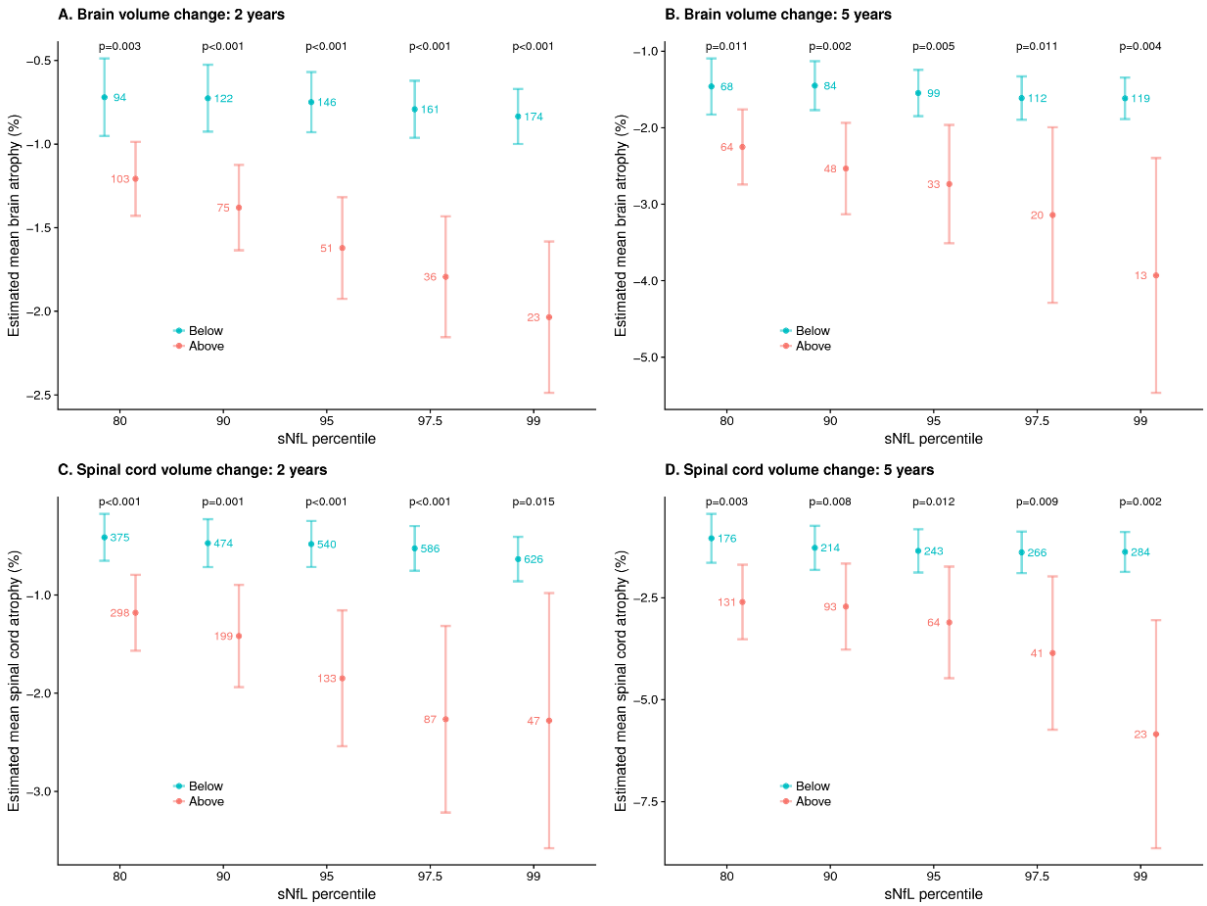

Figure 3. The estimated mean percentage of brain volume change in patients with sNfL above the respective age corrected percentiles gradually increased with increasing sNfL percentile category over 2 (A) and 5 years (B) of observation time. The mean reduction in spinal cord volume over 2 (C) and 5 years (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.

6. Association of sNfL 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 (β_{add}=-0.191%, 95%CI=-0.295--0.086%, p<0.001, n=673 observations and β_{add}=-0.488%, 95%CI=-0.783--0.192%, p=0.001, n=307 observations, respectively, Figure 4). The mean reduction in spinal cord volume over 2 and 5 years gradually increased with increasing sNfL percentile category (Figure 3C and D, 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 Figure 6C and D).

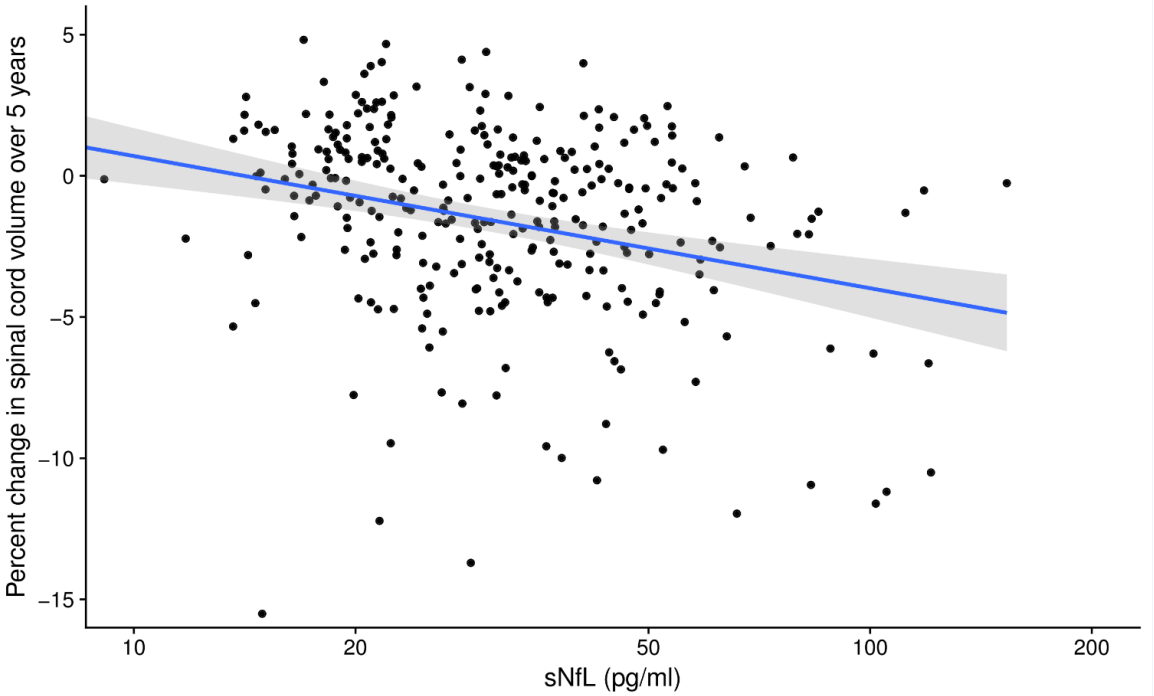

Figure 4. A significant association between sNfL and the change in spinal cord volume over 5 years was found with an estimated additional reduction in spinal cord volume of 0.49% over 5

years per 10 pg/ml increase in sNfL (β_{add}=-0.488%, 95%CI=-0.783--0.192%, p=0.001, n=307 observations).

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 T2 lesions. We complemented our previous observation that the *number* of T2 lesions is associated with sNfL by showing that also T2 lesion *volume,* a more comprehensive measure of brain lesion burden is highly associated with sNfL. This finding further suggests that sNfL may be 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 central nervous system 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 measurable) (Kuhle *et al.* 2017a). 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 T2 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 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 may not astonish 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 MS therapies on sNfL (Kuhle *et al.*, 2016c; Kuhle *et al.*, 2017b).

The release of Neurofilament light chain 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

Cranial image acquisition and data analysis

MRI analysis was performed centrally at the Medical Image Analysis Centre in Basel, blinded with respect to disease subtype, duration and treatment history. The measurement protocol included a three-dimensional T1-weighted scan (MPRAGE, TR/TI/TE/α = 2080 ms/1100 ms/3.93 ms/15°; spatial resolution 0.98×0.98×1 mm3), a double-echo proton density/T2-weighted sequence (TR/TE1/TE2 = 3980 ms/14 ms/108 ms; 0.98×0.98×3 mm3), and a T1-weighted spin echo sequence (TR/TE = 552 ms/ 17 ms; 0.98×0.98×3 mm3). The latter was acquired for 5 min following administration of a single dose (0.1 mM/kg) of contrast agent. Qualitative analysis for the presence of gadolinium enhancement was performed on post-contrast T1-weighted images. Brain lesions were identified and marked by consensus reading on simultaneously viewed T2-weighted and proton density-weighted images (Bendfeldt *et al*., 2009). Additional evaluations were done for new T2 lesions, volume of T2 and T1 gadolinium enhanced lesions. The volumes were measured using an interactive digital analysis program (AMIRA 3.1.1; Mercury Computer System Inc). Brain atrophy was calculated on the 3D T1-weighted data with SIENA (Structural Image Evaluation using Normalization of Atrophy) (Smith *et al.* 2001;Smith *et al.* 2002) between baseline and two-year follow-up, whereas long-term atrophy was calculated between baseline and year five follow-up. SIENAX (an adaptation of SIENA for cross-sectional measurements), improved with lesion filling was used to estimate normalized brain volume (Battaglini *et al.* 2012;Smith et al. 2001;Smith et al. 2002).

Supplementary table 1

Number of visits including serum samples and available MRI data.

	Sum (n)	BL	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	Y10
Visits	**1684**	**257**	**240**	**205**	**179**	**167**	**151**	**143**	**123**	**98**	**93**	**28**
CEL	1190	257	238	201	110	126	135	118	5	0	0	0
T2w lesion volume	1263	257	238	202	175	132	135	119	5	0	0	0
New/enlarging T2w	964	-	238	199	173	125	112	112	5	0	0	0
nBV	1130	256	237	199	174	132	132	0	0	0	0	0
PBVC BL-Y2	197		-	197	-	-	-	-	-	-	-	-
PBVC BL-Y5	132		-	-	-	-	132	-	-	-	-	-
Spinal cord volume	1253	232	224	185	158	118	125	114	97	0	0	0

BL: baseline; Y: year; CEL: contrast enhancing lesions; nBV: normalised brain volume; PBVC: percentage brain volume change.

Supplementary table 2

sNfL percentiles across different ages, derived from a Generalized Additive Model for Location, Scale, and Shape (GAMLSS) on sNfL in healthy controls (samples from baseline and one year follow-up).

Age (years)	**sNfL percentiles (pg/ml)**				
	80th	**90th**	**95th**	**97.5th**	**99th**
30	22.0	25.5	29.3	33.7	40.9
35	24.6	28.5	32.7	37.6	45.7
40	27.6	32.0	36.7	42.2	51.2
45	31.0	35.9	41.3	47.4	57.5
50	34.8	40.3	46.3	53.2	64.5
55	38.8	44.9	51.6	59.3	71.9
60	43.1	49.9	57.3	65.8	79.9
65	47.6	55.1	63.2	72.7	88.2
70	52.2	60.4	69.3	79.7	96.7

sNfL: serum neurofilament light chain.

Supplementary table 3

Demographics and clinical variables at baseline.

Variables	**n (%) / median (IQR)**
Patients	257
Age (years)	44.0 (36.2-53.2)
Sex (females)	179 (69.6)
Diagnosis at BL	
CIS*	11 (4.3)
RRMS**	178 (69.3)
SPMS	54 (21.0)
PPMS	14 (5.4)
Disease duration (years)	10.0 (5.0-18.0)
EDSS	3.0 (2.0-4.0)
Relapses year before BL	
0	182 (70.8)
1	52 (20.2)
2	23 (8.9)
DMT at BL	
Untreated	91 (35.4)
Interferon beta 1b	53 (20.6)
Interferon beta 1a sc	40 (15.6)
Glatirameracetate	33 (12.8)
Interferon beta 1a im	24 (9.3)
Mitoxantrone	7 (2.7)
Azathioprin	6 (2.3)
Other***	3 (1.2)

BL: baseline; CIS: clinically isolated syndrome; DMT: disease modifying treatment; EDSS: expanded disability status scale; im: intramuscular; IQR: interquartile range; PPMS: primary progressive multiple sclerosis; sc: subcutaneous; SPMS: secondary progressive multiple sclerosis. * 3 patients converted to RRMS during follow-up. ** 13 patients converted to SPMS during follow-up. *** Other: One patient on dimethyl-fumarate, and 2 patients on combination therapy of interferon beta 1b and azathioprin.

Supplementary table 4

Univariable and multivariable models testing associations between sNfL and age, sex, EDSS, disease course, recent relapses and DMT treatment status.

Variables (1684 observations)		**sNfL (pg/ml), median, IQR**	**Univariable**			**Multivariable**		
			β_{mult}	**95%CI**	***p***	**β_{mult}**	**95%CI**	***p***
Age		-	1.018	1.011-1.022	<0.001	1.015	1.011-1.020	<0.001
Sex	F (1140)	32.5 (21.8-45.9)	-	-	-	-	-	-
	M (544)	34.7 (25.0-48.3)	1.086	0.971-1.213	0.148	1.025	0.935-1.123	0.599
Disease course	RMS (1209)	29.7 (21.2-42.2)	-	-	-	-	-	-
	PMS (475)	41.9 (31.9-55.7)	1.312	1.198-1.436	<0.001	1.007	0.923-1.098	0.872
EDSS		-	1.094	1.070-1.120	<0.001	1.058	1.033-1.083	<0.001
Recent relapse (<120 days)	No (1545)	32.8 (23.2-45.9)	-	-	-	-	-	-
	Yes (139)	36.8 (24.1-56.3)	1.118	1.034-1.208	0.005	1.144	1.054-1.241	0.001
DMT	Treated (1169)	33.3 (23.0-45.9)	-	-	-	-	-	-
	Untreated (515)	32.4 (23.8-49.2)	0.926	0.851-1.008	0.075	0.953	0.885-1.028	0.215

Estimates (β_{mult}) are multiplicative effects. sNfL: serum neurofilament light chain; CI: confidence interval; F: female; M: male; CIS: clinically isolated syndrome; RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; SPMS: secondary progressive multiple sclerosis; EDSS: Expanded Disability Status Scale; DMT: disease modifying treatment.

Supplementary table 5

Model-predicted marginal means and estimated odds ratio (β_{OR}) including 95% confidence intervals from binominal GEE models: EDSS worsening between the current and subsequent visit (n=1401 observations).

Percentile	**EDSS worsening between current and subsequent visit**					
	Samples (%) with sNfL > percentile	**Above (%, 95%CI)**	**Below (%, 95%CI)**	**β_{OR}**	**95%CI**	***p***
80th	642 (46)	14.1 (11.3-17.4)	9.6 (7.5-12.3)	1.539	1.07-2.22	0.021
90th	432 (31)	16.4 (13.0-20.6)	9.6 (7.7-11.9)	1.858	1.32-2.63	<0.001
95th	288 (21)	16.2 (12.4-21.0)	10.5 (8.6-12.7)	1.654	1.15-2.38	0.006
97.5th	191 (14)	19.0 (14.1-25.1)	10.5 (8.6-12.6)	2.002	1.34-3.00	<0.001
99th	112 (8)	20.7 (13.9-29.7)	10.9 (9.0-13.0)	2.143	1.27-3.61	0.004

Supplementary table 6

Estimated percentage brain volume change over 2 years (β_{add}) in univariable and multivariable linear models testing for associations with *baseline* variables.

Baseline variables (197 observations)		**Univariable**			**Multivariable**		
		β_{add} (%)	**95%CI (%)**	***p***	**β_{add} (%)**	**95%CI (%)**	***p***
sNfL (per 10 pg/ml)		-0.171	-0.226--0.116	<0.001	-0.134	-0.194--0.073	<0.001
Age (years)		-0.006	-0.021-0.009	0.453	0.005	-0.012-0.022	0.550
Sex	F (128)	-	-	-	-	-	-
	M (69)	-0.162	-0.507-0.182	0.354	-0.084	-0.402-0.234	0.603
EDSS		-0.165	-0.264--0.066	0.001	-0.151	-0.271--0.031	0.014
Disease course	RMS (147)	-	-	-	-	-	-
	PMS (50)	-0.086	-0.464-0.292	0.654	0.357	-0.085-0.799	0.113
T2 lesion vol. (per cm^3)		-0.042	-0.066--0.018	<0.001	-0.025	-0.051-0.002	0.071
CEL		-0.342	-0.499--0.0185	<0.001	-0.151	-0.313-0.010	0.067
nBV (per 100 cm^3)		0.201	0.039-0.363	0.015	0.016	-0.185-0.217	0.876

Estimates (β_{add}) are additive effects. CEL: contrast enhancing lesions; CI: confidence interval; EDSS: Expanded Disability Status Scale; F: female; IQR: interquartile range; M: male; sNfL: serum neurofilament light chain.

Supplementary table 7

Model-predicted marginal means and model estimates including 95% confidence intervals of the means from linear models: Estimated mean percent change in brain volume over A. 2 years (197 observations) and B. 5 years (132 observations).

A.

Percentiles	**Estimated mean percent change in brain volume (over 2 years)**					
	Samples n (%) with sNfL > percentile	**Above (%, 95%CI)**	**Below (%, 95%CI)**	**β_{add} (%)**	**95%CI**	***p***
80th	103 (52)	-1.21 (-1.43--0.99)	-0.72 (-0.95--0.49)	-0.49	-0.81--0.17	0.003
90th	75 (38)	-1.38 (-1.63--1.12)	-0.73 (-0.93--0.53)	-0.65	-0.98--0.33	<0.001
95th	51 (26)	-1.62 (-1.93--1.32)	-0.75 (-0.93--0.57)	-0.87	-1.23--0.52	<0.001
97.5th	36 (18)	-1.79 (-2.15--1.43)	-0.79 (-0.96--0.62)	-1.00	-1.40--0.60	<0.001
99th	23 (12)	-2.03 (-2.49--1.58)	-0.83 (-1.00--0.67)	-1.20	-1.68--0.72	<0.001

B.

Percentiles	**Estimated mean percent change in brain volume (over 5 years)**					
	Samples n (%) with sNfL > percentile	**Above (%, 95%CI)**	**Below (%, 95%CI)**	**β_{add} (%)**	**95%CI**	***p***
80th	64 (49)	-2.25 (-2.74--1.76)	-1.46 (-1.83--1.10)	-0.79	-1.40--0.18	0.011
90th	48 (36)	-2.53 (-3.13--1.94)	-1.45 (-1.77--1.13)	-1.08	-1.76--0.41	0.002
95th	33 (25)	-2.74 (-3.51--1.96)	-1.55 (-1.85--1.24)	-1.19	-2.02--0.36	0.005
97.5th	20 (15)	-3.14 (-4.29--1.99)	-1.61 (-1.90--1.33)	-1.53	-2.71--0.35	0.011
99th	13 (10)	-3.93 (-5.46--2.40)	-1.62 (-1.89--1.35)	-2.31	-3.87--0.76	0.004

Supplementary table 8

Model-predicted marginal means and model estimates including 95% confidence intervals of the means from GEE models: Estimated mean percent change in spinal cord volume A. over 2 years (673 observations) and B. 5 years (307 observations).

A.

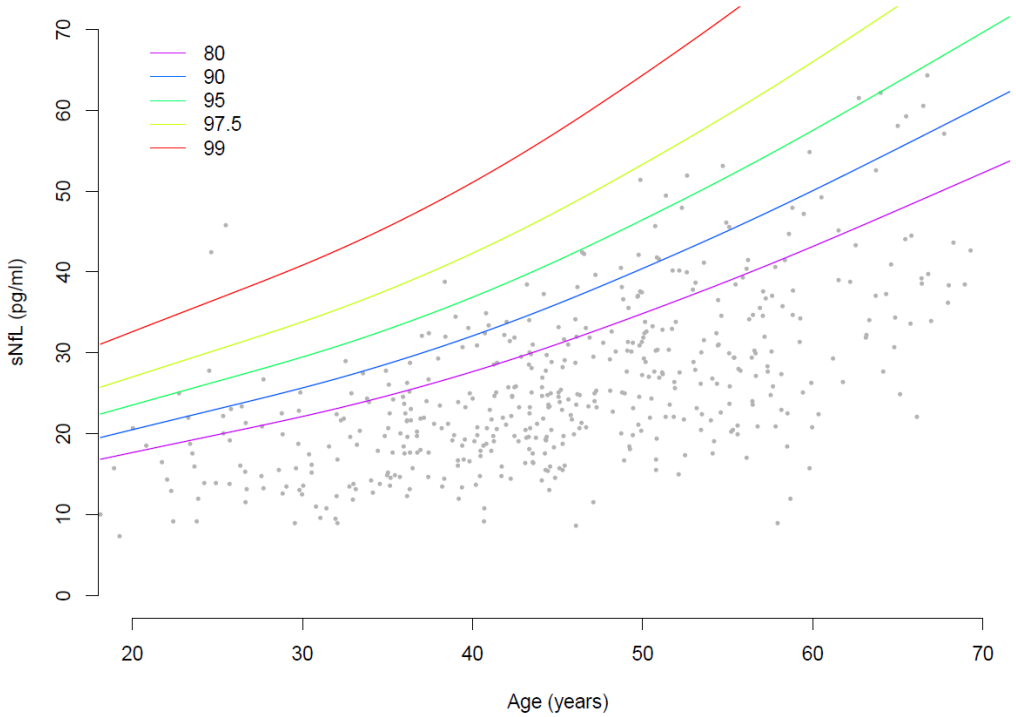
Percentiles	**Estimated mean change in spinal cord volume over 2 years**					
	Samples n (%) with sNfL > percentile	**Above (%, 95%CI)**	**Below (%, 95%CI)**	**β_{add} (%)**	**95%CI**	***p***
80th	298 (44)	-1.18 (-1.57--0.79)	-0.41 (-0.65--0.17)	-0.77	-1.19--0.35	<0.001
90th	199 (30)	-1.42 (-1.94--0.90)	-0.47 (-0.72--0.23)	-0.95	-1.52--0.37	0.001
95th	133 (20)	-1.85 (-2.54--1.16)	-0.48 (-0.71--0.25)	-1.37	-2.11--0.63	<0.001
97.5th	87 (13)	-2.27 (-3.22--1.31)	-0.52 (-0.75--0.30)	-1.74	-2.73--0.75	<0.001
99th	47 (7)	-2.28 (-3.58--0.98)	-0.63 (-0.86--0.41)	-1.65	-2.97--0.32	0.015

B.

Percentiles	**Estimated mean change in spinal cord volume over 5 years**					
	Samples n (%) with sNfL > percentile	**Above (%, 95%CI)**	**Below (%, 95%CI)**	**β_{add} (%)**	**95%CI**	***p***
80th	131 (43)	-2.60 (-3.52--1.69)	-1.04 (-1.64--0.45)	-1.56	-2.6--0.52	0.003
90th	93 (30)	-2.72 (-3.77--1.66)	-1.28 (-1.82--0.74)	-1.44	-2.5--0.38	0.008
95th	64 (21)	-3.11 (-4.47--1.74)	-1.35 (-1.88--0.82)	-1.75	-3.13--0.38	0.012
97.5th	41 (13)	-3.86 (-5.74--1.98)	-1.39 (-1.90--0.88)	-2.47	-4.33--0.61	0.009
99th	23 (8)	-5.84 (-8.64--3.05)	-1.38 (-1.87--0.89)	-4.47	-7.25--1.68	0.002

Supplementary figure 1

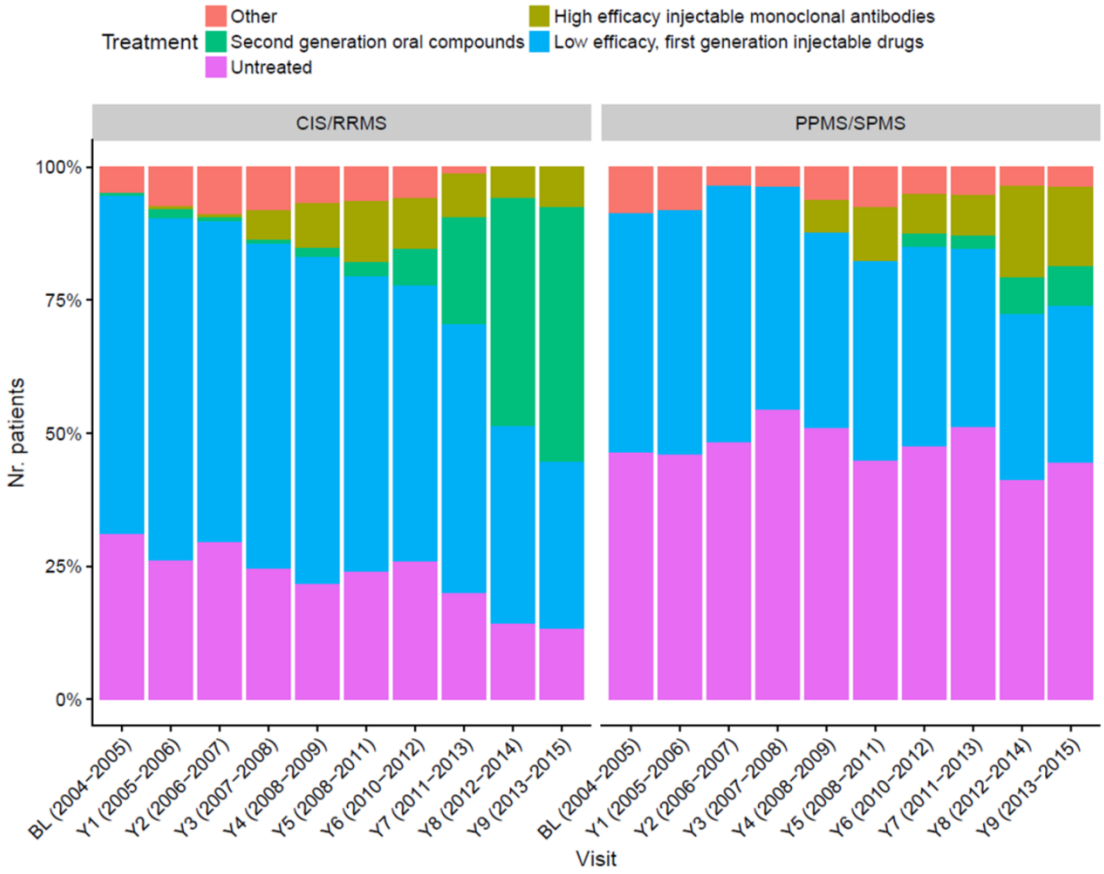
Distribution of sNfL in the healthy controls with age based percentile curves.



Legend: The distribution of serum NfL (sNfL) in healthy controls and its association with age was modelled by means of a Generalized Additive Models for Location, Scale, and Shape (GAMLSS) using a Box-Cox t distribution according to Rigby and Stasinopoulos (Rigby and Stasinopoulos, 2004) and cubic splines. From this model (n=484) percentile curves were obtained (please see the numerical values also in Supplementary Table 2).

Supplementary figure 2

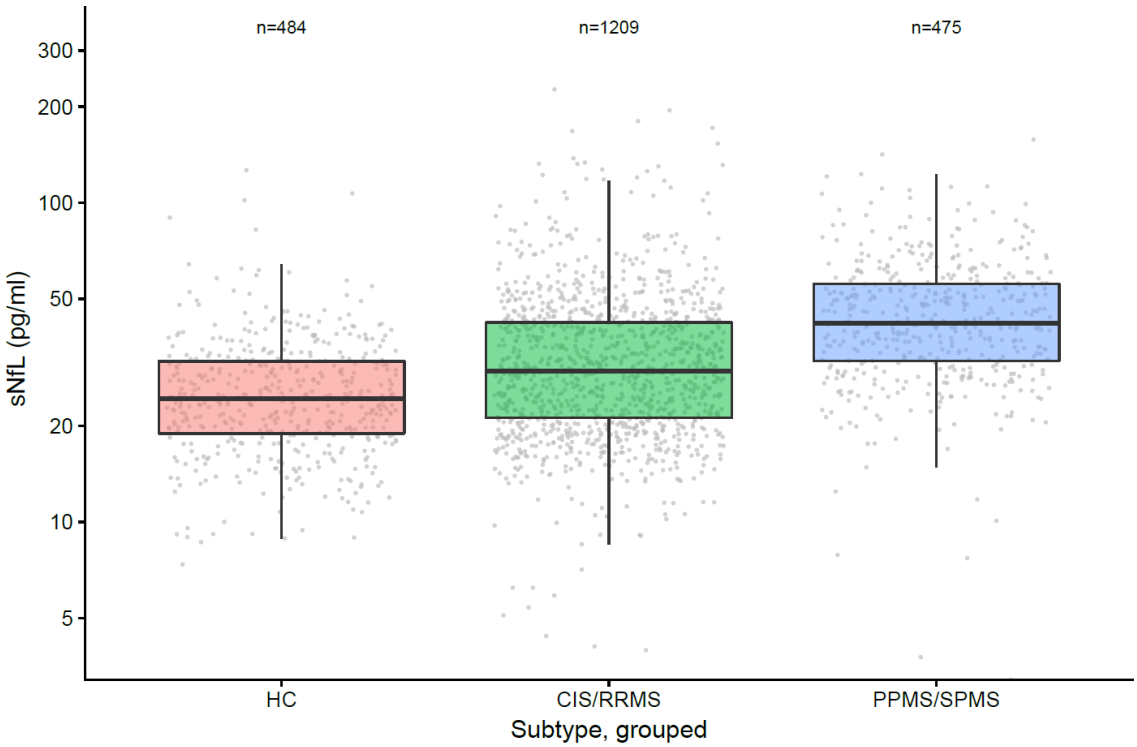
Treatments at respective study years/time periods separated by disease course (percentages). Left: Clinically Isolated Syndrome (CIS)/relapsing remitting MS (RRMS). Right: Primary progressive MS (PPMS)/ secondary progressive MS (SPMS).



Low efficacy, first generation injectable drugs: interferon beta, glatiramer acetate
Second generation oral compounds: Fingolimod, Dimethyl-fumarate, Teriflunomide
High efficacy injectable monoclonal antibodies: Natalizumab, Rituximab
Other: Mitoxantrone, Azathioprin, Interferon beta 1b (s.c.) plus azathioprin, Glatiramer acetate plus Mitoxantrone.

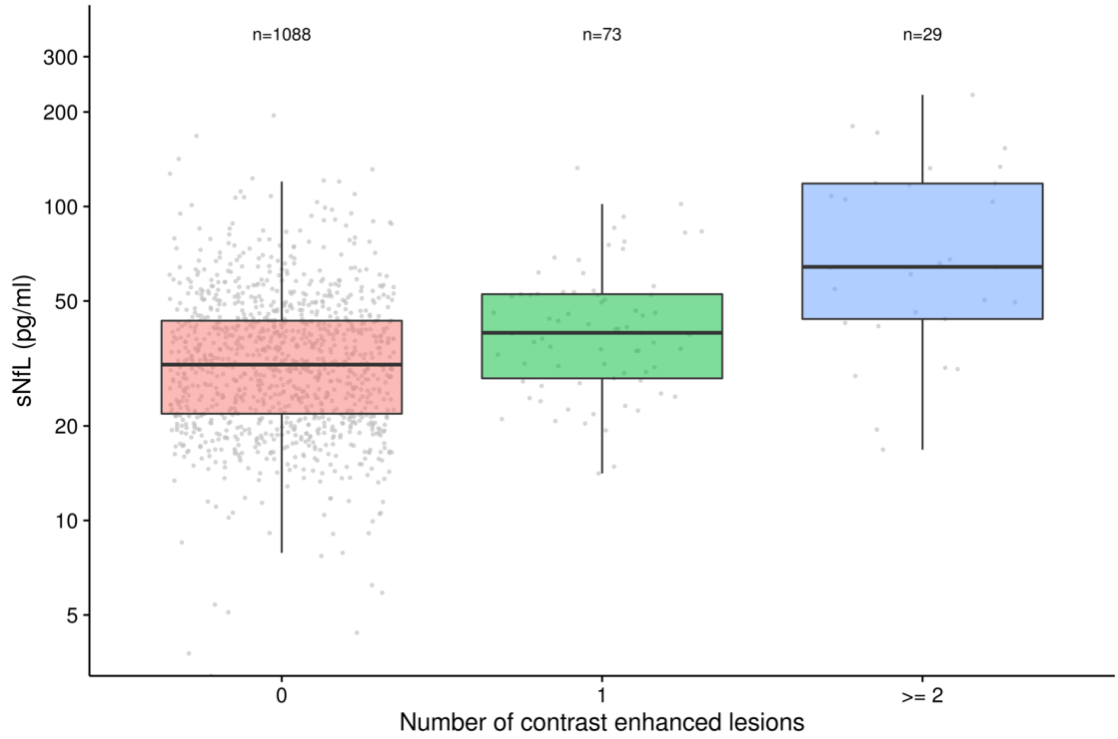
Supplementary figure 3

Serum NfL concentrations in healthy controls, relapsing and progressive multiple sclerosis patients.



Legend: After age correction both RMS (CIS/RRMS) and PMS (PPMS/SPMS) had higher sNfL than HC (RMS: 29.7 (21.2-42.2) pg/ml, β_{mult}=1.263, 95%CI=1.179-1.353, p<0.001; PMS: 41.9 (31.9-55.7) pg/ml, β_{mult}=1.423, 95%CI=1.284-1.576, p<0.001). sNfL concentrations were also higher in PMS as compared to RMS (after age correction: β_{mult}=1.154, 95%CI=1.059-1.258, p=0.001). Numbers in the figure denote the number of samples.

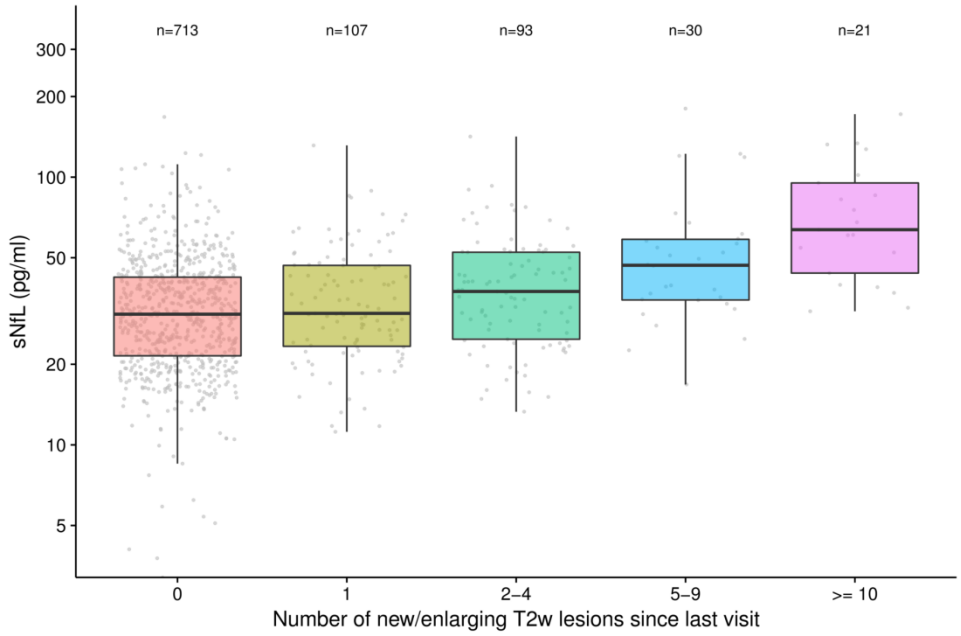
Supplementary figure 4.
Serum NfL concentrations and number of contrast enhancing lesions.



Legend: Univariable analyses showed that sNfL levels were positively associated with the number of contrast enhancing lesions (β_{mult}=1.174, 95%CI=1.105-1.246 per lesion, p<0.001, n=1190 observations). Numbers in the figure denote the number of samples.

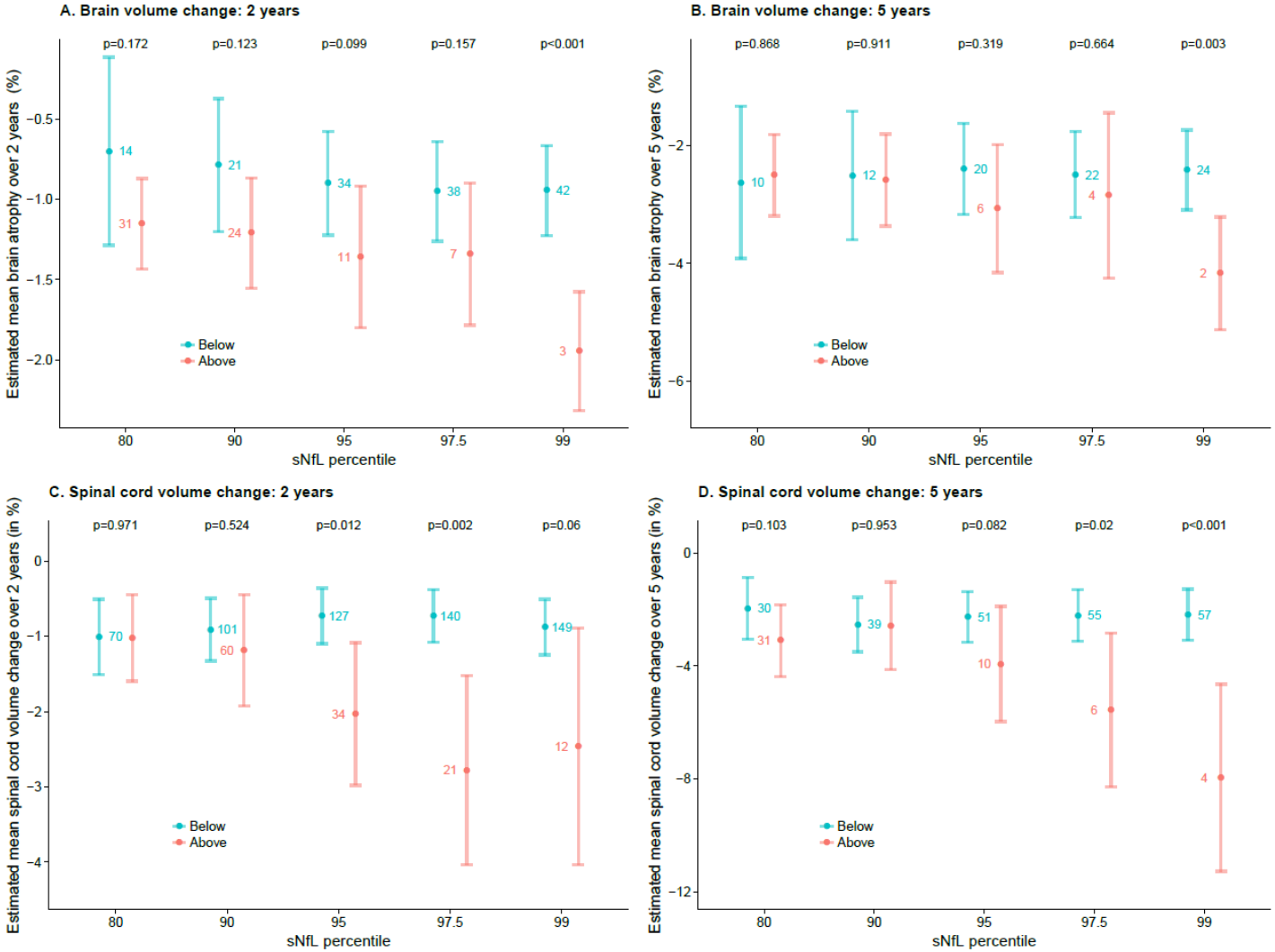
Supplementary figure 5

Serum NfL concentrations and number of new or enlarging T2 hyperintense lesions.



Legend: Univariable analyses showed that sNfL levels were positively associated with the number of new or enlarging lesions (β_{mult}=1.059, 95%CI=1.041-1.078 per lesion, p<0.001, n=964). Numbers in the figure denote the number of samples.

Supplementary figure 6. Estimated mean change in brain volume over 2 years (A) and 5 years (B) and spinal cord volume over 2 years (C) and 5 years (D) against sNfL dichotomized based on age corrected percentile curves from healthy controls in progressive multiple sclerosis patients without contrast enhancing lesions.



Legend: The mean estimated percentage of brain volume change in progressive multiple sclerosis patients without contrast enhancing lesions with sNfL above the 99th age corrected percentile was increased 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. The percentiles were constructed based on HC samples. Numbers in the figure denote the number of samples above or below the respective percentiles of healthy controls.

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Chapter 4: Summary, discussion and future steps

Currently no biomarker is supported by sufficient evidence to be used as a surrogate for clinical or MRI endpoints. Such biomarkers would need to be accurate and reproducible, to be associated with key clinical features and future outcome and, last but not least, to be derived from an easily accessible body fluid allowing repeated measurements over time. Particularly, biofluid markers bear the advantage of measuring ongoing pathologic changes real-time.

Our aim was to develop and explore a highly sensitive biomarker of neuro-axonal damage in MS. The correlation of NfL concentrations in CSF with features of disease activity demonstrated was promising, however the relative invasiveness of lumbar punctures renders longitudinal CSF samplings impractical in routine clinical practice. On the other hand, quantitative analysis of the concentration of NfL in blood was below the sensitivity of existing technologies, given that physiological levels are 50-100 times lower than in CSF. Therefore, it was a primary aim to develop and analytically validate a NfL assay meeting this prerequisite by use of the 4th generation highly sensitive SIMOA platform[2].

In our first study, we showed that NfL can now be reliably measured in serum from MS patients using the SIMOA NfL assay developed during this PhD, even at very low concentration[25,26]. NfL levels in serum increased with age to a similar extent in both healthy controls (HC) and patient cohorts, while no difference was detected between genders. The age association has also been shown for CSF NfL measurements[27] and we speculate that age related brain damage could be responsible for this observation. In this context, it would be important to investigate the impact of comorbidities and vascular risk factors on sNfL levels in both general and MS patient populations. The ability to measure NfL in blood samples is an important prerequisite to follow these questions in large enough and representative populations.

As expected, we observed that NfL levels were considerably lower in serum than in paired CSF samples. However, there was a strikingly positive association between CSF NfL and sNfL, highlighting how serum levels directly reflect CSF NfL concentration. This suggests that neuronal death leads to the progressive release of neurofilaments in the extracellular space, CSF and blood, but how this exactly happens and the mechanisms involved and influencing the passage from CSF to blood are not known yet. For example, it would also be important to investigate the potential mechanisms influencing the molecules half life and influence of blood-brain barrier integrity on the proportion of CSF NfL reaching the peripheral circulation.

Patients had higher sNfL concentration than healthy individuals confirming CSF NfL findings in MS[9-11,28-33] and CIS[34]. Similarly to other neurological diseases including ALS, dementing illnesses and spinal cord injury[20,35,36], we interpret these findings as a consequence of ongoing neuronal damage in the course of MS. This interpretation is further supported by the clear association between sNfL and both brain and spinal cord MRI lesions observed in our, as well as for CSF NfL in previously reported studies[10,17,33,34,37].

In longitudinal repeated measurements age, the presence of a recent relapses and neurological disability as measured by the EDSS were independently associated with levels of sNfL. Notably, sNfL levels were also significantly lower in DMT treated as compared to untreated patients, independently of all other variables. Also in the light of recent data resulting from sNfL analyses from plasma samples demonstrating treatment effects of fingolimod on NfL levels from a randomised controlled trial[38], we believe that the associations of sNfL with DMT treatment status and time since treatment initiation strongly suggest that DMTs are able to reduce NfL release (as shown also for CSF NfL measurements for fingolimod, natalizumab and rituximab[13,14,16-18,39,40]).

In the first study, we finally tested whether sNfL levels above age corrected percentiles derived from HC could predict clinically meaningful events in patients. We found that patients with sNfL levels above certain age adjusted sNfL percentiles derived from healthy controls have a progressively increased risk of both having experienced and experience in the future clinical relapses and disability accumulation. Similarly, the incidence of relapses in the following year and the risk of future EDSS worsening was higher in patients with sNfL values above versus below the respective normative percentiles. These findings suggest high sNfL could be used as an easily available indicator of recently developed neuronal damage, either in clinically silent status or when clinical changes are difficult to interpret and patients report symptoms that are not easily assessed with neurological examinations (e.g. increased fatigue, numbness, fluctuations of previously developed neurological signs or symptoms). They also support the potential use of sNfL as a prognostic marker for the future course of how severe the disease worsening. Two relatively small previous studies have shown that patients with higher CSF neurofilament levels have a worse disease outcome in the long term[41,42]. It is therefore plausible to see a similar association with sNfL and the easily availability of serum and the opportunity to perform repeated measurements in different phases of the disease and under different

treatments and various different treatment options with distinct efficacy versus risk profiles carry obvious and relevant clinical implications.

In summary this study provided a number of important findings: sNfL levels i) can be reliably and reproducibly measured in serum samples from MS patients; ii) are positively associated with age but not gender in independent HC and MS cohorts; iii) closely correlate with NfL concentration in the CSF; iv) are increased in MS patients as compared to HC and positively associated with T2 and gadolinium enhancing lesions in both brain and spinal cord; v) are increased following relapses and in patients with higher EDSS scores, and decreased in DMT treated patients and after starting a new DMT; and vi) are associated with an increased risk of future relapses and EDSS worsening.

In the second study we confirmed in an independent cohort our previous findings[43] that age, recent relapses and concurrent disability are independently associated with sNfL levels in MS[44]. Importantly, sNfL levels were highly increased in the presence of brain CEL and independently associated with the overall T2 lesion volume and brain volume at time of sampling. These findings further supported the hypothesis that sNfL reflects the extent of neuronal loss within the CNS at the time of sampling. We also confirmed that MS patients with higher sNfL levels are at higher risk of experiencing disability worsening in the following year[43] and for the first time we provided consistent evidence for sNfL concentration being a strong predictor of brain atrophy in MS at 2 and 5 years also in a multivariable analysis. Similar strong findings emerged when we analyzed the association between sNfL and spinal cord atrophy[44].

Taken together, these observations show how MS patients with higher sNfL concentrations are at increased risk of experiencing worsening in disability scores and higher rates of brain and spinal cord volume loss in the long term. We therefore believe these patients could benefit from an escalation to more active treatments, before the occurrence of irreversible neuronal damage.

Ongoing and future research and Conclusions

Meanwhile there is an established body of evidence that NfL levels may be decreased by effective DMT in MS, both based on measurement in serum[38,43,45] and in CSF[14,17,18]. On the group level, levels of sNfL decrease typically within 3-6 months, but they do not reach age-matched normal values, thus indicating persistence of continuous neuronal damage[38,43]. Many patients reach the status of NEDA (No Evidence of Disease Activity) or NEPAD (No Evidence of Progression or Active Disease) under DMT[46], but a high proportion of individuals remain

with high levels of NfL: 21.5% of the patients[44] with NEDA had sNfL levels above the 90th age corrected percentile (unpublished). These data indicate that a high number of patients is not considered for appropriate therapy escalation, because they escape detection of subclinical disease activity based on current monitoring algorithms: here measurement of sNfL could be a sensitive therapy monitoring tool for the persistence of subclinical disease activity.

Based on this, in a third and still ongoing study we hypothesized that sNfL will be suitable to monitor not only therapy response, but also the lack of it, and may guide physicians to modify therapy, despite the appearance that a patient may look to have clinically and radiologically 'silent' disease. In a manuscript in preparation, we have investigated this hypothesis for 206 patients under stable (3-24 months) therapy with fingolimod. Here, high levels of NfL occurred despite continuous fingolimod treatment and predicted quantitatively the risk for recurrence of relapse activity 1 (Figure 4A) or 2 years later (Figure 4B) and development of new T2 weighted lesions (Figure 4C), as well as the degree of annualized percentage brain volume loss (Figure 4D) providing initial evidence for sNfL being useful to detect suboptimal treatment response in MS[47].

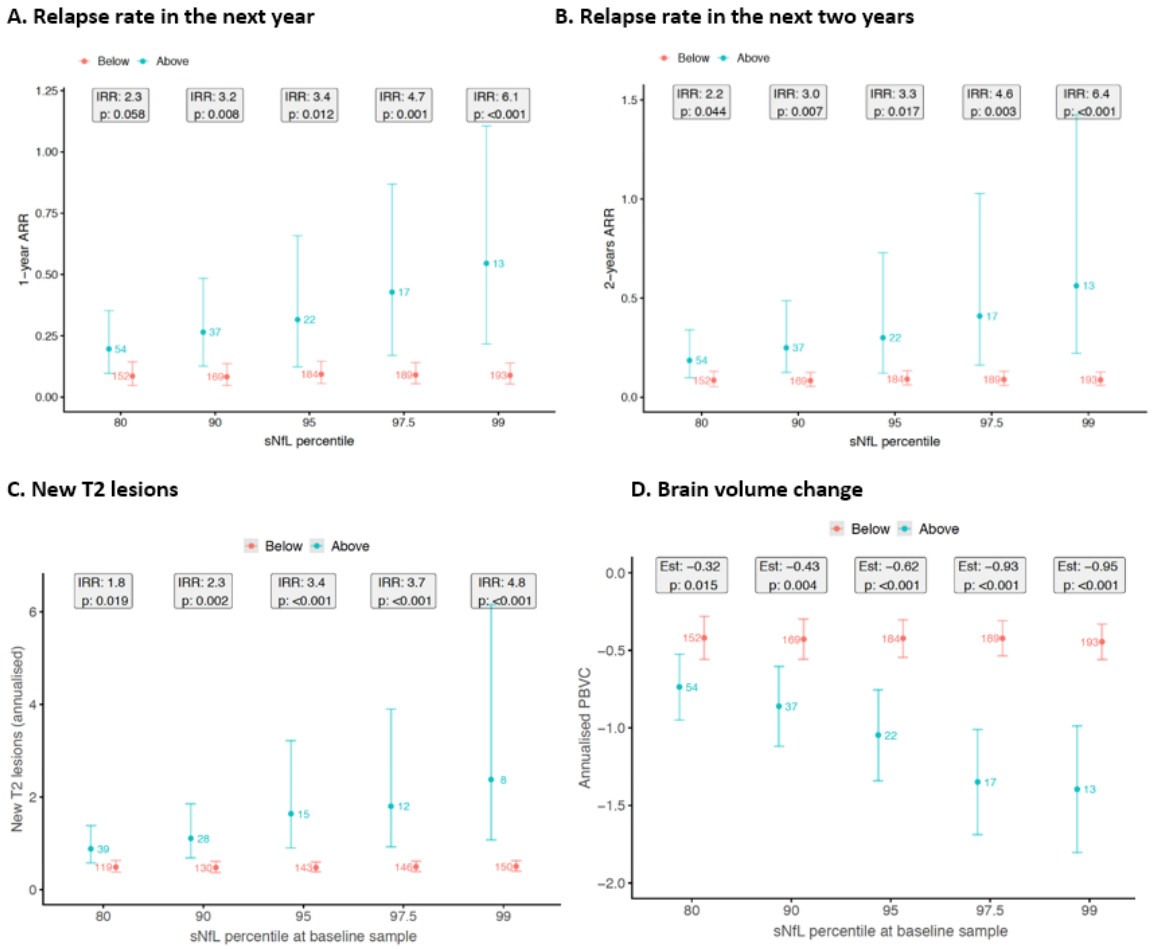

Figure 4. Estimated annualised incidence risk ratios of the annualized relapse rate after one (A) and two (B) years of follow-up, annualized incidence risk ratios for new T2 lesions (C) and annualized brain volume change (D) against sNfL dichotomized based on age-corrected percentile curves from healthy controls. Numbers in the figure denote the number of samples above or below the respective percentiles of healthy controls (according to[44]). Abbreviations: ARR, annualized relapse rate; IRR, incidence rate ratio; PBVC, percentage brain volume change. Numbers in the graph denote the number of patients in each percentile category.

Current evidence for the use of NfL as biomarker in MS is largely restricted to population-based analyses and there are important gaps precluding its use in patient-by-patient based disease monitoring and individual therapeutic decision making[48] that need to be overcome in the next years:

i) NfL levels are independent of the molecular pathways of neuronal damage: for instance, in MS NfL levels cannot be used to differentiate between neuronal damage generated by acute (relapses) or chronic (progression) disease activity. The lack of knowledge on kinetics (turnover rate/half-life time) of NfL in blood circulation for both physiologic and disease conditions is the most prominent hurdle to better understand the relative contribution of each pathogenetic pathway (acute or chronic) to sustain the neurodegenerative process in MS; this is a current impediment for the use of NfL as predictive biomarker for progression, as well as for therapy monitoring[49].

ii) There is no normative data base available that allows to define individual values as being pathologic on the background of a physiologic 2.2%/year increase of levels between 18-70 years[43,44].

iii) The impact of comorbidities on NfL levels in the context of its use as biomarker for MS needs to be further explored. Because NfL is such a sensitive measure, it reports on 'upstream' pathology and increases long before disease is manifest clinically[50]. Thus, the contribution of neurologic, psychiatric and non-communicable diseases needs to be factored into the assessment of sNfL levels.

The successful closure of these gaps will be essential if sNfL levels are to be established as the first biofluid marker to quantify present and to predict future neurodegenerative processes in

MS, and to support therapeutic decision making in individual patients for personalised medicine.

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** denotes equal contribution*

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Appendix A: Publications

** denotes equal contribution*

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Review articles

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