Diffuse Axonal and Tissue Injury in Patients With Multiple Sclerosis With Low Cerebral Lesion Load and No Disability

Nicola De Stefano, MD; Sridar Narayanan, MSc; Simon J. Francis, BSc; Steve Smith, DPhil; Marzia Mortilla, MD; M. Carmela Tartaglia, BSc; Maria L. Bartolozzi, MD; Leonello Guidi, MD; Antonio Federico, MD; Douglas L. Arnold, MD

Background: Although in situ pathological studies and in vivo magnetic resonance (MR) investigations have shown that axonal injury can be significant in the early stages of multiple sclerosis (MS), diffuse axonal injury is generally considered a secondary event. Cerebral axonal damage can be specifically assessed in vivo by measuring levels of brain N-acetylaspartate (NAA, a specific index of axonal integrity detected by MR spectroscopy). Other new MR measurements such as magnetization transfer ratio (MTr) or computed estimation of brain volume can provide less specific indexes of tissue damage.

Objective: To determine whether diffuse axonal and tissue injury is present in patients with definite MS who do not show clinically significant disability.

Methods: We measured brain NAA levels (normalized to creatine [Cr]), MTr values, and cerebral volumes in patients with definite MS who had low T2-weighted MR imaging lesion volumes and no clinical disability, and also in age-matched healthy control subjects.

Results: Values of central brain NAA/Cr and MTr in normal-appearing white matter were significantly lower in the MS patients than in controls (P<.001). In contrast, total brain volumes were not significantly different between these groups. Similar results were found for MS patients with early disease (duration, <3 years) and with a particularly low cerebral T2-weighted MR imaging lesion load (≤2 cm³).

Conclusions: Cerebral NAA/Cr and MTr values are diffusely decreased in MS patients with early disease, low demyelinating lesion load, and no significant disability. This suggests that axonal and/or tissue injury begins very early in the course of MS and might be at least partially independent of cerebral demyelination.

Arch Neurol. 2002;59:1565-1571

MULTIPLE SCLEROSIS (MS) is an inflammatory demyelinating disease of the central nervous system that causes severe clinical disability in young adults. Traditionally, impairment of the central nervous system and the related loss of function have been considered to be largely due to the demyelination and consequent delay or block of electrical conduction by axons that are otherwise substantially preserved. In the past decade, however, in vivo magnetic resonance (MR) spectroscopy (MRS) studies of N-acetylaspartate (NAA) and in situ postmortem studies have demonstrated that sparing of axons is only relative in MS and injured or transected axons are a common finding in this disorder. This has led to a reconsideration of the role of axonal injury in MS and, in particular, its relevance to clinical disability.

There is an increasing agreement that axonal loss plays a major role in the pathology of MS and there exists both in vivo and in situ evidence that axonal injury can be significant from the early stages of the disease. However, the mechanisms by which axonal injury occurs are not fully understood. For example, if axonal injury is simply a bystander effect secondary to demyelination, then the degree of axonal injury should be strongly related to the degree of demyelination. However, several experimental studies suggest that axonal injury and dysfunction in MS may be independent of the degree of demyelination and might begin to accumulate before clinical disability is evident.

Axonal injury inside and beyond MS lesions can be evaluated with MRS by measuring brain levels of NAA (an amino acid localized almost exclusively in neurons and axons in the mature central nervous system) and in situ postmortem studies have demonstrated that sparing of axons is only relative in MS and injured or transected axons are a common finding in this disorder. This has led to a reconsideration of the role of axonal injury in MS and, in particular, its relevance to clinical disability.

There is an increasing agreement that axonal loss plays a major role in the pathology of MS and there exists both in vivo and in situ evidence that axonal injury can be significant from the early stages of the disease. However, the mechanisms by which axonal injury occurs are not fully understood. For example, if axonal injury is simply a bystander effect secondary to demyelination, then the degree of axonal injury should be strongly related to the degree of demyelination. However, several experimental studies suggest that axonal injury and dysfunction in MS may be independent of the degree of demyelination and might begin to accumulate before clinical disability is evident.
strated to be sensitive in detecting early pathologic changes in brains of patients with MS. Thus, our goal in the present study was to assess in vivo whether diffuse cerebral axonal and tissue injury accrues in nondisabled MS patients who exhibit little evidence of focal demyelination. To do this, we evaluated values of NAA-creatine (Cr), MTr, and total cerebral volumes in the brains of a selected group of patients with established MS who showed low volumes of cerebral T2-weighted (T2-W) lesions on conventional MR imaging and absence of disability at clinical examination.

SUBJECTS AND METHODS

STUDY POPULATION

Sixty patients (41 women and 19 men; age range, 18-54 years; mean, 35 years) with clinically definite MS but without clinical disability (Expanded Disability Status Scale [EDSS], 0-2) were chosen from the population followed up at the MS clinics of the Montreal Neurological Institute and Hospital, Montreal, Quebec (MNH, n=26), and of the Institute of Neurological Sciences of the University of Siena, Siena, Italy (Siena; n=34). Patients from both sites had a relatively short disease duration (range, 0.4-13.0 years; median, 2.7 years) and were all classified as having the relapsing-remitting form of the disease. All patients were relapse- and drug treatment-free for at least 1 month before study entry. The ethics committees of both institutions approved the study. Informed consent was obtained from all participating subjects.

MR EXAMINATIONS

All patients were examined using the same MR protocol, which included combined proton MR imaging and MRS imaging examinations of the brain. A transverse dual-echo, turbo spin-echo sequence (repetition time, 2075 milliseconds; first echo time, 30 milliseconds; second echo time, 90 milliseconds; 256 x 256 matrix; 1 signal average; and 250-mm field of view) yielding proton density and T2-W images with 50 contiguous 3-mm slices was acquired parallel to the plane connecting the anterior and posterior commissures. Subsequently, an MT sequence was performed to acquire 2 transverse T1-W, gradient-echo images, 1 without (No Sat) and 1 with (Sat) MT saturation pulses (repetition time, 35 milliseconds; echo time, 10 milliseconds; 256 x 256 matrix; 1 signal average; and 250-mm field of view). This sequence yielded image volumes of 50 slices, 3 mm thick, oriented to match the proton density-T2 acquisition exactly. The MT pulse was a 1.2-millisecond on-resonance (radio-frequency field strength, 20 µT) placed just before each slice-selective excitation. The MR images were used to select an intracranial volume of interest (VOI) for spectroscopy measuring approximately 100 mm anteroposterior x 20 mm craniocaudal x 90 mm left to right. This was centered on the corpus callosum to include mostly white matter of both hemispheres. Two-dimensional spectroscopic images were obtained using a 90°-180°-180° pulse sequence (repetition time, 2000 milliseconds; echo time, 272 milliseconds; 250-mm field of view; 32 x 32 phase-encoding steps; and 1 signal average per step) as previously described. Water suppression was achieved by placing frequency-selective excitation pulses at the beginning of the MRS imaging sequence. Before the water-suppressed acquisition, another MRS image was acquired without water suppression (repetition time, 850 milliseconds; echo time, 272 milliseconds; 250-mm field of view; and 16 x 16 phase-encoding steps) to allow for B0 homogeneity correction.

MR DATA ANALYSIS

Lesion Volumes and MT

The T2-W lesions were classified by a single observer (S.J.F.) with the use of a user-supervised thresholding technique. Lesion borders were determined primarily on proton density-weighted images, but information from T2-W and T1-W images were also considered. As the aim of the study was to study a population with established MS and low disease burden, MS patients were included only if they had a T2-W lesion volume of less than 20 cm3 (about 1.3% of the total brain tissue volume). This condition was met in 57 of the 60 MS patients; thus, data from 3 patients were excluded from the analysis. Percentage difference MTr images were calculated on a voxel-by-voxel basis according to the following equation:

\[ MTr = 100 \times (\text{No Sat} - \text{Sat}) / \text{No Sat} \]

(after thresholding above the noise background), as previously described. Once T2-W lesions and normal-appearing brain were classified, the MTr values of these regions were calculated. Only values of MTr in the normal-appearing white matter (NAWM) were considered here. These were calculated by taking consistent samples of white matter from 5 regions (corona radiata and centrum semiovale, frontal lobe, genu of corpus callosum, splenium of corpus callosum, and occipital lobe) (Figure 1). The MTr for NAWM was then obtained by averaging the mean MTr from each region. Values of MTr for NAWM in the control group were obtained in the same way.

Proton-MRS Imaging

Postprocessing of the raw proton-MRS imaging data was performed as previously described. Metabolite resonance intensities of NAA were determined automatically from peak areas relative to a spline-corrected baseline and expressed as ratios to Cr (Figure 2). The resonance intensity of intravoxel Cr has been widely used as an internal standard in MRS studies in vivo, as it is relatively equally present in all brain cells and tends to be stable in nonacute pathology. Changes in apparent brain Cr concentrations have been reported in MS in recent MRS studies attempting absolute quantitation. However, all current quantitative approaches have important limitations when applied to clinical studies and, in MS patients, have shown discrepant results in lesions and NAWM. In vitro MRS, which does not suffer from the limitations of in vivo quantitation, has demonstrated that Cr does not change in normal-appearing tissues of the brains of MS patients. Thus, as lesions accounted for only a minimal portion of the large central VOI (mean, 1.5% [range, 0%-3%]; data not shown) in this group of MS patients, changes in Cr, although possible, are unlikely and results were expressed as the intravoxel ratio of NAA to Cr. The relative NAA/Cr values of the whole brain region were obtained by averaging the NAA/Cr for all the voxels in the spectroscopic VOI for each subject. Spectra at the edges of the VOI affected by chemical shift artifacts associated with selective excitation were deleted before averaging.

Total Brain Volumes

On T1-W MR images, normalized volumes of the whole of the brain parenchyma were measured using a method for brain volume measurement (the cross-sectional version of the SIENA software [SIENAX; available at: http://www.fmrib.ox.ac.uk/analysis/research/siena/]) (Figure 3). SIENAX uses a method to extract the brain and skull from the MR images, as previously described. A tissue segmentation program is then used to segment the extracted brain image into brain tissue, cerebrospinal fluid, and background, yielding an estimate of total volume of less than 20 cm3 (about 1.3% of the total brain tissue volume).
brain tissue volume. The original MR images are 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 factor for each subject. The estimate of brain tissue volume for a subject is then multiplied by the normalization factor to yield the normalized brain volume (NBV).

**STATISTICAL ANALYSIS**

At each site, MR data of MS patients were compared with those of an age-matched healthy control (HC) group: 21 subjects at MNH (13 women and 8 men; age range, 22-57 years [mean, 35 years]) and 21 at Siena (12 women and 9 men; age range, 21-52 years [mean, 35 years]). Comparisons were made between corresponding HC populations of each site (ie, Siena-HC vs MNH-HC) and between the whole group of MS patients and HC subjects of both sites. In the latter case, MR data were standardized at each site using a z score transformation relative to the corresponding HC group. This allowed avoiding potentially spurious results due to machine-related differences between sites. The nonparametric Kruskal-Wallis 1-way analysis of variance on ranks was used for the statistical analysis, and values were considered significant at the .05 level. SYSTAT software (Version 9 for Windows; SPSS Inc, Chicago, Ill) was used to perform statistical calculations.

**RESULTS**

The comparison of MR measurements from each center showed that NAA/Cr and NBV values of the HC groups were not different between the 2 sites (NAA/Cr in Siena-HC,
3.06 ± 0.2 and in MNH-HC, 3.14 ± 0.16; NBV in Siena-HC, 1467 ± 41 cm³ and in MNH-HC, 1476 ± 68 cm³; $P < 0.001$ for both). However, white matter MTr values were significantly lower in the Siena-HC group than in the MNH-HC group (MTr in Siena-HC, 35.3 ± 0.9 and in MNH-HC, 36.4 ± 0.4; $P < 0.001$). This was probably due to the sensitivity of MTr measurements to subtle differences in hardware between MR scanners. However, as mentioned before, all MR data measurements were standardized at each site using a z score transformation to correct for differences in HC subjects at different sites and to allow comparisons between the whole groups of HC subjects and MS patients.

In the whole group of MS patients without clinical disability, the standardized levels of central brain NAA/Cr were significantly lower than those of HC subjects ($P < 0.001$, Figure 4). Similarly, standardized MTr values were lower in the NAWM of MS patients than in the white matter of HC subjects ($P < 0.001$, Figure 4). However, while the fully automated estimation of standardized NBV showed a trend toward decreased values in the MS group with respect to the age-matched HC group, this did not reach statistical significance ($P = 0.07$, Figure 4).

When similar analyses were performed in MS patients grouped according to duration of disease, 36 pa-
patients with early disease duration (<3 years) still showed significantly lower brain NAA/Cr and MTr values than did HC subjects (P = .001 and P = .004, respectively; Figure 5). Furthermore, a subgroup of 26 patients with minimal lesion volume (~2 cm³ of T2-W MR imaging lesions, about 0.15% of the total brain volume) also showed significantly low NAA/Cr and MTr (P < .05 for both; Figure 6). In both subgroups, patient values of NBV were not different from those of HC subjects (P > .50 in both subgroups).

Because NAA is localized to neurons and axons in adult human brain and correlates strongly with axonal density, levels of brain NAA detected by proton-MRS imaging can be interpreted, with some rare exceptions, as a surrogate of neuronal and axonal integrity. Large decreases of NAA have been observed in numerous spectroscopic studies inside and beyond MS lesions and have been demonstrated to occur, to a lesser degree, also in the NAWM of MS patients from the early disease stages. Results of the present study extend these previous observations by showing that significant decreases of NAA can be detected in the NAWM of MS patients with very low disease duration, in the absence of substantial focal brain demyelination and before permanent clinical disability becomes evident. Given that NAA decreases were confined to demyelinating lesions in brains of patients with clinically isolated syndromes who subsequently developed MS, our observations also suggest that diffuse cerebral axonal injury rapidly accumulates in the early stages of the disease.

In addition to decreases of NAA/Cr, we also found decreases of MTr in the NAWM in this group of nondisabled MS patients. Magnetization transfer imaging of the brain is based on the interactions between the free water protons and protons attached to macromolecules, and a low MTr indirectly reflects tissue (matrix) damage. Several studies have demonstrated marked MTr reductions in lesions and NAWM of patients with MS. As recent studies have shown that focal MTr decreases in NAWM can occur before lesion appearance on conventional MR imaging, a low MTr in the NAWM may reflect subtle, microscopic, or molecular pathology of myelin in macroscopically normal white matter. Edema, astrocytic proliferation, perivascular inflammation, and demyelination may all contribute to a decreased amount of water bound to macromolecules in the NAWM, and, as a consequence, reduced MTr. These pathologic features, however, are not prominent in the NAWM in early MS. A potential mechanism for subtle molecular alteration of myelin remains to be determined, but its presence is suggested by the fact that MTr values are decreased in the white matter of patients with MS who have completely normal results of conventional MR imaging of their brain. In addition, since MTr decreases in postmortem brain of MS patients also correlate with axonal loss, it may be possible that membrane alterations associated with axonal injury also contribute to the decreases in MTr.

In this cross-sectional study, we did not find significant differences in NBV between MS patients and age-matched HC subjects, although there was a trend suggesting the presence of modest brain atrophy in the patient group as a whole. Significant atrophy has been recently reported in the brains of MS patients in both cross-sectional and longitudinal studies that used automated or semiautomated measures of brain volume.

©2002 American Medical Association. All rights reserved.
particular, significant losses of brain volume have been found in MS patients with mild disability, and significantly increased rates of ventricular enlargement have been reported in early-stage MS patients and in patients with clinically isolated syndromes who later developed MS. Differences between these published data and those presented herein could be the result of the lower sensitivity of cross-sectional measurements of total brain volume with respect to measurements of atrophy rates and the particularly mild clinical condition of the patients included in our study. Indeed, by showing a trend toward NBV decreases in MS patients with a low lesion load and the absence of disability, we suspect that the present results are not really in conflict with previous findings. Notably, the differences in the relative magnitude of decreases in NAA/Cr and NBV in our study suggest that decreases in NAA (which can result from axonal dysfunction, decreases in axonal density, and axonal loss), and decreases in NBV (which reflect a less pathologically specific tissue loss) do not always occur in parallel. Brain atrophy should be considered a later event that is not necessarily proportional to axonal injury. It follows that in vivo measures of total axonal injury and loss should be based on measurements of both decreases in brain volume and decreases of NAA density in remaining brain tissue.

By restricting our analysis to MS patients with a low volume of T2-W lesions, we sought evidence that the abnormalities in NAWM could occur independently of focal demyelinating lesions. As reported in a number of previous studies, decreases of NAA and MTr are more pronounced inside demyelinating lesions than in the NAWM of MS patients. However, the presence of significant decreases of NAA/Cr and MTr in our subgroup of patients in whom lesions occupied less than 0.2% of the total brain tissue (the patient subgroup with $\leq 2$ cm$^3$ of T2-W MR imaging lesions) suggests that axonal and tissue injury in MS might accrue, at least in part, independently of focal cerebral demyelination. Since walleterian degeneration of axons traversing lesions is unlikely to account for much of the NAA decrease seen in these patients with very low lesion loads, the diffuse axonal abnormality must result from nonlesional abnormalities associated either with subtle myelin abnormality that is not visible on conventional MR imaging or with subtle axonal abnormality, possibly due to the indirect effects of inflammation. That diffuse decreases of NAA are to some extent reversible with immunomodulatory therapy is consistent with a role for inflammation in these decreases.

How can widespread axonal injury occur in the brains of MS patients without clinical evidence of disability? Experimental and functional MR imaging data may provide an answer. Neurons can function as dynamic electric machines with electroresponsive properties that change in response to pathologic insults. However, in the initial disease stages, both adaptive cortical reorganization (by the “unmasking” of latent pathways) and ion channel redistribution can achieve complete functional recovery and cause neuronal degeneration to remain subclinical. Development of permanent disability occurs later, when a threshold of axonal loss is reached and compensatory resources of the central nervous system are exhausted. Thus, axonal injury does occur in MS, even in the absence of clinical disability, and can be detected and monitored by pathologically specific MR measures.

In conclusion, results of our study indicate that cerebral NAA/Cr and MTr values are diffusely decreased in brains of patients with early MS, minimal focal T2-W lesion volume, and no clinical evidence of disability. This finding suggests that axonal and/or tissue injury might be to some extent independent of focal cerebral demyelination and is probably initially well compensated for by brain plasticity. Decreases of total brain volume do not necessarily occur in parallel with decreases of NAA and, therefore, both NAA and brain atrophy should be assessed to determine the true total extent of axonal injury and loss.

Accepted for publication May 29, 2002.

Author contributions: Study concept and design (Drs De Stefano and Arnold, Mr Narayan, and Ms Tartaglia); acquisition of data (Drs De Stefano, Mortilla, Bartolozzi, Guidi, and Arnold; Messrs Narayan and Francis; and Ms Tartaglia); analysis and interpretation of data (Drs De Stefano, Smith, Federico, and Arnold and Messrs Narayan and Francis); drafting of the manuscript (Drs De Stefano, Smith, and Federico and Messrs Narayan and Francis); critical revision of the manuscript for important intellectual content (Mr Narayan and Drs Mortilla, Bartolozzi, Guidi, and Arnold); statistical expertise (Drs De Stefano, Smith, and Bartolozzi and Mr Narayan); administrative, technical, and material support (Messrs Narayan and Francis and Drs Mortilla, Bartolozzi, and Federico); and study supervision (Dr Arnold).

This study was supported by grants from the Multiple Sclerosis Society of Canada (Toronto, Ontario) and the Medical Research Council of Canada (Ottawa, Ontario). Dr De Stefano was supported by a PAR (Progetto Ateneo di Ricerca) grant of the University of Siena. Dr Federico was supported by a grant from MURST (Ministero dell’Università e della Ricerca Scientifica e Tecnologica, Rome, Italy). Dr Arnold was supported by grants from the Multiple Sclerosis Society of Canada and the Medical Research Council of Canada.

Corresponding author and reprints: Nicola De Stefano, MD, Institute of Neurological Sciences, Viale Bracci 2, 53100, Siena, Italy (e-mail: destefano@unisi.it).