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Serum Neurofilament Identifies Patients With Multiple Sclerosis With Severe Focal Axonal Damage in a 6-Year Longitudinal Cohort

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Abstract

Background and Objectives

Immunomodulatory therapies reduce the relapse rate but only marginally control disability progression in patients with MS. Although serum neurofilament light chain (sNfL) levels correlate best with acute signs of inflammation (e.g., relapses and gadolinium-enhancing [Gd+] lesions), their role in predicting progressive biology and irreversible axonal damage is less clear. We aimed to determine the ability of sNfL to dissect distinct measures of disease severity and predict future (no) evidence of disease activity (EDA/no evidence of disease activity [NEDA]).

Methods

One hundred fifty-three of 221 patients with relapsing-remitting MS initially enrolled in the Neurofilament and longterm outcome in MS cohort at the MS outpatient clinic of the University Medical Center Mainz (Germany) met the inclusion criteria for this prospective observational cohort study with a median follow-up of 6 years (interquartile range 4–7 years). Progressive disease forms were excluded. Inclusion criteria consisted of Expanded Disability Status Scale (EDSS) assessment within 3 months and MRI within 12 months around blood sampling at baseline (y0) and follow-up (y6). EDSS progression at y6 had to be confirmed 12 weeks later. sNfL was measured by single-molecule array, and the following additional variables were recorded: therapy, medical history, and detailed MRI parameters (T2 hyperintense lesions, Gd+ lesions, and new persistent T1 hypointense lesions).

Results

Patients experiencing EDSS progression or new persistent T1 lesions at y6 showed increased sNfL levels at y0 compared with stable patients or patients with inflammatory activity only. As a potential readily accessible marker of neurodegeneration, we incorporated the absence of persistent T1 lesions to the NEDA-3 concept (NEDA-3^{T1}: n = 54, 35.3%; EDA^{T1}: n = 99, 64.7%) and then evaluated a risk score with factors that distinguish patients with and without NEDA-3^{T1} status. Adding sNfL to this risk score significantly improved NEDA-3^{T1} prediction (0.697 95% CI 0.616–0.770 vs 0.819 95% CI 0.747–0.878, $p < 0.001$). Patients with sNfL values ≤8.6 pg/mL showed a 76% risk reduction for EDA^{T1} at y6 (hazard ratio 0.244, 95% CI 0.142–0.419, $p < 0.001$).

Discussion

sNfL levels associate with severe focal axonal damage as reflected by development of persistent T1 lesions. Baseline sNfL values predicted NEDA-3^{T1} status at 6-year follow-up.

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Glossary

AUC = area under the curve; **CIS** = clinically isolated syndrome; **EDSS** = Expanded Disability Status Scale; **FLAIR** = fluid-attenuated inversion recovery; **GD+** = gadolinium enhancing; **NaloMS** = Neurofilament and longterm outcome in MS; **NEDA** = no evidence of disease activity; **ROC** = receiver operating characteristic; **RRMS** = relapsing-remitting MS; **sNfL** = serum neurofilament light chain; **SPMS** = secondary progressive MS.

Both immune-mediated focal inflammatory and diffuse chronic neurodegenerative processes characterize MS pathology.[1,2] Especially accumulating neuronal cell death is crucial for individual patients' outcome, underlining the importance of early identification of patients at risk for future disease progression. Serum neurofilament light chains (sNfLs) are an emerging marker in MS[3-6] and other chronic[7] and acute[8,9] neurologic diseases. Although sNfL levels are clearly associated with acute inflammatory activity, insights into the value for dissection of focal vs diffuse axonal damage are limited. Specifically, at the cross-sectional level, sNfL is strongly associated with clinical relapses,[10-12] number of T2 hyperintense[3,4,12] or gadolinium-enhancing (Gd+) lesions,[3,4,12] and T2 lesion volume.[3,4] Moreover, there is some additional predictive capacity of sNfL for inflammatory activity such as clinical relapses,[12,13] T2 lesion volume change,[3,14] development of new T2 hyperintense lesions,[15] and increase in the number of Gd+ lesions,[16] mostly within the next 1–2 years. In contrast, data on association with measures of disability progression such as the Expanded Disability Status Scale (EDSS) increase are conflicting. Some studies observed a weak association with EDSS,[12,17,18] whereas others report no association with disability status.[3,19,20] Another approach to monitor the progressive component of the disease relies on MRI parameters depicting neurodegeneration such as gray matter fraction and percentage brain volume loss. sNfL is moderately associated with current normalized brain volume[13,21] and future percentage change in brain volume.[3,13,15,22] Nonetheless, these MRI parameters lack the broad availability of high-resolution scanners and standardization necessary to guarantee widespread applicability and are therefore not routinely used outside of specialized centers.

Composite scores to define outcome measures capturing various aspects of the disease (e.g., no evidence of disease activity [NEDA]) become increasingly appealing in guiding treatment decisions in patients under modern immunomodulatory therapies.[23] NEDA-3 status, the most commonly used approach, is defined as an absence of clinical relapses, EDSS progression, and new T2 hyperintense/Gd+ lesions over the last year.[24] More refined considerations have led the NEDA-4 concept, which additionally incorporates brain atrophy.[25] However, because of the pitfalls mentioned before, this concept is mostly reserved for clinical trial settings or specialized centers. Most notably, there is not yet a consensus on an MRI-derived atrophy threshold on an individual basis.[26,27] Other easily evaluable markers of irreversible tissue destruction such as T1-weighted hypointense lesions (the so-called black holes), reflecting areas of focal neuronal loss, might represent an alternative approach to assess irreversible axonal damage. Patients with black holes harbor an increased risk of disability accumulation,[28,29] and increased sNfL levels are associated with the number of T1 hypointense lesions[30-32] and increase in T1 lesion volume.[33,34] In the current study, we aimed to disentangle the relationship between sNfL and development of new T1 hypointense lesions as a marker for focal axonal damage and to assess incorporation of T1 hypointense lesions into the NEDA-3 status.

Methods

NaloMS Cohort

We prospectively included patients with relapsing-remitting MS (RRMS) or clinically isolated syndrome (CIS), retrospectively classified according to the 2017 McDonald criteria,[35] between October 2010 and July 2016 in the Neurofilament and longterm outcome in MS (NaloMS)[36] cohort at the Department of Neurology MS outpatient clinic at the University Medical Center Mainz (Germany). For the current analysis, we selected patients with available MRI at y0 and follow-up (y6), within 12 months of serum sampling for sNfL measurement (n = 153 of 221). In addition, EDSS assessment had to be performed within ± 3 months of serum sampling. Patients with progressive disease forms at screening were excluded. At study entry and y6, venous blood was collected for determination of sNfL, and the following variables were recorded: EDSS score, immunomodulatory therapy, medical history, and MRI parameters (T2 hyperintense lesions, Gd+ lesions, and new persistent T1 hypointense lesions). Between study entry and y6, the patients underwent regular visits within our outpatient clinic as deemed necessary by the treating physician.

Standard Protocol Approvals, Registrations, and Patient Consents

The study protocol was approved by the Ethics Committee of the Aerztekammer Rheinland Pfalz and conducted in accordance with the Declaration of Helsinki; all patients gave written informed consent. We followed TRIPOD guidelines for prediction model development and reporting (for details, see eTable 1, links.lww.com/NXI/A769).

Clinical and MRI End Points

We aim to assess the predictive ability of sNfL with respect to no evidence of disease activity status, including development of new persistent T1 hypointense lesions (NEDA-3^{T1}). NEDA-3^{T1} status was defined as the absence of clinical relapse, MRI activity (T2 hyperintense lesions, Gd+), EDSS progression, and the absence of new persistent T1 hypointense lesions (T1) 1 year before follow-up. EDSS worsening within the 12 months

before y6 was defined as an EDSS increase of at least 1.5 points for patients starting at EDSS 0, at least 1.0 EDSS points for patients with an initial EDSS between 1 and 4.5, and at least 0.5 points for patients starting with an EDSS ≥5.[37,38] EDSS worsening had to be confirmed 12 weeks after y6 assessment. In addition, the last available MRI at y6 (for our purposes, at y6 means within ± 12 months) was assessed with regard to the presence of Gd+ lesions, the occurrence of new or enlarging T2 hyperintense lesions, and the presence of new persistent T1 hypointense lesions. All new T1 hypointense lesions had to be confirmed on the next sequential MRI scan (persistent).

sNfL Measurements

For details on sNfL measurements, we refer to previously published protocols.[3,8] In short, we spun the collected venous blood at 1,300*g* at room temperature for 15 minutes, a maximum 2 hours after sampling, and stored it locally at −80°C. sNfL levels were determined in duplicates by SiMoA HD-1 (Quanterix, Billerica, MA) using the Neurofilament-Light Advantage Kit (Quanterix) according to the manufacturer's instructions. Intra-assay coefficient of variation (CVs) above 20% were measured twice. The mean intra-assay CV was 6.2%. Two low and high controls, consisting of recombinant human sNfL antigen, were included in each sample run to monitor plate-to-plate variation (low: mean 8.8 pg/mL, interassay CV 13.6%; high: mean 192.7 pg/mL, interassay CV 13.3%). sNfL measurements were performed in a blinded fashion without information about clinical data.

Quantification of White Matter Lesion and Gray Matter Volume

The quantification of T2 lesion volume was performed as previously described.[39] In brief, the volumes were determined using the cross-sectional pipeline of the lesion segmentation toolbox[40] of the Statistical Parametric Mapping software. Three-dimensional fluid-attenuated inversion recovery images were coregistered to 3D T1-weighted images and bias corrected. After partial volume estimation, lesion segmentation was performed with 20 different initial threshold values for the lesion growth algorithm.[40] We evaluated the optimal threshold for each patient (κ value, dependent on image contrast) and calculated average values. Then, we applied a uniform κ value of 0.1 in all patients to automatically estimate the lesion volume and filling of 3D T1-weighted images. Subsequently, the filled 3D T1-weighted images and the native 3D T1-weighted images were segmented and then normalized to Montreal Neurological Institute space. To increase reliability, we inspected the quality of the segmentations visually.

Statistics

We used RStudio version 1.2.5033 (RStudio Inc) with R version 3.6.3 (R Foundation for Statistical Computing, Austria), SPSS version 24 (IBM Corporation, Armonk, NY), and MedCalc version 19.2.1 (MedCalc Software Ltd, Ostend, Belgium) for statistical analysis. Normal distribution was evaluated by Kolmogorov-Smirnov and Shapiro-Wilk tests. Non-normally distributed variables underwent Mann-Whitney or Kruskal-Wallis tests with Bonferroni correction for multiple testing, where appropriate. Conversely, normally distributed data were analyzed by taking advantage of the Student *t* test or 1-way/2-way analysis of variance (ANOVA). Within-subject factors over time were analyzed by mixed linear models or 2-way mixed ANOVA after log transformation of sNfL values to normalize the distributions, following 1-way ANOVA with the Tukey post hoc test for multiple comparisons. Bivariate Pearson correlation was calculated for the association between sNfL values and clinical/MRI parameters at y0 and y6 and is displayed in a correlogram using the R packages Hmisc[41] and corrplot.[42] Linear regression analysis was used to compare the variance of the dependent variable (sNfL) by covariates in the model. Differences in proportions were analyzed by the χ^2 test. Predictive capacity of sNfL for NEDA-3^{T1} status was assessed by sensitivity, specificity, positive predictive value, and negative predictive value. Multiple binominal regression analyses were conducted including variables with $p < 0.05$ at the univariate level and additional clinically relevant variables. Receiver operating characteristic (ROC) curves were drawn and calculated for estimation of prognostic information for y0 sNfL on experiencing NEDA-3^{T1} status at y6. Area under the curves (AUCs) derived from ROC analysis and Kaplan-Meier survival analysis were compared using the MedCalc for Windows software. All statistical analysis was performed using the original data without modifications. *p* Values <0.05 were considered statistically significant.

Data Availability

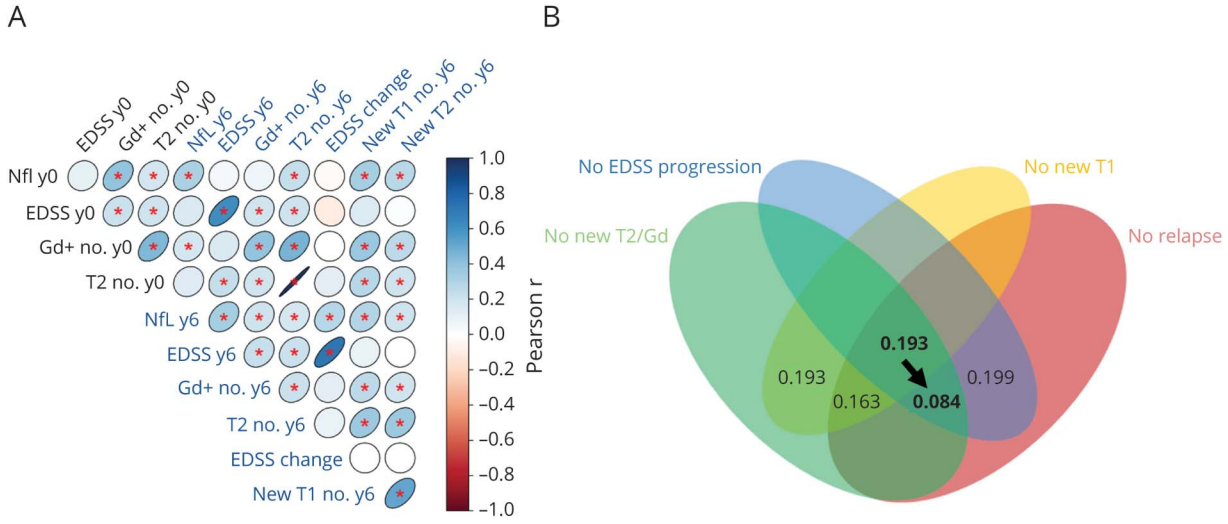
Data not provided in the article because of space limitations may be shared (anonymized) by the corresponding author on the reasonable request of any qualified investigator for purposes of replicating procedures and results.

Results

sNfL Levels Predict the Future Development of New Persistent T1 Hypointense Lesions

The NaloMS cohort is a prospective observational cohort designed to assess the long-term relevance of sNfL as a biomarker in patients with MS. MRI data obtained within ± 12 months of sNfL assessment were available for 153/221 patients with CIS and RRMS with a median follow-up of 5.7 years (range 4.3–7.3 years). First, we built a univariate correlation matrix of different markers of disease activity to unravel associations between sNfL and clinical and MRI parameters (Figure 1A). sNfL levels at study entry correlated with signs of inflammatory activity at baseline such as number of Gd+ lesions ($r = 0.391$, $p < 0.001$), T2 hyperintense lesion number ($r = 0.185$, $p = 0.022$), and T2 hyperintense lesion number at y6 ($r = 0.232$, $p = 0.004$). Moreover, development of new T2 hyperintense lesions after 6 years correlated with y0 sNfL values ($r = 0.280$, $p < 0.001$). Whereas a significant correlation between sNfL and y0 EDSS ($r = 0.104$, $p = 0.199$) and y6 EDSS ($r = 0.053$, $p = 0.5131$) and EDSS change over time ($r = -0.024$, $p = 0.769$) was lacking, we observed a correlation between y0 sNfL and development of new T1 hypointense lesions ($r = 0.336$, $p < 0.001$). This observation shows that although initially high y0 sNfL values reflect current inflammatory activity, they also have a predictive value for the future development of new T1 hypointense and T2 hyperintense lesions.

Figure 1 Association of sNfL With Severe Focal Neuronal Damage Reflected by Persistent T1 Hypointense Lesions in Patients With MS



(A) Correlation matrix showing the correlation coefficients of the associations between log-transformed y0 serum neurofilament light chain (sNfL) and clinical and MRI parameters at baseline and 6-year follow-up. Ellipses are used as glyphs for correlations. With increasing absolute value of *r*, the minor axis of the ellipse decreases, whereas a correlation with *r* close to 0 approximates more and more to a circle. The glyphs of the negative correlation coefficients are plotted as a reflection of their positive counterparts. Because it is difficult to distinguish small correlation coefficients from the ellipse shape, Pearson *r* is additionally translated into a color pallet ranging from deep red ($r = -1$) to dark blue ($r = +1$). Correlations with $p < 0.05$ were considered statistically significant, and the corresponding ellipse is marked with *. (B) The Venn diagram shows the corrected R^2 values of different linear regression models, all with y0 sNfL as the dependent variable, to determine which combination of NEDA criteria best explains the variance of y0 sNfL. For example, the model in the middle considered the combination of all possible criteria, namely, absence of clinical relapse, absence of new T2 hyperintense lesion/GD+ lesions, absence of EDSS progression, and the absence of new persistent T1 hypointense lesions on follow-up MRI. This is visually indicated by the area being proportionally included in all 4 ellipses. The corrected R^2 value of this specific model was 0.193. The black arrow illustrates the drop in R^2 from 0.193 to 0.084 only due to the exclusion of absence of new persistent T1 hypointense lesions from this model. Accordingly, the second model can only explain less of the y0 sNfL variance. EDSS = Expanded Disability Status Scale; NfL = neurofilament light chain; NEDA = no evidence of disease activity; EDSS change = difference between y0 EDSS and y6 EDSS; Gd+ no. = number of GD+ MRI lesions; sNfL = serum neurofilament light chain; T2 no. = number of hyperintense lesions in T2-weighted MRI sequences; new T1 no. = number of new persistent hypointense lesions in T1-weighted MRI sequences at y6 compared with y0; y0 = year 0/baseline; y6 = year 6/follow-up.

Next, we performed multiple linear regression analysis with sNfL as the dependent variable and different combinations of activity criteria and age as independent variables to determine whether y0 sNfL predicts future inflammatory (relapses and new or enlarging T2 hyperintense or Gd+ lesions) or progressive (EDSS worsening and new T1 hypointense lesions) processes (Figure 1B). R^2—the proportion of variance in y0 sNfL levels that can be explained by the independent variables—dropped from 0.193 after inclusion of 4 NEDA criteria (relapse, subclinical MRI activity [T2 hyperintense/Gd+], EDSS progression, and new T1 hypointense lesions) to 0.084 after removal of the new T1 hypointense lesions criteria. This emphasizes that high y0 sNfL levels are a strong predictor for the future development of new hypointense T1 lesions at y6 (Figure 1B).

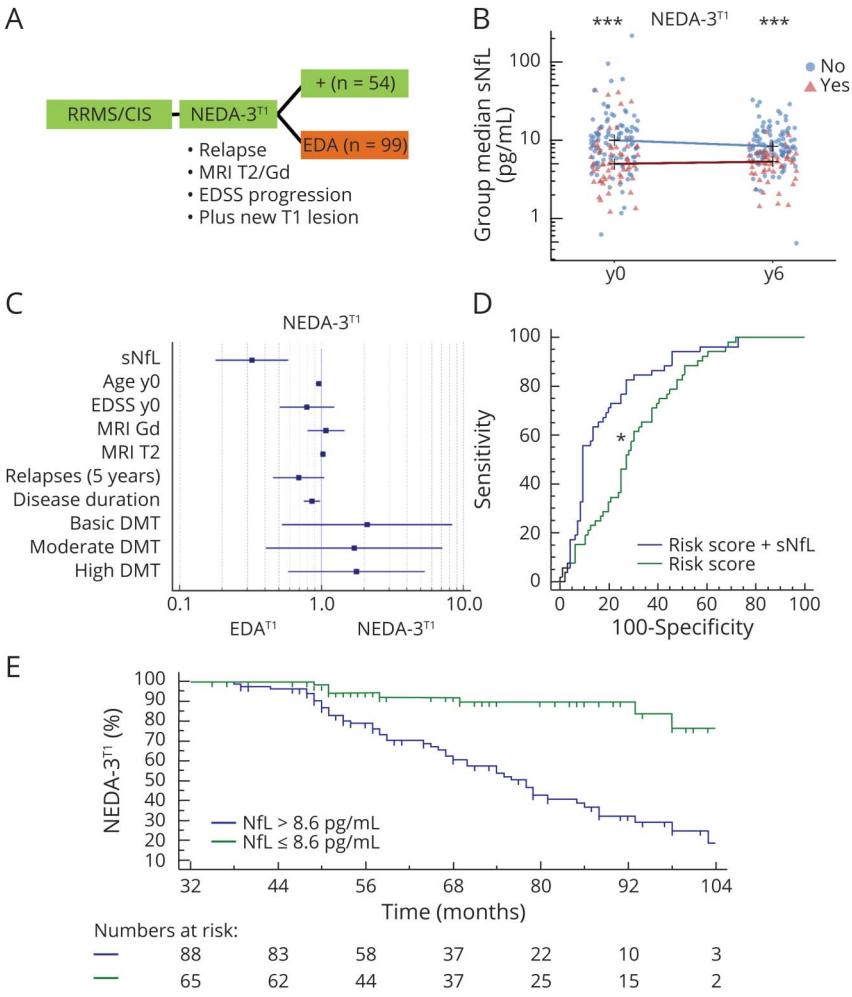
Longitudinal sNfL Kinetics Identify Patients Reaching NEDA-3^{T1} Status

NEDA-3 status is one of the most commonly used composite scores, both as an outcome measure in treatment trials and to guide treatment decisions in the clinic. Patients with NEDA-3 must not have clinical relapse, EDSS progression, or new T2/Gd+ lesions on MRI scans in the past 12 months.[23,24] We next evaluated the utility of adding new T1 hypointense lesions, a potential readily accessible marker of neurodegeneration, to the established NEDA-3 concept (the so-called NEDA-3^{T1}; Figure 2A). Patients with evidence of disease activity and development of new T1 hypointense lesions (EDA^{T1}) had increased sNfL levels at y0 (10.2 pg/mL [5.8–14.8]) and y6 (8.2 pg/mL [6.3–12.3]) compared with patients with NEDA-3^{T1} status at y0 (4.9 pg/mL [3.3–8.1], $p < 0.001$ compared with EDA^{T1}) and y6 (5.4 pg/mL [4.1–6.8], $p < 0.001$ compared with EDA^{T1}). Notably, patients fulfilling NEDA-3^{T1} status at y6 showed unchanged sNfL levels (y0, 4.9 pg/mL (3.3–8.1); y6, 5.4 pg/mL (4.1–6.8); Wilcoxon test, $p = 0.955$), whereas patients with EDA^{T1}, probably in terms of regression to the mean,[4] showed a trend toward a decrease in sNfL levels from 10.2 pg/mL (y0, 5.8–14.8) to 8.2 pg/mL (y6, 6.3–12.3; Wilcoxon test, $p = 0.095$; Figure 2B). Together with the aforementioned strong correlation of sNfL with inflammatory activity such as gadolinium enhancement and T2 hyperintense lesions at y0, this is an important point for inflammation-initiated neuronal loss associated with disability progression (EDSS worsening) and focal neuronal damage (new T1 hypointense lesions).

Y0 sNfL Levels Predict NEDA-3^{T1} Status After 6-Year Follow-up

Next, we systematically assessed factors associated with NEDA-3^{T1} status at y6 (Table 1). Overall, 54 of 153 patients

Figure 2 Y0 sNfL Levels Identify Patients With Future Focal Neuronal Damage and Predict NEDA-3^{T1} Status



(A) Trial profile: 153 patients with RRMS and clinically isolated syndrome (CIS) were prospectively followed within the NaloMS cohort. Of these, 54 fulfilled the NEDA-3^{T1} criteria, whereas 99 patients showed clinical relapse, new T2 hyperintense/Gd+ lesions on MRI, EDSS progression, or new persistent T1 hypointense lesions on y6 MRI, defined as EDA^{T1}. (B) y6 sNfL showed a trend toward a drop from initially high y0 sNfL values to lower values in patients with evidence of disease activity (EDA^{T1}) according to the NEDA-3^{T1} criteria (y0 sNfL 10.2 pg/mL (5.8–14.8) to y6 sNfL 8.2 (6.3–12.3); Wilcoxon test, $p = 0.095$). Stable patients reaching NEDA-3^{T1} criteria, showed unchanged y6 sNfL values (y0 sNfL 4.9 pg/mL (3.3–8.1) y6 sNfL 5.4 pg/mL (4.1–6.8); Wilcoxon test, $p = 0.955$). EDA^{T1} patients had higher sNfL levels at both y0 and y6 compared with NEDA-3^{T1} patients (y0, $p < 0.001$; y6, $p = 0.001$). (C) Binary logistic regression model with no evidence of disease activity (NEDA-3^{T1}) as the dependent variable revealed reduced y0 sNfL (OR 0.883, 95% CI 0.819–0.952, $p < 0.001$) and reduced disease duration (OR 0.851, 95% CI 0.755, 0.974) as predictors for NEDA-3^{T1} status at y6. In contrast, age (OR 0.956, 95% CI 0.914–1.000, $p = 0.050$), y0 EDSS (OR 0.810, 95% CI 0.519–1.264 $p = 0.353$), number of Gd+ lesions at y0 (OR 1.148, 95% CI 0.851–1.549, $p = 0.366$), number of T2 hyperintense lesions at y0 (OR 1.024, 95% CI 0.982–1.068, $p = 0.270$), relapses within the last 5 years (OR 0.670, 95% CI 0.441–1.017, $p = 0.670$), basic disease-modifying therapy (DMT) (OR 1.935, 95% CI 0.491–7.627, $p = 0.345$), moderate DMT (OR 1.591, 95% CI 0.382–6.633, $p = 0.524$), and high DMT (OR 1.763, 95% CI 0.582–5.341, $p = 0.316$) were not predictors of NEDA-3^{T1} status at y6. (D) By adding sNfL to a risk score (incorporating age, Gd enhancement at baseline, T2 hyperintense lesions at baseline, y0 EDSS, relapses within the last 5 years, and disease duration), the AUC for prediction of NEDA-3^{T1} status could be significantly improved from 0.704 (95% CI 0.627–0.776, $p < 0.009$) for the risk score alone (green line) to 0.820 (95% CI 0.749–0.879, $p < 0.001$) after additional incorporation of y0 sNfL (blue line, p for difference between the AUC of the risk score ± y0 sNfL, $p < 0.001$). (E) Survival analysis with regard to time fulfilling NEDA-3^{T1} status in patients with sNfL >8.6 pg/mL (blue line) compared with patients with sNfL ≤8.6 pg/mL (green line). Patients with sNfL ≤8.6 pg/mL were more likely to stay on NEDA-3^{T1} at y6 (HR 0.244, 95% CI 0.142–0.419 vs HR 4.101, 95% CI 2.387–7.048, log-rank test $p < 0.001$). The median time until EDA was 78 months (95% CI 68–86) for patients with sNfL >8.6 pg/mL compared with 93 months (95% CI 81–103) for patients with lower sNfL values. *$p < 0.05$, ***$p \leq 0.001$. EDSS = Expanded Disability Status Scale; EDSS change = difference between y0 EDSS and y6 EDSS; Gd+ no. = number of gadolinium-enhancing MRI lesions; NfL = neurofilament light chain; sNfL = serum neurofilament light chain; T2 no. = number of hyperintense lesions in T2-weighted MRI sequences; new T1 no. = number of new persistent hypointense lesions in T1-weighted MRI sequences at y0 compared with y6; y0 = year 0/baseline; y6 = year 6/follow-up.

(35.3%) showed NEDA-3^{T1} status at y6. Patients with NEDA-3^{T1} had a lower age, fewer Gd+ lesions, and a lower y0 EDSS compared with EDA^{T1} patients. Moreover, patients reaching NEDA-3^{T1} at y6 had a lower y6 EDSS, a lower disease duration, fewer new T1 hypointense lesions, fewer Gd+ lesions, and lower clinical relapse activity compared with EDA^{T1} patients. Twenty-one patients in the EDA^{T1} group (21.2%) showed transition to secondary progressive MS (SPMS), whereas in the NEDA-3^{T1} group, all patients were still classified as patients with RRMS ($p < 0.001$; for details, see Table 1).

These factors, unbalanced at the univariate level, and additional known risk factors for inflammatory activity and disability progression were incorporated into a logistic regression model to unravel the effects of potential confounders on the ability of sNfL to predict NEDA-3^{T1} status at y6. After multivariable correction, only decreased sNfL levels (OR 0.883,

Table 1 Factors Associated With NEDA-3^{T1} Status at 6-Year Follow-up

	EDA	NEDA-3^{T1}	*p* Value
N	99	54	
Age at diagnosis (y)	32.3 (25.5–41.7)	31.0 (24.3–40.8)	0.577
y0 sNfL (pg/mL)	10.2 (5.8–14.8)	4.9 (3.3–8.1)	**<0.001**
y0 age (y)	37.0 (27.7–45.1)	31.4 (24.9–41.4)	0.054
y6 sNfL (pg/mL)	8.2 (6.3–12.3)	5.4 (4.1–6.8)	**0.001**
y6 age (y)	43.5 (33.7–50.9)	38.6 (30.4–46.6)	**0.029**
Female	70 (70.7)	39 (72.2)	0.843
Disease course at baseline			
RRMS	99	54	
Smoking	27 (27.3)	9 (16.7)	0.141
Pack-years	16.61 (±13.48)	15.2 (±11.07)	0.769
OCB	87 (87.9)	49 (90.7)	0.789
EDSS			
y0	1.0 (1–2.5) 1.49 (±1.31)	1.0 (0–1.5) 0.94 (±0.91)	**0.019** **0.007**
y6	2.0 (1–3.5) 2.51 (±1.88)	1.0 (0–1.5) 0.95 (±0.82)	**0.000** **<0.001**
Disease duration (y)	8.1 (6.2–12.9)	6.9 (6.0–8.1)	**0.015**
y0 MRI			
Gd enhancement	30 (30.3)	11 (20.4)	0.185
Gd lesion number	1.09 (±2.91)	0.39 (±0.88)	**0.028**
T2 lesion number	16.97 (±12.95)	14.22 (±10.3)	0.152
T2 lesion volume (mL)	2.24 (1.1–6.3)	1.44 (0.7–4.6)	0.117
y6 MRI			
Gd enhancement	6 (6.1)	0 (0)	0.090
Gd lesion number	0.20 (±1.01)	0 (0)	**0.049**
New T1 lesion	35 (35.4)	0 (0)	**<0.001**
New T1 lesion number	0.84 (±1.66)	0 (0)	**<0.001**
New/enlarging T2 lesions number	1.48 (±2.42)	0 (0)	<0.001
T2 lesion volume (mL)	3.21 (1.4–10.9)	1.81 (0.8–5.1)	**0.013**
DMT			0.113
No DMT	18 (18.2)	9 (16.7)	
Basic	15 (15.2)	10 (18.5)	
Moderate	33 (33.3)	26 (48.1)	
High	33 (33.3)	9 (16.7)	
y6 DMT	80 (81.6)	45 (83.3)	1.000
Disease course—y6			**<0.001**
RRMS	78/78.8)	54 (100.0)	
SPMS	21 (21.2)	0 (0)	
NEDA status—y6			
Clinical relapse	22 (22.2)	0 (0)	**<0.001**
MRI activity	49 (49.5)	0 (0)	**<0.001**
EDSS progression	58 (58.6)	0 (0)	**<0.001**
Relapse activity—y6			
No of relapses last 5 years	1.02 (±0.24)	0 (0)	**0.017**
No of relapses last year	0.24 (±0.58)	0 (0)	**<0.001**

Abbreviations: basic DMT = interferons and glatiramer acetate; DMT = disease-modifying therapy; EDSS = Expanded Disability Status Scale; Gd = gadolinium; high DMT = natalizumab, rituximab, fingolimod, ocrelizumab, daclizumab, alemtuzumab, and mitoxantrone; NEDA = no evidence of disease activity; OCB = oligoclonal band; moderate DMT = teriflunomide and dimethyl fumarate; SPMS = secondary progressive MS; sNfL = serum neurofilament light chain; y0 = study entry; y6 = follow-up; RRMS = relapsing-remitting MS.
Data are presented as number (percentages), median (interquartile range 25th-75th percentile), or mean (±SD), as appropriate.
Statistically significant differences with *p* values < 0.05 are shown in bold.

95% CI 0.819–0.952, $p = 0.001$) and shorter disease duration (OR 0.851, 95% CI 0.744–0.974, $p = 0.019$) were associated with NEDA-3^{T1} status at y6 (Figure 2C). The factors differing between NEDA-3^{T1} and EDA^{T1} were incorporated into a composite risk score including age, y0 Gd enhancement on MRI, y0 number of T2 hyperintense lesions on MRI, y0 EDSS, relapses within the last 5 years, and disease duration. Y0 sNfL exhibited an ROC-AUC for prediction of NEDA-3^{T1} status of 0.737 (95% CI 0.658–0.806, $p < 0.001$). After adding sNfL to the aforementioned cumulative risk score, the ROC-AUC could be significantly improved from 0.704 (95% CI 0.623–0.776, $p < 0.001$) for the risk score alone to 0.820 (95% CI 0.749–0.879, $p < 0.001$) after additional consideration of y0 sNfL (p for difference between the AUC of the risk score ± y0 sNfL, $p < 0.001$, Figure 2D, eTable 2, links.lww.com/NXI/A769).

To generate precise information that can be applied in the clinical setting, we derived the sNfL cutoff for prediction of NEDA-3^{T1} status at y6 from the Youden index of the sNfL ROC-AUC, which was found to be 8.6 pg/mL. For prediction of NEDA-3^{T1} status, this cutoff exhibits an 85.2% sensitivity and 58.6% specificity. Table 2 displays detailed diagnostic test effectiveness of sNfL for prediction of NEDA-3^{T1} status at y6.

This sNfL cutoff value of 8.6 pg/mL was next assessed by Kaplan-Meier analysis using an independent statistical approach. To this end, the patient cohort was grouped into

Table 2 Diagnostic Test Effectiveness of y0 sNfL for Prediction of NEDA-3^{T1} Status

NEDA-3^{T1} status						
Risk score	**AUC 95% CI**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**NPV (95% CI**	**PPV (95% CI)**	**Accuracy**
sNfL y0	0.737 (0.658–0.806)	85.2 (72.9–93.4)	59.6 (49.4–69.3)	88.1 (79.2–93.5)	53.5 (46.9–60.0)	68.6
Risk score (age + MRI-Gd + MRI-T2 + EDSS + relapses (y6) + disease duration	0.704 (0.627–0.776)	88.5 (76.6–95.6)	49.0 (38.6–59.4)	82.4 (71.2–89.8)	44.3 (39.1–49.7)	62.9
Plus sNfL y0	0.820 (0.749–0.879)	82.7 (69.7–91.8)	72.9 (62.9–81.5)	85.7 (77.1–91.5)	53.8 (46.6–60.9)	76.4

Abbreviations: AUC = area under the curve, derived from the receiver operating characteristic curve; EDSS, Expanded Disability Status Scale; Gd, gadolinium; NEDA-3, no evidence of disease activity–3 (clinical relapse, new or enlarging T2 hyperintense lesions, gadolinium-enhancing lesions, and EDSS progression); NEDA-3^{T1}, NEDA and progression (clinical relapse, new or enlarging T2 hyperintense lesions, gadolinium-enhancing lesions, EDSS progression, and new persistent T1 hypointense lesion/signs of brain atrophy on year 6 MRI); NPV = negative predictive value; PPV = positive predictive value; sNfL y0, serum neurofilament light chain at study entry.

patients with sNfL levels >8.6 pg/mL and patients with levels ≤8.6 pg/mL. Patients with sNfL above 8.6 pg/mL had a 70% risk increase for occurrence of disease activity (EDA^{T1}) at y6 compared with patients with lower sNfL (hazard ratio [HR] 1.698, 95% CI 1.127–2.557, log-rank test, $p = 0.011$). In line with this, the median time until EDA^{T1} was reduced from 93 months in patients with y0 sNfL ≤8.6 pg/mL (95% CI 81–103) to 78 months (95% CI 68–86) in patients with sNfL >8.6 pg/mL. Taken together, y0 sNfL >8.6 pg/mL is associated with a considerably shorter time until occurrence of EDA^{T1} (Figure 2E).

Discussion

Although an elevation of sNfL certainly reflects acute focal lesion development in patients with MS, its practical value for monitoring neurodegenerative processes and predicting long-term outcome of patients is still unclear. We show here that elevated y0 sNfL values are particularly associated with future irreversible focal neuronal damage, as measured by development of new persistent T1 hypointense lesions. These lesions reflect tissue destruction in the CNS and are linked to disability progression in the long-term.[43] Postmortem analyses revealed substantial axonal loss in persistent T1 hypointense lesions with a decrease in axonal density with increasing T1 hypointensity.[44] In contrast, lesions with histopathologic signs of remyelination decreased in T1 hypointensity over time.[45]

Our study emphasizes a link between increased sNfL values with current and future permanent focal axonal damage. This is specifically reflected by the fact that when current inflammatory activity reaches a certain threshold, it is able to cause irreversible tissue destruction associated with future development of black holes. This hypothesis is further underlined by both (1) neuropathologic studies demonstrating a close association between inflammation and neurodegeneration in MS[46] and (2) clinical data showing that an early therapeutic intervention with disease-modifying therapies reduced the risk of disability progression and conversion into SPMS years later.[47] In line with a previous single report in a cohort of 23 patients (which was thus limited by the low number of patients and a mixture of patients with progressive MS and RRMS) that demonstrated an association between sNfL values and future increased T1 lesion volume after hematopoietic stem cell transplantation,[33] our study adds significant evidence that sNfL is able to predict development of new persistent T1 hypointense lesions.

An important motivation for our study was that routine assessment of neurodegenerative processes by conventional clinical and MRI measures is still challenging.[24] Nonetheless, monitoring of the neurodegenerative component of MS is crucial to appropriately evaluate treatment response and enable treatment escalation. We here provide evidence that initially elevated sNfL levels influence the development of future focal neuronal loss, as assessed by persistent T1 hypointense lesions, which are linked with disability outcomes in MS.[29] We therefore propose to include the absence of new persistent T1 hypointense lesions, as a measure for progressive MS pathology, into the NEDA-3 concept (NEDA-3^{T1}) to equally weigh neurodegenerative and inflammatory disease components for guidance of treatment decision. Importantly, sNfL was able to predict NEDA-3^{T1} status at median 6-year follow-up, which remained significant after multivariate correction. In addition, we showed a superior predictive capacity for adding T1 hypointense lesions to a composite risk score; patients with sNfL levels equal to or below 8.6 pg/mL showed a 76% risk reduction for evidence of disease activity and development of new persistent T1 hypointense lesions at y6 compared with patients with sNfL values above 8.6 pg/mL. These findings expand previous reports showing a predictive capacity of sNfL for NEDA-3 status.[34,48] However, these reports are limited to measurements in CSF[48] and a follow-up of up to 4 years.[34] In patients under alemtuzumab treatment, 2 cohorts with limited numbers of patients, but high-frequency sNfL assessment, showed low sNfL concentrations at the individual level when NEDA-3 status was achieved; these levels were also significantly lower than in EDA patients when compared between groups.[49,50] Importantly, the authors report individual sNfL spikes in patients who still formally meet the NEDA-3 criteria. If these transient spikes do indeed reflect neuroaxonal damage, it is

most likely due to neurodegenerative processes, which are not currently captured in the primarily inflammation-dominated NEDA-3 criteria.[49] This observation further supports our proposed NEDA-3^{T1} concept to equally balance inflammatory and progressive components of MS pathology.

Nevertheless, our results should be interpreted with caution due to the relatively small cohort size, the lack of external validation of our predictive models, and the specific characteristics of our cohort, such as a relatively mild disease course (mean EDSS of 2 in the EDA^{T1} group at y6). The feasibility of implementing the NEDA-3^{T1} concept via inclusion of persistent T1 hypointense lesions and the value of sNfL for prediction of NEDA-3^{T1}status and the development of normative data for sNfL all need to be addressed in future studies to foster their way into clinical practice.

As a perspective, sNfL could also be useful as a biomarker for neuroprotection or remyelination. Various modern DMTs show beneficial effects on T1 hypointense lesions,[51] likely through anti-inflammatory effects. However, therapeutic approaches that act on the proliferation and differentiation of oligodendrocyte progenitors to promote remyelination are still urgently needed.[52] Among other agents, brain-derived neurotrophic factor has been discussed for years, as it shows various beneficial effects in different mouse models, but is associated with certain limitations.[53] Recently, it was reported that regulatory T cells (Tregs) can promote oligodendrocyte differentiation in mice in addition to their immune surveillance function.[54] Treatment with autologous Tregs in RRMS is possible and, as far as can be deduced from a small number of participants, appears to be reasonably safe.[55] Agents that indirectly increase the number and function of Tregs, such as propionic acid,[56] are also already being tested in clinical practice.

Taken together, we here demonstrate that sNfL is associated with severe focal neuronal damage and improves the monitoring of progressive MS pathology. In addition, sNfL is able to predict NEDA-3^{T1} status and thereby identifies patients with a high probability of inflammatory-driven secondary focal neuronal injury, which is associated with disability progression in the long run.

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Disclosure

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Appendix *(continued)*

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