Axonal Damage in Multiple Sclerosis

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CHAPTER 5

Neurofilament Light Antibodies in Serum Reflect Response to Natalizumab Treatment in Multiple Sclerosis

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Abstract
Increased levels of antibodies to neurofilament light protein (NF-L) in biological fluids have been found to reflect neuroinflammatory responses and neurodegeneration in multiple sclerosis (MS).
The objective of this study was to evaluate whether levels of serum antibodies against NF-L correlate with clinical variants and treatment response in MS.
Autoantibody reactivity to the NF-L protein was tested in serum samples from patients with relapsing-remitting MS (RRMS) and secondary progressive MS (SPMS). Two other cohorts of RRMS patients under treatment with natalizumab were analysed cross-sectionally and longitudinally. The follow-up samples were taken at 6, 12, 18 and 24 months after treatment, and the NF-L antibody levels were compared against baseline levels.
NF-L antibody levels were higher in MS clinical groups than healthy controls and in RRMS compared to SPMS patients ($p<0.001$). NF-L antibody levels were lower in natalizumab treated than in untreated patients ($p<0.001$). In the longitudinal series, NF-L antibody levels decreased over time and a significant difference was found following 24 months of treatment compared with baseline measurements ($p=0.001$).
In conclusion, drug efficacy in MS treatment indicates the potential use of monitoring the content of antibodies against NF-L as a predictive biomarker of treatment response in MS.

Introduction
Neuroinflammatory and neurodegenerative processes lead to neuroaxonal damage, the morphological substrate of irreversible disability in multiple sclerosis (MS). Neuronal damage in the central nervous system (CNS) correlates with increased amounts of cytoskeletal proteins released into the extracellular environment. This in turn may induce immune reactivity to both intracellular and surface neuronal proteins present in blood and cerebrospinal fluid (CSF) from patients with neurological disorders (1). Therefore, neuronal cytoskeleton-derived proteins such as neurofilaments and the specific antibodies to these proteins represent potential indicators for diagnosis and monitoring of neurological diseases (2, 3).
Neurofilament (NF) proteins consisting predominantly of NF light (NF-L), medium (NF-M) and heavy (NF-H) chains and alpha-internexin are major structural components of neurons (4). The overexpression, aggregation and abnormal phosphorylation of these proteins are associated with axonal pathology in neurological diseases (5-7). Axonal damage and the subsequent release of NF into the extracellular fluids could lead to T cell-dependent specific antibody generation which may contribute to the neurodestructive processes and disability in MS (8-10). Intrathecal synthesis of antibodies against NF-L correlates very well with magnetic resonance imaging (MRI) markers of cerebral atrophy in MS patients (11), supporting the idea that axonal damage occurs at early stages of the disease while immune responses to NF are also significantly up regulated in serum of patients with progressive MS (12, 13).
Studies on the immune response to neurofilaments have given general indications that NF-L antibodies could function as biomarkers for the staging and phenotypic characterisation of MS and other neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) and stroke (8, 12-17). Little is understood about the significance of this immunological signature in the prognosis or response to treatment in MS. Although previous studies showed the presence of immune responses to neurofilaments in different courses of MS, inconclusive and variable results have been shown. Anti-

NF-L immunoglobulin (Ig) G are elevated in MS patients with primary progressive MS (PPMS) and SPMS (13) but are also incremented in clinically isolated syndrome (CIS) early converted into MS (14) and RRMS (11). However, other studies report no difference in the NF-L antibody status in MS patients with different disease courses (8). Monoclonal antibodies such as natalizumab, which inhibits leukocyte migration across the blood-brain barrier by blocking adhesion of activated T lymphocytes to brain endothelial cells, has proven to be an efficient drug in RRMS (18). The phase III monotherapy trial, the Natalizumab Safety and Efficacy in Relapsing Remitting Multiple Sclerosis (AFFIRM) study shows that treatment with natalizumab reduces the annual relapse rate and the accumulation of neurological disability (19).
Here we have evaluated levels of serum antibodies against NF-L in RRMS patients treated with natalizumab compared with untreated patients, and tracked the NF-L antibody levels in serial samples during the treatment and in MS patients at different stages of the disease. Antibodies to NF-L were measured by enzyme-linked immunosorbent assay (ELISA) using a mouse recombinant NF-L protein that has 98% sequence identity to human NF-L. The results from this study show a relationship between antibodies to NF-L protein and disease stage as well as a correlation between levels of NF-L-specific antibodies and the therapeutic efficacy of natalizumab in MS.

Materials and methods

Ethics statement

This study was approved by the local ethical committees: The North London REC 2 (reference 10/H0724/36) (cohorts 1 and 2), Hospital Universitario La Fe Committee (cohort 3) and the University College London Hospitals Committee and National Research Ethics Committee (cohort 4). The natalizumab treatment in cohort 3 was provided according to the European Medicines Agency (EMA) guidelines and NICE guidelines TA127. All participants provided written informed consent to the study.

Study population

Eighty-eight MS patients (62 RRMS and 26 SPMS from three different centres) were included in the study. The RRMS patients comprised three different cohorts: patients treated with natalizumab (cross-sectional study and longitudinal study) and a group of untreated patients. The demographical and clinical information are summarised in Table 1. Briefly, patients with RRMS were recruited at The Royal London Hospital, UK in 2011. Eligible patients had a diagnosis of RRMS according to the McDonald criteria (20). Untreated patients (cohort 1) had received no disease-modifying therapy for at least 1 year at the time of blood draw, and 17 of these had never received any treatment. Patients in cohort 2 had already been started on natalizumab before blood sampling. The serum samples were taken on average 1.1 years after treatment initiation. Cohort 3 consisted of RRMS patients recruited between 2007 and 2009 from La Fe Polytechnic and University Hospital in Valencia, Spain. Eligible patients had a diagnosis of RRMS (20) and a brain MRI scan demonstrating at least nine T2-hyperintensive lesions and at least one gadolinium-enhancing lesion (21). All patients from cohort 3 were receiving between one and four different disease-modifying drugs. Patients with high disease activity or rapidly evolving severe RRMS were started on natalizumab and enrolled in the study. Serial serum samples were taken at baseline and at months 6, 12, 18 and 24 after the initiation of treatment. A brain MRI scan was performed at baseline and yearly thereafter. Cohort 4 consisted of SPMS patients recruited from the National

Hospital for Neurology and Neurosurgery and from the Royal Free Hospital, London, UK in 2010. Eligible patients had a diagnosis of SPMS, defined as a period of deterioration, independent of relapses, sustained for at least 6 months, and that followed a period of RRMS. As controls, healthy donors were included in the study. Clinical data were not available to the investigators who performed the assays.

Table 1. Demographic data and clinical characteristics of MS patients and controls

MS patients	RRMS untreated Cohort 1	Natalizumab treatment (cross-sectional study) Cohort 2	Natalizumab treatment (longitudinal study) Cohort 3	SPMS Cohort 4	Healthy controls
n	22	16	24	26	24
Gender female/ male	14/8	6/10	19/5	17/9	17/7
Age[a] (years)	35.6 ± 8.4	33.3 ± 9.0	39.4 ± 8.2	47.6 ± 7.2	37.9 ± 8.7
Duration of disease[a] (years)	5.8 ± 6.6	4.3 ± 3.1	7.2 ± 5.8	7.5 ± 4.9	NA
EDSS[b] baseline	2.0 (1.0-4.6)	4.0 (3.0-5.5)	3.5 (2.6-4.3)	6.0 (5.2-6.5)	NA
EDSS[b] after treatment	NA	3.75 (2.0-5.3)[c]	2.7 (2.5-3.8)[c]	NA	NA
Time since last relapse[a] (months)	8.2 ± 7.4	11.8 ± 6.0	NA	NA	NA
NF-L antibody levels[d]	1.61 (1.5-1.7)	0.98 (0.3-1.3)	0.94 (0.7-1.1)[e]	1.21 (1.1-1.3)	0.81 (0.6-0.9)

[a]*Data are presented as mean ± SD.*
[b]*Data are presented as median and interquartile range 25th to 75th centile.*
[c]*12 months after treatment.*
[d]*Data are presented as median OD values of NF-L antibody levels and interquartile range 25th to 75th centile measured by ELISA at 450 nm.*
[e]*Baseline.*
EDSS, Expanded Disability Status Scale; SD, standard deviation; OD, optical density; NA, not applicable.

Antigen

Full-length recombinant mouse neurofilament light (rmNF-L) was produced as previously described (22). Briefly, the gene coding for the NF-L protein was amplified by polymerase chain reaction (PCR) using the primers 5'-CATATGAGTTCGTTCGGCTAC-3' and 5'-GGATCCTCAATCTTTCTTCTTAGC-3' comprising *Nde*I and *Bam*HI restriction sites respectively. The PCR product was digested and ligated in the expression vector pET15b (Novagen, USA). The pET15b plasmid was transformed into BL21:DE3 and the fusion protein was purified on Ni^{2+}-NTA-beads (Pharmacia, Uppsala, Sweden). Expression of the fusion protein was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG, VWR, The Netherlands). The supernatant containing rmNF-L was applied to a HiTrap Chelating HP column (GE Healthcare, UK) and the eluted fractions were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing rmNF-L, as determined by western blotting, were stored at 4°C until use. To assess the specificity of the antibodies to the rmNF-L protein we first examined responses to proteins applied to western blots. Antibodies raised to rmNF-L did not recognise NF-M and NF-H in Biozzi ABH mice spinal cord and brain tissues. The data revealed a band at 68 kDa, the molecular weight for NF-L, while no reactivity was observed

to NF-M (100 kDa) and NF-H (200 kDa) (Supp. Fig. 1). Monoclonal antibodies to rmNF-L were cross-reactive to NF-L in mouse and human CNS tissues.

ELISA
Serum samples from RRMS, SPMS and healthy donors were tested using ELISA. Briefly, Nunclon plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 10 µg/mL rmNF-L in carbonate buffer pH 9.6. Plates were washed in phosphate buffered saline (PBS) and blocked with 2% bovine serum albumin / PBS. After blocking, serum samples at 1:100 dilution were incubated for 1 h at room temperature. After washing in PBS-Tween 0.1%, the plates were incubated for 1 h at room temperature with horseradish-peroxidase (HRP)-conjugated goat anti-human IgG (Sigma, UK). The reaction was developed with 3,3',5,5'-tetramethel-benzidine (TMB) substrate (Thermo Fisher Scientific, UK) and stopped by the addition of 2 M hydrochloric acid. Results were normalised by subtracting the absorbance derived from uncoated wells and pooled serum samples. The absorbance was measured at 450 nm using a Synergy HT microplate reader (Bio-Tek instruments, VT). Cohorts 1, 2, 4 and healthy donors were analysed at the same time. Serial samples from cohort 3 were assessed in parallel.

Statistics
Comparisons between different groups of MS patients and controls were performed using the non-parametric Mann–Whitney U test. Box plots indicate the median, the interquartile range (25th to 75th percentile) and the 90th and 10th percentiles. P values <0.05 were considered as statistically significant. To discriminate the reactivity of different serum samples from MS patients against NF-L recombinant protein, receiver-operating characteristic (ROC) curves and area under the curve (AUC) were performed to calculate the optimal cut-off values (95% confidence interval (CI)) for sensitivity and specificity. The Spearman rank correlation coefficient was used to evaluate the relation between the levels of NF-L antibodies and clinical parameters. Statistical analysis was performed using SigmaPlot software 11 (Systat, San Jose, CA).

Results

Levels of NF-L antibodies are higher in patients in the relapsing-remitting stage of MS
Serum samples of MS patients, including RRMS and SPMS subjects (cohorts 1 and 4), were analysed with regard to the NF-L protein reactivity and compared with controls. The mean intra-assay coefficient of variation of optical densities was 9.5% for all samples measured. Box plots show the median and interquartile range (IQR) and the scatter plots show individual data and the mean optical density (OD) value. Levels of NF-L antibodies were significantly elevated in MS compared with healthy subjects ($p<0.001$). The median absorbance values for antibodies to NF-L were 1.61 (IQR 1.47 to 1.74) in RRMS and 1.21 (IQR 1.10 to 1.33) in SPMS patients and 0.81 (IQR 0.57 to 0.93) in healthy subjects (Fig. 1). RRMS had significantly higher serum levels of antibodies to NF-L than SPMS patients ($p<0.001$). No correlations between NF-L antibody levels and age were determined in the different groups of MS patients, RRMS ($r=-0.19$, $p=0.38$), SPMS ($r=-0.08$, $p=0.6$), or in the healthy controls ($r=-0.25$, $p=0.23$).

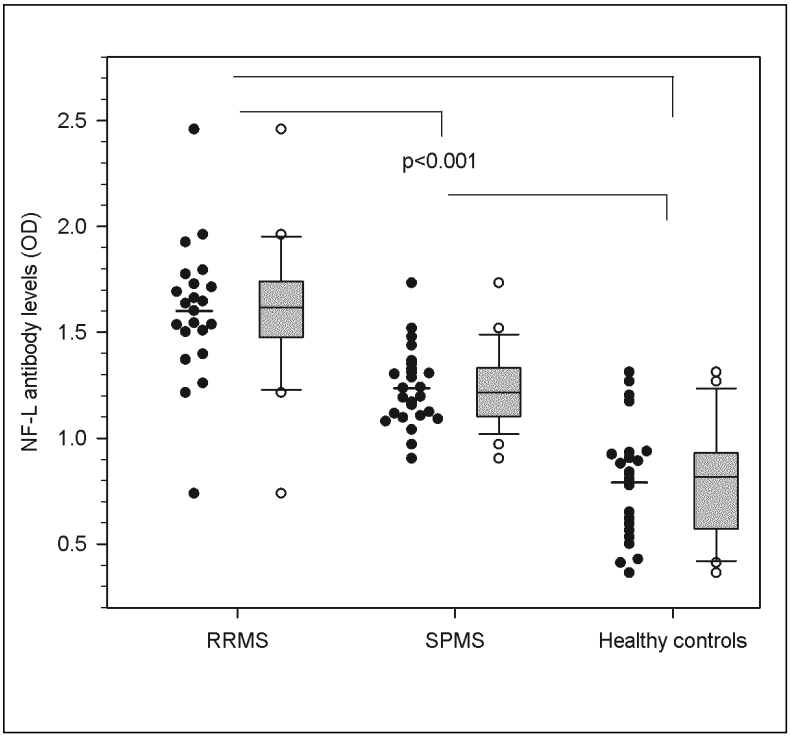

Figure 1. Levels of NF-L antibodies in MS patients at different stages of disease progression. *Samples from patients with RRMS (n=22) or SPMS (n=26) and healthy controls (n=24) were examined by ELISA at 450 nm. NF-L serum antibody levels were significantly higher in RRMS than SPMS (p<0.001). Analysis of individual groups of MS patients also showed significant differences in NF-L antibodies when compared to healthy controls (p<0.001). Box plots show the median OD values of NF-L antibody levels and IQR (25th to 75th centile) and whiskers indicate the 90th and 10th centiles. Dots represent individual samples and lines show the mean OD values.*

NF-L antibody levels do not correlate with neurological disability

The relationship between the level of NF-L antibodies and the Expanded Disability Status Scale (EDSS) scores was analysed in the different groups of MS patients. Median EDSS scores were 2.8 (IQR 1.5 to 4.9) in RRMS (cohorts 1 and 2), 3.5 (IQR 2.6 to 4.3) in RRMS (cohort 3) and 6.0 (IQR 5.2 to 6.5) in SPMS patients (cohort 4). Linear regression analysis showed no correlation between serum levels of NF-L antibodies and disability status in all groups assessed: r=-0.23, p=0.16 in RRMS (cohorts 1 and 2); r=-0.16, p=0.43 in RRMS (cohort 3) and r=-0.31, p=0.12 in SPMS (cohort 4) (Fig. 2). No correlation was found between NF-L antibody levels and disease duration.

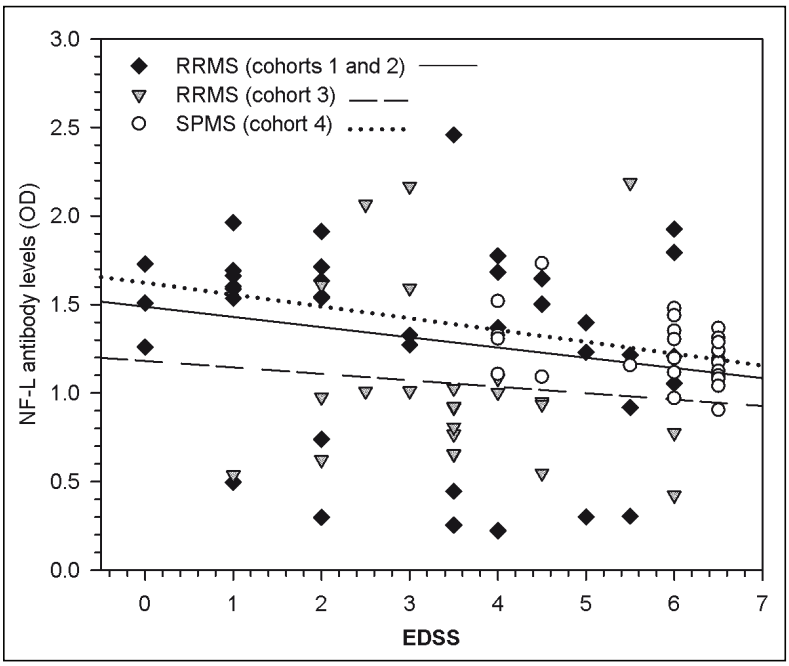

Figure 2. NF-L antibody levels vs neurological disability. *Serum NF-L antibody levels were analysed in the different groups of patients with various degrees of MS-related disability rated according to the EDSS. These serum samples were subjected to NF-L-specific ELISA and the results represent the optical density at 450 nm. Linear regression analysis showed no correlation between NF-L antibody levels and disability in the different groups of MS patients: r=-0.23, p=0.16 in RRMS (cohorts 1 and 2, n=38); r=-0.16, p=0.43 in RRMS (cohort 3, n=24) and r=-0.31, p=0.12 in SPMS (cohort 4, n=26).*

Levels of serum NF-L antibodies decrease in patients on treatment with natalizumab

We comparatively measured the levels of NF-L antibodies in natalizumab treated and untreated patients. NF-L antibody levels were significantly lower in treated than in untreated patients (p<0.001, Fig. 3A). The median absorbance values for antibodies to NF-L were 1.61 (IQR 1.47 to 1.74) in untreated and 0.98 (IQR 0.30 to 1.31) in treated patients. ROC analysis revealed a cut-off value of 1.34 with 86.36% sensitivity and 81.25% specificity in differentiating natalizumab treated from untreated patients. Treated MS patients showed a decrease in relapse rate post-natalizumab, from 3.2 ± 2.1 to 0.25 ± 0.4. The time from last relapse was 8.2 ± 7.4 and 11.8 ± 6.0 months for untreated and treated patients respectively. A negative correlation between time since last relapse and NF-L levels (r=-0.34, p=0.03) in RRMS patients was observed (Fig. 3B).

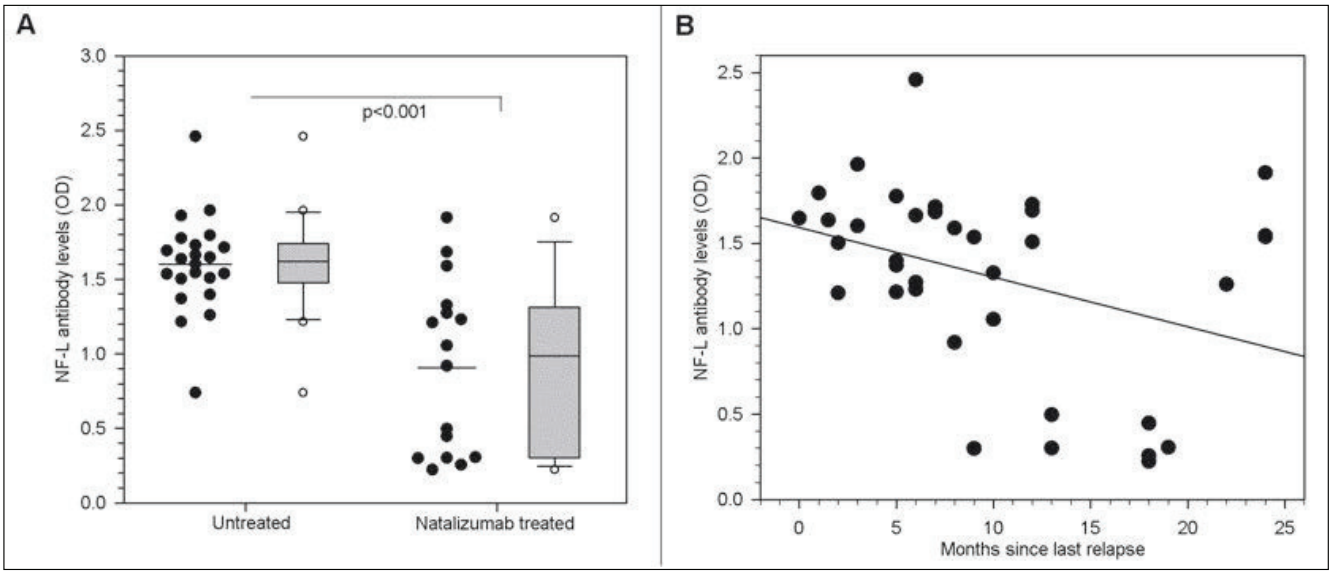

Figure 3. Levels of NF-L antibodies in MS patients treated with natalizumab. A*) Significant decreased levels of NF-L antibodies were determined in serum samples from RRMS patients following treatment with natalizumab (n=22), compared with patients that did not undergo treatment (n=16) (p<0.001). Box plots show the median OD values of NF-L antibody levels measured by ELISA at 450 nm and IQR (25th to 75th centile) and whiskers indicate the 90th and 10th centiles. Scatter plots represent individual samples and lines indicate the mean OD values.* ***B****) A negative correlation between time since last relapse and NF-L antibody levels in the total group of RRMS is shown (r=-0.34, p=0.03).*

We also monitored the expression of serum NF-L antibodies in MS patients during a follow-up period while on treatment with natalizumab to ascertain whether changes in the level of this biological marker can signal treatment response. Serum levels of antibodies against NF-L decreased over time from baseline values in longitudinal measurements performed in MS patients treated with natalizumab (Fig. 4A). The levels of NF-L antibodies started to decrease gradually during the follow-up measurements. After 24 months of treatment, a significant reduction of NF-L antibodies was observed (p<0.001). The median absorbance values for antibodies to NF-L were 0.94 (IQR 0.68 to 1.06) at baseline, 0.77 (IQR 0.58 to 1.07) at 6 months, 0.87 (IQR 0.54 to 1.08) at 12 months, 0.90 (IQR 0.45 to 1.28) at 18 months and 0.55 (IQR 0.37 to 0.74) at 24 months after natalizumab treatment. Individual patient analysis showed that in 86.6% of the patients the level of NF-L antibodies were diminished at some extent after 24 months of treatment compared with baseline

levels (p=0.001, Fig. 4B). All patients had at least one relapse in the year before starting natalizumab (mean 2 ± 1); while 80% of patients remained relapse-free in the first year of treatment (mean 0.21 ± 0.4 relapses) and 95.8% in the second year (mean 0.04 ± 0.2). Moreover, 12 months after the start of natalizumab treatment, 50% of patients showed a decrease in EDSS score average and a reduction of the number of contrast-enhancing lesions on MRI (1.79 ± 2.4 to 0.083 ± 0.28) compared with the contrast MR appearance before the initiation of treatment.

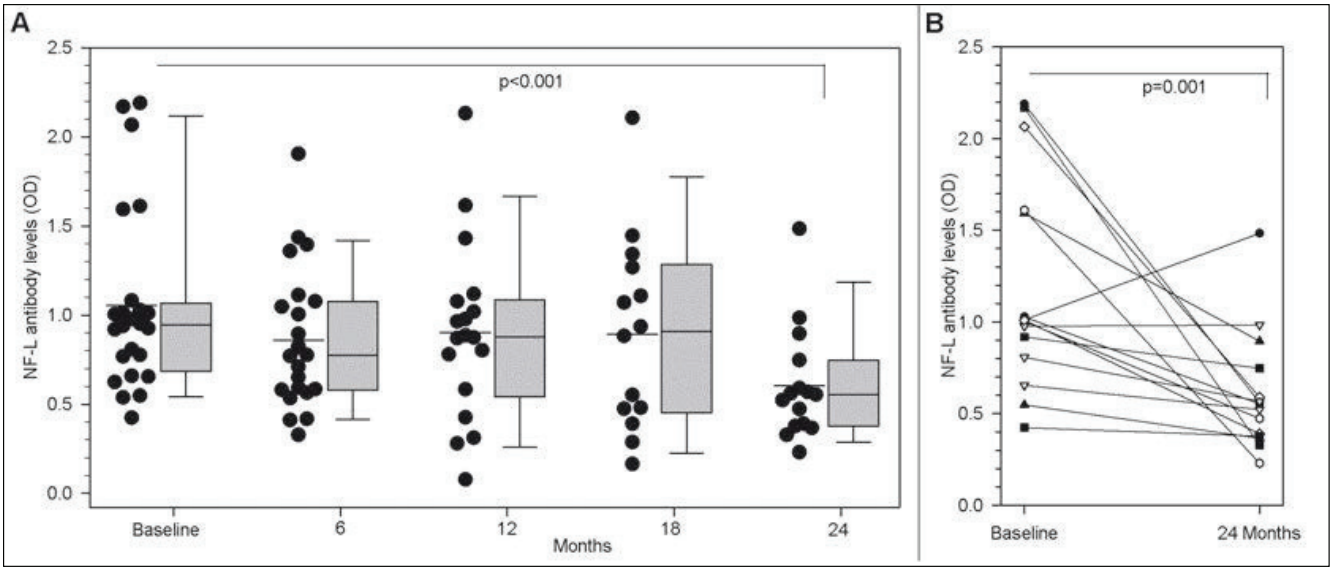

Figure 4. NF-L antibody levels compared at baseline and at different time points during the treatment with natalizumab. *The antibody responses to NF-L were evaluated in a follow-up study in RRMS patients that received treatment with natalizumab. OD was measured at 450 nm using NF-L-specific ELISA.* ***A****) Analysis of group responses at 0 (basal samples) (n=24), 6 months (n=23), 12 months (n=18), 18 months (n=14) and 24 months (n=15) after natalizumab treatment, showed a significant difference in NF-L-specific antibody responses following 24 months of treatment (p<0.001). Box plots show the median OD values of NF-L antibody levels and IQR (25th to 75th centile) and whiskers indicate the 90th and 10th centiles. Dots represent individual samples and lines show the mean OD values.* ***B****) Individual patient analysis showed that in 86.6% of the patients the level of NF-L antibodies decreases at some extent after 24 months of treatment compared to baseline levels (n=15), p=0.01.*

Discussion

Neurodegeneration is associated with altered expression and aberrant localisation of neuronal proteins that may be associated with a specific immune response directed against protein targets (23). The overexpression and accumulation of cytoskeletal components of neurons like NF-L proteins and the immune response against them may reduce the detection yield in most ELISA-based methods used for measurements in biological fluids (24).

Available data show that the release of neuronal antigens such as NF-L into the CSF of MS patients has a significant potential to become an informative biomarker for the monitoring of axonal damage and disease progression (3). While CSF remains the biofluid of choice because of its privileged anatomic location and for the search of by-products of neurodegeneration, it is clear that serial lumbar punctures to monitor disease progression or to evaluate treatment response are largely impractical, particularly in advanced and frail patients (25). In line with our work, current strategies for biomarker discovery and validation may progressively privilege easily accessible and cost-effective measurements in peripheral blood.

This is likely to be the most reasonable strategy as blood contains a wider variety of protein and cell immune mediators not only related to CNS pathology but also to systemic inflammation that may have an impact on the brain.
Our data show higher levels of NF-L antibodies in an early phase of MS, when the clinical development is dominated by relapses and remissions. In a later stage, when the clinical picture edges towards a more progressive disease, NF-L antibodies appeared to decline. Other studies show similar findings with increased levels of NF-L antibodies within an early MS (14), while higher autoantibodies targeting NF-L in PPMS and SPMS are also reported (13, 15). The shift from a relapsing-remitting to a secondary progressive disease mode is characterised by a possible change of the inflammatory environment and by a different degree of neuroaxonal loss (and of consequent neurofilaments release), possibly driving the differential expression of antibodies against NF-L observed in the two phases of MS. If the increase of antibodies against NF-L reflects the surge of NF-L antigens in biological fluids, then neuroaxonal degeneration must be a very early event in the development of MS, as reported in RRMS (26, 27). Since SPMS is a phase of irreversible accumulation of neurological disability, one would also expect a significant level of neurodegeneration, neurofilament pathology and immune response to it. The change of expression of antibodies against NF-L may instead be intrinsically linked to the type of immune response in each phase of the disease. In line with other studies, we found no association between EDSS scores and NF-L antibody levels (13). However, the observation that NF-L antibodies were negatively correlated with time since last relapse, suggests their possible biological role in the pathogenesis of the disease. In this study we also found differences in the levels of NF-L antibodies between the two RRMS groups (cohorts 1 and 3) that may result from previous multiple treatments administered to patients of cohort 3 before starting natalizumab, which is correlated with lower levels of NF-L antibodies (8).
Here, serum samples from MS patients on natalizumab were analysed longitudinally to evaluate the immune response to NF-L as a measure of response to treatment. Licensed treatments for MS using monoclonal antibodies show satisfactory results with regard to different parameters of disease progression in RRMS (28, 29). Natalizumab is the first α4 integrin antagonist in a new class of selective adhesion molecule inhibitors with anti-inflammatory and neuroprotective properties (30). Large-scale trials into the drug's efficacy in RRMS have disclosed a reduced risk of sustained progression of disability over 2 years (19). The release of NFs into biological fluids and the immune response against it are considered good biomarkers of axonal degeneration and disease progression. Thus, the levels of antibodies against NF-L might be a potential biomarker of treatment response complementary to other clinical and paraclinical assessments. In line with this working hypothesis, we show that antibodies against NF-L declined in a 2-year treatment period compared with their baseline levels. This decrease was associated with a reduction of disease burden as measured by functional rating scales and MRI. Interestingly, NF-L antibody levels in natalizumab treated MS patients from two different cohorts showed to be consistently lower than basal samples or untreated patients. The use of two independent groups of MS patients demonstrated a good correlation of the NF-L antibodies profiles. Although the benefit of the natalizumab treatment was achieved after 1 year of treatment, there seemed to be a time-dependent reduction of NF-L immunity in treated patients that is measurable after 24 months, suggestive of a possible cumulative effect of the treatment in the first 2 years or with a change in the avidity of these antibodies. Nevertheless, a decrease

in the level of NF-L antibodies was observed as early as 6 months after the initiation of the treatment. Individual analysis of the patients showed that most likely the changes in the levels of these antibodies are linked to previous or future relapses. Interestingly, the few patients that experienced a relapse after the first year of treatment showed an increased level of NF-L antibodies during this period that eventually decreased after 24 months. Our results are in line with studies reporting significant lower levels of NF-L protein in CSF from MS patients following treatment with natalizumab (31, 32) and reduction of antibodies to neurocytoskeletal proteins in MS patients receiving therapy (33). Current data also show that natalizumab treatment reduces the mean relapse rate, decreases the mean EDSS score and reduces contrast-enhancing lesions on MRI. Clearly, these parameters might be a reflection of the levels of both NF-L protein and antibodies to NF-L in the blood. These results support NF-L antibodies in serum as an easily measurable biomarker with a potential predictive value of disease activity and treatment outcome in MS which could be used along other clinical and paraclinical readouts of disease progression in this condition. Previously we showed that NF-L antibody levels in serum can be used to monitor disease progression in neurodegenerative diseases like ALS (16). Follow up studies in a large cohort of patients that allow a patient by patient analysis of the relationship between NF-L antibody levels and clinical response to treatment would be of interest for future studies.
In summary, data presented here provide supportive evidence that the level of serum autoantibodies to NF-L protein is a good indicator of disease activity in MS. Our study also adds information about the association between natalizumab therapy and the status of NF-L antibodies in serum. This finding may also be applicable to other neurological conditions characterised by axonal loss and immune response to NF-L release.

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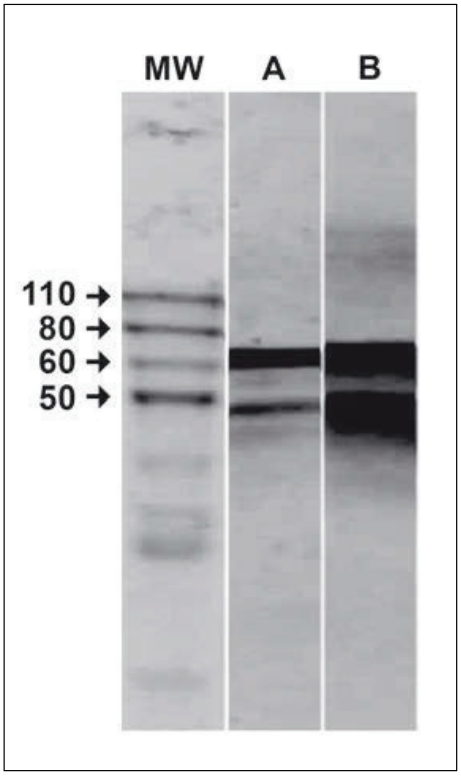
Conflict of interest
None declared.

Data sharing statement
Anonymised data about clinical samples are available upon request to the corresponding author.



***Supplementary Figure 1**. Serum from rmNF-L immunised Biozzi ABH mice was tested on spinal cord (**A**) and brain (**B**) homogenates from ABH mice. The western blot shows that immune serum react specifically to NF-L as judged by the correct molecular weight (68 kDa) and degradation products in the 45-50 kDa range. MW: molecular weight.*

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