Progressive Gray Matter Damage in Patients With Relapsing-Remitting Multiple Sclerosis

A Longitudinal Diffusion Tensor Magnetic Resonance Imaging Study

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Background: Diffusion tensor magnetic resonance imaging (DT MRI) has the potential to provide in vivo information about tissue microstructure. In multiple sclerosis (MS), DT MRI has disclosed the presence of occult structural damage in the normal-appearing brain tissues.

Objective: To investigate whether DT MRI is sensitive to longitudinal changes of brain damage that may occur beyond the resolution of T2-weighted images in patients with relapsing-remitting MS.

Design: Twenty-six untreated patients with relapsing-remitting MS were followed up for 18 months. Dual-echo, DT and postcontrast T1-weighted MRIs of the brain were obtained at baseline and then every 3 months. Mean diffusivity ($D\bar{\circ}$) histograms of normal-appearing gray (GM) and white matter were produced. Total T2-hyperintense and T1-hypointense lesion volumes; normalized whole brain tissue, GM, and white matter volumes; percentage brain volume change between the study entry and exit images; average lesion $D\bar{\circ}$; and fractional anisotropy were also calculated.

Results: During the study period, a significant decrease of normalized whole brain tissue, average lesion fractional anisotropy and normal-appearing GM $D\bar{\circ}$ histogram peak height, and a significant increase of average normal-appearing GM $D\bar{\circ}$ and T2-hyperintense lesion volumes were observed. Changes of normal-appearing GM diffusivity were independent of the concomitant changes of normalized whole brain tissue and GM volumes.

Conclusions: The DT MRI findings show progressive microstructural changes in the normal-appearing GM of patients with untreated relapsing-remitting MS. Such changes do not reflect a concomitant development of brain atrophy and confirm the importance of GM pathology in MS.

Arch Neurol. 2005;62:578-584

IN PATIENTS WITH RELAPSING-REMITTING multiple sclerosis (RRMS), serial T2-weighted and enhanced T1-weighted magnetic resonance images (MRIs) of the brain disclose disease activity with a greater sensitivity than the clinical assessment of relapses.1 This has made conventional MRI (cMRI) a valuable tool to monitor the efficacy of experimental treatments in large-scale clinical trials of MS.1 Nevertheless, a discrepancy between clinical and MRI findings has emerged in patients with RRMS, calling for new MRI markers to monitor MS evolution more accurately.2

Against this background, diffusion tensor (DT) MRI is a very promising technique, because it enables reliable estimates of the extent of structural changes occurring within and outside T2-visible MS lesions to be obtained.3 Diffusion is the microscopic random translational motion of molecules. Although diffusion is inherently a 3-dimensional process, in some tissues such as brain white matter (WM) with an oriented microstructure, the molecular mobility is not the same in all directions. This property is called anisotropy, and it results in a variation of the measured diffusivity with tissue measurement direction. Because WM fiber tracts consist of aligned myelinated axons, hindrance of water diffusion is much greater across than along the major axis of axonal fibers. Under these conditions, a full characterization of diffusion can only be found in terms of a tensor, a $3 \times 3$ matrix, where the on-diagonal elements represent the diffusion coefficients along the axes of the reference frame, whereas the off-diagonal elements account for the correlations between molecular displacement along orthogonal directions. From the tensor, it is possible to derive some scalar indices. These include the mean diffusivity ($D\bar{\circ}$) (equal to one third of the trace of the diffusion tensor), which is affected

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by cellular size and integrity, and the fractional anisotropy (FA), which reflects the degree of alignment of cellular structures within fiber tracts and their structural integrity. Using histogram analysis, D and FA maps of large portions of the brain and gray matter (GM) and WM compartments can be obtained.

To our knowledge, only 3 small preliminary studies, all based on region-of-interest (ROI) analysis, have investigated the sensitivity of DT MRI to disease changes for a maximum period of 12 months. This longitudinal, histogram-based study was performed to address the following questions: (1) Is DT MRI sensitive to MS-related changes for an 18-month follow-up? (2) Are changes of DT MRI histogram-derived metrics correlated with clinical and cMRI findings in patients with RRMS?

METHODS

PATIENTS

Patients had to have clinically definite MS\textsuperscript{10} for at least 1 year and an RR course.\textsuperscript{11} Other inclusion criteria were the absence of previous and present treatment with disease-modifying agents and the ability to undergo serial MRI. The study duration was 18 months. All patients were fully informed about available disease-modifying treatments, but did not start any of them for medical or personal reasons. In case disease-modifying treatment needed to be initiated during the study period, it was decided a priori that those patients would have been excluded from the present analysis.

Clinical follow-up consisted of neurological visits with a rating of the Expanded Disability Status Scale (EDSS)\textsuperscript{13} every 3 months, within 3 days from the acquisition of the MRIs. All patients underwent evaluation by a single neurologist (R.C.), who was unaware of the MRI findings. In case of symptoms suggestive of a clinical relapse, patients were instructed to contact the same neurologist for additional visits and treatment decisions. Clinical relapses were always treated with intravenous methylprednisolone sodium succinate, $1 \text{ g/d for 3 to 5}$ consecutive days (with no subsequent tapering of therapy). At the study exit, patients were considered clinically worsened if their EDSS score increased by $1$ point or more when compared with study entry; in case of recent relapses, EDSS deterioration had to be confirmed by an additional visit after 1 month. All patients signed an informed consent before study initiation. The study design was approved by the local ethical committees of the participating institutions.

IMAGE ACQUISITION

At a mean $+80$ of every $90 \pm 10$ days, brain MRI was performed using a 1.5-T scanner during a regular course of maintenance treatment (the total number of scans per patient was $7$). During each session, the following sequences were acquired: (1) dual-echo turbo spin-echo (repetition time [TR], $3300$ milliseconds; first echo time [TE], $16$ milliseconds; second TE, $98$ milliseconds; echo train length, $5$); (2) balanced-gradient spin-echo echoplanar (interecho spacing, $0.8$ milliseconds; TE, $123$ milliseconds), with diffusion gradients applied in $8$ noncollinear directions, chosen to cover $3$-dimensional space uniformly\textsuperscript{13,14} (a detailed description of this sequence is given elsewhere\textsuperscript{9}); and (3) postcontrast T$1$-weighted conventional spin-echo (TR, $768$ milliseconds; TE, $15$ milliseconds), $5$ minutes after the intravenous administration of $0.1 \text{ mmol/kg gadopentetate dimeglumine}$.

For dual-echo and T$1$-weighted images, $24$ contiguous axial slices were obtained with $5$-mm thickness, $256 \times 256$ matrix size, and $250 \times 250$-mm field of view. The slices were positioned to run parallel to a line that joins the most inferoanterior and interoposterior parts of the corpus callosum. For DT MRIs, $10$ axial contiguous sections with $5$-mm thickness, $128 \times 128$ matrix, and $250 \times 250$-mm field of view were acquired, with the same orientation as the dual-echo images, positioning the penultimate caudal section to match exactly the central section of the dual echo set. This brain portion was chosen because the periventricular region is a common location for MS lesions. For follow-up images, patients were carefully repositioned in the scanner following published guidelines.\textsuperscript{15}

IMAGE ANALYSIS

Two experienced observers (C.O.-G. and M.R.), without knowing to whom the images belonged, identified by consensus the hyperintense lesions on proton density–weighted and T$1$-hypointense and gadopentetate-enhancing lesions on T$1$-weighted images. T$2$-weighted images were always used to increase confidence in lesion identification. The numbers of total and new enhancing lesions and new T$1$-hypointense and T$2$-hyperintense lesions were counted by the same observers by consensus. Blinding to patient identity and image order was maintained during lesion identification, except for the count of new lesions on serial images, when the observers had to be unblinded to the image order. Total gadopentetate-enhancing, T$2$-hyperintense, and T$1$-hypointense lesion volumes were measured by a single observer, using a local thresholding technique for lesion segmentation.\textsuperscript{16}

On T$1$-weighted images, normalized volumes of the whole brain tissue (WBT), WM, and GM were measured using a fully automated method, the cross-sectional version of the Structural Imaging Evaluation of Normalized Atrophy (SIENA) software (SIENAX; available from the FSL-FMRIB software library, University of Oxford, Oxford, England).\textsuperscript{17};\textsuperscript{18} First, SIENAX uses a brain extraction tool method to extract the brain and skull from MRIs, as extensively described elsewhere.\textsuperscript{18} A tissue segmentation program\textsuperscript{18} is then used to segment the extracted brain image into WM, GM, cerebrospinal fluid, and background, yielding an estimate of the absolute volumes of brain tissue compartments. Original MRIs are subsequently registered to a canonical image in a standardized space (using the skull image to provide the scaling cue), a procedure that provides a spatial normalization scaling factor for each subject. The estimated WBT, WM, and GM absolute volumes for a subject are then multiplied by the normalization factor, to yield a normalized parenchymal volume of these tissue compartments. Longitudinal percentage brain volume changes (PBVC) between baseline and study exit scans were also estimated using the SIENA method.\textsuperscript{17}

From DT MRIs, D and FA maps were created and coregistered to the dual-echo images following a method that has been described elsewhere.\textsuperscript{30} Lesion outlines on proton density–weighted images were automatically transferred onto the coregistered D and FA images, and the area, D, and FA of each lesion were measured. Then, for each patient, the average lesion $D$ and FA, weighted by lesion area,\textsuperscript{31} were calculated.

Normalized histograms of normal-appearing WM (NAWM) and GM (NAGM) $D$ maps were created as previously described.\textsuperscript{32} The segmentation of NAWM and NAGM was obtained using an automated technique based on FA thresholding, which has been previously validated in healthy control subjects and patients with MS.\textsuperscript{30,21} Because FA maps are used for the segmentation process, NAWM and NAGM histograms were not derived from them. For all histograms, the average $D$ values and heights and locations of their peaks were calculated. Given the strong correlation existing between average $D$
and the respective histogram peak location, the latter quantity was a priori excluded from the analysis, to minimize the number of comparisons and, therefore, reduce the risk of type 1 errors.

**STATISTICAL ANALYSIS**

A mixed random-effect model with autoregressive terms to adjust for autocorrelation and time dependence was used to analyze the changes of MRI-derived variables over time. Because 11 tests were planned, a Bonferroni-corrected P value of .0045 was defined as the significance cutoff. Correlations were evaluated using the Spearman rank correlation coefficient. Only those DT MRI-derived variables with a significant change during the study period entered a subsequent multivariable analysis to evaluate whether such changes were dependent on those of other cMRI metrics (T2 lesion volume, number of new gadopentetate-enhancing lesions, gadopentetate-enhancing lesion volume, normalized WBT, and GM and WM volumes). The choice of independent variables was made a priori, and it was based on biological considerations.

**RESULTS**

Twenty-eight patients with RRMS were enrolled in the study. However, 2 of them started treatment with interferon beta and azathioprine after 6 and 9 months of follow-up and, as a consequence, they were excluded from the present analysis. The completed study cohort included 26 patients with RRMS (18 women and 8 men). Their mean age was 36.0 years (range, 25-50 years); mean disease duration, 10.0 years (range, 1-15 years); and median EDSS scores, 1.5 (range, 0.0-4.0) at study entry and 2.0 (range, 0.0-4.0) at study exit. Reasons for not initiating disease-modifying treatment included patient refusal (10 cases), medical contraindications (6 cases), no relapses in the preceding year (6 cases), and a history of depression (4 cases). During the follow-up, 16 patients (62%) experienced 36 relapses. At the final visit, 7 patients (27%) were considered clinically worsened. A total of 177 MRI sessions of the expected 182 were performed. In 4 patients, 5 MRIs were performed while the patients were receiving steroid treatment. The results of the present study did not change when these images were excluded from the analysis (data not shown).

**Figure 1** displays the cMRI-detectable disease activity at each time point of the study. In 5 patients (19%), no new gadopentetate-enhancing lesions were seen at any time point; in only 1 patient did no new gadopentetate-enhancing, T1-hypointense, or T2-hyperintense lesion develop during the entire follow-up.

The **Table** reports cMRI and DT MRI findings at baseline and final follow-up visit. Mean PBVC between the baseline and study exit images was -0.91% (SE, 0.27%). Time-trend analysis revealed a significant increase of T2-hyperintense lesion volume (β = 657; P < .001) and average NAGM D (β = 0.006; P = .001) during the entire study period (**Figure 2**). The on-study decreases of normalized WBT volume (β = -5.0; P < .001), NAGM D histogram peak height (β = -1.03; P < .001), and average lesion FA (β = -0.002; P < .001) were also found to be significant (**Figure 3**). This was not the case for the decreases of normalized WM and GM volumes. No significant correlations were found between the observed percentage changes of the individual variables, with the exception of that between T2-visible lesion volume change and NAGM D histogram peak height (r = -0.66; P = .001). No significant correlations were found between PBVC and the corresponding percentage changes of T2-visible lesion volume or DT MRI-derived variables.

Three different multivariable analyses were performed in which average NAGM D, NAGM D histogram...
peak height, and average lesion FA were the dependent variables. In all of these analyses, new gadopentetate-enhancing lesions, T2-visible lesion volume, gadopentetate-enhancing lesion volume, and normalized WBT, WM, and GM volumes at each time point were the independent variables. The on-study increase of average NAGM \(D\) (\(p=0.000003; P<.001\)) and the on-study decrease of NAGM \(D\) histogram peak height (\(p=-0.004; P<.001\)) were found to be correlated with the concomitant change of T2-weighted lesion volume. The changes of average lesion FA were not significantly correlated with any of the tested covariates.

The observed on-study changes of cMRI and DT MRI variables did not differ between patients who did and did not experience clinical relapses during the study, or between patients who were and were not considered clinically worsened at study exit (data not shown).

**Comment**

Diffusion tensor MRI studies of MS have provided important pieces of information on the pathological heterogeneity of T2-visible lesions and have confirmed that disease-related damage does not spare brain tissues that appear normal on conventional MRIs. Brain DT histogram-derived metrics have been found to differ between patients with MS and healthy subjects, as well as among patients with MS and varying disease phenotypes and disability. To the best of our knowledge, only a few preliminary studies have investigated whether DT MRI is sensitive to longitudinal changes of microstructural MS damage. All these studies were conducted using ROI analysis and small samples of patients.

In this longitudinal study, we interrogated the ability of DT MRI and histogram analysis to provide new insights into the evolution of MS tissue damage during an 18-month period. Histogram analysis has several advantages over ROI analysis, including the limited human intervention, the reduced postprocessing time, and the ability to provide overall estimates of disease burden in the brain and NAWM and NAGM taken in isolation. Patients underwent imaging every 3 months, and a time-trend analysis of data was performed to reduce the possible confounding effect of the short-term variability of MRI measures at a 2–time point (study entry vs study exit) analysis.

Conventional MRI changes over time only consisted of a significant increase of the T2-hyperintense lesion volume. This agrees with the concomitant, MRI-detectable disease activity, which showed an average accumulation of about 20 new T2-visible lesions per patient during the 18-month follow-up. It is interesting that T1-hypointense lesion volume did not change significantly during this period, suggesting that, at least for RRMS, cMRI-derived measures thought to reflect severe WM damage (T1 “black holes”) may lack sensitivity to disease-related changes during relatively short periods of time. That the observed decreases of normalized WM and GM volumes were not significant, whereas the change of normalized WBT volume was, suggests that the process of GM/WM segmentation could increase measurement variability, thus decreasing the statistical power for detecting longitudinal changes. Appropriate imaging-

### Table. Conventional and DT MRI Findings From 26 Patients With RRMS at Study Entry and Exit

<table>
<thead>
<tr>
<th>MRI Findings, Mean (SD)</th>
<th>Study Entry</th>
<th>Study Exit</th>
<th>(P) Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2-visible lesion volume, mL</td>
<td>12.1 (8.9)</td>
<td>15.9 (9.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>T1-visible lesion volume, mL</td>
<td>2.5 (2.8)</td>
<td>2.8 (2.8)</td>
<td>.08</td>
</tr>
<tr>
<td>WBT volume, mL</td>
<td>1520.5 (95.9)</td>
<td>1490.5 (72.5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>WM volume, mL</td>
<td>827.1 (68.3)</td>
<td>805.8 (49.7)</td>
<td>.10</td>
</tr>
<tr>
<td>GM volume, mL</td>
<td>693.4 (65.9)</td>
<td>684.6 (43.5)</td>
<td>.11</td>
</tr>
<tr>
<td>Average lesion (\bar{D}), mm(^2)/s</td>
<td>1.00 (0.08)</td>
<td>1.02 (0.07)</td>
<td>.01</td>
</tr>
<tr>
<td>Average lesion FA‡</td>
<td>0.28 (0.03)</td>
<td>0.27 (0.02)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Average NAGM (\bar{D}), mm(^2)/s</td>
<td>1.09 (0.06)</td>
<td>1.14 (0.07)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Average NAWM (\bar{D}), mm(^2)/s</td>
<td>0.85 (0.02)</td>
<td>0.86 (0.03)</td>
<td>.54</td>
</tr>
<tr>
<td>NAGM (\bar{D}) peak height</td>
<td>62.3 (9.7)</td>
<td>55.3 (11.1)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>NAWM (\bar{D}) peak height</td>
<td>152.9 (22.6)</td>
<td>152.1 (24.3)</td>
<td>.53</td>
</tr>
</tbody>
</table>

Abbreviations: \(\bar{D}\), mean diffusivity; DT, diffusion tensor; FA, fractional anisotropy; GM, gray matter; MRI, magnetic resonance imaging; NA, normal-appearing; RRMS, relapsing-remitting multiple sclerosis; WBT, whole brain tissue; WM, white matter.

†Calculated for time-trend analysis. Boldface type indicates significant \(P\) values after Bonferroni correction.

‡Expressed as a dimensionless index.

![Figure 2. Histograms of mean diffusivity (\(\bar{D}\)) values from the normal-appearing white matter (A) and gray matter (B) of 26 patients with relapsing-remitting multiple sclerosis at study entry and after 18 months. At study exit, a decrease of the normal-appearing gray matter histogram peak height and a shift of its shape toward the right end, suggesting an increased number of pixels with higher \(\bar{D}\) values can be observed. Analysis is described in the “Statistical Analysis” subsection of the “Methods” section.](http://archneur.jamanetwork.com/pdfaccess.ashx?url=/data/journals/neur/12044/)
A reimagining reproducibility studies in patients and healthy subjects are warranted to clarify this issue, given the increasing use of MRI measures of atrophy to monitor MS irreversible damage. The most novel and intriguing finding of this study is the detection of a significant progressive increase of the NAGM water $D$ over time, which was correlated with the concomitant increase of T2-weighted lesion volume, but not with the variations of normalized WBT, WM, and GM volumes. This suggests a progressive accumulation of GM damage already in the RR phase of the disease, which might be one of the factors responsible for the development of brain atrophy and for some of the clinical manifestations of the disease such as neuropsychological impairment. Previous cross-sectional DT MRI studies showed that the average $D$ of NAGM from patients with MS was higher than that of GM from matched controls, and that NAGM changes were more pronounced in patients with the most disabling forms of the disease. A moderate correlation between cognitive impairment and NAGM $D$ in mildly disabled patients with RRMS has also been found. Our results also agree with cross-sectional data of positron emission tomography, functional MRI, and quantitative structural MRI studies, which show functional and structural abnormalities of the NAGM of patients with MS and various disease phenotypes.

With these findings in mind, the major issue to be addressed is the definition of the factors that might be responsible for the progressive increase of GM water diffusivity in MS. Given the florid activity seen on cMRIs in the WM of these patients, the most readily apparent explanation for this finding might be a progressive development and accumulation of new MS lesions in the NAGM. Pathological studies have shown that GM lesions represent a significant portion of the overall brain lesion burden in MS. In addition, 2 postmortem studies have convincingly shown that (1) demyelinated lesions of the cerebral cortex from patients with MS harbor transected dendrites, transected axons, and apoptotic neurons, and (2) there is a 30% to 35% neuronal loss in the thalamus of patients with MS. Such destructive components of GM pathology of MS would inevitably lead to increased tissue permeability to water molecular motion and, as a consequence, contribute to the increased NAGM $D$. An alternative, but not mutually exclusive, explanation of the observed evolution of $D$ changes in the NAGM might be a progressive retrograde degeneration of neurons secondary to the damage of the fibers traversing WM lesions. That retrograde degeneration might contribute to the observed progressive NAGM damage is supported by the significant relationship between T2-visible lesion volume accumulation and increased NAGM $D$. Since partial volume effects secondary to GM atrophy have the potential to cause an NAGM diffusivity increase (due to the inclusion of voxels partially contaminated by cerebrospinal fluid), we also measured the changes of normalized WBT and GM volumes over time and found that these volumes and NAGM diffusivity changes are not correlated, indicating that the development of brain or GM atrophy did not significantly influence DT MRI findings. As a consequence, we can also reasonably exclude that the progressive GM damage found in these patients is attributable to partial volume averaging secondary to atrophy.

The lack of significant changes of NAWM $D$ values during the study period is somewhat counterintuitive. Using ROI analysis, Werring et al reported an esti-
mated rate of 1.4% increase per month for diffusivity values in 19 NAWM regions that remained unaffected by T2-visible lesions in a 1-year period in 5 patients with MS. In addition, Werring et al and Rocca et al reported a significant, progressive increase of diffusivity during short periods in NAWM areas where new T2-visible lesions subsequently developed. There are at least 3 plausible explanations for this finding. First, by using histogram analysis, we might have lost sensitivity to modest changes occurring in some NAWM regions. This is in keeping with the results of previous cross-sectional, ROI-based, DT MRI studies, showing abnormal $D$ values in some, but not all, of the assessed NAWM areas. Second, the pathological substrates of MS brain damage may have a variable impact on diffusivity measurements. Demyelination and axonal loss are likely to be associated with a net loss of restricting barriers to water molecular motion, whereas gliosis and cytotoxic edema might decrease water diffusivity. Finally, progressive water diffusivity changes in the NAWM might have not been detected simply because pixels belonging to newly formed, T2-visible WM lesions (where significant $D$ changes are known to occur in comparison with previously normal tissue) were necessarily excluded from the analysis. Methodological studies investigating the stability of DT MRI-derived quantities over time are also needed to clarify whether any measurement variability may decrease the sensitivity for detecting changes of NAWM diffusivity. However, that we found significant DT MRI changes in only 1 of the 2 brain tissue compartments studied supports the hypothesis that nonbiological factors should play a minor role, if any.

To avoid the potential effects of disease-modifying treatment on DT MRI findings, we studied only patients who remained untreated during the study period, with the exception of steroid administration in case of relapses. However, this might represent a potential source of bias. The study was planned and conducted when, in Italy, injectable interferon beta-1a and beta-1b were the only licensed treatments for RRMS with clinical evidence of disease activity in the 2 years preceding treatment initiation. Thus, one might argue that the patients enrolled in this study represent a cohort with a relatively benign disease course, and that our findings cannot be generalized to the overall RRMS population. Nevertheless, we believe this may not be the case, because about 60% of the patients experienced at least 1 relapse during the study period, about 30% of them showed a confirmed 1-point increase of the EDSS score at study exit, and, on average, they had an MRI-detectable disease activity that was within the expected ranges.

We were disappointed that we did not find significant correlations between DT MRIs and clinical changes over time. This is likely due to the relatively short duration of the study, to the small EDSS range of this patient cohort, and to the many limitations of EDSS. However, the lack of a correlation between clinical and DT MRI findings during this 18-month follow-up does not exclude that DT MRI might provide useful prognostic markers for clinical evolution during a longer follow-up, as recently shown for magnetization transfer MRI.

Accepted for Publication: August 13, 2004.
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Funding/Support: This study was supported by an educational grant from the European Neurological Society, Le Kremlin Bicêtre, France (Dr Oreja-Guevara), and in part by a grant from the Fondazione Italiana Sclerosi Multipla, Genoa, Italy (contract 2000/R/37).

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