Original Research Paper

Serum neurofilament light levels correlate with change of olfactory function in multiple sclerosis

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Abstract

Background: Serum neurofilament light chain levels (sNfL) and impairment of olfactory function emerge as biomarkers in multiple sclerosis (MS). However, the relation between sNfL and olfactory function in MS has not been investigated yet.

Objective: We aimed to determine whether sNfL levels correlate with olfactory function in relapsing–remitting (RR) MS.

Methods: We annually measured sNfL and olfactory function (Sniffin' Sticks test: Threshold (T) and combined discrimination-identification (DI) score) in 80 RRMS patients and compared sNfL to T and DI scores.

Results: T scores significantly correlated with sNfL levels at simultaneous measurement (–1.5 points, 95% CI: –2.6–0.5 per 10 pg/ml sNfL increase; $p < 0.001$ per 10 pg/ml sNfL increase), but not at temporally distant measurement. Patients with ≥ 2 sNfL measures above the 75th percentile displayed significantly larger DI decrease (median 3.0 points, IQR 2.0–4.5) compared to patients with no or only one sNfL measure above the 75th percentile (0.0, IQR –0.5–0.5, $p < 0.001$ and 1.0, IQR 0.0–3.30, $p = 0.008$, respectively). 13–18% of the variance in T and 22% in DI decrease could be predicted from sNfL levels.

Conclusions: sNfL correlates with different qualities of olfactory function in patients with RRMS further strengthening the value of olfactory function as a biomarker of inflammation and axonal damage in MS.

Introduction

Predicting short- and long-term disease course is highly challenging in multiple sclerosis (MS). The increasing number of disease-modifying treatment (DMT) options available necessitates the development of reliable biomarkers for that purpose.

Impairment of olfactory function is a characteristic feature in MS with different modalities reflecting different aspects of MS pathology.[1–8] The capacity to correctly identify odours (identification) and to discriminate them (discrimination) is affected in MS patients displaying both clinical and paraclinical signs of MS-associated neurodegeneration such as physical or cognitive disability progression, brain atrophy and reduced retinal thickness.[5,6,9] On the other hand, olfactory threshold is transiently impaired in active MS and during acute relapse resolving in phases of clinical stability, which indicates an association with short-term inflammatory activity.[5,6,10]

Neurofilament light chain (NfL) is a major component of the neuronal cytoskeleton and general marker of axonal injury.[11] Measured in the CSF, NfL levels are associated with the occurrence of relapses, neurological disability, MRI lesions and treatment status in MS.[12,13] The ultra-sensitive single molecule array (Simoa) technology enables reliable quantification of NfL in serum (sNfL) with

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concentrations in serum and CSF highly correlated.[14] sNfL levels have been shown to increase after the occurrence of clinical relapses, decrease after DMT initiation, to be associated with various MRI parameters (T2 lesion load, occurrence of new/enlarging T2 lesions or contrast enhancing lesions, brain and spinal cord volume loss) and to predict short-term disease activity.[12,13,15,16] Thus, sNfL is increasingly propagated as a suitable biomarker of axonal injury in MS.

However, the relation between sNfL and olfactory function in MS has not been investigated so far. In the present study, therefore, we aimed to determine whether sNfL levels correlate with different modalities of olfactory function in a cohort of relapsing–remitting (RR) MS patients.

Methods

For the present study, we identified patients from a prospective, observational study on olfactory function in RRMS at the MS Clinic of the Department of Neurology at the Medical University Innsbruck with available serum samples at baseline and at least two annual follow ups.[5,6] RRMS patients were diagnosed according to the 2010 McDonald criteria and aged between 18 and 65 years.

Clinical study visits were conducted at baseline (Y0) and after one (Y1), two (Y2) and three years (Y3) of follow up. Demographic data, neurological and treatment history including DMT and occurrence and date of relapses was obtained from each participant at every visit. A relapse was defined as patient-reported symptoms or objectively confirmed neurological signs typical of an acute CNS inflammatory demyelinating event with duration of at least 24 hours in the absence of fever or infection and separated from the last relapse by at least 30 days.[17] Expanded disability status scale (EDSS) score was obtained at every visit.[18] If a relapse had occurred within six months before the scheduled visit, EDSS was only considered when confirmed after six months. EDSS progression was defined as a confirmed EDSS increase of 0.5 or more sustained for at least 12 months as compared to baseline.

DMT status was classified at each visit as following: (1) 'no DMT' defined as patients receiving no DMT, (2) 'moderately effective DMT' defined as patients receiving either interferon beta preparations, glatirameracetate, dimethylfumarate or teriflunomide, or (3) 'highly effective DMT' defined as patients receiving either natalizumab, fingolimod or alemtuzumab. Additionally, we classified at year 1, 2 and 3: (1) 'no switch' defined as patients remaining in the same DMT group as at the previous visit or (2) 'initiation/escalation' defined as patients switching from no DMT to moderate effective DMT or from moderate effective DMT to high effective DMT since the previous visit.

Olfactory function assessment

Olfactory function was assessed at each visit using the extended version of the Sniffin' Sticks test (Burghart Medizintechnik, Wedel, Germany) according to the manufacturer's instruction including change of testing sticks every six months.[19] The normative values are based on data from 3000 healthy subjects.[20] The Sniffin' Sticks is a three stages test (threshold, discrimination and identification) based on pen-like odour-dispensing devices. The maximum score at each stage is 16 points and reflects optimal olfactory function. Lower scores are associated with impaired ability to identify and discriminate odours and an increased threshold for odour perception. Discrimination and identification were summed in a composite score (DI score).[5,6,9] Based on our previous studies, DI worsening was defined as a decrease in DI score ≥ 2 points sustained for at least 12 months as compared to baseline.[5,6]

At every visit, olfactory testing was postponed for four weeks, if the patient had a relapse or received corticosteroids within four weeks or if upper respiratory tract infections were present at the time of assessment.

sNfL assessment

Peripheral blood (8 ml) was taken by venipuncture at the day of the study visits Y0, Y1, Y2 and Y3. Serum was then immediately stored at –80°C according to international consensus guidelines.[21] We measured sNfL concentrations on a Quanterix SR-X[TM] analyser by a commercially available Simoa[TM] NF-light® Advantage Kit according to the manufactures' instructions. For sNfL measures we included additional 30 serum samples from age-matched healthy controls (defined as patients without any neurological disease and without a history of head trauma within three months prior to serum sampling). Intra-assay variability and inter-assay variability of the assay were 5.9%. The analytical sensitivity was 0.34 pg/ml. All samples produced signals above the analytic sensitivity of the assay.

The investigators performing the sNfL testing were blinded to clinical and olfaction parameters and the investigators assessing clinical parameters and olfaction parameters were blinded to sNfL results.

Ethics

The study was approved by the ethics committee of the Medical University Innsbruck (ethical approval number: AM3743-281/4.3) and all participants gave written informed consent before inclusion into the study.

Statistics

Statistical analysis was performed using SPSS 25.0 (SPSS Inc, Chicago, IL, USA). Categorical variables were expressed in frequencies and percentages, continuous variables were tested for normal distribution by the Kolmogorov–Smirnov test and displayed as mean and standard deviation (SD) or median and inter-quartile range (IQR) as appropriate.

Untransformed sNfL levels were used in all analyses. Several subjects were missing sNfL measurements at some timepoints, and these subjects were removed from analyses related to that specific timepoint. To assess the effect of missing sNfL values, we performed sensitivity analyses with multiple imputation using the missing not at random (MNAR) approach.[22] In some analyses, sNfL values were dichotomized into levels above or below the 75th percentile.

Univariate comparisons were done by Chi-square-test/Fisher's exact test, Mann–Whitney U test, Kruskal–Wallis test or independent t-test (with Welch's correction in case of unequal standard deviations between the groups) as appropriate. Repeated measurements were analysed by ANOVA or Wilcoxon-signed-rank test as appropriate. Univariate correlations were calculated by Pearson or Spearman test as appropriate.

To assess the potentially time-dependent association between sNfL and olfactory function, we performed multiple linear regression models adjusted for sex, age and disease duration at Y0 regarding threshold and DI scores at each time of assessment including each individual sNfL measurement during the observation period. In order to quantify the additional variance explained by adding sNfL levels to the multiple regression models, we reported the R^2 from full models and the change of R^2 in the absence of sNfL. A p-value < 0.05 was considered statistically significant. We used Bonferroni correction to correct for multiple testing.

Results

Of 151 patients enrolled in the original OCT-OLF-MS study, we finally included 80 RRMS patients in the present study. Ten patients were lost to follow up (eight before the first follow up visit; two before the second follow up) and were therefore not eligible for statistical analysis, 61 patients had fewer than two serum samples for sNfL measurement available. Serum was available for sNfL measurement in 80/80 patients at Y0, in 72/80 at Y1, in 73/80 at Y2 and in 75/80 at Y3. Sensitivity analyses regarding missing sNfL values did not show a significant effect of missing sNfL values on our results.

Characteristics of the final study cohort are given in Table 1. At Y0, 77.5% of patients were treated and the proportion of treated patients increased to 91.2, 95.0 and 97.5% at Y1, Y2 and Y3, respectively. Also, the proportion of patients treated with highly effective DMTs (natalizumab, fingolimod and alemtuzumab) increased from 33.8% at baseline to 48.8, 51.2 and 53.8 at Y1, Y2 and Y3.

The median sNfL levels were significantly higher in the MS cohort compared to age-matched healthy controls (Table 1) and did not significantly change during the observation period ($p = 0.235$) but showed a considerable individual variability (Figure 1(a) and (b)).

Patients suffering EDSS progression during the observation period had significantly higher median sNfL levels compared to patients without EDSS progression at Y1 (10.3 pg/ml v. 5.6; $p = 0.007$), Y2 (9.8 v. 5.5; $p = 0.011$), and Y3 (8.6 v. 4.8; $p = 0.033$), but not at Y0 (7.6 v. 5.6; $p = 0.171$). Mean annualized relapse rate (ARR) during the observation period significantly correlated with sNfL levels at all timepoints (Y0: $r_s = 0.287$, $p = 0.010$, Y1: $r_s = 0.237$, $p = 0.023$, Y2: $r_s = 0.220$, $p = 0.029$, Y3: $r_s = 0.209$, $p = 0.031$).

Regarding DMT status, median sNfL levels did not significantly differ between patients receiving no DMT, moderate-effective DMT and high-effective DMT (6.9 pg/ml v. 5.6 v. 6.4, respectively; $p = 0.215$). When comparing sNfL levels according to DMT switching status, we found significantly higher median sNfL values at the sampling before DMT initiation/escalation in switchers compared to non-switchers (10.3 pg/ml v. 5.5, $p < 0.001$), while there was no significant difference after DMT initiation/escalation (6.7 pg/ml v. 6.5,

Table 1. Characteristics of relapsing–remitting multiple sclerosis (RRMS) patients and healthy controls.

	RRMS ($n = 80$)	HC ($n = 30$)	*p*-value
Female[a]	67 (83.8)	16 (53)	0.002[e]
Age (years)[b]	32.8 (7.6)	33.5 (7.9)	0.705[f]
Disease duration (years)[b]	4.6 (5.0)	NA	
RRMS[a]	80 (100)	NA	
Annualized relapse rate during observation period[b]	0.33 (0.39)	NA	
EDSS at baseline[c]	1.5 (0–6.5)	NA	
EDSS progression during observation period[a]	24 (30.0)		
Threshold baseline[d]	5.75 (4.25–7.0)		
DI-score baseline[d]	27.0 (22.25–29.0)		
sNfL baseline (pg/ml)[d]	6.7 (4.5–10.1)	4.3 (3.5–6.3)	<0.001[g]
DMT received at baseline[a]	62 (77.5)	NA	
Interferon beta	8 (10.0)	NA	
Glatirameracetate	12 (15.0)	NA	
Dimethylfumarate	15 (18.8)	NA	
Fingolimod	7 (8.8)	NA	
Natalizumab	20 (25.0)	NA	
DMT status switchers			
Initiation/Escalation	49 (61.3)	NA	

[a]*n* (%); [b]mean (standard deviation); [c]median and range; [d]median and interquartile range; [e]Fisher's exact test; [f]independent t-test; [g]Mann–Whitney U test.

DI: sum score of odour discrimination and identification; DMT: disease modifying treatment; EDSS: expanded disability status scale; sNfL: serum neurofilament light chain; RRMS: relapsing–remitting multiple sclerosis.
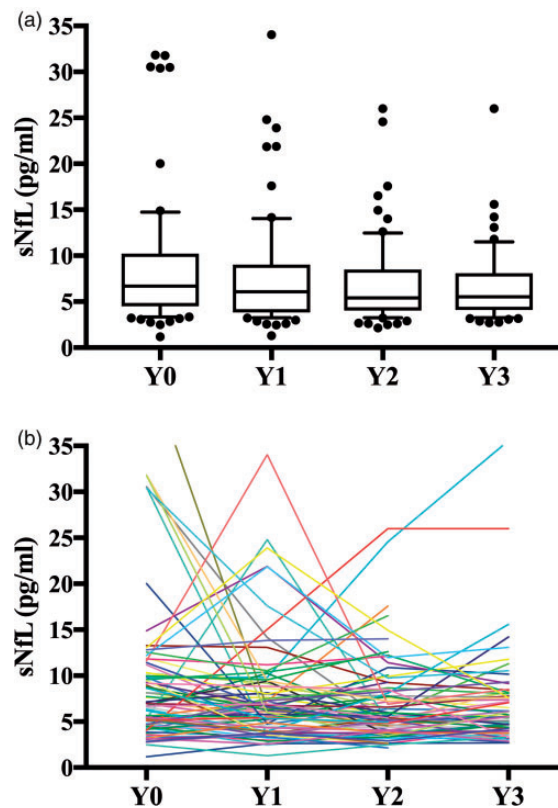
Figure 1. (a) Median serum neurofilament light chain (sNfL) levels and (b) individual sNfL trajectories for each patient.

$p = 0.811$). Median sNfL levels decreased by 3.6 pg/ml (IQR 2.2–5.4) from prior to post DMT initiation/escalation.

Olfactory threshold

During the observation period, median olfactory threshold scores did not change (median change = 0.0, IQR –1.5–1.25, $p = 0.659$). The within-subject stability was low ($r = 0.27$; $p = 0.523$) and 55% of patients had improved threshold scores from baseline to Y3.

When analysing sNfL levels correcting for sex, age and disease duration, we found a significant correlation between median sNfL concentration and median threshold scores at the respective point of sNfL measurement with an increase of 10 pg/ml in sNfL transferring to a mean reduction in threshold scores between 1.2 and 1.6 points ($p < 0.001$, Table 2). However, there was no correlation between sNfL levels at a given timepoint and threshold scores obtained at another time of measurement. sNfL levels accounted for 13–18% of the threshold score variance at the respective point of sNfL measurement (Table 2).

Patients suffering a relapse resulting in sustained EDSS progression during the observation period ($n = 24/80$, 30%) showed significantly higher sNfL levels (10.6 pg/ml) at the point of measurement after occurrence of the relapse compared to patients with a relapse not resulting in sustained EDSS progression (32/80, 40%: 7.5 pg/ml, $p = 0.032$) and patients without a relapse (24/80, 30%: 5.6 pg/ml, $p = 0.009$) at the respective point of measurement (Figure 2(a)). The difference between patients with a relapse not resulting in sustained EDSS progression and patients without a relapse was not statistically significant. These differences were not present at temporally distant measurements.

Regarding olfactory threshold, patients without a relapse during the observation period displayed significantly higher threshold scores (7.00, IQR 5.50–8.00) compared to patients suffering a relapse with or without sustained EDSS progression (3.75, IQR 2.25–5.00, $p = 0.008$ and 4.25, IQR 3.25–4.25, $p = 0.032$, respectively) at the respective point of measurement, while there was no significant difference between patients suffering a relapse with and without sustained EDSS progression (Figure 2(b)). Again, these differences were not present at temporally distant measurements.

Table 2. Multivariate linear regression models predicting olfactory threshold by sNfL levels.

	n	Threshold Y0 Estimate (95% CI) [a]	p-value [b]	R² change (R²)	Threshold Y1 Estimate (95% CI) [a]	p-value [b]	R² change (R²)	Threshold Y2 Estimate (95% CI) [a]	p-value [b]	R² change (R²)	Threshold Y3 Estimate (95% CI) [a]	p-value [b]	R² change (R²)
Y0 sNfL	80	**-1.4 (-2.8—-0.5)**	**<0.001**	**0.137 (0.259)**	-0.7 (-1.1—0.0)	0.099	0.079 (0.179)	-0.2 (-1.1—0.9)	0.354	0.011 (0.139)	0.0 (-0.8—0.9)	0.928	0.001 (0.073)
Y1 sNfL	72	-0.1 (-0.9—0.3)	0.756	0.001 (0.118)	**-1.2 (-2.7—-0.3)**	**<0.001**	**0.142 (0.268)**	-0.8 (-1.2—0.0)	0.067	0.117 (0.176)	0.1 (-0.7—0.3)	0.853	0.001 (0.113)
Y2 sNfL	73	0.2 (-0.8—0.2)	0.653	0.003 (0.101)	-0.1 (-0.8—1.1)	0.815	0.001 (0.087)	**-1.6 (-2.4—-0.8)**	**<0.001**	**0.179 (0.238)**	-0.7 (-1.2—0.0)	0.089	0.108 (0.219)
Y3 sNfL	75	0.0 (-0.9—0.9)	0.998	0.001 (0.071)	0.0 (-0.8—0.9)	0.990	0.001 (0.076)	-0.2 (-0.9—1.0)	0.837	0.002 (0.078)	**-1.2 (-2.6—-0.4)**	**<0.001**	**0.162 (0.257)**

The estimate corresponds to the mean change in olfactory threshold per 10 pg/ml increase in NfL. R^2 change indicates the additional variance explained by adding sNfL levels to the multiple regression models.
[a]corrected for age, sex and disease duration at baseline; [b]corrected for multiple testing.
sNFL: serum neurofilament light chain; 95% CI: 95% confidence interval; Y0: baseline; Y1: year 1; Y2: year 2; Y3: year 3.
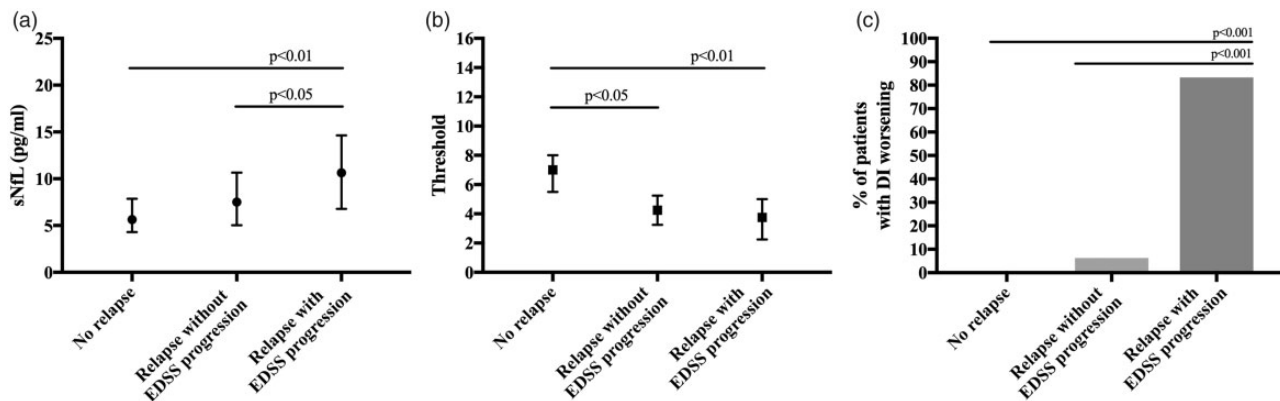
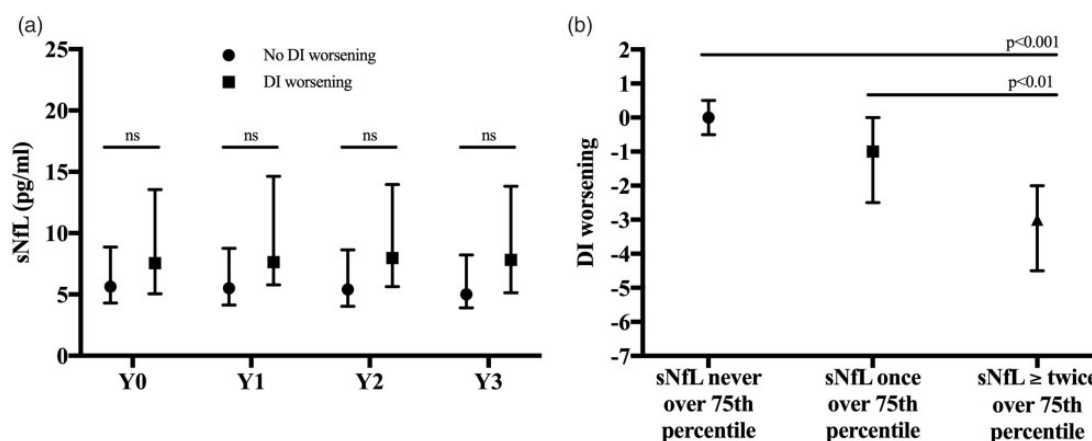**Figure 2.** (a) Serum neurofilament light chain (sNfL) levels, (b) olfactory threshold scores and (c) sum score of discrimination and identification (DI) worsening after occurrence of a relapse with or without expanded disability status scale (EDSS) progression. sNfL and threshold scores at the point of measurement after occurrence of a relapse are depicted as median and interquartile range. *p*-values were calculated by Kruskal–Wallis test ((a) and (b)) and chi-square-test (c).

Odour discrimination/identification score

Median DI scores did significantly decrease during the observation period (median change = −1.5, IQR −2.5–0.5, $p < 0.001$) with high within-subject stability ($r = 0.80$; $p < 0.001$) and 3.8% of patients improving in DI scores.

After correction for sex, age and disease duration, we did not find any significant association between median sNfL levels at a single point of measurement and DI scores, although there was a trend towards higher sNfL levels at Y1 and Y2 correlating with lower DI scores at Y2 and Y3 (Table 3). In the multivariate model, sNfL levels accounted for 3–16% of the variance in DI score at the respective point of sNfL measurement (Table 3).

Median sNfL levels did not significantly differ at any single point of measurement in patients presenting DI worsening during the observation period (22/80, 27.5%) compared to patients without DI worsening (Figure 3(a)).

Next, we compared the amount of DI decrease according to the observed frequency of sNfL levels above the 75th percentile in repeated measurements. Patients with ≥ 2 sNfL measures above the 75th percentile displayed significantly larger decrease in DI (median 3.0 points, IQR 2.0–4.5) compared to patients with no or only one sNfL measure above the 75th percentile (0.0, IQR −0.5–0.5, $p < 0.001$ and 1.0, IQR 0.0–3.30, $p = 0.008$, respectively) (Figure 3(b)).

In a multivariate model correcting for sex, age and disease duration, the number of sNfL measures

above the 75th percentile accounted for 22% of the variance in DI decrease.

DI worsening was significantly more frequent in patients suffering a relapse resulting in sustained EDSS progression during the observation period ($n = 20/24$, 83.3%) as opposed to patients with a relapse not resulting in sustained EDSS progression ($n = 2/32$, 6.3%, $p < 0.001$) and patients without a relapse ($n = 0/24$, 0%, $p < 0.001$) (Figure 2(c)).

Discussion

sNfL is emerging as the first blood-based biomarker of axonal damage in MS. While sNfL levels have been associated with various clinical and MRI outcome measures, no published studies have investigated the relation of sNfL levels and different modalities of olfactory function yet.

Our study shows that sNfL is significantly and independently associated with olfactory function in MS with two key findings: (a) impairment of olfactory threshold is correlated with increased sNfL levels at simultaneous measurement (with an increase of 10 pg/ml in sNfL transferring to a mean reduction in thresh old score of 1.5 points) but not at timely distant measurements, and (b) patients with sNfL levels repeatedly (at least two times) above the 75th percentile displayed significantly larger DI decrease (median 3.0 points) compared to patients with no or only one sNfL measure above the 75th percentile.

Impairment of olfactory threshold is transiently occurring in temporal proximity to relapses but not related to neurodegeneration (i.e. brain atrophy and

Table 3. Multivariate linear regression models predicting DI score by sNfL levels.

	n	DI score Y0			DI score Y1			DI score Y2			DI score Y3		
		Estimate[a] (95% CI)	p–value[b]	R^2 change (R^2)	Estimate[a] (95% CI)	p–value[b]	R^2 change (R^2)	Estimate[a] (95% CI)	p-value[b]	R^2 change (R^2)	Estimate[a] (95% CI)	p-value[b]	R^2 change (R^2)
Y0 sNfL	80	−1.2 (−2.9–0.3)	0.234	0.053 (0.194)	−1.3 (−2.7–0.2)	0.207	0.072 (0.212)	−1.2 (−2.6–0.1)	0.193	0.091 (0.239)	−1.2 (−2.5–0.0)	0.095	0.155 (0.243)
Y1 sNfL	72	−1.5 (−3.8–1.7)	0.533	0.023 (0.166)	−1.5 (−3.5–1.3)	0.445	0.043 (0.184)	−1.6 (−2.5–0.0)	0.079	0.157 (0.249)	−1.6 (−2.5–0.0)	0.069	0.163 (0.256)
Y2 sNfL	73	−1.5 (−2.6–0.6)	0.484	0.038 (0.145)	−1.5 (−2.8–0.5)	0.524	0.032 (0.139)	−1.6 (−2.6–0.6)	0.512	0.076 (0.222)	−1.5 (−3.1–0.0)	0.073	0.156 (0.232)
Y3 sNfL	75	−1.3 (−2.1–0.3)	0.440	0.040 (0.169)	−1.2 (−2.3–0.4)	0.467	0.039 (0.157)	−1.2 (−1.9–0.3)	0.347	0.058 (0.177)	−1.1 (−2.0–0.1)	0.097	0.158 (0.217)

The estimate corresponds to the mean change in olfactory threshold per 10pg/ml increase in NfL. R^2 change indicates the additional variance explained by adding sNfL levels to the multiple regression models.
[a]corrected for age, sex and disease duration at baseline; [b]corrected for multiple testing.
DI: sum score of discrimination and identification; sNFL: serum neurofilament light chain; 95% CI: 95% confidence interval; Y0: baseline; Y1: year; Y2: year 2; Y3: year 3.

Figure 3. Relation of serum neurofilament light chain (sNfL) levels and worsening sum score of discrimination and identification (DI). (a) Median sNfL levels and interquartile range in patients with and without occurrence of DI worsening during the observation period. (b) The degree of DI worsening depending on the number of times sNfL levels occurred above the 75th percentile in a single patient. p-values were calculated by Wilcoxon-signed-Rank-test (a) and Kruskal–Wallis test (b). Y0: baseline; Y1: year 1; Y2: year 2; Y3: year 3.

retinal nerve fibre layer atrophy) and, thus, is suggested as a biomarker of short-term inflammatory activity.[5,6,9,10] Pathophysiologically, threshold is thought to be a function of more peripheral parts of the olfactory system and potentially affected by a bystander inflammation in the olfactory tract during phases of clinical disease activity possibly via demyelination.[23] Our results support this concept as we found increased sNfL levels to be correlated with reduced threshold scores at simultaneous but not at temporally distant measurements. Also, threshold seems to reflect the presence of inflammation as it differentiates between patients with and without a recent relapse irrespective of subsequent EDSS progression. On the other hand, sNfL concentration may represent the degree of axonal damage as it distinguishes between relapses with and without subsequent EDSS progression.

Deterioration of odour discrimination and identification is irreversibly occurring in association with clinical (i.e. EDSS, cognitive function) and paraclinical (i.e. brain and retinal nerve fibre layer atrophy) measures of neurodegeneration.[1–6,9] Discrimination and identification can be summed in the composite score (DI score) providing better correlation to clinical variables than each subscore alone.[6] Since DI rely on complex cortical functions, they are likely to be mainly affected by neurodegenerative processes.[9,23,24] Thus, DI score advocated as a biomarker of neurodegeneration in MS. sNfL levels have been reported to predict brain and spinal cord atrophy over five years more reliably than T2 lesions or T1 contrast enhancing lesions suggesting that sNfL is a more accurate indicator of ongoing axonal loss than MRI measures of acute and chronic lesional activity.[15] Strengthening this hypothesis, the degree

of decrease in DI score was correlated with the number of sNfL measures exceeding the 75th percentile. This suggests that DI worsening is resulting from continuously ongoing axonal damage which is reflected by repeatedly elevated sNfL rather than by a single sNfL level. Correspondingly, DI worsening occurred in 83% of patients suffering a relapse with incomplete remission of symptoms (i.e. resulting in sustained EDSS progression), but only in 6% of relapses with complete remission and in no patient without a relapse. However, only 22% of the variance in DI decrease could be predicted from sNfL levels. This is very similar to the reported percentage of brain atrophy variation explained by annual sNfL levels.[25] Thus, a large proportion of axonal loss remains uncaptured by annual sNfL measurement.

In our highly treated cohort, we did not find significantly different sNfL concentrations between patients receiving no DMT, moderate-effective DMT and high-effective DMT. However, sNfL levels were significantly higher at the sampling before a DMT initiation/escalation, and this difference subsequently disappeared after the initiation/escalation. Median sNfL decrease after DMT initiation/escalation was 4 pg/ml, but we did not have the power to investigate differences further.

The strengths of this study were the longitudinal study design with annual measurement of sNfL and olfactory function in a well characterized cohort with three year clinical and olfactory outcomes. However, our study has several limitations. Importantly, we used a commercially available Simoa assay for sNfL measurement, which differs in some aspects from home-brew assays used in previous studies.[12,14–16,26] In our personal experience, the commercially available assay produces lower absolute sNfL concentrations (by about 50–70%) compared to home-brew assays, although using the same antibody. The reason for that is not yet completely known, but the employment of different calibrators (recombinant human NfL v. bovine NfL) may be one driving factor. Therefore, comparison of absolute sNfL levels between studies using different sNfL assays is so far limited. To enable implementation of sNfL into clinical routine, harmonization of testing assays is obviously essential. Further, not all patients enrolled in the original study had serum samples available. However, we performed sensitivity analyses comparing clinical and olfactory outcomes in patients included and excluded from the present study and did not find any significant differences rendering a potential bias unlikely. Also,

serum samples were not available for all subjects for each time point throughout the observation period which resulted in lower participant counts for some analyses. However, we performed sensitivity analyses regarding missing sNfL values and did not detect a significant effect of missing sNfL values on our results. This is a highly treated cohort of patients, which potentially limits the ability to detect effects of sNfL on olfactory function. The variability in sNfL values was lower by Y1, especially after the initiation/escalation of DMT, thereby potentially limiting the predictive ability in this cohort. We did not have concurrent CSF samples with our serum samples, and therefore cannot comment on additional associations of CSF NfL levels in our cohort. Moreover, our results cannot be applied to patients with progressive courses of MS since they were excluded from our study.

In conclusion, sNfL is correlated with change of different aspects of olfactory function in patients with RRMS adding evidence to strengthen its value as biomarker in MS. While olfactory threshold seems to reflect the presence of inflammation and possibly demyelination, discrimination and identification may represent the degree of axonal damage. However, further studies are needed to prove replicability and clinical value.

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