

# Antibodies to neurofilament light as potential biomarkers in multiple sclerosis

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## ABSTRACT

**Background and objective** The concentration of neurofilament light (NfL) protein in cerebrospinal fluid (CSF) and blood is widely considered as a quantitative measure of neuro-axonal injury. Immune reactivity to NfL released into extracellular fluids induces specific autoantibody response. We investigated the levels and avidity of antibodies to NfL in patients with multiple sclerosis (MS) treated with disease-modifying therapies (DMTs) and their correlation with disease worsening and NfL protein concentration.

**Methods** We conducted a prospective longitudinal study in 246 patients with MS (125 DMT-treated and 121 untreated at baseline). Serum levels of NfL antibodies, antibody avidity and immune complexes were determined by ELISA. NfL protein was measured using the Simoa platform. Clinical variables were tested for their association with the measured parameters in multivariate generalised estimating equation models.

**Results** Multivariate analysis showed that levels of NfL antibodies were higher in progressive MS compared with clinically isolated syndrome (CIS)/relapsing remitting multiple sclerosis (RRMS) ( $p=0.010$ ). Anti-NfL levels drop with increasing disability score (Expanded Disability Status Scale (EDSS)) ( $p=0.002$ ), although conversely, were significantly elevated in CIS/RRMS after a recent EDSS increase ( $p=0.012$ ). Patients receiving DMTs showed decreased levels of anti-NfL ( $p=0.008$ ), high-avidity antibodies ( $p=0.017$ ) and immune-complexes compared with untreated CIS/RRMS. Patients with MS switching to natalizumab showed lower levels of anti-NfL but higher immune complexes compared with healthy controls ( $p=0.0071$ ). A weak association was observed between the levels of NfL protein and NfL antibodies.

**Conclusions** These results support the potential usefulness of quantifying antibody response to NfL as potential markers of progression and treatment response in MS and need to be considered when interpreting peripheral blood NfL levels.

## INTRODUCTION

The axonal cytoskeleton protein neurofilament light (NfL) is released and accumulated in extracellular fluids after neuronal damage, being a hallmark of neurodegeneration.<sup>1–7</sup> This may well trigger or boost specific immune reactivity to neurofilament (Nf)

proteins in blood and cerebrospinal fluid of patients with multiple sclerosis (MS). Studies on autoimmunity to Nfs have shown the link of NfL-IgG-positive patients with central nervous system (CNS) disorders and neurodegenerative diseases.<sup>8–14</sup>

Antibodies to neuronal antigens might modulate disease activity; either providing protection by clearing neurotoxic aggregates<sup>15</sup> or contributing to the exacerbation of inflammatory responses and axonal pathology.<sup>11 16 17</sup>

Our previous observations indicate that increased levels of auto-antibodies to NfL are associated with the progression of amyotrophic lateral sclerosis and decrease in response to treatment in patients with MS.<sup>18 19</sup> Likewise, NfL antibodies induced exacerbation of neurological disease in animal models and axonal loss in spinal cord co-cultures and neurons.<sup>20</sup>

To qualitatively study the antibody function to NfL, we aimed to determine their avidity, which counts for the strength and stability of the antibody–antigen interaction and has been used as a measure of functional maturation of the humoral immune response.<sup>21–23</sup> The affinity degree of antibodies has also been associated with defective homeostatic pathways and formation of immune complexes, suggesting a functional role in disease.<sup>24 25</sup>

Here, we used a longitudinal MS cohort to examine the serum levels of total and high-avidity antibodies against NfL and their relationship with the NfL protein levels, which has not been addressed before. We compared the anti-NfL levels in patients with untreated MS and patients switching to a new disease-modifying therapy (DMT) during a follow-up period to establish whether changes in NfL antibodies and NfL-immune complexes could signal treatment response and disease progression.



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## METHODS

### Standard protocol approvals and patient consents

Serum samples were provided by the Neurologic Clinic and Policlinic, University Hospital Basel (Switzerland) as part of the Swiss Multiple Sclerosis Cohort Study (SMSC) described before.<sup>1</sup> Informed consent was obtained from all participants.

### Study population

A prospective observational study was carried out in a longitudinal Swiss Multiple sclerosis Cohort (n=246). Longitudinal serum samples were available at baseline and at follow-up (FU) visits; FU1 (7±4 months) and FU2 (16±6 months).

The inclusion criteria for this study were as follows: a diagnosis of clinically isolated syndrome (CIS, n=14); relapsing remitting multiple sclerosis (RRMS, n=184); primary progressive multiple sclerosis (PPMS, n=20) or secondary progressive multiple sclerosis (SPMS, n=28); initiation of DMT treatment or switching to a new DMT and availability of demographic and clinical data at time of sample collection.<sup>1 26</sup>

Untreated samples were taken before DMT initiation (or switch). From the untreated patients at baseline (n=121), 98 started DMT and 23 did not receive any treatment. Patients switching to a second-line DMT received the following: natalizumab (n=21), rituximab (n=16) or fingolimod treatment (n=136) and 11 patients did not change DMT. Healthy controls (HC) (n=45) were selected based on the inclusion criteria of no diagnosis of MS or other neurological disease. They were collected and provided by the Neurologic Clinic and Policlinic, University Hospital Basel, as previously reported.<sup>1</sup> Demographic data and clinical characteristics of patients with MS and healthy controls are described in [table 1](#).

### Detection of antibodies to NfL protein

Sera were tested by ELISA using the methodology previously described.<sup>18 19</sup> Nunc-Immuno microtiter 96-well solid plates (Thermo Fisher Scientific, UK) were coated with 2 µg/mL recombinant human-NfL (Progen, Germany) and blocked with 2% bovine serum albumin. Samples were tested in triplicates at 1:100 dilution and antibody binding was detected with horseradish-peroxidase-conjugated goat anti-human IgG (whole molecule) (Sigma, UK). The reaction was developed with TMB 3,3',5,5'-tetramethyl-benzidine substrate (Thermo Fisher Scientific, UK). The absorbance was measured at 450 nm using a Synergy HT microplate reader (Bio-Tek instruments, VT).<sup>18</sup> Results were normalised by subtracting the absorbance derived from uncoated wells and pooled serum samples. Accuracy of sequential ELISA measurements was tested by the inclusion of a standard curve with a known sample, using a pool of human sera containing high levels of NfL antibodies. For comparison purposes, data were transformed to arbitrary units (ArbUnits) in the range (0, 1000) according to the standard curve.

### Avidity determination of NfL antibodies

For a qualitative evaluation of anti-NfL, their avidity was calculated as a measure of the stability of antigen-antibody complexes.<sup>27</sup> Avidity was determined by elution assays on NfL precoated ELISA plates. After incubation with serum samples, plates were washed extensively with phosphate buffered saline (PBS)-Tween 0.1% and incubated for 20 min at room temperature with sodium thiocyanate (NaSCN) 1 M, used as a chaotropic agent to disrupt immune complexes, as previously described.<sup>23 25 28</sup> A duplicate of each sample was included in the same plate for quantification of total and high-avidity NfL antibodies, calculated as the amount of residual antibodies after PBS or NaSCN incubation, respectively. The reaction was developed as described above. The avidity index was calculated as the percentage of antibodies bound to the antigen-coated plate after elution in comparison to the total binding in absence of NaSCN.<sup>25 29</sup>

### Immune complexes detection

Immune complexes were measured using a polyclonal antibody to human NfL protein. Nunclon plates were coated overnight at 4°C with 0.5 µg/mL anti-human NfL (Abbexa, Cambridge, UK), followed by incubation with serum samples and anti-human IgG.<sup>18</sup> The proportional amount of IgG in immune complexes (pg/mL) was quantified by interpolation of optical densities from the standard curve. The IgG standard curve was generated using anti-human IgG (Fc specific) antibody (Sigma, UK) to capture purified human IgG (Sigma, UK) serially diluted (1:2) from 375 ng/mL to 0.02 ng/mL.

All assays were carried out in triplicates. The intra-assay coefficients of variation (%CVs) were 6.48% and 7.52% for detection of NfL antibodies and immune complexes, respectively. The interassay %CVs were 15.92% and 6.05% for detection of NfL antibodies and immune complexes, respectively. The lower limit detection of immune complexes was 300 pg/mL and the upper limit was 9000 pg/mL.

### Detection of NfL protein

NfL was measured at the Neurologic Clinic and Policlinic, University Hospital Basel (Switzerland) using Simoa technology as previously reported.<sup>1</sup> Briefly, NfL concentrations were measured in serum samples using the capture and biotinylated monoclonal antibodies 47:3 and 2:1 from UmanDiagnostics (Umea, Sweden)<sup>1 3 30</sup> and calibrator from the NfL assay, transferred onto the Simoa platform. The capture antibody was conjugated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-activated paramagnetic beads (Quantarix). The assay was run on a Simoa HD-1 instrument (Quantarix) using a two-step Assay Neat 2.0 protocol. Calibrators (neat) and serum samples were diluted 1:4 and measured in duplicate. Standard NfL was obtained from UmanDiagnostics. Calibrators ranged from 0 pg/mL to 2000 pg/mL for serum measurements.

**Table 1** Demographic data and clinical characteristics of the population in study

	MS cohort starter	MS cohort non-starter	All MS	HC
N	212	34	246	45
No. of samples				
1	0 (0.0)	0 (0.0)	0 (0.0)	45 (100)
2	12 (5.7)	6 (17.6)	18 (7.3)	0 (0.0)
3	200 (94.3)	28 (82.4)	228 (92.7)	0 (0.0)
Gender=M	61 (28.8)	23 (67.6)	84 (31.1)	14 (31.1)
Age	40.6 (32.8–48.8)	54.5 (49.2–60.9)	42.2 (33.6–51.4)	43.5 (35.1–50.2)
MS subtype				
CIS	14 (6.6)	0 (0.0)	14 (5.7)	0 (0.0)
RRMS	184 (86.8)	0 (0.0)	184 (74.8)	0 (0.0)
PPMS	2 (0.9)	18 (52.9)	20 (8.1)	0 (0.0)
SPMS	12 (5.7)	16 (47.1)	28 (11.4)	0 (0.0)
HC	0 (0.0)	0 (0.0)	0 (0.0)	45 (100.0)
Disease duration(Y)	6.6 (1.6–14.3)	15.3 (7.9–23.7)	7.4 (1.8–15.3)	
EDSS	2.5 (1.5–3.5)	4.8 (3.6–6.0)	3.0 (1.5–4.0)	
DMT at baseline				
Interferon beta-1a (Avonex)	23 (10.8)	0 (0.0)	23 (9.3)	
Interferon beta-1b	29 (13.7)	3 (8.8)	32 (13.0)	
Glatiramer acetate	31 (14.6)	0 (0.0)	31 (12.6)	
Fingolimod	10 (4.7)	0 (0.0)	10 (4.1)	
Azathioprine	5 (2.4)	0 (0.0)	5 (2.0)	
Natalizumab	30 (14.2)	0 (0.0)	30 (12.2)	
Mitoxantrone	7 (3.3)	3 (8.8)	10 (4.1)	
Interferon beta-1a (Rebif)	10 (4.7)	1 (2.9)	11 (4.5)	
Rituximab	1 (0.5)	0 (0.0)	1 (0.4)	
Dimethyl fumarate	2 (0.9)	0 (0.0)	2 (0.8)	
Study medication	0 (0.0)	4 (11.8)	4 (1.6)	
Treatment naive	64 (30.2)	0 (0.0)	64 (26.0)	
No treatment	0 (0.0)	23 (67.6)	23 (9.3)	
Switch to				
Interferon beta-1a (Avonex)	11 (5.2)		11 (4.5)	
Interferon beta-1b	15 (7.1)		15 (6.1)	
Glatiramer acetate	13 (6.1)		13 (5.3)	
Fingolimod	136 (64.2)		136 (55.3)	
Natalizumab	21 (9.9)		21 (8.5)	
Rituximab	16 (7.5)		16 (6.5)	
Relapse within 60 days before BL=Yes	57 (26.9)		57 (23.2)	
Days BL sample to switch	41.0 (5.0–93.8)		41.0 (5.0–93.8)	
Days BL to first FU sample	217.0 (183.5–365.0)	363.5 (335.2–371.8)	224.0 (188.0–368.0)	
Days BL to second FU sample	511.0 (385.0–699.0)	731.0 (664.5–748.8)	543.0 (387.0–725.0)	
Days first to second FU sample	265.5 (176.0–364.0)	366.5 (334.5–380.2)	308.0 (176.8–365.8)	
FU time from BL sample (days)	1436.5 (1070.0–1642.0)	897.0 (715.5–1094.2)	1324.0 (966.8–1600.2)	
FU time from last sample (days)	790.0 (371.8–1119.2)	348.0 (0.0–377.5)	741.0 (358.2–1097.8)	

Categorical variables are described by counts and percentages (%), continuous and ordinal variables by median and IQR.

BL, baseline; CIS, clinically isolated syndrome; DMT, disease-modifying treatment; EDSS, Expanded Disability Status Scale; FU, follow up; HC, healthy controls; M, males; MS non-starters, patients with progressive MS who were either untreated or had not changed DMT; MS starters, patients starting or switching to a new DMT after baseline sampling; PPMS, primary progressive MS; RRMS, relapsing remitting MS; SPMS, secondary progressive MS.

### Statistical analysis

Categorical variables were described by counts and percentages, and continuous variables by median and

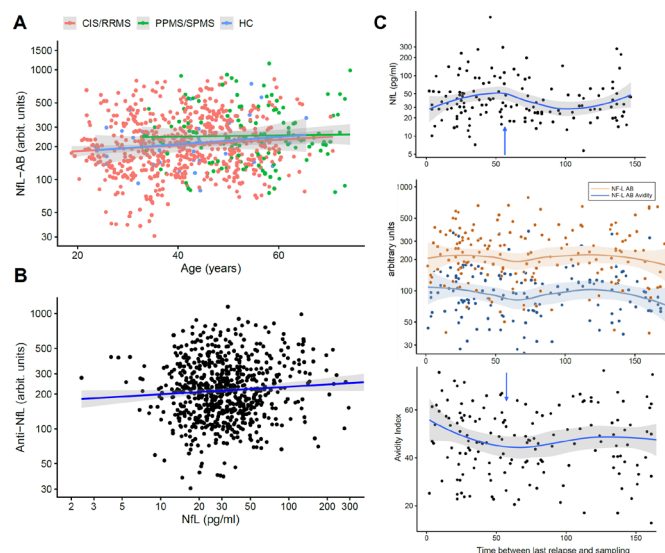
IQR (table 1). The associations between clinical parameters and NfL antibody levels and antibody avidities were modelled with linear generalised estimating equation

(GEE) models while accounting for within-patient correlation. Unless not specified otherwise, NfL levels and all NfL antibody measures were log-transformed prior to analysis to meet the normality assumption. For all models with log-transformed end points, the estimates (regression coefficients) were back-transformed to the original scale; 95% CI and p values are presented. The effects are multiplicative, that is, an estimate of 1.05 means an increase of 5% for each increase in the predictor by one unit (if continuous) or whether the indicated level is present instead of the basic level (if the variable is categorical), respectively. The following variables were tested for association with log-transformed NfL protein/ anti-NfL using the GEE model, accounting for multiple samples per patient: age, gender, disease subtype (CIS/RRMS vs PPMS/SPMS), recent relapses (within 60 days before sampling), Expanded Disability Status sSale (EDSS) at time of sampling, recent EDSS increase (ie, increase in EDSS since previous visit of  $\geq 1.5$  points from an EDSS score of 0.0;  $\geq 1.0$  point from an EDSS score of 1.0–5.5 or  $\geq 0.5$  point from an EDSS score  $\geq 6.0$ ), DMT treatment status (treated vs untreated). The associations between the individual parameters and anti-NfL measures were first investigated in individual univariate models without and with correcting for age. Finally, all the variables were included in the multivariate model and missing data were not imputed. The quality of all GEE models was investigated by visually inspecting residuals and quantile–quantile plots. Model selection as well as the selection of the correlations structure was performed based on the quasi-likelihood under the independence model criterion.<sup>31</sup> The association between time under treatment and NfL antibody at follow-up was investigated using linear GEE models with log-transformed NfL antibody at follow-up as dependent variables and time under treatment and baseline NfL antibody as a covariates. All analyses were conducted using the statistical software R.<sup>32</sup>

## RESULTS

### Study population

Demographics and clinical features are summarised in [table 1](#). The MS cohort comprised of 246 patients with MS (125 DMT-treated and 121 untreated at baseline) classified as follows: 184 RRMS, 14 CIS, 28 SPMS, 20 PPMS and 45 HC. The median age was 42.2 years; IQR=33.6–51.4 in patients with MS and 43.5 years ; IQR=35.1–50.2 in HC. MS cohort starters were defined as patients starting or switching to a new DMT after baseline sampling (n=212) and non-starters (patients with progressive MS) who were either untreated or had not changed DMT (n=34). The mean EDSS score of patients with MS was  $3.0\pm 1.7$ , in the MS cohort starter group it was  $2.7\pm 1.6$  and  $5\pm 1.5$  in non-starters. The mean duration of disease of patients with MS was  $10.0\pm 9.6$  years; for the MS cohort starter group  $8.9\pm 8.7$  years and  $17.1\pm 11.07$  years in non-starters. The median time between baseline sampling and DMT initiation in the starters group was 41 days; IQR=5.0–93.8.

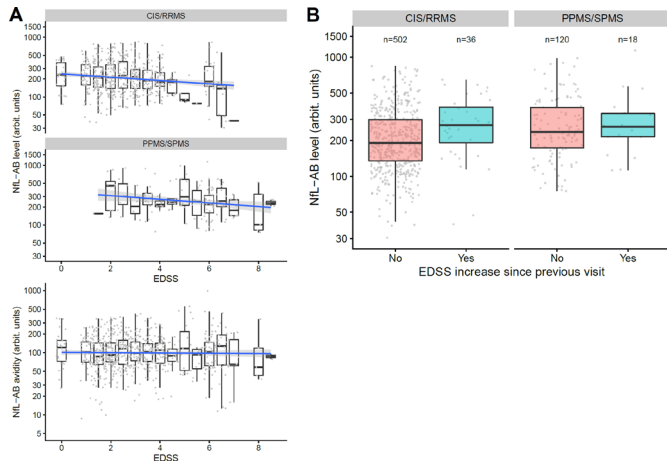


**Figure 1** Association between anti-NfL and NfL protein levels and anti-NfL vs age by MS subtype. (A) Anti-NfL vs age by MS subtype. Both anti-NfL level and NfL avidity tended to increase with age in patients with MS (<1% per year). This slope is not related to the MS subtype (interaction not significant). (B) The scatter plot shows the linear regression of correlation between NfL antibodies and NfL protein in all samples from patients with MS. The grey band indicates the 95% CI. (C) Level of NfL, NfL antibodies and avidity in samples taken within 150 days after a relapse. The plots show the association between NfL protein levels with disease relapses (peak day 60) (upper panel). The opposite trend is observed about day 60 in the avidity index of NfL antibodies (lower panel). CIS, clinically isolated syndrome; HC, healthy control; MS, multiple sclerosis; NfL-AB, neurofilament light antibodies; PPMS, primary progressive MS; RRMS, relapsing remitting MS; SPMS, secondary progressive MS.

### Correlation between levels of NfL antibodies and NfL protein

Both, NfL antibodies levels and antibody avidity tended to increase with age in patients with MS and healthy subjects, showing an increment of <1% per year. The interaction of age with MS subtype was not significant ([figure 1A](#)). Total and high-avidity NfL antibodies levels increased 0.6% per year (estimate=1.006, 95% CI 1.000 to 1.013, p=0.046) and 0.9% per year (estimate=1.009, 95% CI 1.001 to 1.016, p=0.018), respectively. Analysis of all MS samples showed a very weak association between NfL antibody and NfL protein levels. The linear correlation of non-transformed data is displayed in [figure 1B](#), and the GEE model analysis of the association between  $\log_{10}$  (anti-NfL) and  $\log_{10}$  (NfL protein) showed an estimate=0.059, 95% CI 0.013 to 0.105, p=0.012. To investigate if any time lag could be relevant, the levels of NfL protein at the current visit and anti-NfL at the subsequent visit, were compared. No association between the two parameters became apparent at the available sampling date and no interaction was found with MS subtype.

However, levels of NfL antibodies and NfL protein tend to correlate negatively with relapse of disease. [Figure 1C](#) helps to visualise a maximum in the NfL-protein levels roughly 60 days after a relapse while the avidity index

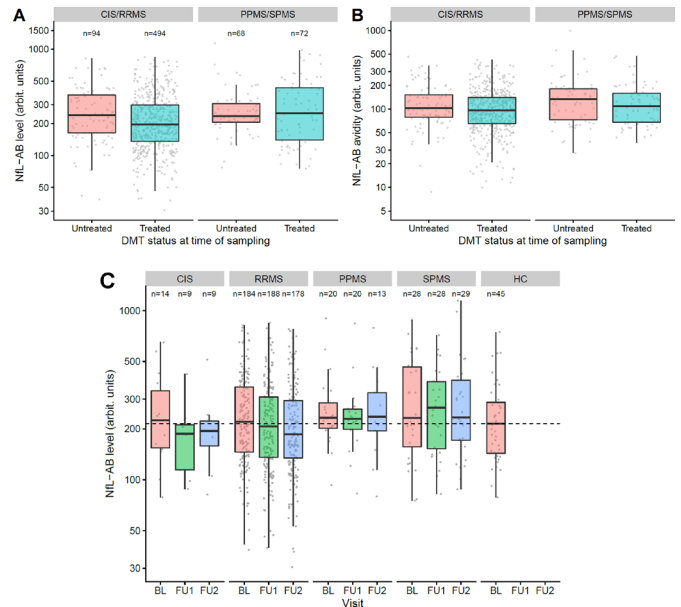


**Figure 2** Association between NfL antibodies and EDSS. (A) Anti-NfL levels tended to decrease with increasing EDSS. The decrease becomes significant when correcting for age and MS subtype (upper panels). Lower panel shows no correlation between the avidity of anti-NfL vs EDSS. (B) Increase of NfL antibody levels after recent EDSS progression by subtype. In CIS/RRMS, anti-NfL levels were significantly higher after an EDSS increase since the last visit (x-axis: EDSS; y-axis: antibody levels). CIS, clinically isolated syndrome; EDSS, Expanded Disability Status Scale; MS, multiple sclerosis; n, number of samples; NfL-AB, neurofilament light antibodies; PPMS, primary progressive MS; RRMS, relapsing remitting MS; SPMS, secondary progressive MS.

of NfL-antibodies shows a minimum at the same time point. Quite opposite, by day 110 after a relapse there was a trend in NfL protein to decrease and NfL antibodies and avidity to increase. Of note, patients with untreated RRMS and with a recent relapse (<60 days before sampling) showed elevated anti-NfL levels (median=271, (IQR=182–373)) compared with those without a recent relapse (median=225, (IQR=164–339)).

The univariate analysis indicates that anti-NfL levels tended to be higher in PPMS/SPMS than in HC (estimate=1.165, 95% CI 1.007 to 1.347, p=0.040 without age correction and estimate=1.104, 95% CI 0.954 to 1.278, p=0.183 when adjusted for age) and not significant between CIS/RRMS and HC (estimate=0.969, 95% CI 0.840 to 1.119, p=0.672). The quantification of high-avidity antibodies discriminated better the MS subtype groups (estimate=1.31, 95% CI 1.058 to 1.642, p=0.014) compared with total antibody levels (estimate=1.23, 95% CI 0.992 to 1.525, p=0.059).

In untreated samples, anti-NfL levels tended to decrease with increasing EDSS (estimate=0.953, 95% CI 0.914 to 0.994, p=0.026). However, the disability status was not correlated with the avidity of antibodies (estimate=0.960, 95% CI 0.920 to 1.002, p=0.064) (figure 2A). Conversely, in CIS/RRMS, anti-NfL levels were significantly higher after an EDSS increase since the last visit (figure 2B). The median anti-NfL in CIS/RRMS was 190.9 (IQR=135.2–299) vs 268.8 (IQR=193–382.2) after no recent and a recent EDSS increase respectively, estimate=1.109, 95% CI 1.014



**Figure 3** Levels of total and high-avidity NfL antibodies in patients with MS by subtype. (A) Decrease in NfL antibody levels. Anti-NfL levels were approximately 8% lower in DMT-treated patients compared with the untreated ones. (B) Decrease in avidity is seen in patients with CIS/RRMS after treatment. The untreated samples were taken from patients starting or switching treatment, who tend to be more active at the first sampling time point. (C) Serum NfL antibody levels in untreated patients over time were higher in progressive MS (PPMS/SPMS) compared with CIS, RRMS and HC. Dotted line indicates the median for the healthy controls group. Lines in the boxes denote the median of the corresponding sample time (FU1: 217.0 (183.5–365.0) days and FU2: 511.0 (385.0–699.0) days after baseline). BL, baseline; CIS, clinically isolated syndrome; DMT, disease-modifying therapy; FU1, first follow-up; FU2, second follow-up; HC, healthy control; MS, multiple sclerosis; n, number of samples; NfL-AB, neurofilament light antibodies; PPMS, primary progressive MS; RRMS, relapsing remitting MS; SPMS, secondary progressive MS.

to 1.214, p=0.024. There was no significant change in the avidity of antibodies after a recent EDSS increase.

**Anti-NfL level of patients with MS with and without treatment by subtype**

NfL antibody levels were approximately 8% lower in patients with treated CIS/RRMS compared with untreated. Significant increased anti-NfL levels were found in patients with untreated CIS/RRMS compared with patients receiving DMT (estimate=0.916, 95% CI 0.859 to 0.977, p=0.008 (figure 3A) as well as high-avidity anti-NfL (estimate=0.929, 95% CI 0.875 to 0.987, p=0.017) (figure 3B) (table 2). The differences of NfL antibody levels and avidity in patients with treated PPMS/SPMS versus patients with untreated PPMS/SPMS were not significant (table 2).

**Multivariate model for anti-NfL**

In the multivariate analysis, all variables were included. Table 2, shows the estimates of the GEE model analysing

**Table 2** Association between serum levels of (log) NfL antibodies (and avidity) and different variables, tested in the multivariate model in the MS cohort

Multivariate model	NfL antibodies			High-avidity NfL antibodies		
	Estimate	95% CI	P value	Estimate	95% CI	P value
<b>Variables</b>						
<b>Age</b>	1.008	(1.001 to 1.015)	0.031	1.01	(1.002 to 1.018)	0.018
Adding log(NfL)	1.008	(1.000 to 1.015)	0.037	1.01	(1.002 to 1.018)	0.016
<b>PPMS/SPMS vs CIS/RRMS</b>	1.353	(1.074 to 1.703)	0.01	1.287	(1.015 to 1.632)	0.037
Adding log(NfL)	1.359	(1.080 to 1.712)	0.009	1.279	(1.007 to 1.625)	0.044
<b>Gender</b>						
Male vs Female (log NfL Ab)	0.898	(0.768 to 1.050)	0.176	0.868	(0.734 to 1.026)	0.096
<b>Disability status EDSS</b>	0.932	(0.892 to 0.974)	0.002	0.947	(0.902 to 0.995)	0.031
<b>Recent relapse, &lt;60 days ago</b>						
patients with MS, Yes vs No	0.963	(0.888 to 1.046)	0.373	0.95	(0.878 to 1.028)	0.202
<b>After recent EDSS increase</b>	1.132	(1.028 to 1.247)	0.012	1.07	(0.963 to 1.189)	0.207
Adding: log(NfL)	1.127	(1.022 to 1.241)	0.016	1.076	(0.969 to 1.194)	0.17
Disease duration	1.121	(1.018 to 1.234)	0.02	–	–	–
<b>DMT treated vs untreated</b>	0.931	(0.865 to 1.003)	0.058	0.94	(0.878 to 1.007)	0.079
Adding log(NfL)	0.941	(0.878 to 1.009)	0.086	0.929	(0.870 to 0.992)	0.028
Patients with MS (univariate)	0.914	(0.861 to 0.971)	0.003	0.924	(0.871 to 0.981)	0.009
CIS/RRMS only (univariate)	0.916	(0.859 to 0.977)	0.008	0.929	(0.875 to 0.987)	0.017
PPMS/SPMS only (univariate)	0.988	(0.782 to 1.249)	0.921	0.878	(0.690 to 1.118)	0.292

NfL antibodies and NfL avidity, levels in serum.

CIS, clinically isolated syndrome; DMT, disease-modifying treatment; EDSS, Expanded Disability Status Scale; Estimate, regression coefficient; NfL, neurofilament light; PPMS, primary progressive multiple sclerosis; RRMS, relapsing remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis.

the association between log(anti-NfL) and log(high-avidity anti-NfL) and clinical variables. Antibody levels and avidity tend to slightly increase with age (estimate=1.008, 95% CI 1.002 to 1.015,  $p=0.031$  and estimate=1.010, 95% CI 1.002 to 1.018,  $p=0.018$  respectively). Levels of NfL antibodies were significantly higher in patients with progressive MS (PPMS/SPMS vs CIS/RRMS, estimate=1.353, 95% CI 1.074 to 1.703,  $p=0.010$ ) and the correlation increased when disease duration ( $p=0.008$ ) and log(NfL) ( $p=0.009$ ) are included in the model. The anti-NfL level by MS subtype group and over time is illustrated in [figure 3C](#). Similarly, avidity of NfL antibodies were also higher in patients with progressive MS (PPMS/SPMS vs CIS/RRMS, estimate=1.287, 95% CI 1.015 to 1.632,  $p=0.037$ ).

Antibody levels tended to drop with increasing EDSS (estimate=0.932, 95% CI 0.892 to 0.974,  $p=0.002$ ). Conversely, NfL antibody levels were significantly higher in CIS/RRMS after a recent EDSS increase: (estimate=1.132, 95% CI 1.028 to 1.247,  $p=0.012$ ), after correcting for log(NfL) ( $p=0.016$ ) and after including disease duration ( $p=0.020$ ) ([table 2](#)). Likewise, level of total anti-NfL tended to be higher in untreated patients compared with DMT-treated patients (estimate=0.931, 95% CI 0.865 to 1.003,  $p=0.058$ ). After correcting

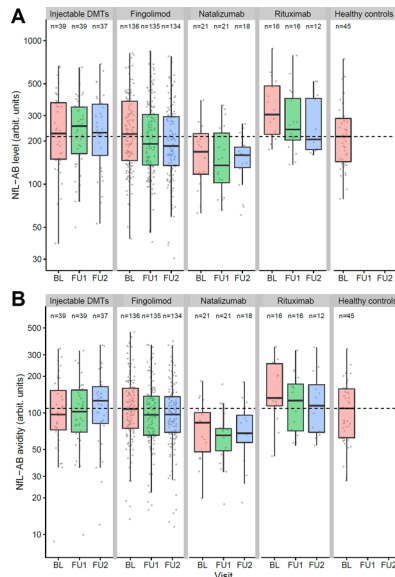
for log(NfL), the level of high-avidity NfL antibodies discriminated better the groups of patients under treatment versus the untreated ones (estimate=0.929, 95% CI 0.870 to 0.992,  $p=0.028$ ).

#### Change of levels of NfL antibodies by (switch to) treatment and over time

GEE analysis of longitudinal samples, including time under treatment and covariates, indicates that total and high-avidity anti-NfL levels tended to decrease during natalizumab, rituximab and fingolimod treatment, except for injectable DMTs ([figure 4A](#)).

High-avidity NfL antibodies decreased in about 81.2%, 76.1% and 69.8% of patients treated with rituximab, natalizumab and fingolimod, respectively. Decrement of high-avidity NfL antibodies was more evident after FU1 particularly in patients treated with natalizumab and rituximab but remained unchanged in patients treated with injectable DMTs ([figure 4B](#)).

Notably, baseline NfL antibody levels were much lower in patients starting natalizumab than in HCs but higher in patients starting rituximab. These levels were similar in patients initiating fingolimod and injectable DMTs and HCs.

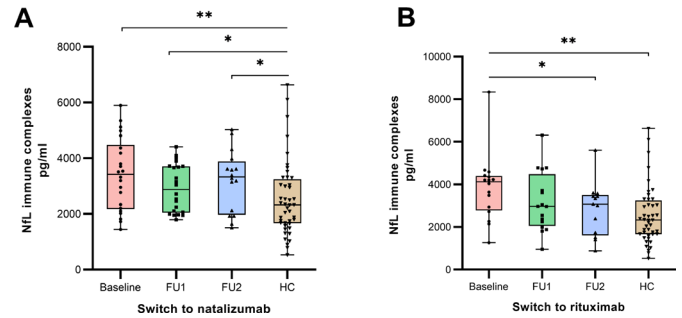


**Figure 4** Serum level of NfL antibodies in patients starting or switching to a new DMT. (A) Total NfL antibodies and (B) high-avidity NfL antibody levels tended to decrease over time after natalizumab, rituximab and fingolimod treatment. Patients switching to natalizumab showed lower level of antibodies than healthy controls. At baseline, patients switched to rituximab (PPMS/SPMS and RRMS) had higher levels of antibodies than baseline natalizumab (RRMS). Patients receiving injectable DMTs treatment had NfL levels that remained approximately stable over time. Dotted line indicates the median for the healthy controls group. Lines in the boxes denote the median of the corresponding sample time (BL: baseline, FU1: 217.0 (183.5–365.0) days and FU2: 511.0 (385.0–699.0) days after baseline). Injectable DMTs include interferon beta-1a (Avonex), interferon beta-1b, glatiramer acetate, mitoxantrone, interferon beta-1a (Rebif) and other study medications. The x-axis indicates time after treatment initiation. DMT, disease-modifying therapy; FU1, first follow-up; FU2, second follow-up; HC, healthy control; MS, multiple sclerosis; n, number of samples; NfL-AB, neurofilament light antibodies; PPMS, primary progressive MS; RRMS, relapsing remitting MS; SPMS, secondary progressive MS.

### NfL immune complexes detection

The possible aggregation between NfL protein and specific antibodies was examined by quantifying the immune complexes formation. Results show that in contrast to anti-NfL levels, immune complexes were higher in patients starting natalizumab compared with HCs. [Figure 5A](#) shows the level of immune complexes for natalizumab at baseline (median=3418, (IQR=2178–4478)) vs HC (median=2326, (IQR=1664–3242)), ( $p=0.0071$ ); FU1 after treatment (median=2879, (IQR=2045–3712)) vs HC ( $p=0.041$ ) and FU2 after treatment (median=3330, (IQR=1973–3884)) vs HC ( $p=0.029$ ).

Likewise, NfL immune complexes tended to decrease as the treatment progresses. [Figure 5B](#) shows the difference in patients that switched to rituximab at baseline (median=4119 (IQR=2776–4398)) vs FU2 (median=3069, (IQR=1614–3508)), ( $p=0.043$ ) and vs HC ( $p=0.0013$ ).



**Figure 5** Level of NfL immune complexes in serum of patients switched to a new DMT. NfL immune complexes were higher in patients switching to a new DMT. (A) Patients starting natalizumab compared with HCs. Natalizumab at baseline vs HC ( $p=0.0071$ ); FU1 vs HC ( $p=0.04$ ) and FU2 vs HC ( $p=0.029$ ). (B) NfL immune complexes tended to decrease as the treatment progresses. The box plot shows the difference in patients that switched to rituximab at baseline vs FU2 ( $p=0.043$ ), baseline vs HC ( $p=0.0013$ ). Lines in the boxes denote the median of the corresponding sample time (FU1: 217.0 (183.5–365.0) days and FU2: 511.0 (385.0–699.0) days after baseline). Comparisons between two groups were performed using the non-parametric Mann-Whitney U test. Box plots indicate the median, the IQR (25th–75th percentile), whiskers represent the maximum and minimum values. \* $p$  values  $< 0.05$  and \*\* $p < 0.01$  were considered as statistically significant. BL, baseline; DMT, disease-modifying therapy; FU1, first-follow-up; FU2, second follow-up; HCs, healthy controls; NfL, neurofilament light.

The level of NfL immune complexes decreased in 100% of patients treated with rituximab and in 61.9% of patients treated with natalizumab. Levels of immune complexes were positively correlated with high-avidity antibodies mainly in PPMS/SPMS ( $r=0.531$ ,  $p=0.0092$ ), weakly correlated in RRMS ( $r=0.23$ ,  $p=0.037$ ) but uncorrelated with antibodies in HC.

### DISCUSSION

Autoimmune reactivity to NfL released during axonal damage may not only represent a biomarker of neurodegeneration but also play an important role in the pathogenesis of disease. [5 6 9 11 15 16 29 33 34](#)

We found that levels of NfL protein were very weakly correlated with the levels of NfL specific antibodies and also observed a trend for an inverse correlation between these two parameters and a recent relapse. High-frequency sampling data would be needed to study the dynamic of the different parameters and a possible lag-time needed to allow the expansion of specific humoral response once NfL is released.

The antibody levels against NfL tended to increase with age in patients with MS. Importantly, we observed a negative correlation between the level of NfL antibodies and the EDSS. This effect could be mediated by low-avidity NfL antibodies given the lack of association of EDSS with the avidity of antibodies. In contrast, a significant positive association between the incidence of NfL antibodies and a recent EDSS increase was found. Of note in patients

with RRMS, a recent EDSS increase was correlated with augmented levels of total NfL antibodies but not associated with their avidity, which can be related to the time needed for affinity maturation to occur. This suggests a possible protective role of NfL antibodies or a cumulative effect in PPMS/SPMS in contrast with an actual inflammatory process in active RRMS that seems to be reflected in an EDSS increase. The correlation with a recent EDSS increase could indicate a recent neuronal damage, where the measurement of NfL antibodies might have a diagnostic potential and be an additional marker to monitor neurodegeneration.

On the contrary, as reported previously, the NfL protein levels in the SMSC cohort were positively correlated with the EDSS, although they were not significantly associated with a recent EDSS worsening.<sup>1</sup> Of note, the covariates: age, MS subtype, EDSS and EDSS increase since last visit remained significant when NfL is added to the model, suggesting that the two measures are more likely independent.

Total and high-avidity NfL antibodies were significantly elevated in SPMS/PPMS in comparison to CIS/RRMS. High-avidity antibodies tended to discriminate better the MS subtype groups; therefore avidity determination might complement anti-NfL levels for a better diagnostic performance. Patients with SPMS/PPMS with long disease duration and accumulated disability might have developed higher affinity antibodies after longer exposure to the NfL released into the CNS and blood. High-avidity antibodies exhibit greater and maximal binding to the target antigen and very low cross-reactivity to other molecules and therefore are possibly involved in axonal degeneration compared with low avidity antibodies.<sup>29</sup>

Analysis of anti-NfL in patients switching treatment showed that CIS/RRMS-treated patients display lower levels and avidity in comparison to the untreated ones. Untreated patients, who are mostly before starting or switching to a new drug, typically show more active disease at the first sampling time point. In particular, the avidity of NfL antibodies decrease after the first follow-up. Thus, in addition to the anti-NfL levels, this parameter might also be useful to predict response to treatment.

Except for injectable DMTs, the decrease in NfL antibodies with time since the start of new treatment appeared similar across different DMTs like natalizumab, rituximab and fingolimod. It would be important to increase the sample size and the follow-up period to further investigate changes in the different drug subgroups.

The rise of autoimmune reaction against NfL might also result in the formation of stable anti-NfL–NfL protein immune complexes generated at optimal antibody:antigen ratios.<sup>35</sup> Immune complex formation and aggregation could also mask the detection of free Nf protein and antibodies.<sup>36 37</sup> This can explain why patients switching to natalizumab displayed lower levels of NfL antibodies than healthy controls but more elevated NfL immune complexes. Likewise, the lower levels of total and high-avidity NfL antibodies observed at baseline in

patients switching to natalizumab (RRMS) compared with patients switched to rituximab (PPMS/SPMS) and fingolimod, could be related with the degree of disease activity in the different groups and with the formation of immune complexes. The decrease of NfL antibodies after treatment with natalizumab is in line with previous observations. The augment in the levels of these antibodies at the second follow-up are most likely linked to future relapses as reported in previous studies.<sup>18</sup> Of note, no changes were observed in the levels of total IgG during the follow-up period in natalizumab and rituximab treated patients (data not shown).

A positive correlation between high avidity antibodies and immune complex formation was observed in patients with MS but not in HC. This could inform about the stability of the complexes in diseased patients which might be correlated with differences in the potential to mediate effector mechanisms compared with natural antibodies, whose main function is to ensure specific homeostasis by reacting to self-antigens.<sup>38</sup> Further investigation is needed to address the formation of immune complexes in correlation with disease relapses. Neuron specific antibodies and immune complexes might also play a pathogenic role by interacting with Fc receptors in microglia and astrocytes promoting the release of inflammatory mediators and activation of complement, which could exacerbate the disease.<sup>39</sup>

The analysis of different IgG subclasses will help to differentiate potential pro-inflammatory NfL antibodies related to disease progression. The evaluation of the clinical significance of NfL antibody and avidity determination will help to discriminate between natural antibodies to NfL in healthy individuals and potential pathogenic antibodies in patients with MS.

Overall results reveal that Nf antibody response is a fluctuating dynamic process that most likely depends on time and concentration of the target protein released after neuronal damage. The clinical applicability of measuring the serum levels of NfL antibodies and high-avidity antibodies as biomarkers could be complemented with the measurement of immune complexes. In this study we have shown that not only the level of Nf antibodies but also their avidity increase in progressive MS and those levels decrease after treatment. The very weak correlation found between the NfL protein and antibodies against NfL indicate that immune reactivity to the protein can be an independent and complementary marker in MS.

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**Data availability statement** Data are available upon reasonable request. Anonymized data can be requested from the corresponding author.

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