Serum neurofilament light chain is a biomarker of acute and chronic neuronal damage in early multiple sclerosis

Nelly Siller, Jens Kuhle, Muthuraman Muthuraman, Christian Barro, Timo Uphaus, Sergiu Groppa, Ludwig Kappos, Frauke Zipp and Stefan Bittner

Abstract

Background: Monitoring neuronal injury remains one key challenge in early relapsing-remitting multiple sclerosis (RRMS) patients. Upon axonal damage, neurofilament – a major component of the neuro-axonal cytoskeleton – is released into the cerebrospinal fluid (CSF) and subsequently peripheral blood.

Objective: To investigate the relevance of serum neurofilament light chain (sNfL) for acute and chronic axonal damage in early RRMS.

Methods: sNfL levels were determined in 74 patients (63 therapy-naive) with recently diagnosed clinically isolated syndrome (CIS) or RRMS using Single Molecule Array technology. Standardized 3 T magnetic resonance imaging (MRI) was performed at baseline and 1–3 consecutive follow-ups (42 patients; range: 6–37 months).

Results: Baseline sNfL correlated significantly with T2 lesion volume ($r = 0.555$, $p < 0.0001$). There was no correlation between baseline sNfL and age, Expanded Disability Status Scale (EDSS) score or other calculated MRI measures. However, T2 lesion volume increased ($r = 0.67$, $p < 0.0001$) and brain parenchymal volume decreased more rapidly in patients with higher baseline sNfL ($r = -0.623$, $p = 0.0004$). Gd-enhancing lesions correlated positively with sNfL levels. Initiation of disease-modifying treatment led to a significant decrease in sNfL levels.

Conclusion: sNfL indicates acute inflammation as demonstrated by correlation with Gd+ lesions. It is a promising biomarker for neuro-axonal damage in early multiple sclerosis (MS) patients, since higher baseline sNfL levels predicted future brain atrophy within 2 years.

Keywords: Multiple sclerosis, neurodegeneration, neurofilament, biomarker, clinical progression, MRI, disease activity

Correspondence to:
S Bittner
Department of Neurology, University Medical Center of the Johannes Gutenberg University Mainz, 55131 Mainz, Germany.
stefan.bittner@unimedizin-mainz.de

Nelly Siller
Muthuraman Muthuraman
Timo Uphaus
Sergiu Groppa
Frauke Zipp
Stefan Bittner
Department of Neurology and Focus Program Translational Neuroscience (FTN), Research Center for Immunotherapy (FZI), Rhine-Main Neuroscience Network (rmn²), University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

Jens Kuhle
Christian Barro
Ludwig Kappos
Neurologic Clinic and Policlinic and Departments of Medicine, Biomedicine and Clinical Research, University Hospital Basel, University of Basel, Basel, Switzerland

Introduction

Neurofilament as a major component of the axonal cytoskeleton is released into the interstitial fluid, cerebrospinal fluid (CSF) and subsequently serum in low levels upon neuro-axonal damage occurring in neurological disorders.[1] Modern magnetic resonance imaging (MRI) techniques and immunohistochemical studies have revealed neuronal damage in early stages of the disease course of patients with clinically isolated syndrome (CIS) or relapsing-remitting multiple sclerosis (RRMS).[2–4] Based on the apparent molecular mass of the mammalian subunits, three major subunits of neurofilament have been defined: light (65−70 kDa), medium (140–160 kDa) and heavy chain (200–220 kDa). Neurofilament light chain (NfL) has been studied most extensively and an association with acute neuro-axonal damage was demonstrated for NfL which is elevated in CSF during all stages of multiple sclerosis (MS) with a heightened (10 times) increase in times of acute relapses.[5]

Advances in analytic sensitivity using Single Molecule Array (Simoa) technology provides the basis for assessment of NfL levels in serum samples (sNfL) as an important step towards its use as a biomarker in clinical routine.[6] Indeed, NfL levels in serum and CSF are highly correlated and sNfL in RRMS patients is increased compared to healthy

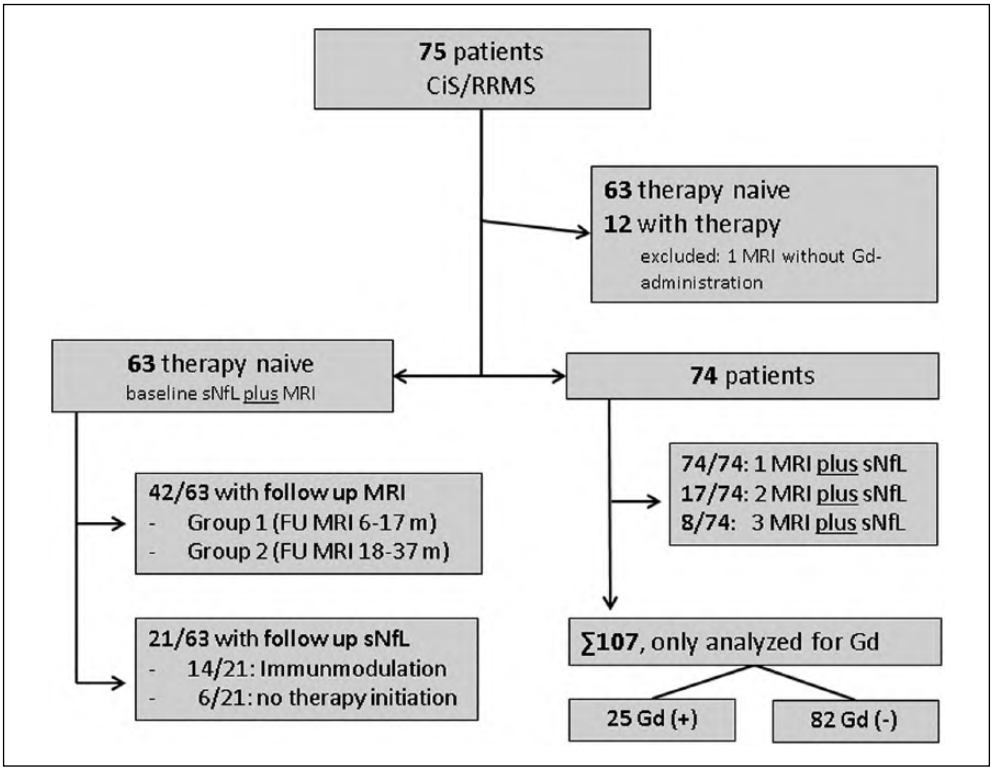

Figure 1. Study design. sNfL was determined in serum of 63 therapy-naive patients; standardized MRI was performed at baseline. 1−3 consecutive MRI follow-ups (FU) in 42 patients after 637 months were conducted. Follow-up MRIs were classified into two groups, one with MRI after less than 18 months ($n = 38$, median = 11 months) and one with MRI at least 18 months later ($n = 33$, median = 24 months). Correlation between sNfL levels and gadolinium (Gd)-enhancing lesions was evaluated in 74 patients, 62 of the naive cohort and an additional 12 patients (one patient in the therapy-naive cohort refused gadolinium administration). Follow-up MRIs with consecutive serum samples were included leading to 107 sNfL values and MRI scans.

controls.[7,8] A recent study using this technology in MS has proposed sNfL as a biomarker to monitor or potentially even predict neuronal damage in MS patients,[8] while it is still largely unclear, whether sNfL could predict neuronal damage in very early MS or CIS. Therefore, we investigated the association between sNfL and clinical and MRI measurements in a therapy-naive cohort of CIS and RRMS patients in the very early stage of disease.

Methods

Study design and population

In all, 74 patients (63 therapy naive) with recently diagnosed CIS or RRMS were included in the study (see also Figure 1 for study design), which was approved by the local ethics committees; all patients provided written informed consent. sNfL was determined by a previously described Simoa NfL assay.[8] Measurements were performed in a completely blinded fashion, since two centres (University Medical Center Mainz and University Hospital Basel) were involved in the study. Serum samples were obtained in Mainz, pseudonymized, and sent and analysed without clinical data or other information. Standardized 3 T MRI protocol was performed at baseline; 42 patients underwent 1−3 consecutive follow-ups after 6–37 months. Follow-up MRIs were classified into two groups, one with MRI after less than 18 months ($n = 38$, median: 11 months) and one with MRI at least 18 months later ($n = 33$, median: 24 months). Imaging was performed using sagittal three-dimensional (3D) T1-weighted magnetization-prepared rapid gradient-echo (MP-RAGE) sequence (TE/TI/TR = 2.52/900/1900 ms, flip angle = 9°, field of view (FOV) = 256 × 256 mm^2, matrix size = 256 × 256, slab thickness = 192 mm, voxel size = 1 × 1 × 1 mm^3), sagittal 3D T2-weighted fluid-attenuated inversion recovery (FLAIR) sequence (TE/TI/TR = 388/1800/5000 ms, echo-train length = 848, FOV = 256 × 256 mm^2, matrix size = 256 × 256, slab thickness = 192 mm, voxel size = 1 × 1 × 1 mm^3). MRI data were obtained and analysed by blinded raters

who had no further information about the sNfL levels or clinical data of the investigated patients.

Correlation between sNfL levels and gadolinium (Gd)-enhancing lesions was evaluated in all 74 patients: 62 of the naive cohort and an additional 12 patients with therapy already at baseline. One patient in the treated cohort did not receive gadolinium and could therefore not be included in the correlation analysis between NfL and Gd-enhancing lesions. Altogether, 107 sNfL values and corresponding MRI scans were available for analysis.

Consecutive serum samples after a median 13 months were available in 21 patients, 14 patients had started an immunomodulatory therapy and 6 had not. One patient quit immunomodulatory therapy after 4 weeks and was added to the latter group, assuming no long-term therapeutic effect of dimethyl fumarate after this short period of time. Informed consent was obtained from all patients and the study was approved by local Medical Ethics Committee.

MRI analysis

Initially, lesion maps were drawn on T2-weighted 3D FLAIR images using the MRIcron software (http://www.mccauslandcenter.sc.edu/mricro/mricron/). Using the lesion segmentation toolbox (LST) which is part of the statistical parameter mapping (SPM8) software, 3D FLAIR images were co-registered to 3D T1 images and bias corrected. After partial volume estimation (PVE), lesion segmentation was performed with 20 different initial threshold values for the lesion growth algorithm. By comparing automatically and manually estimated lesion maps, the optimal threshold (κ value) for lesion detection was determined for each patient and used for automatic lesion volume estimation and lesion filling. Subsequently, the filled 3D T1 images as well as the native 3D T1 images were segmented into grey matter, white matter and CSF and normalized to Montreal Neurological Institute (MNI) space. Finally, the quality of the segmentations was visually inspected.

Serum NfL measurements

Serum samples were collected by treating physicians during the past few years at the University Medical Center Mainz. Samples were processed at room temperature within 2 hours. Serum samples were spun at 2000*g* at room temperature for 10 minutes, aliquoted in polypropylene tubes and stored at −80°C. Serum NfL was measured by Simoa NfL assay as described previously.[8] Bovine lyophilized NfL was obtained from UmanDiagnostics. Calibrators ranged from 0 to 2000 pg/mL. Batch prepared calibrators were stored at −80°C. Inter-assay coefficients of variation (CV) were 4%, 2%, and 3% for control samples with mean concentrations of 13, 33 and 283 pg/mL, respectively. The mean intra-assay CV of duplicate determinations for concentration was 4%. Repeated measuring was performed for few samples with intra-assay CV above 20%.

Statistical analysis

Summary statistics are presented as mean ± SD, median (range) and percentage, where applicable. For univariate analysis, the Mann–Whitney *U* test and chi-square test were used as appropriate. For correlation, non-parametric Spearman analysis was used. Multivariate regression analysis was performed with Gd lesion number as dependent variable and sNfL, age, sex and so on as independent variables. Receiver operating characteristic (ROC) analysis was used to estimate the predictive discriminating values. For every individual cut-off point, there is a pair of diagnostic sensitivity and specificity values. To construct a ROC graph, we plotted these pairs of values with 1 − specificity on the x-axis and sensitivity on the y-axis. From the ROC curves, we estimated Youden's index, which is defined for all points of an ROC curve, and the maximum value of the index may be used as a criterion for selecting the optimum cut-off point between two variables. The index is represented graphically as the height above the 50% chance line, and it is also equivalent to the area under the curve (AUC) represented by a single operating point. *p*-values less than <0.05 were considered statistically significant. All statistical analyses were performed by means of SPSS 23.0 package software (IBM, Armonk, NY, USA). Prediction accuracy of brain atrophy by NfL was calculated using support vector machine (SVM). SVM is a powerful tool for nonlinear prediction between two variables.[9] In short, the algorithm looks for an optimal threshold between the two variables by maximizing the correlation between classes' closest points. In most cases, the linear prediction is not ideal so a projection into a higher-dimensional space is performed where the data points effectively become linearly correlated. Here, we have used the radial basis function kernel for this projection due to its good performance as discussed previously[9] and used the grid search (min = 1; max = 10) to find the few optimal input parameters namely C (Type of regression algorithm; 1 to 1000) and gamma (0.25). The selection was checked by 10-fold cross validation by taking 70% of the data for training and 30% for testing.

Table 1. Detailed characteristics of patient data and MRI parameters for treatment-naive cohort.

Variable	Naive cohort[a]	*p* value[b] (rho value)
n	63	
sNfL (pg/mL)	36.26 (24.1–63.2)	
Age	35 (27–46)	0.405 (0.107)
Female	44 (69.8%)	
DD (months)	1 (0–2)	0.350 (0.120)
EDSS	1 (0–2)	0.568 (0.073)
Gap time (blood withdrawal-MRI; days)	53 (6–137)	0.137 (−0.189)
LV (mL)	1.76 (0.8–4.39)	<0.0001* (0.555)
GMV (mL)	620.6 (569.7–673.5)	0.757 (−0.040)
WMV (mL)	568.8 (534.3–623.3)	0.500 (0.087)
BP (mL)	1187.2 (1124.5–1281.2)	0.857 (0.023)
TV (mL)	1412.5 (1336.4–1523.9)	0.488 (0.089)

sNfL: serum neurofilament light chain; MRI: magnetic resonance imaging; EDSS: Expanded Disability Status Scale; DD: disease duration at time point of serum sampling; LV: lesion volume; GMV: grey matter volume; WMV: white matter volume; BP: brain parenchyma; TV: total volume.
[a]Unless noted otherwise, data reported as median (range).
[b]Derived from non-parametric correlation (Spearman-rho) with sNfL.
*Statistically significant.

Table 2. Detailed characteristics of patient data and MRI parameters for patients included in the gadolinium-enhancing lesion analysis.

Variable	Gadolinium cohort[a]		*p* value
n	25 (+)	82 (–)	
NfL (pg/mL)	63.2 (37.6−159)	28.1 (18.8−37.6)	<0.0001[b],*
Age	36 (28−46)	40 (29−47)	0.614[b]
Female	20 (80%)	56 (68%)	0.320[c]
DD (months)	1 (1−4.5)	8 (1−20.5)	0.045[b],*
EDSS	1 (0−2)	1 (1−2)	0.576[b]
Gap time (blood withdrawal-MRI; days)	8 (−20 to 54)	0 (−3.2 to 23)	0.449[b]

NfL: neurofilament light chain; MRI: magnetic resonance imaging; EDSS Expanded Disability Status Scale; DD: disease duration at time point of serum sampling
[a]Unless noted otherwise, data reported as median (range).
[b]Derived from Mann–Whitney *U* test.
[c]Derived from chi-square test.
*Statistically significant.

Results

Baseline sNfL levels significantly correlate with baseline T2 lesion volume and T2 lesion volume change in MS patients

To assess the clinical value of measuring sNfL levels in early MS patients, we focused on therapy-naive patients ($n=63$) with newly diagnosed CIS/RRMS. The median disease duration from first symptoms onset was 1 month. See Tables 1 and 2 for detailed patient characteristics. The median NfL serum level in our naive cohort at baseline was 36.26 (24.1−63.2) pg/mL. In our therapy naive group, 43 patients had been diagnosed with RRMS and 20 patients reached criteria for CIS. RRMS patients had significantly higher median (45.33 pg/mL) sNfL levels in comparison to CIS patients (median = 28.39 pg/mL, $p<0.05$; Supplementary Figure 1). There were no significant correlations between baseline NfL and disease duration ($r=0.120$, $p=0.350$), Expanded Disability Status Scale (EDSS) score ($r=0.073$, $p=0.568$) or age ($r=0.107$, $p=0.405$) in our cohort.

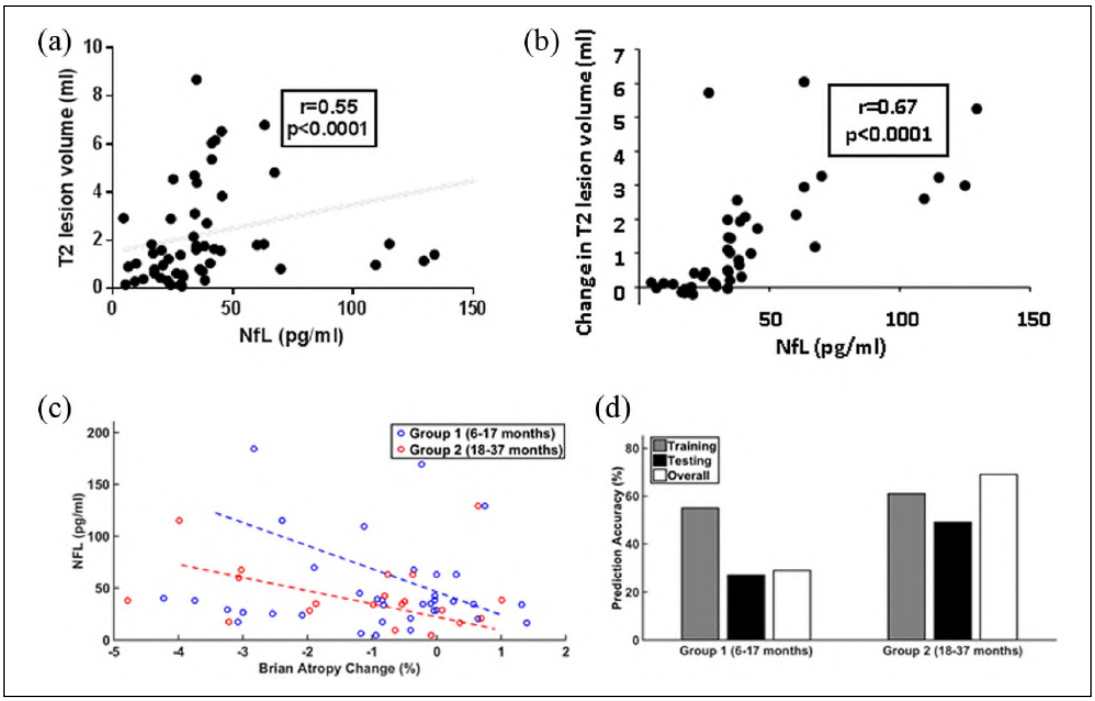

Figure 2. Correlation of baseline sNfL levels with T2 lesion volume at baseline and brain atrophy rates after 1 and 2 years of follow-up. (a) Baseline sNfL correlated significantly with T2 lesion volume ($r=0.55$, $p<0.0001$). One outlier was excluded due to technical difficulties: movement artefacts during acquisition prevented further analysis. The regression analysis refers to all data points; however, for improved visualization the x/y axes were adjusted leading to 9 data points out of range. (b) Baseline sNfL correlated significantly with T2 lesion volume change over time ($r=0.67$, $p<0.0001$), comparing T2 lesion volume at baseline with MRI follow-up after 6–37 months. (c) Brain parenchyma decreased more rapidly in patients with higher baseline sNfL. Analysis was performed after a median MRI follow-up of 11 months (Group 1, 6–17 months, $n=38$) and after 24 months (Group 2, >18 months, $n=33$). (d) Non-linear prediction (SVM) showed 69.2% prediction accuracy (70% training and 30% testing) of brain atrophy after median 24 months with baseline sNfL.

Baseline sNfL correlated significantly with T2 lesion volume (Figure 2(a), $r=0.555$, $p<0.0001$) and change in T2 lesion volume over time (Figure 2(b), $r=0.67$, $p<0.0001$). Additional MRI measures did not show any significant correlation with baseline sNfL (white matter volume ($r=0.087$, $p=0.5$), grey matter volume ($r=-0.040$, $p=0.757$), brain parenchyma ($r=0.023$, $p=0.857$) or total volume ($r=0.089$, $p=0.488$)).

Baseline sNfL levels predict brain atrophy progression

As neurofilament levels are thought to reflect ongoing neuro-axonal damage, we assessed whether patients with higher sNfL levels would suffer from increased brain atrophy at later time points. Indeed, over the next 6–37 months, brain parenchyma decreased more rapidly in patients with higher baseline sNfL (all patients: $r=-0.623$, $p=0.0004$; MRI group 1: $r=-0.652$, $p=0.0003$; MRI group 2: $r=-0.415$, $p=0.002$, Figure 2(b)). Predictive analysis using SVM was performed to assess prediction accuracy of sNfL for subsequent brain atrophy (Figure 2(c)). In our cohort, baseline sNfL levels showed only a low prediction accuracy of brain atrophy after 1 year (group 1, 29.4%), compared to relatively high prediction accuracy (69.2%) after 2 years (group 2). These findings might reflect the temporal latency of structural MRI quantification towards neurodegenerative processes.

Association between sNfL and gadolinium-enhancing lesions

Elevations of sNfL levels reflect neuro-axonal damage, mediated both by acute inflammatory damage in an acute relapse and 'chronic' ongoing neurodegenerative processes. We first addressed the relation between Gd+ MRI lesions and sNfL levels. For this analysis, all available MRIs (both baseline and follow-up data) with corresponding serum samples were pooled. Median NfL serum levels in patients with Gd+ lesions were 63.2 (37.6–159) pg/mL and 28.1 (18.8–37.6) pg/mL in patients without acute inflammation ($p<0.0001$, Figure 3(a)). There was a positive

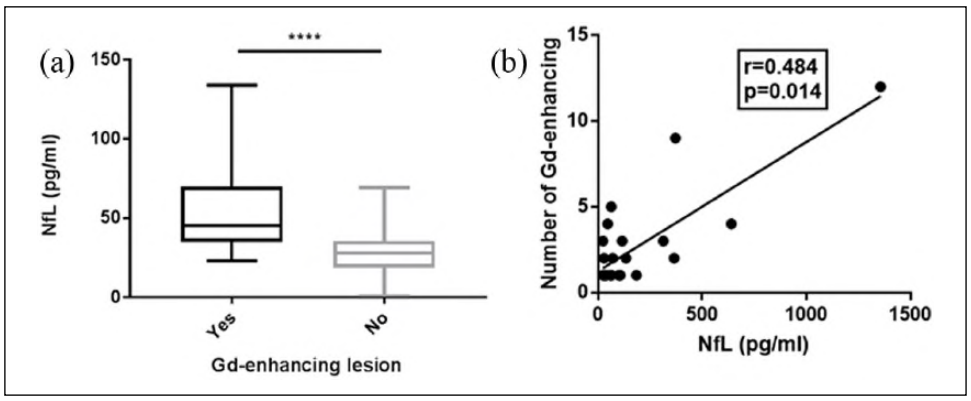

Figure 3. Association between sNfL levels and gadolinium-enhancing lesions. (a) Median sNfL in group with Gd-enhancing lesion was 45.3 pg/mL, $n=19$ (−6 outliers), median NfL in group without Gd-enhancing lesion was 27.9 pg/mL, $n=79$ (−3 outliers), $p<0.0001$. Boxes include median, 25th and 75th percentiles. (b) Positive correlation, using non-parametric Spearman correlation, between number of Gd-enhancing lesions and baseline serum NfL levels in 25 patients ($r=0.484$, $p=0.014$).

correlation between the number of Gd-enhancing lesions and serum NfL levels ($r=0.484$, $p=0.014$, Figure 3(b)). We found no significant differences in age, sex, EDSS score or gap time between blood withdrawal and MRI assessment between the two groups. Disease duration was median 8 months in patients without Gd-enhancing lesions and only 1 month in the group with Gd-enhancing lesions ($p=0.045$). Multivariate regression analysis confirmed sNfL as the only parameter influencing Gd lesion numbers ($p<0.001$) while age, sex, disease duration or EDSS score were not significant. ROC analysis was performed to determine the predictive discriminating value of sNfL. sNfL below or above 34 pg/mL predicted gadolinium enhancement in MRI with a sensitivity of 84% and specificity of 66% (AUC = 0.845); the Youden index was 0.53. In clinical practice, where repetitive MRI assessments are common in young MS patients, sNfL measurements might therefore be a supportive tool to decide which patient needs gadolinium application and when MRI without gadolinium is supported.

Effect of immunomodulatory therapy on sNfL levels: longitudinal changes of sNfL over time

Longitudinal serum samples were available for a limited number of patients ($n=21$) within our naive cohort (follow-up serum sample after median 13 months), allowing us to address the impact of therapy initiation on sNfL levels. In total, 14 out of 21 newly diagnosed patients started an immunomodulatory therapy after baseline sNfL measurement, whereas 7 patients remained therapy naive. Five patients started with dimethyl fumarate, six with β-interferons, two with glatiramer acetate and one patient with teriflunomide. Median baseline sNfL was not significantly different between groups (61.57 pg/mL in the therapy group, 34.94 pg/mL in the naive group, $p=0.488$). Median change in NfL in the therapy group was −34.2 pg/mL, whereas the therapy naive group had a median reduction of only −3.7 pg/mL ($p=0.0074$, Figure 4(a) and (b)) in the second sNfL analysis. Of note, while a correlation between treatment initiation and reduced sNfL levels is highly probable, one has to point out significant differences concerning age, sex and disease duration in the groups (age: 40 vs 50 years, $p=0.020$; sex: 8 females and 6 males vs 7 females, $p=0.05$; disease duration: 1 month vs 8 months, $p=0.04$).

Discussion

The search for valid biomarkers for patients with MS has been unsatisfying throughout many years. Particularly at the beginning of the disease, prospective biomarkers could facilitate therapy decisions, permit the detection of neuroimmunological activity before structural damage becomes prominent[10] and therefore lead to better patient outcome and decreased incidence of transition to progressive disease course in the long term.[11] So far, female sex, pure sensory onset, lower age at onset and other prognostic factors are associated with a benign course in early RRMS patients.[12] However, availability of prognostic parameters in addition to MRI measurements in clinical practice is limited. Easily accessible, reliable and sensitive biomarkers are therefore needed.

In our study, we were able to show in a cohort of newly diagnosed CIS/RRMS patients that sNfL levels

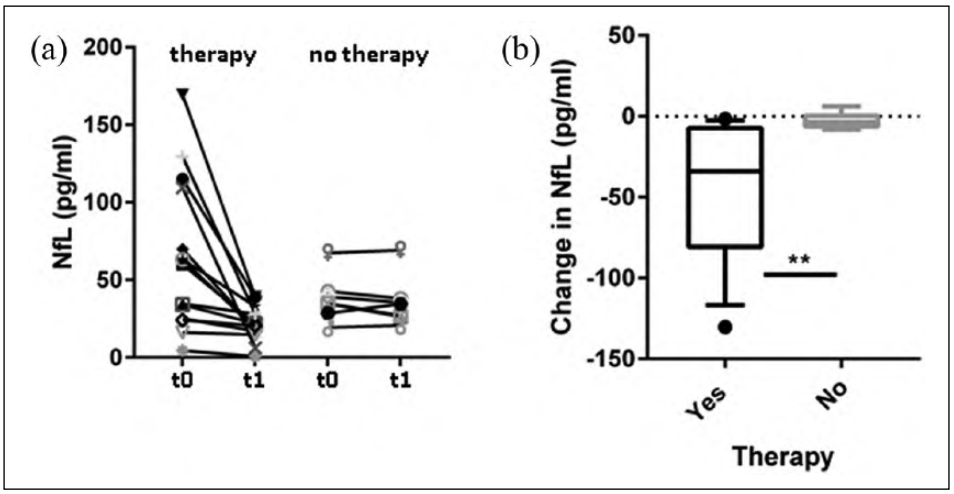

Figure 4. Effect of immunomodulatory therapy on sNfL levels. (a) 14 therapy-naive patients who started an immunomodulatory therapy in comparison to 7 naive patients staying without therapy, each with a consecutive serum sample (both groups median 13 months after baseline; $t0$ = baseline, $t1$ = follow-up). Immunomodulatory therapy was started median 12 months before second serum sample. (b) sNfL decreases significantly after initiation of any immunomodulatory therapy (** $p = 0.0074$, obtained by Mann–Whitney U test).

at the time point of diagnosis correlate significantly with T2 lesion volume at baseline and at any time point with the presence and number of Gd-enhancing lesions. Longitudinal investigations showed a decrease in sNfL after initiating disease-modifying therapies. Patients with higher baseline sNfL showed a more rapid decrease in brain volume and a more rapid increase in T2 lesion volume after 2 years. Acute inflammatory neuronal damage, illustrated by Gd-enhancing lesions, was clearly associated with sNfL levels. Strong correlation of T2 lesion volume and of brain atrophy rates with baseline sNfL lead to the question whether treatment decisions in individual patient care may be adjusted to neuronal damage already in the beginning of the disease.

Recently, a pilot study in 22 versus 20 patients from a phase II trial of riluzole as add-on treatment to weekly interferon-β (IFN-β)-1a addressed, for the first time, the potential of using sNfL as a biomarker for neuronal damage in MS. Interestingly, sNfL levels correlated with EDSS score, neuropsychological outcome and Gd-enhancing lesions and predicted whole brain atrophy at 1 and 2 years, possibly reflecting progressive neuronal loss and ongoing disease activity.[13] In a larger study assessing two independent Swiss cohorts, these initial findings were largely confirmed and expanded as sNfL levels correlated with EDSS assessments, presence of relapses and Gd-enhancing lesions. Disease-modifying treatments reduced sNfL levels and patients with higher sNfL levels were at risk of future relapses and EDSS progression.[8] While these findings are indeed promising, this study addressed mostly patients with established, ongoing disease (disease duration ~7 years and EDSS ~3.0), in contrast to our investigation focusing on very early MS patients (disease duration ~1 month and EDSS ~1.0).

So far, MRI-derived metrics are most commonly used in clinical practice to monitor subclinical disease activity and – so far only in study cohorts – progressive brain atrophy. Other parameters such as NEDA (no evidence of disease activity, that is, no relapse, EDSS worsening or new MRI lesions) have been suggested as novel monitoring compound parameters for treating MS patients.[14,15] However, a recent study showed that clinical and MRI data included in NEDA after 2 years did not correlate with the long-term outcome (>90% of the subjects had a 10-year follow-up) in a cohort of actively treated MS and CIS patients ($n = 517$).[16] The authors questioned both annual MRI assessments for monitoring in individual MS care and commonly used clinical and MRI features over 2 years in most randomized clinical trials. Disability accumulation in patients despite meeting NEDA criteria was possibly overlooked due to a lack of spinal cord MRI and brain atrophy evaluation. While it is important to point out that in contrast to MRI, NfL levels cannot provide localization information about the specific site of axonal damage, a contribution of spinal cord damage to sNfL levels was recently depicted by Disanto et al.[8]

We found a significant reduction of sNfL in patients that started immunomodulatory treatment in comparison to patients that stayed naive. Reduction of NfL in

CSF of RRMS patients under second line/escalation therapies natalizumab,[17] fingolimod[18] and rituximab[19] and recently also in plasma for fingolimod[20] has been described before. Our cohort was too small to detect differences in sNfL decrease between different drugs in the therapy group or to address NfL levels of patients with insufficient treatment responses.

Several questions remain that need to be addressed in larger cohorts of early RRMS patients with longitudinal sNfL and MRI measurements. Is it possible to monitor individual treatment responses via sNfL? Can we predict neuronal loss or even transition into long-term progressive disease course in the very early stage of disease and match immunomodulatory therapy regimes? Our results underline the potential value of NfL as a biomarker for neuronal damage in MS patients and extend these findings to a group of patients with very early disease course of RRMS and CIS. Serial measurements of serum NfL levels might be used to monitor the extent of inflammatory-driven neuronal damage and could be useful for treatment decisions. Larger, prospective studies are warranted.

Acknowledgements

The authors thank Cheryl Ernest for proofreading and editing the manuscript. N.S. and J.K. contributed equally as first authors. F.Z. and S.B. contributed equally as senior authors.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This work was supported by the German Research Council (DFG, CRC-TR-128 to S.G., F.Z. and S.B.).

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