ABSTRACT

Background: Brain volume loss (BVL) is a key outcome in MS trials. Natalizumab is highly effective on inflammation with moderate impact on atrophy.

Objective: To explore BVL in patients receiving natalizumab with an emphasis on grey matter (GM).

Methods: We performed a retrospective post-hoc analysis of BVL in 38 patients receiving natalizumab for 3 years using longitudinal Voxel-Based-Morphometry (VBM) and Freesurfer.

Results: Significant BVL was observed during first year: Brain-Parenchymal-Fraction (BPF): -1.12% ($p<0.001$); White-Matter-Fraction (WMF): -0.9% ($p=0.001$); Grey-Matter-Fraction (GMF): -1.28% ($p=0.002$). GM loss was found using VBM in bilateral cerebellum, cingulum, left>right fronto-parietal cortex, right>left hippocampus, and left caudate. Freesurfer showed significant volume losses in subcortical GM, brainstem, and cerebellum, and cortical thinning in the left insula. In the second year only WMF decrease (-0.6%; $p=0.015$) was observed with no VBM changes, although Freesurfer, detected significant volume loss in thalamus, hippocampus and cerebellum. Baseline gadolinium-enhancement influenced WMF and BPF changes during the first year, but not GMF. Patients with confirmed EDSS worsening at 3 years had lower baseline GMF and left thalamus volume, and greater BVL over follow-up.

Conclusion: BVL develops mainly during the first year of natalizumab therapy. GM changes are independent of baseline inflammation and correlate with disability.

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INTRODUCTION

Magnetic Resonance Imaging (MRI) is the main paraclinical tool for diagnosis, prognosis and monitoring treatment response in Multiple Sclerosis (MS), and a key outcome measure in clinical trials[1]. MRI provides indications on demyelinating, inflammatory and neurodegenerative processes[2], and it can be used as surrogate for clinical events[3]. MRI-derived brain volume loss (BVL) can be used as a marker of neurodegeneration, is detected in the early stages of the disease[4, 5] and is associated with long term disability[6]. Grey matter (GM) damage, a key component of neurodegeneration[7], has also shown significant associations with disability[8-10].

Clinical trials have reported positive effects on BVL of some drugs but not others[11] and a recent meta-analysis showed an additive effect of BVL on active T2 lesions to predict the impact of treatment on disability progression at 2 years[12].

Natalizumab has a potent anti-inflammatory effect[13, 14] but its impact on neurodegeneration outcomes, such as BVL, has not been convincingly shown[13,15], probably due to a pseudoatrophy effect mostly affecting white matter (WM)[16].Thus, to better delineate the impact of natalizumab on neurodegeneration, the investigation of GM volume and its correlation with clinical outcomes such as disability is warranted.

The present study aims to explore longitudinal global and regional brain volume changes in MS patients receiving natalizumab for at least 36 months. Cortical and subcortical GM volumes and Cortical Thickness (CTh) were determined with the FreeSurfer software and the Voxel Based Morphometry (VBM) approach implemented in the Statistical Parametric Mapping (SPM) software was used. A description of such changes will be complemented with clinical and radiological correlations, with an emphasis on disability progression.

METHODS

Clinical Data

A consecutive cohort of MS patients receiving natalizumab 300mg IV every four weeks for at least 36 consecutive months as of December 2013 was selected. Clinical assessments were performed three-monthly by a trained neurologist including MS relapses, progressive multifocal leukoencephalopathy symptoms and EDSS. Confirmed EDSS worsening was defined as an increase of 1 point when baseline EDSS <5.5 or an increase of 0.5 point if baseline EDSS $\geq$5.5, sustained up until the 36 month follow-up time-point.

The study was approved by the local ethics committee and the patients signed an informed consent.

MRI Data

MRI Acquisition

Patients underwent MRI within three months of natalizumab initiation (baseline) and at 12, 24 and 36 months of follow-up. Images were acquired in a 1.5T scanner (Siemens Symphony, Erlangen, Germany) using an MS protocol including: pre- and post-gadolinium (Gd) T1-weighted (2D-T1) and Magnetization-Prepared Rapid Acquisition Gradient Echo (MPRAGE) images. (*MRI Appendix)*.

WM lesion delineation and MPRAGE masking

Hypointense lesions on 2D-T1 images were visually identified and marked-off by a trained neurologist using a semi-automated edge-finding tool (Jim Version 6.0, Xinapse Systems, Northants, UK; http://www.xinapse.com). Binary lesion-masks for each patient were created using JIM6 and the lesion volume (LV) was calculated. 2D-T1 images (and corresponding lesion masks) were co-registered, to the MPRAGE using SPM (SPM8, Wellcome Department of Cognitive Neurology, London, UK, http://www.fil.ion.ucl.ac.uk/spm). The co-registered lesion-masks were visually checked for errors and used in the segmentation step to prevent misclassification of WM lesions in the MPRAGE images.

VBM: Longitudinal processing

Longitudinal processing stream implemented in VBM8 was used for assessing GM changes between time points. Briefly, all images of each subject were co-registered to correct for differences in position and distribution of intensity non-uniformities. Spatial normalization parameters were estimated for the baseline image only and applied to all images[17]. Segmentation of GM, WM, cerebrospinal fluid -CSF-, lesion masks, and modulation were performed. Finally, smoothing using a 10mm full width at half maximum Gaussian kernel was performed prior to statistics. Global, tissue-specific volumes as well as the corresponding fractions (GMF=GM/TIV, WMF=(WM+LV)/TIV, BPF=(GM+WM+LV)/TIV) were also obtained. The percentage of GMF, WMF and BPF change between time points was derived from the following formula:

100*[TissueFraction(Timepoint1)-TissueFraction(Timepoint2)/TissueFraction(Timepoint1)])

FreeSurfer

Global and regional CTh as well as cortical and subcortical GM volume measures were obtained at baseline, 12, 24 and 36 months of follow-up on the MPRAGE images by means of the longitudinal stream[17] included in the FreeSurfer analysis suite (release v5.1.0, http://surfer.nmr.mgh.harvard.edu/). FreeSurfer estimated regional volumes were normalized for estimated TIV. Mean CTh measures for each lobe were calculated as the sum of the thickness of individual cortices included in each lobe, divided by the number of cortices included. The percentage of regional volume/CTh change between time points was calculated as mentioned above in the VBM section. WM structures were not included in this study.

Statistical Analysis

VBM- and FreeSurfer-derived global and tissue-specific volumes

After checking for normality of data distribution, associations between baseline clinical variables, discrete tissue fractions values (GMF, WMF, BPF), FreeSurfer regional GM volumes (cortical, subcortical) and mean lobar CTh were tested. Bivariate correlations were evaluated using the Pearson or the Spearman rank correlation coefficients. Partial correlations were assessed using covariates of interest. A t-test for paired samples was used to search for differences of GMF/WMF/BPF in each pair of consecutive time points and a t-test for independent samples was used to check differences between groups. Statistical significance was set at $p<0.05$. Given the exploratory nature of the study and the use of discrete data, significance values are reported without correction for multiple comparisons to avoid type II errors. Statistical Package for the Social Sciences (SPSS, Version 21, Chicago, Illinois, USA) and Graphpad Prism5 (Graphpad Software Inc. www.graphpad.com) were used.

VBM Analysis

We evaluated regional baseline GM differences between groups for gender (male vs. female), and presence of Gd-enhancement (Gd- vs. Gd+), using a two-sample t-test model, and correlations with demographic and clinical data (age, disease duration (DD), baseline EDSS and EDSS at last visit) using a Multiple Regression Model. Longitudinal regional GM changes between time points were assessed using a Flexible Factorial Design[19], including age and DD as covariates. Differences were considered significant at $p<0.05$and $k=10$ voxels. We have used the FWE-corrected p value to avoid false positives. For each correlation both direct and inverse contrasts were probed. The localization of areas of GM atrophy was defined using the Anatomic Automatic Labelling[20] tool from SPM8. To evaluate differences in GM changes between groups we produced a “difference image” using SPM8, subtracting the smoothed images between time points, and comparing the differences between groups (two-sample t-test model) or the association with an explanatory variable (Multiple Regression Model) as appropriate.

RESULTS

Sample selection and composition

A total of 74 patients receiving natalizumab for at least 36 consecutive months were identified, 38 had MRI scans that were suitable for VBM and FreeSurfer analysis at baseline, 37 at month 12 (1 excluded scan because it was acquired in a different scanner), 32 at month 24 (6 excluded scans because they were acquired in a different scanner) and 20 at month 36 (18 excluded scans because they were acquired in a different scanner). Six patients (15.8%) experienced confirmed EDSS worsening at 36 months. Baseline characteristics of included and excluded patients are shown in Table 1.

Baseline cross-sectional analysis

VBM-derived global and tissue-specific volumes

Older age at baseline was correlated with a lower GMF ($r=-0.631$, $p<0.001$; adjusting for DD partial $r=-0.608$, $p=0.009$). No significant correlations of age with WMF ($r=0.158$, $p=0.34$) or BPF ($r=-0.288$, $p=0.79$) were observed. No differences in global or tissue-specific fractions were observed between male and female patients (mean GMF 0.466 vs. 0.469, $p=0.64$; WMF 0.333 vs. 0.331, $p=0.78$; BPF 0.799 vs. 0.800, $p=0.92$). Longer DD was associated with lower global and tissue-specific fractions: GMF ($r=-0.387$, $p=0.01$), WMF ($r=-0.315$, $p=0.054$), and BPF ($r=-0.501$, $p=0.001$), but only GMF remained statistically significant after adjusting for age (partial $r=-0.36$, $p=0.045$). Higher baseline EDSS scores were associated with a lower GMF (Rho=-0.312, $p=0.028$), BPF (Rho=-0.269, $p=0.051$), but no correlations were observed between EDSS and WMF (Rho=-0.089, $p=0.29$). We found no differences in baseline global or tissue-specific fractions between patients with or without Gd-enhancement

at baseline (mean GMF 0.471 vs. 0.464, p=0.31; WMF 0.329 vs. 0.335, p=0.41; BPF 0.800 vs. 0.800 p=0.98).

VBM-derived analysis

We only found a significant association between age and GM concentration in the right supramarginal gyrus (T=6.77, MNI coordinates (48,-34,4) p<0.005) (older age implies lower GM concentration). No statistically significant regional GM differences were observed for other variables.

FreeSurfer-derived parameters

Cortical, subcortical and cerebellar volumes. Significant negative correlations were observed between age and left hippocampal and amygdalar volumes, as well as with bilateral cortical volumes, although these correlations did not persist after adjusting for DD. DD was found to negatively correlate with bilateral cortical and subcortical GM volumes, and with a number of individual subcortical structures. Baseline EDSS was also negatively correlated with bilateral cortical and subcortical GM volumes and with bilateral cerebellar cortical volume, as well as with a number of individual subcortical structures (Table 2).

Cortical Thickness measures. Age was correlated with left mean hemispheric CTh, especially with left insular and left frontal CTh which survived after adjusting for DD. DD was correlated with bilateral mean CTh, especially within the parietal and occipital lobes. No significant correlations were observed with baseline EDSS (Table 2).

Longitudinal analysis: description of changes in brain fractions and regional GM

VBM-derived global and tissue-specific volumes

Statistically significant decreases were observed during the first year in GMF (-1.28%, p=0.002), WMF (-0.9%, p=0.001) and BPF (-1.125%, p<0.001). During the second year, only

a significant WMF loss was observed (-0.6%, p=0.015; GMF -0.43%, p=0.4; BPF -0.62%, p=0.17) (Figure 1).

VBM-derived analysis

Areas of significant GM decrease after 12 months were located in bilateral cerebellum, bilateral cingulum, left>right parietal-frontal cortex, right>left hippocampus, and left caudate (Table 3, Figure 2). No further changes were observed in years 2 and 3.

FreeSurfer-derived parameters

Cortical, subcortical and cerebellar volumes. Significant volume decreases during the first year were found in the brainstem, in a number of subcortical GM structures and the cerebellum bilaterally, and in the left hemisphere cortex. During the second year, we only found significant decreases in the left thalamus, and bilaterally in the cerebellar cortex and hippocampus. During the third year, only a significant decrease within the cerebellar cortex was found bilaterally (Table 4).

Cortical thickness measures. Only a significant decrease was observed within the left insula during the first year of treatment (p=0.024). No further changes were observed in years 2 and 3.

Longitudinal analysis: correlations.

VBM-derived global and tissue-specific volumes

Age, gender and baseline EDSS were not correlated with changes in any tissue-specific fraction for any of the three one-year periods. Patients with Gd-enhancement at baseline had greater WMF losses during the first year (mean WMF change: Gd- -0.09% vs. Gd+ -1.77%, p=0.002) whereas no differences were found for GMF (mean GMF change: Gd- -0.9% vs.

Gd+ -1.2%, p=0.59); a trend was observed for BPF (mean BPF change: Gd- -0.5% vs. Gd+ -1.4%, p=0.057). No differences between Gd- and Gd+ groups were observed for any fraction in the second or third year of follow-up. Patients with confirmed EDSS worsening had lower baseline GMF (worsening EDSS: 0.451 vs. stable/improved EDSS: 0.471, p=0.025) and higher baseline WMF (worsening: 0.354 vs. stable/improved: 0.328, p=0.012), whereas no differences were found in baseline BPF (p=0.64). Patients with confirmed EDSS worsening had greater GMF loss (mean change -1.97% vs. 0.42%, p=0.012) and BPF loss (mean change -1.26% vs. 0.19%, p= 0.02) during the third year (Figure 3).

VBM-derived analysis

No statistically significant associations were observed between regional GM changes and baseline clinical variables, baseline Gd-enhancement or EDSS change status.

FreeSurfer-derived parameters

Cortical, subcortical and cerebellar volumes. Male patients had greater left cerebellar cortex volume loss during the first year (-2.3% vs -0.73%, p=0.025) but no other significant associations with regional volume changes were observed; age and Gd status at baseline were not associated with any FreeSurfer-derived regional volume changes in any of the three one-year periods. Patients who experienced confirmed EDSS worsening had smaller baseline left thalamus volume (0.395 vs 0.443, p=0.037) and larger volume losses during the first year in left pallidum (-12.5% vs -4.4%, p=0.055), bilateral cortical (-3.18% vs -0.19%, p=0.014), and total GM (-3.0% vs -0.51%, p=0.019). No significant associations were found during the second or third one-year periods.

Cortical thickness measures. No CTh changes in any of the three one-year periods were found to be significantly correlated with gender, age, DD, baseline EDSS or Gd status. Patients with confirmed EDSS worsening had larger bilateral mean CTh decreases during the first year (-2.18% vs 0.01%, p=0.049). No other significant associations were found.

DISCUSSION

In this study we present a cross-sectional and longitudinal VBM and FreeSurfer study describing global and regional GM volume and CTh changes in patients receiving natalizumab as a retrospective post hoc analysis in a sub-cohort of patients with 3D-T1 MRI. Our results provide evidence that significant whole brain, WM and GM loss (a widespread damage which is affecting infratentorial, subcortical and cortical structures) is present during the first year of treatment with natalizumab. WM changes are in close relation with Gd-enhancement at baseline, but this is not the case for GM. It also seems that atrophy slows during the second and third year of treatment (but it is still significant for WM, during the second year, and cerebellum GM until the third year of treatment). GM changes (but not WM) are the only to be significantly associated with clinical parameters (e.g., disability).

The present findings are also consistent with our two previous reports on the same cohort. In a one-year follow-up study using 2D-T1 scans and a segmentation approach we have previously shown that early changes in the first year of treatment with natalizumab were mainly due to WM volume loss (pseudoatrophy effect) and were influenced by baseline Gd-enhancement, whereas no significant GM volume changes were found[16]. In the 3-year follow-up study of this same cohort using 2D-T1 images analysed with SIENA, the influence of baseline MRI activity in whole BVL was also significant all through the second year, with a marginal association of BVL and disability[21]. Changes in black-hole volume or new T2/Gd+ lesions were not significantly correlated with disability progression in this cohort (data not shown).

In the present study we have used a different approach to evaluate regional BVL in a subcohort for which 3D-T1 studies were available with a voxel-to-voxel and surface-based analysis, to focus on GM volume and CTh changes and their association with disability.

Patterns of regional GM atrophy have been widely described using VBM, both in cross-sectional[22] and longitudinal[23] analyses, although a distinctive GM pattern associated with a

specific stage has not been elucidated yet, probably due to the high heterogeneity of the disease. Our findings are in agreement with the areas found by Lansley et al. in a recent metanalysis of VBM studies which reported significant GM loss in bilateral thalamus, basal ganglia, bilateral pre and post central cortex and cingulum[9]. FreeSurfer results seem to add a more detailed depiction of subcortical GM volume loss during the same period of time. These areas are strongly interconnected with other brain regions[8] and have also been correlated with disability [24,25].

In our cohort, composed mainly of young RRMS patients with moderate disability, VBM results showed that only global GM volume was significantly correlated with baseline EDSS. Using FreeSurfer, we did find an association between disability and regional GM volumes including cortical hemispheric, subcortical and bilateral cerebellum areas, which is in agreement with previous studies[26] showing that correlations with clinical status are mostly involving GM and not WM[10].

First year GM atrophy could be theoretically attributed to real neurodegenerative processes such as Wallerian and axonal degeneration, in our VBM analysis we found no differences during the second or third year, maybe indicating a slowdown of atrophy during this period, as previously documented with the use of disease modifying therapies [27,28]. Using FreeSurfer we were able to find GM loss during the second year in a few restricted areas (left thalamus and bilateral hippocampus), while cerebellum cortex volume loss was significant all through the 36 months of study, maybe indicating that this software might be more sensitive subtle changes.

Although we could not find any particular longitudinal GM region loss associated with confirmed EDSS worsening using VBM, probably due to small sample size and small proportion of patients who experienced EDSS worsening, it was interesting to note that patients who deteriorated had lower baseline GMF than those who remained

stable/improved, findings that have been reported in previous studies[29,30]. These patients also experienced greater GMF and BPF loss in the third year of follow-up. This is also in line with a recent 10 year follow-up study in 81 MS patients showing that patients with disability progression developed significantly larger whole brain, cortical and putamen volume loss compared to patients without disability, with no differences in WM or lesion volume[31]. Whether the observed stabilization of GM damage is due to a neuroprotective effect of natalizumab deserves further investigation in appropriately designed studies. Some studies have already pointed to this hypothesis using MRI[32,33] , with recent studies using spectroscopy showing that natalizumab increases the levels of N-acetylaspartate, creatine and phosphocreatine, maybe indicating an enhanced axonal metabolism in natalizumab-treated patients[34] and reduction of WM damage during the first year of treatment using diffusion tensor imaging[35]. Finally, body fluid biomarkers in a progressive MS study with patients treated with natalizumab also showed a reduction in intrathecal inflammation (decreased levels of osteopontin) accompanied by decreases in other markers of neurodegeneration (neurofilament light chain) and tissue damage (increases in MTR in cortical GM and normal-appearing WM)[36]. Nonetheless, it is worth mentioning that these findings cannot be used to predict treatment response to natalizumab and they are, at present, only valid for group analyses purposes.

As a small exploratory study with a retrospective post-hoc analysis, our findings are limited by the initial small sample size and posterior longitudinal case attrition due to acquisition in different scanners precluding valid comparisons. Enough evidence is available that using different scanners in longitudinal analysis may hinder the results, and we decided to exclude these images to improve reliability and consistency of the results, at expenses of losing sample size[37]. Also, as we reported the significant values without correction for multiple comparisons to avoid type II errors, number of false positives or false negatives could be inflated. A control group (healthy controls and/or patients on different disease modifying therapy) would have been useful to truly determine the possible impact of natalizumab in the

suppression of BVL, and particularly GM atrophy observed during the second and third year of treatment. Regional correlations with physical disability (eg. MSFC 9HPT, 25FWT or Functional Scores), cognitive impairment, fatigue, and quality of life scales would also had been interesting to explore.

In conclusion, patients receiving natalizumab develop significant global BVL during the first year of treatment. WM volume changes, occurring throughout the first 24 months, are influenced by Gd-enhancement at baseline, but GM volume changes, seem to be independent of baseline inflammation and appear to be correlated with disability. Further prospective studies in larger samples are warranted to investigate whether global or regional BVL may be useful to predict treatment response to natalizumab.

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