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CSF levels of neurofilament and tau correlate with brain atrophy in natalizumab-treated multiple sclerosis

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Author contributions

J. Mellergård contributed to the design of the study, collection, analyses and interpretation of data, drafting and revision of the manuscript.

A. Tisell contributed to the design of the study, collection, analyses and interpretation of data, drafting and revision of the manuscript.

I. Blystad contributed with plaque contamination analyses of the ¹H-MRS data.

A. Grönqvist contributed to the analyses of brain volume change.

K. Blennow contributed with the analyses and interpretation of markers of neurodegeneration and revision of the manuscript.

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C. Dahle contributed to the design of the study, selection and examination of patients, interpretation of data and revision of the manuscript.

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J. Ernerudh contributed to the design of the study, interpretation of data, drafting and revision of the manuscript.

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Disclosure of conflict of interest

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Dr. Tisell has nothing to disclose.

Dr. Blystad has nothing to disclose.

Mr. Grönqvist has nothing to disclose.

Dr. Blennow reports personal fees from IBL International, personal fees from Fujirebio Europe, personal fees from Roche Diagnostics, personal fees from Eli Lilly, outside the submitted work.

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J. Ernerudh has nothing to disclose.

ABSTRACT

Background: Brain atrophy is related to clinical deterioration in MS, but its association to intrathecal markers of inflammation or neurodegeneration is unclear. We aimed to investigate if cerebrospinal fluid (CSF) markers of inflammation or neurodegeneration are associated with brain volume change in natalizumab-treated MS, and if this change is reflected in non-lesional white matter metabolites.

Methods: 25 patients with natalizumab-treated MS were followed for three years with assessment of percentage brain volume change (PBVC) and absolute quantification of metabolites with proton magnetic resonance spectroscopy (^1H -MRS). Analyses of inflammatory (IL1- β , IL-6, CXCL8, CXCL10, CXCL11, CCL22) and neurodegenerative (neurofilament light protein (NFL), glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), tau proteins) markers were done at baseline and 1-year follow-up.

Results: The mean decline in PBVC was 3% at 3-year follow-up, although mean ^1H -MRS metabolite levels in non-lesional white matter were unchanged. CSF-levels of NFL and tau at baseline correlated negatively with PBVC over three years ($r=-0.564$, $p=0.012$ and $r=-0.592$, $p=0.010$, respectively).

Conclusions: A significant 3-year whole-brain atrophy was not reflected in mean metabolite-change of non-lesional white matter. In addition, our results suggest that CSF-levels of NFL and tau correlate with brain atrophy development and may be used for evaluating treatment response in inflammatory active MS.

INTRODUCTION

Irreversible neuroaxonal damage is well established to constitute the pathological basis for progressive neurological decline in multiple sclerosis (MS)(1). The accumulation of neuroaxonal damage relates to brain atrophy development and measures of brain volume loss, as a proxy for brain atrophy, thus appear a promising method to assess risk for clinical deterioration and evaluate treatment response in MS(2, 3). However, there are aspects to be considered if measures of brain volume are used for disease monitoring. Firstly, a decline in brain volume is an end-stage phenomenon only to be detected retrospectively(4). Bio-markers that may predict such future atrophy development are therefore needed to make early intervention possible. Secondly, loss of brain volume due to the resolution of inflammatory edema during treatment, *i.e.* pseudoatrophy, may aggravate the interpretation of true coincident neuroaxonal loss(5). In this perspective, magnetic resonance spectroscopy (¹H-MRS) complements brain volume measurements regarding ongoing pathological processes in vivo.

In this longitudinal study, 25 patients with natalizumab-treated MS were followed for 3 years with MRI including ¹H-MRS metabolite concentrations in normal appearing white matter (NAWM) and measurements of percentage brain volume change (PBVC). The main aim was to investigate if intrathecal markers of neurodegeneration or inflammation were associated with change in brain volume. As a complement to the evaluation of brain volume change, we also aimed to study NAWM ¹H-MRS-spectra investigating possible reflections of brain volume loss at the metabolite level.

METHODS

Patients. Natalizumab treatment (300 mg IV once a month) was initiated in 25 patients with relapsing MS according to general guidelines based on clinical and MRI parameters (Table 1). The study was observational and set up in a clinical setting and for ethical reasons there was no placebo-treated group. All included patients fulfilled the McDonald criteria for MS(6) and were consecutively recruited from the Department of Neurology at the University Hospital, Linköping. MRI-examinations were performed before starting treatment (mean 1.6 ± 2.0 (SD) months), *i.e.* 'baseline', and after 1-year of treatment (mean 12.6 ± 1.1 (SD) months), and after three years of treatment (mean 37.2 ± 1.2 (SD) months). 11 patients experienced a relapse within 3 months prior baseline MRI. The 3-year follow-up MRI was done at least 3 months after latest relapse. CSF sampling was obtained before start of natalizumab treatment (mean 1.6 ± 2.5 (SD) months) and after 1-year of treatment (mean 12.4 ± 1.4 (SD) months). Neurological examination was done by a neurologist (MV, CD or JM) including definition of EDSS at baseline and at 1- and 3-year follow-up. From the patient cohort in this study the effect of 1-year

natalizumab treatment on CSF cytokine and chemokine levels (9 patients)(7) and on CSF levels of neurodegenerative markers (20 patients)(8) has been published before. Baseline and 1-year follow-up data on ¹H-MRS and clinical scoring after natalizumab treatment (23 patients) have also been published previously(8). The study was approved by The Regional Ethics Committee in Linköping (D nr M180-07 T130-09) and written consent was obtained from participants.

INSERT TABLE 1 ABOUT HERE

CSF biomarkers. Handling of CSF, and assessment of cytokine concentrations using a multiple bead kit (Invitrogen® Cytokine Ultrasensitive Human 10-plex Panel) was done as previously described(7). An in-house assay was used to measure concentrations of CXCL10, CXCL11 and CCL22(7). The samples were analysed on a Luminex¹⁰⁰ instrument (Biosource, CA, USA), and data acquired using the StarStation 2.0 software (Applied Cytometry Systems, Sheffield, UK). Cytokine and chemokine values under the detection limit were assigned to half the value of the lowest standard point. CSF myelin basic protein (MBP) was analysed using a sandwich ELISA (Active MBP ELISA, Diagnostic Systems Laboratories Inc., Webster, TX). The lower limit of detection for this assay was 0.1 ng/mL. CSF total tau (tau) and tau phosphorylated at threonine 181 (P-tau) were determined using the Luminex xMAP technology and the INNOBIA AlzBio3 kit (Innogenetics Zwijndrecht, Belgium) as previously described(9). CSF neurofilament light protein (NFL) and glial fibrillary acidic protein (GFAP) were analysed using previously described ELISAs(10, 11).

MRI data acquisition. All examinations were performed using an Achieva 1.5 T MR scanner (Philips, Best, The Netherlands), and an eight channels SENSE head coil. A standard MRI protocol with T₂ weighted (T2W) and T₁ weighted (T1W) sequences including pre- and post-gadolinium (GD) and fluid attenuation inversion recovery (FLAIR) images was used for clinical radiological evaluation by a neuroradiologist (IB). Acquisition of R₁, R₂ (1/T2), PD and placement of ¹H-MRS-voxels were done as previously described(8) (Figure 1). In the initial phase of the project (for the first 24 patients at baseline, and the first 9 patients at the 1-year follow-up) a single MRS voxel was acquired. In a later phase of the project (for the last 3 patients at baseline, the last 18 patients at the 1-year follow-up and for all 25 patients at 3-year follow-up), the examination time allowed for a second MRS voxel, which was added and placed using the same criteria, but lateral and opposite to the first MRS voxel.

Absolute quantification of MRS data. The MRS spectra were analysed using LCModel ver 6.2-4G (S. Provencher, Canada)(12), with a spectral basis set obtained from Dr M. Ljungberg at Sahlgrenska Academy (Gothenburg, Sweden). Absolute aqueous fraction concentrations mMaq were estimated using the method described previously(13). The internal water signal was used as an internal reference. The metabolites total creatine (tCr), total choline (tCho), *myo*-Inositol (*mIns*), total N-acetylaspartate (tNA), the sum of glutamate and glutamine (tGlx) were analysed(14) (Figure 1). In order to avoid bias towards high concentrations, no measurements were excluded from the subsequent statistical analysis based on the Cramer Rao low bounds estimation in the LCModel analysis(15). The spectra were visually inspected for improper residuals and large artifacts in the spectral region of interest (4.0-0.2 ppm). However, no spectra were excluded due to such residuals or artifacts.

INSERT FIGURE 1 ABOUT HERE

Calculation of percentage brain volume change (PBVC). PBVC calculations were based on segmentation of the axial FLAIR volume. Firstly, we carried out a registration of the FLAIR volumes for the different time points, using Analyze ver 10 (AnalyzeDirect, USA). The Analyze software was also used to cut border slices that differed after the registration. Secondly, the parenchymal tissue and lateral ventricles were segmented using the 'Image Foresting Transform' (IFT)(16). Thirdly, the parenchymal volume was calculated as the subtraction between segmented brain volume including ventricles minus segmented ventricular volume. To calculate PBVC, the difference between parenchymal volumes at time points was normalized to baseline parenchymal volumes.

Statistics. Pairwise comparisons of clinical scoring variables were analysed using Wilcoxon signed rank test. Pairwise comparisons of ¹H-MRS metabolites and MRI-variable R₂ were analysed using paired samples t-test. Differences in PBVC (baseline compared with 1- and 3-year follow-up) were calculated using one sample t-test. Differences in PBVC at 1- and 3-year follow-up between patients with or without GD+ lesions at baseline MRI were analysed using unpaired samples t-test. Correlation analyses between intrathecal markers and change in ¹H-MRS metabolites or PBVC were performed using Spearman correlation coefficient. A possible association between age and levels of NFL, tau or PBVC was investigated using Spearman or Pearson correlation coefficients, respectively. Correlation analyses between PBVC and ratios of ¹H-MRS metabolites were done using Pearson correlation coefficient. Significance values for correlation coefficients are reported without correction for multiple comparisons to avoid type II errors(17). All statistical calculations were performed in

Statistica 12 software (Statsoft Inc., OK, USA) or Graphpad Prism 5 (GraphPad software Inc., CA, USA). $p < 0.05$ was considered statistically significant.

RESULTS

Clinical data. During the 3-year follow-up time there were in total 12 relapses among 6 patients (Table 2). Steroid treatment (oral) was given only to the patient that had in total 5 relapses (at every relapse). At 1-year follow-up only one patient had a relapse within 6 months prior to the follow-up sample. The mean annual relapse rate during the whole 3-year follow-up period was 0.15 (12 relapses/77.5 person-years). There was an improvement in clinical scoring variables during the follow-up time and EDSS remained unchanged (Table 2).

INSERT TABLE 2 ABOUT HERE

¹H-MRS metabolite concentrations. Mean ¹H-MRS-metabolite concentrations were stable during the whole 3-year follow-up period (Table 3). Since the ratio between *m*Ins and tNA in NAWM has been proposed as a predictive measure of brain volume loss(18), we calculated ratios of these metabolites at baseline, 1- and 3-year follow-up. No difference in these ratios was however found (data not shown). *m*Ins/tNA ratios were then tested for correlations versus PBVC from baseline to 1- and 3-year follow-up, respectively. However, no correlations were found (data not shown). The free water content in examined voxels, represented by R_2 , did not change from baseline to 1- or 3-year follow-up (data not shown). Data on ¹H-MRS-metabolite concentrations at baseline and 1-year follow-up has previously been published for this patient cohort(8). In order to evaluate our previous findings in this cohort, *i.e.* an association between 1-year change in ¹H-MRS metabolites and levels of markers of intrathecal inflammation(8) we extended the correlation analyses to the total follow-up period of three years. However, no correlation between levels of cytokines or chemokines after 1-year of natalizumab treatment and change in ¹H-MRS metabolite concentrations between one to three years of treatment was found (data not shown).

INSERT TABLE 3 ABOUT HERE

MRI data and PBVC. In 23 of 25 patients data on gadolinium enhanced (GD+) lesions at baseline were available. Seven of these 23 patients (30%) showed GD+ lesions at baseline. At one and three years of treatment no patient showed GD+ lesions (data on 25 patients was available for both occasions). Five patients (20%) showed either at least one new appearing or enlarged lesion on T2W at 1-year follow-up. At 3-year follow-up, 6 patients (24%) showed either at least one new or enlarged lesion on T2W. There was a significant brain volume

loss as measured by a decrease in PBVC between baseline and 3-year follow-up (mean $97.0\% \pm 2.1$ (SD)); thus a mean difference in PBVC compared with baseline of -3.0% , $p < 0.0001$, Figure 2A). There was also a significant decrease in PBVC already at 1-year follow-up compared with baseline (mean $98.2\% \pm 1.6$ (SD)); thus a mean difference in PBVC compared with baseline of -1.8% , $p < 0.0001$, Figure 2A). When excluding patients with relapses during follow-up, similar findings were obtained, the mean PBVC from baseline to 1-year changed to 98.1% (excluding 2 patients) and from baseline to 3-year follow-up changed to 96.7% (excluding 6 patients). There was no difference in PBVC during follow-up comparing patients with or without new or enlarged lesions on T2W.

Based on the presence of GD+ lesions at baseline, the evolution of PBVC from baseline to 3-year follow-up was then studied. No statistically significant differences in PBVC at 1- or 3-year follow-up according to the presence or absence of GD+ lesions at baseline were however found, although patients with GD+ lesions at baseline tended to decrease more in PBVC than patients with no baseline GD+ lesions ($p=0.078$, Figure 2B).

INSERT FIGURE 2 ABOUT HERE

PBVC and intrathecal markers of inflammation and neurodegeneration. Correlation analyses showed that NFL at baseline correlated with PBVC during the 3-year follow-up period ($r=-0.564$, $p=0.012$, Figure 2C). Furthermore, NFL-levels at 1-year follow-up and PBVC the following two years tended to correlate ($r=-0.409$, $p=0.073$, $n=20$). Levels of tau at baseline correlated with PBVC at 3-year follow-up ($r=-0.592$, $p=0.010$, Figure 2D) and 1-year follow-up ($r=-0.607$, $p=0.008$, $n=18$). Excluding patients with relapses during follow-up yielded similar results in the correlation analyses as in the whole patient group. As to baseline NFL and PBVC during the 3-year follow-up (excluding 3 patients), showed a tendency to a correlation ($r=-0.492$, $p=0.053$, $n=16$). For baseline tau, the same analysis (excluding 4 patients) showed a correlation versus PBVC over three years ($r=-0.634$, $p=0.015$, $n=14$). There was no correlation between age on one hand and NFL, tau or PVBC on the other hand (data not shown). Levels of CXCL11 at 1-year follow-up correlated with PBVC the following two years ($r=-0.4502$, $p=0.046$, $n=20$). No other significant correlations between CSF-levels of markers of inflammation (IL-1 β , IL-6, CXCL8, CXCL10, CXCL11, CCL22) or neurodegeneration (GFAP, MBP, tau, P-tau) at baseline or 1-year follow-up versus corresponding PBVC at 1- or 3-year follow-up were found (data not shown).

PBVC and change in ^1H -MRS metabolites. PBVC from baseline to 1- and 3-year follow-up correlated with change in tNA concentration during the same time period ($r=-0.403$, $p=0.046$ and $r=-0.522$, $p=0.008$, respectively, Table 4). There was also a correlation between PBVC from 1- to 3-year follow-up versus change in MRI-variable R_2 ($r=0.518$, $p=0.008$, Table 4) as well as a tendency for a correlation between PBVC and change

in R_2 over three years ($r=0.375$, $p=0.065$, Table 4). No other correlations between changes in ^1H -MRS metabolite concentrations and corresponding PBVC were found (Table 4).

INSERT TABLE 4 ABOUT HERE

DISCUSSION

This paper is the first to report a 3-year longitudinal study on brain volume change in MS and its association to markers of neurodegeneration and inflammation as well as NAWM metabolite levels. Assessment of metabolite concentrations in NAWM were used as a complement to brain volume measures to analyse possible parallel ongoing *in vivo* processes during the follow-up time. We show that brain volume loss continues throughout the 3-year follow up period despite an effective anti-inflammatory treatment with natalizumab(7), but the cohort size did not permit well-powered true multivariate modelling of this brain volume outcome. Parallel with this brain volume loss, ¹H-MRS metabolites in NAWM were stable. Our results indicate that CSF-levels of NFL and tau are associated with the extent of future brain volume loss in inflammatory active MS.

The total brain volume decrease over three years was 3 %, as measured by the decline in PBVC. The loss was most evident (-1.8%) during the first year of treatment, and slightly more pronounced when excluding patients with relapses during follow-up. The latter finding suggests that a decline in PBVC may be alleviated by relapse-related edema. The rate of brain volume loss in normal aging is estimated to be 0.1-0.3 % per year and in MS 0.5-1.35 % per year(2, 19). There is only one previous report on natalizumab-treated MS-patients followed for three years, showing a total mean decline in PBVC of 2.1% during the 3-year follow-up(20). In addition, there are two reports on PBVC in natalizumab-treated MS where patients were followed for 18 months, showing a mean decrease in PBVC of -1.18 % and -2.5 %, respectively(21, 22). Taken together, our data are well in line with earlier studies, although results were not identical. A main explanation to variations is probably patient heterogeneity since age, disease duration and baseline EDSS differed between studies. However, a consistent finding in all studies (including our) was the pattern of an accentuated brain volume loss during the first year of treatment. This rapid initial brain volume change is considered to represent non-tissue related brain volume loss (pseudotrophy), in particular resolution of edema, although it may mask ongoing “true” concurrent tissue-related brain volume loss (as loss of myelin, axons and glial cells). The inflammatory edema linked to pseudotrophy is suggested to originate mainly from focal white matter lesions, whereas tissue-related brain volume loss is proposed to be a diffuse process affecting both white and gray matter(23). In accordance with this view, we found no changes in ¹H-MRS metabolite concentrations or in free water content (R_2) in NAWM at 1-year follow-up. This indicates that neither changed metabolite composition nor water content in NAWM could account for brain volume loss linked to pseudotrophy. During the follow-up period of three years, the predominant non-tissue related brain volume loss during the first year of treatment is likely replaced by brain

volume loss due to tissue-related damage, *i. e.* irreversible neuroaxonal damage. Since mean metabolite concentrations in NAWM were unchanged during the entire follow-up period, tissue-related brain volume loss in our cohort did not seem to take place in NAWM. Collectively, our data indicate that the observed decline in brain volume, irrespective of tissue-related or linked to pseudoatrophy, is not NAWM-associated but may instead be explained by processes affecting lesional white matter or possibly grey matter. The view of lesional white matter being affected is supported by an autopsy study on global brain pathology showing that patients with relapsing MS (as in our present study) mainly presented new and active focal demyelinating lesions in white matter, while patients with progressive MS showed a diffuse inflammatory reaction in NAWM as well as cortical demyelination(24).

Correlation analyses showed that a higher rate of brain volume loss was associated with increasing tNA concentrations in NAWM. The explanation to this observation may be that the viability of axons (reflected by tNA levels) increases with the resolution of edema in adjacent or distant lesions. This is supported by the fact that concentrations of tNA may fluctuate over time and thus a decline in tNA levels could indicate a possible reversible axonal dysfunction rather than a permanent axonal loss(25).

The potentially most useful finding in this study was the association between brain volume loss over three years and elevated levels of NFL and tau in CSF at baseline. Neurofilaments are major components of axonal cytoskeleton proteins. Inflammatory demyelination may cause disruption of axonal integrity and release of neurofilaments in the extracellular fluid. CSF-levels of NFL, the smallest of three neurofilament chains, has thus been linked to inflammation and ongoing axonal damage(26-28). Tau protein is primarily found in neuronal structures and promote axonal stability and intraneuronal transport(29). Combined analysis of neurofilament and tau has been shown to predict conversion from clinically isolated syndrome to MS (30). As with NFL, tau protein is considered to be released into CSF after axonal damage. However, in contrast to NFL(8, 27, 31), data on tau in MS are inconsistent(32, 33). In line with our previous reported findings in this cohort (8), mean levels of NFL decreased (897 to 351 ng/L, $p=0.0002$) whereas mean levels of tau were almost identical (44.2 to 44.3 ng/L, $p=0.049$) after 1-year natalizumab treatment. At 1-year follow-up only one patient increased in NFL levels and 6 patients increased in tau levels, but none of these patients had a relapse during the first year of treatment. We here report that CSF levels of NFL and tau were associated with long-term brain volume loss. This finding implicates that these markers reflect the baseline level of intrathecal inflammation with axonal damage that

within three years account for tissue-related brain volume loss, *i. e.* brain atrophy. In this perspective levels of NFL and tau are associated with axonal damage secondary to inflammation, suggesting that they may be used as markers for response of anti-inflammatory treatment.

To conclude, this 3-year longitudinal study of natalizumab-treated MS show that despite unchanged metabolite levels in NAWM, there is a continuous significant brain volume loss throughout the 3-year follow-up time. Furthermore, levels of NFL and tau in CSF were associated with the rate of brain volume loss indicating that NFL and tau may be used as markers for brain atrophy development and possibly also for evaluating anti-inflammatory treatment response in MS.

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FIGURE LEGENDS

Figure 1

In the left column typical proton magnetic resonance spectroscopy (^1H -MRS) voxel of interest (VOI) placements are showed on a sagittal FLAIR image, for baseline, 1-year follow-up and 3-year follow-up. In the right column corresponding spectra are presented. *mIns* = *myo*-Inositol; tCho = total choline; tCr = total creatine; Glx = the sum of glutamate and glutamine; NAAG = N-acetylaspartylglutamate; NAA = N-acetylaspartate. The sum of NAAG and NAA constitute tNA = total N-acetylaspartate.

Figure 2

Percentage brain volume change (PBVC) from baseline to follow-up after three years of natalizumab treatment. $n=25$ (A). PBVC at 1- and 3-year follow-up according to presence of gadolinium enhanced lesions at baseline. 1 y GD+/- represents PBVC at 1-year follow-up and 3 y GD+/- represents PBVC at 3-year follow-up (B). Mean values are shown and errors bars represent SD.

Correlation analysis between NFL levels at baseline and PBVC from baseline to 3-year follow-up (C), and between tau levels at baseline and PBVC from baseline to 3-year follow-up (D). A linear regression with 95 % confidence interval is shown. Open dots represent patients with relapses during follow-up. r =Spearman correlation coefficient.

Baseline

1-year follow-up

3-years follow-up

A **B**

	GD- (n=18)	GD+ (n=7)	p
Baseline	100	100	
1-year follow-up	98.5±1.6	97.6±1.4	0.195
3-year follow-up	97.4±2.0	95.8±1.8	0.078

C

D

Table 1 Patient characteristics at baseline

No. of subjects	25
Median age, years (range)	38 (22-47)
Sex (M/F)	11/14
Median disease duration, years (range) ^a	9.3 (0.9-23)
Diagnosis (RRMS / PRMS)	23/2
EDSS (no. of subjects)	
0-3.5	22
4.0-5.5	1
6.0-7.0	2
Median EDSS	2.5
Treatment ^b (no. of subjects)	
Interferon- β	20
Glatiramer acetate	1
No treatment	4
Median number of relapses last two years (range)	2 (0-8)
No. of patients with relapse within last month before baseline sample/no. of patients treated with steroids	4/4
GD+ lesions at baseline (no. of patients) ^c	
0 lesion	16
1 lesions	5
3 lesions	1
6 lesions	1

^a Median number of years from first symptoms of MS to inclusion. ^b Treatment within 3 months before the baseline CSF sample. ^c No. of gadolinium enhanced lesions at baseline MR, n=23 because lack of data in two patients. M/F = male/female; RRMS = relapsing-remitting MS; PRMS = progressive MS with superimposed relapses; EDSS = Expanded Disability Status Scale.

Table 2 Clinical data at baseline and after one and three years of natalizumab treatment

Clinical parameters	Baseline ^a	1-year follow-up ^a	3-year follow-up	p
EDSS	2.5 (0-7)	2.0 (0-6.5)	2.5 (0-6.5)	0.307
MSSS	3.9 (0.2-8.9)	3.1 (0.2-7.9)	3.0 (0.1-7.6) ^b	0.009
MSIS-29				
physical	2.2 (1.0-4.4)	1.5 (1.0-4.2)	1.7 (1.0-4.1) ^b	0.012
psychological	2.1 (1.0-4.6)	1.6 (1.0-4.6)	1.8 (1.0-3.7) ^b	0.003
SDMT	49 (24-69)	53 (3-74)	56 (39-71) ^c	0.001
Cumulative no of relapses during follow-up (no. of patients)				
0 relapse	NA	23	19	NA
1 relapse	NA	1	4	NA
2 relapses	NA	1	0	NA
3 relapses	NA	0	1 ^d	NA
5 relapses	NA	0	1 ^e	NA

Median values are given and range within parenthesis. p refers to Wilcoxon signed rank test comparing baseline and 3-year follow-up. n = 25 unless stated otherwise. ^a For 23 out of 25 patients, clinical data at baseline and 1-year follow-up have been published before (Mellergard et al 2012). ^b n=22, ^c n=21. ^d This patient had a discontinuation of treatment for 2 months after 22 months of natalizumab treatment (because of conversion to positive JC-virus serology). ^e This patient had a discontinuation of treatment for 4 months after 20 months of natalizumab treatment (because of suspicion of lack of effect). After discontinuation of natalizumab this patient was started on fingolimod therapy after a three months washout period, but because of side effects and recurrence of neuroinflammation, treatment with natalizumab was re-started after 2 weeks. CSF = cerebrospinal fluid; EDSS = Expanded Disability Status Scale; MSSS = Multiple Sclerosis Severity Score; MSIS-29 = Multiple Sclerosis Impact Scale; SDMT = Symbol Digit Modalities Test.

Table 3 MRS metabolite levels and MRI variable R2 at baseline and after one and three year of natalizumab treatment

	Baseline (n=25)		1-year follow-up (n=25)		3-year follow-up (n=25)		Change during treatment (baseline to 3-year follow-up)			
	Mean	SD	Mean	SD	Mean	SD	Mean diff	CI		p
tNA	11.93	1.38	11.65	1.18	11.96	1.26	0.03	-0.29	0.36	0.841
tCr	6.98	0.54	6.93	0.44	6.81	0.42	-0.18	-0.40	0.04	0.110
tCho	2.85	0.37	2.83	0.23	2.87	0.29	0.03	-0.07	0.12	0.553
<i>m</i> Ins	7.14	1.18	7.14	1.26	7.05	1.24	-0.09	-0.58	0.39	0.694
tGlx	12.62	2.16	12.46	2.16	12.13	1.47	-0.50	-1.56	0.57	0.344
R ₂	11.11	0.70	10.99	0.55	10.97	0.60	-0.14	-0.36	0.07	0.178

3-year mean difference in metabolite concentrations are presented with a 95% confidence interval (CI) and p values were calculated using paired samples t-test. All concentration values are presented in units of mM aq. tNA = total N-acetylaspartate; tCr = total creatine; tCho = total choline; *m*Ins = *myo*-inositol; tGlx = the sum of glutamate and glutamine; R₂ = MRI variable 1/T2.

Table 4 Correlation analysis between percentage brain volume change (PBVC) and ¹H-MRS metabolite change

PBVC (%)	¹ H-MRS metabolite change (Δ)						
Baseline to 1-year follow-up	Baseline to 1-year follow-up						
		ΔtNA	ΔtCr	ΔtCho	Δ <i>m</i> Ins	ΔtGlx	ΔR ₂
	r	-0.40	-0.10	0.01	-0.39	-0.01	0.27
	p	0.046	0.618	0.950	0.057	0.970	0.184
	1-year to 3-year follow-up						
1-year to 3-year follow-up		ΔtNA	ΔtCr	ΔtCho	Δ <i>m</i> Ins	ΔtGlx	ΔR ₂
	r	0.11	0.23	-0.34	0.33	0.25	0.52
	p	0.606	0.266	0.097	0.112	0.219	0.008
Baseline to 3-year follow-up	Baseline to 3-year follow-up						
		ΔtNA	ΔtCr	ΔtCho	Δ <i>m</i> Ins	ΔtGlx	ΔR ₂
	r	-0.52	0.07	-0.38	0.09	-0.02	0.38
	p	0.008	0.748	0.062	0.662	0.942	0.065

Pearson correlation coefficients are used. Significant correlations in bold. n=25. tNA = total N-acetylaspartate; tCr = total creatine; tCho = total choline; *m*Ins = *myo*-Inositol; tGlx = the sum of glutamate and glutamine; R₂ = MRI-variable (1/T₂).