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Imaging axonal damage of normal-appearing white matter in multiple sclerosis

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Summary

The current study was designed to determine the relative distribution of decreases of N*-acetylaspartate (NAA), a marker of axonal damage, between lesions and normal-appearing white matter of patients with established multiple sclerosis and to test for associations between changes in the ratio of NAA to creatine/phosphocreatine (NAA : Cr) in those compartments and changes in disability. Data were collected from a 30-month longitudinal study of 28 patients with either a relapsing course with partial remissons and no progression between attacks (relapsing/remitting) (11 patients) or a course of progressively increasing disability, following a period of relapsing/remitting disease (secondary progressive) (17 patients). Proton magnetic resonance spectroscopic imaging (MRSI) and conventional MRI examinations were performed at 6–8-month intervals with concurrent clinical assessments of disability. General linear models were used to test associations between MRSI, MRI, lesion volume and clinical data. Analysis confirmed that the NAA : Cr ratio is lower in lesions than in the normal-appearing white matter (–15.3% in relapsing/remitting multiple sclerosis and –8.8% in secondary progressive multiple sclerosis). The lower NAA : Cr ratio per unit lesion volume previously observed for secondary progressive relative to relapsing/remitting patients was found to result from a lower ratio (8.2%,* $P < 0.01$*) in the normal-appearing white matter rather than from any differences within lesions. The importance of changes in the normal-appearing white matter was emphasized further with the observation that the NAA : Cr ratio in the normal-appearing white matter accounted for most of the observed 15.6% (*$P < 0.001$*) decrease in the NAA : Cr ratio in the brains of relapsing/remitting patients over the period of study. The decrease in the NAA : Cr ratio in normal-appearing white matter correlated strongly (*$P < 0.001$*) with changes in disability in the relapsing/remitting subgroup. These results add to data suggesting that axonal damage or loss may be responsible for functional impairments in multiple sclerosis. The accumulation of secondary axonal damage in the normal-appearing white matter may be of particular significance for understanding chronic disability in this disease.*

Keywords: multiple sclerosis; magnetic resonance spectroscopy; pathology; white matter; axons

Abbreviations: AC = anterior commissure; Cr = creatine/phosphocreatine; EDSS = Expanded Disability Status Scale; MRS = magnetic resonance spectroscopy; MRSI = MRS imaging; NAA = *N*-acetylaspartate; PC = posterior commissure; SPM = statistical parametric mapping

Introduction

MRI can demonstrate macroscopic lesions associated with multiple sclerosis with high sensitivity, but lesions on standard T_2-weighted MRI lack specificity for underlying pathological processes such as inflammation, demyelination or axonal loss. The pathophysiological heterogeneity of T_2-weighted lesions is important for the understanding of why identically appearing lesions may have different clinical consequences and must contribute to the weakness of the association between T_2-weighted lesion volumes and clinical disability (Paty *et al.*, 1993; Filippi *et al.*, 1995). Such conventional MRI is also insensitive to any microscopic changes (e.g. axonal damage or very small foci of inflammation or

demyelination) that may extend beyond the borders of macroscopic lesions. Because the relative volume of the normal-appearing white matter is so much larger than that of lesions, even modest diffuse pathological changes in normal-appearing white matter could make major contributions to clinical disability.

Although more cumbersome to implement and interpret than MRI, magnetic resonance spectroscopy (MRS) is a potentially useful method for characterization of pathology in multiple sclerosis as it provides chemical indices that are associated with relatively specific pathological changes. In addition, MRS also can detect axonal damage or loss in normal-appearing white matter on conventional T_2-weighted images in brains of patients with multiple sclerosis (Arnold *et al*., 1992; Davie *et al*., 1994; Husted *et al*., 1994; De Stefano *et al*., 1995*a*).

The relevance of measures of axonal damage to understanding functional impairment in multiple sclerosis is suggested by several MR observations. MRS studies of patients recovering from large, single demyelinating lesions have shown a strong, inverse correlation between axonal damage as assessed by decreases in the relative concentration of brain *N*-acetylaspartate (NAA) and functional impairment or disability (De Stefano *et al*., 1995*a*). However, while relative NAA decreases can be reversible in resolving lesions over relatively short periods of time (Arnold *et al*., 1992; Davie *et al*., 1994; De Stefano *et al*., 1995*b*), the observation of low ratio of NAA to creatine/phosphocreatine (NAA : Cr) in patients with long-standing disease (Arnold *et al*., 1990), the inexorable clinical progression of multiple sclerosis and the associated increase in total lesion load suggest that over longer periods, progressive decreases in brain NAA should be found. In a preliminary single voxel study we found evidence for such progression over 18 months in one group of multiple sclerosis patients (Arnold *et al*., 1994).

In a more recent study (Matthews *et al*., 1996) we reported that the decrease in brain NAA : Cr ratio per unit T_2-weighted lesion volume is greater in patients with secondary progressive (progressively increasing disability following a period of relapsing/remitting) than relapsing/remitting (relapsing with partial remissions and no progression between attacks) disease. This latter (single voxel) MRS study prompted us to question the relationship between the distribution of chemical changes associated with axonal damage and the distribution of lesions. It was impossible to determine from the data in our earlier study whether the changes observed were the result of a lower NAA : Cr ratio in the lesions or in the normal-appearing white matter of secondary progressive patients relative to relapsing/remitting patients. We therefore worked to develop a quantitative approach to the combined analysis of longitudinal MRI, MR spectroscopic imaging (MRSI), and clinical data.

Using serially collected MRSI data from 28 patients with multiple sclerosis (11 relapsing/remitting, 17 secondary progressive), we applied random-effects general linear models to answer four questions: (i) Does the difference between relapsing/remitting and secondary progressive patients in the relationship between the T_2-weighted lesion volume and the brain NAA : Cr ratio (Matthews *et al*., 1996) arise from differences in the relative NAA : Cr ratio within lesions or within the normal-appearing white matter? (ii) Is there a significant difference between relapsing/remitting and secondary progressive patients in the overall rate of progression of the NAA : Cr ratio with time? (iii) If so, does this difference arise from differences in the lesions or in the normal-appearing white matter? (iv) Is the decrease of the NAA : Cr ratio in normal-appearing white matter relevant to clinical disability?

Methods

Study population

Twenty-eight patients with clinically definite multiple sclerosis (Poser *et al*., 1983) were chosen from the population followed in the Montreal Neurological Hospital multiple sclerosis clinic. Patients were classified according to clinical course as having either recurrent relapses with partial remission and no progression between attacks (relapsing/remitting course) or secondary, chronic progressive disease with progression in the absence of discrete relapses after an early history of relapsing/remitting disease (secondary progressive course). We attempted to match the two patient groups on the basis of disability and to choose groups with disability levels relevant to current clinical therapeutic trials. This had the effect of selecting for patients with intermediate Expanded Disability Status Scales (EDSS) (Kurtzke 1983). These patients were followed for 30 months with five MRI examinations and with neurological disability evaluations performed every 6–8 months. Normal control subjects were hospital and laboratory workers in good health. This study was approved by the Montreal Neurological Hospital Ethics Committee and informed consent was obtained from all participating subjects.

MRI examinations

Conventional proton MRI and MRSI examinations of the brain were obtained in a single session for each examination using a Philips Gyroscan operating at 1.5 T (Philips Medical Systems, Best, The Netherlands). A sagittal survey image was used to identify the anterior commissure (AC) and posterior commissure (PC). Multislice images were obtained in coronal and transverse planes, perpendicular and parallel to the AC–PC line, respectively (TR = 2116, TE = 30,78, slice thickness 5.5 mm). These images were used to position an intracranial volume of interest for spectroscopy parallel to the AC–PC line and centred on the corpus callosum craniocaudally. The volume of interest measured ~105 mm anteroposterior × 20 mm craniocaudal × 95 mm left–right and was kept constant in size and position after the first examination.

Proton spectra were acquired using a 90°–180°–180° sequence for volume selection (Ordidge *et al*., 1987) (TR = 2000, TE = 272). Magnetic field homogeneity was optimized to a linewidth of ~5 Hz over the volume of interest using the proton signal from water. Water suppression was achieved by selective inversion of the water resonance prior to volume selection using an adiabatic inversion pulse and adjustment of the waiting time so that the spectrum was acquired when the water signal passed through zero (Luyten *et al*., 1989).

MRSIs were generated by two-dimensional phase-encoding (250 × 250 mm field of view, 32 × 32 phase encoding steps, one signal average per step). After a water-suppressed acquisition was completed, another MRSI was acquired without water suppression using TR = 850, TE = 272, field of view = 250 × 250 mm, and 16 × 16 phase encoding steps.

Post-processing

Post-processing of the raw data was done on a SUN/SPARC system using SUNspec1 software (Philips Medical Systems). The non-water-suppressed MRSIs were interpolated to 32 × 32 steps. A mild Gaussian *k*-space filter and an inverse two-dimensional Fourier transformation was then applied to both the water-suppressed and unsuppressed MRSI. Artefacts present in the time domain water-suppressed signal due to static magnetic field inhomogeneities and time-varying gradients were corrected by dividing the water-suppressed MRSI signal by the non-water-suppressed signal (den Hollander *et al*., 1991). This does not affect relative signal intensities. The resulting time domain signal was left-shifted and subtracted from itself to improve water suppression (Roth *et al*., 1980). To enhance the resolution of the spectral peaks, a Lorentzian-to-Gaussian transformation was applied prior to Fourier transformation in the spectral domain. The result was 1024 voxels (32 × 32) each containing data ready for quantification and subsequent generation of the MRSIs. The nominal voxel size in plane was about 8 × 8 × 22 mm, giving a resolution of about 12 × 12 × 22 mm after *k*-space filtering.

Peaks for NAA and Cr were detected using locally developed software. Each resonance intensity was determined from the area of each peak which was bounded below by a locally estimated baseline depending on the neighbourhood at the wings of each peak. Chemical shifts were calculated relative to the NAA resonance at 2.0 p.p.m.

Multiple sclerosis lesion classification was performed using locally developed software offering the ability to toggle between the proton density and T_2-weighted images (to allow easier discrimination between grey matter and CSF). Semi-automatic outlining tools facilitated definition of lesion volumes (Kamber, 1991). To use them, the operator established a contrast threshold for the lesion edge based on a linear map of signal intensities in a slice. The edge of the lesion was then automatically traced in two dimensions to define the lesion outline. This task was performed for each lesion and corrected by an expert (S.N.) as necessary. Lesion volumes were calculated by multiplying two-dimensional lesion areas by the slice thickness.

All images for the five time points were transformed into a standard anatomical space (Talairach and Tournoux, 1988; Evans *et al*., 1992; Narayanan *et al*., 1997) for statistical analysis.

Statistics

Our goal was to quantify the relationship between chemical pathology measured using the NAA : Cr ratio and several other independent variables, including the spatial distribution of T_2-weighted hyperintense lesions, clinical subgroup (relapsing/remitting versus secondary progressive), clinical disability (EDSS), and duration of disease. The changes in the NAA : Cr ratio over time were also of interest. An appropriate way to do this is to use general linear models (Kleinbaum *et al*., 1988). However, two features of the longitudinally collected images need to be incorporated into the statistical analysis. First, for physiological and also instrumental reasons, MRS images are 'smooth', i.e. they change gradually because the voxels are large with blurred margins and the 'edges' of pathological change are not sharply defined. Thus, signals coming from voxels close to each other in space and time are strongly correlated. Secondly, because there are variations between individual study subjects that cannot be accounted for by any known characteristics or independent variables, the average resonance intensity of any individual MRSI can be higher or lower than the group average.

Although the general linear approach of statistical parametric mapping (SPM) could have been employed to address both of these two features of longitudinally collected image data (Friston *et al*., 1995), we chose to adopt an alternative approach which is an extension of the random-effects models (Laird and Ware, 1982; Jennrich and Schluchter, 1986) to spatial and temporal cases (Fu *et al*., 1996). Differences between the two approaches and the reason we chose to base our analyses on a random-effects model are discussed below.

In our random-effects model approach, the NAA : Cr ratio is modelled as a linear function of a group of independent variables as follows.

$$\text{NAA : Cr}_{ivt} = \mathbf{X}_{ivt}\times\beta + \mu_i + e_i, \qquad (1)$$

where X_{ivt} is the design matrix containing the independent variables for subject *i*, voxel *v*, and time *t*. β is a vector of coefficients quantifying the dependence of relative NAA intensity (NAA : Cr) on independent variables included in the design matrix. μ_i is the random effect for individual subject *i*. The variable e_i is used to represent the measurement errors and spatial-temporal correlation in serial images, i.e. it is used to account for 'bleeding' of the MR signal due to the point spread function and the gradual nature of changes of the NAA : Cr ratio in brain.

There are two particular advantages of the statistics we

Table 1 *Clinical disability of patients before and during the study*

Multiple sclerosis	EDSS score*							
	–30	–18	–6	0	+7.5	+15	+22.5	+30
Relapsing/remitting	4.50 ± 4.24	5.30 ± 2.79	5.22 ± 2.45	4.82 ± 2.25	4.63 ± 3.10	5.05 ± 3.24	4.82 ± 2.98	4.86 ± 2.87
Secondary progressive	4.65 ± 3.01	5.43 ± 2.70	5.94 ± 1.68	5.97 ± 1.70	6.03 ± 1.63	6.12 ± 1.72	6.26 ± 1.33	6.32 ± 1.54

*Months before (minus) and after (plus) starting the study; data are presented as means ± 2 SDs.

applied here for a natural history trial. First, statistical efficiency is gained by taking into consideration the intra-subject correlation with random-effects models. Secondly, loss to follow-up and missing data, a common problem in longitudinal studies, can be handled properly.

Examples of how the general linear model described above can be used to obtain statistical inferences follow. When the correlation between the EDSS and the NAA : Cr ratio is of interest, a general linear model of the following form can be used:

$$\text{NAA : Cr} = \text{EDSS}$$

In this formulation, the equals sign means the item to its left can be modelled as a linear function of items to its right. Every item to the right of the equals sign (here EDSS only) is included in the design matrix in equation (1) as an independent variable. If the correlation is suspected to be different between subgroups, we can use the following more complex model to test for correlation within specific subgroups:

$$\text{NAA : Cr} = \text{Subgroup} + \text{EDSS} + \text{Subgroup} \times \text{EDSS}$$

This model has separate correlation parameters for each of the different subgroups. We can also determine if the difference between subgroups in the correlation between the EDSS and the NAA : Cr ratio is statistically significant. The design matrix for this model includes the clinical subgroup, EDSS score, and their interaction. Confounding effects, e.g. partial volume effects from ventricles (Collins *et al*., 1993), can be controlled for by considering them explicitly as additional variables in the general linear models.

As all images are already in standard space, the normal spatial variation of metabolite concentration can be controlled for in the random-effects model by matching voxel locations, i.e. using voxel location as an independent variable. Advantages of voxel-location matching, issues related to model fitting and statistical significance tests for random-effects models have been discussed in detail in an earlier methodology paper (Fu *et al*., 1996). It is worth emphasizing that, in this study, the precision of all general linear models has been improved significantly by voxel-location matching.

To further clarify our approach, we can contrast it with the widely used SPM approach. In SPM (Friston *et al*., 1995), univariate general linear models are applied independently to each individual voxel in images. This gives several images of spatially distributed linear parameters of interest. Spatial correlation in images is then taken into consideration by adjusting the threshold level for statistical significance. The results are expressed as the spatially distributed parametric maps which are so very informative for functional imaging studies.

Random effect models, by contrast, take into consideration the spatial correlation between voxels based on multivariate statistics. They summarize imaging data and clinical information in a few parameters which can be easily interpreted as discussed above and below in the Results section. Thus, results derived from random-effects models are arguably more suitable for highly clinical–pathological studies. It is important to note that currently the random-effects models approach can only be applied to images with low spatial resolution (e.g. MRSI) due to its computational load.

Results

Patient characterization

The study group included 11 patients with relapsing/remitting multiple sclerosis, 17 patients with secondary progressive multiple sclerosis, and 12 normal control subjects. The EDSS ranged from 2.5 to 7.5 in the relapsing/remitting patients and 4–8 in the secondary progressive patients with medians of 5.0 and 6.0, respectively. Relapsing/remitting patients had well-defined relapses followed by at least partial remission, without clinical evidence for progression between attacks. Secondary progressive patients had a prior history of relapsing/remitting disease, but had gone on to show progression without discrete relapses. EDSS scores for the two groups for the 30 months of the study and the 30 months prior to entry into the study are shown in Table 1.

Quantitative lesion-volume measurements

At the beginning of the study, the average total cerebral lesion volume was 42.7 cm^3 in relapsing/remitting patients and 20.0 cm^3 in secondary progressive patients. The difference in lesion volumes is significant ($P < 0.05$). After 30 months follow-up, the average total lesion volume was 58.3 cm^3 and 30.5 cm^3 in the relapsing/remitting and secondary progressive patient groups, respectively.

The average lesion volumes within the MRSI slice were 15.8 cm^3 in relapsing/remitting and 9.0 cm^3 in secondary progressive patients at the beginning of the study and these

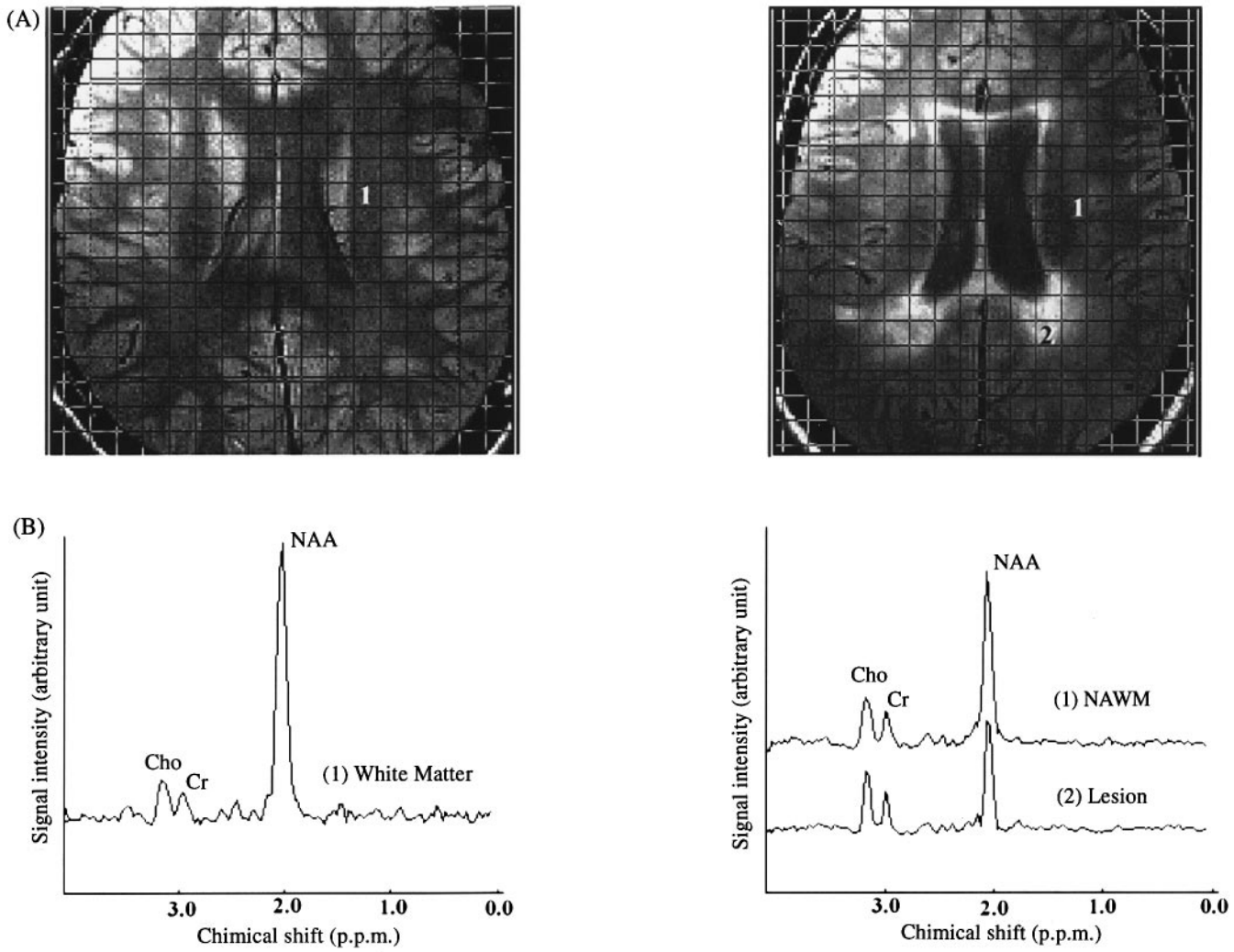

Fig. 1 T_2-weighted MRI (**A**) and proton spectra (**B**) from a normal control subject (*left*) and a multiple sclerosis patient (*right*). The proton spectra were selected from the voxels indicated in the MRI (**1** in the normal control, **1** and **2** in the multiple sclerosis patient). The NAA : Cr intensity ratio is lower in voxels within the normal-appearing white matter (NAWM) of multiple sclerosis patients than in those from normal white matter of the control subjects and even lower in voxels from lesions in the patients.

increased to 20.4 cm^3 (relapsing/remitting) and 13.7 cm^3 (secondary progressive) by the end of the study.

Averaged MRSI results

Conventional MR and single-slice MRS images were obtained for each patient at 6–8 month intervals. Representative examples of T_2-weighted MRI and spectra from selected voxels from a normal control and a multiple sclerosis patient are shown in Fig. 1. There was variation in the NAA : Cr resonance intensity ratio across the MRS images from both normal control subjects and multiple sclerosis patients, with lower NAA : Cr ratio values in the region of the ventricles. (Observation of lower periventricular NAA : Cr ratios in normal control subjects suggests that this change results from a combination of spatial heterogeneity in NAA and Cr within the brain substance or a systematic underestimation of the NAA : Cr ratio when the signal-to-noise ratio is lowered by ventricular partial volume effect.) The NAA : Cr signal intensity ratios were consistently lower in images from multiple sclerosis patients than in those from control subjects. At the start of the study, the average NAA : Cr ratio through the image volume was 3.98 $\pm$ 0.36 for relapsing/remitting and 3.44 $\pm$ 0.10 for secondary progressive patients (5.0 $\pm$ 0.13 for control subjects, $P < 0.0001$ compared with multiple sclerosis patients).

General linear models for multimodal analysis of MRI, MRSI and clinical data

Analysis of the spatial variation in the NAA : Cr ratio as a function of the distribution of lesions

We first wished to determine whether the difference between relapsing/remitting and secondary progressive patients in the relationship (Lesion Volume)/(NAA : Cr) that we previously

Table 2 *Results from model describing the spatial variation in the NAA : Cr ratio as a function of lesion distribution*

Independent variable	Coefficient (β) estimate	Standard error	Comparison with normal-appearing white matter in relapsing/remitting MS	Significance of change (*P*-value)
NAWM (secondary/progressive MS)	–0.41	0.16	–8.2%	<0.01
Lesion (relapsing/remitting MS)	–0.77	0.15	–15.3%	<0.001
Lesion (secondary/progressive MS)	–0.44	0.16	–8.8%	<0.01
Ventricle	–0.22	0.11	–4.4%	<0.05

The model is NAA : Cr ratio = Subgroup + Lesion + Subgroup × Lesion + Ventricle. MS = multiple sclerosis; NAWM = normal-appearing white matter.

reported in a single voxel MRI/MRS study (Matthews *et al*., 1996) arose as a consequence of differences between the two patient groups in the relative NAA : Cr ratio within lesions or of differences within the normal-appearing white matter. To do this, the relationship between the distribution of T_2-weighted hyperintense signal from multiple sclerosis lesions on the MRI and the NAA : Cr ratio in co-registered MRSI voxels was evaluated for the two clinical subgroups using the following model (1):

$$\text{NAA : Cr} = \text{Subgroup} + \text{Lesion} + \text{Subgroup} \times \text{Lesion} + \text{Ventricle}$$

where Subgroup equals 0 or 1 (for relapsing/remitting and secondary progressive, respectively) and Lesion and Ventricle are the proportions of multiple sclerosis lesions and ventricles, respectively, in an individual voxel of the MRSI. Inclusion of the Ventricle term allowed correction for artifacts from partial volume effects of the ventricular CSF in the MRSI slice.

Fitting of the model with the image data from all five time points (Table 2) confirmed previous studies (Arnold *et al*., 1992) demonstrating that the NAA : Cr ratio is lower in lesions than in the surrounding normal-appearing white matter. As well, application of the model demonstrated a significant difference in the extent of the decrease of the NAA : Cr ratio in normal-appearing white matter in the two patient groups: the NAA : Cr ratio was 8.2% lower ($P < 0.01$) in the normal-appearing white matter of secondary progressive than in the normal-appearing white matter of relapsing/remitting patients.

Changes in the NAA : Cr ratio over time

The changes in the brain NAA : Cr ratio with time in the voxels of the serially obtained images from the two patient subgroups were estimated using the following linear model (2).

$$\text{NAA : Cr} = \text{Subgroup} + \text{Time} + \text{Subgroup} \times \text{Time} + \text{Ventricle}$$

where Subgroup equals 0 or 1 (for relapsing/remitting and secondary progressive, respectively) and Time is the examination number in the series of five studies.

This model demonstrated a difference between patients in the two subgroups in the time course of changes in the NAA : Cr ratio (Table 3). There was a significant decrease in the NAA : Cr ratio of 6.2% per year or 15.6% over the 30 month study period ($P < 0.001$) for the relapsing/remitting patients. No significant changes were found for the secondary progressive patients over the course of the study.

We then asked whether the observed decrease in the NAA : Cr ratio with time for the relapsing/remitting subgroup might have resulted solely from the observed increases in lesion volume, as the NAA : Cr ratio was shown to be lower in lesions than in normal-appearing white matter. Such an effect of increases in lesion volume on the brain NAA : Cr ratio might be less apparent for secondary progressive patients despite the increase in lesion volume in these patients, because both the total lesion load and the difference between the NAA : Cr ratio in lesions and in normal-appearing white matter are smaller.

This hypothesis was tested using the following model (3):

$$\text{NAA : Cr} = \text{Subgroup} + \text{Time} + \text{Subgroup} \times \text{Time} + \text{Lesion} + \text{Subgroup} \times \text{Lesion} + \text{Ventricle}$$

where the terms are as defined above. Model (3) established a correlation between the changes in lesion volume and the NAA : Cr ratio over time for the relapsing/remitting group (Table 4), but showed that increases in the lesion volume alone accounted for only a small proportion of the observed changes in the NAA : Cr ratio with time; comparison of the coefficients (β) expressing the changes of NAA : Cr ratio for model 2 (6.2%) in which the possible effects of increases in lesion volume are ignored and model 3 (5.8%) (in which the possible effects of increasing lesion volume are explicitly considered) indicates that the larger volume function of a focally decreased NAA : Cr ratio in the white matter arising from an increase in lesion volume accounts for only ~0.4% of the observed decrease in the NAA : Cr ratio (Tables 3 and 4).

To confirm that the NAA : Cr ratio in the normal-appearing white matter was decreasing with time, an independent analysis of data selected only from voxels in the normal-appearing white matter of the relapsing/remitting patients was performed. A significant decrease ($P < 0.001$) in the NAA : Cr ratio of 16% over the study period was found in these voxels confined to normal-appearing white matter.

Table 3 *Results from model describing changes in the NAA : Cr ratio over time*

Independent variable	Coefficient (β) estimate	Standard error	Comparison with normal-appearing white matter in relapsing/remitting MS	Significance of change (*P*-value)
Time (in relapsing/remitting MS)	−0.3	0.05	−6.2(%) *	<0.001
Time (in secondary/progressive MS)	0.07	0.03	+1.3(%) *	Not significant

The model is NAA : Cr ratio = Subgroup + Time (years) + Subgroup × Time (years). MS = multiple sclerosis. *Percentage change per year.

Table 4 *Results from model describing changes in the NAA : Cr ratio over time adjusted for lesion volume evolution*

Independent variable	Coefficient (β) estimate	Standard error	Comparison with normal-appearing white matter in relapsing/remitting MS	Significance of change (*P*-value)
Time (in relapsing/remitting MS)	−0.30	0.06	−5.8*	<0.001
Time (in secondary/progressive MS)	0.07	0.03	+1.4*	Not significant
Lesion (in relapsing/remitting MS)	−0.64	0.14	$-12.7^{\dagger}$	<0.001
Lesion (in secondary/progressive MS)	0.40	0.16	$-8.0^{\dagger}$	<0.05

The model is NAA : Cr ratio = Subgroup + Time (years) + Subgroup × Time (years) + Lesion + Subgroup × Lesion. MS = multiple sclerosis. *Percentage change per year. †Effect of lesion on NAA : Cr ratio for all time points.

Table 5 *Results from model describing the relationship between the NAA : Cr ratio in brain and disability*

Independent variable	Coefficient (β) estimate	Standard error	Significance of change (*P*-value)
EDSS (in relapsing/remitting MS)	–0.17	0.06	<0.005
EDSS (in secondary/progressive MS)	0.004	0.08	Not significant

The model is NAA : Cr ratio = Subgroup + EDSS + Subgroup × EDSS. MS = multiple sclerosis.

The relationship between the brain NAA : Cr ratio and disability over the period of study

The statistical power of any test of the correlation between clinical disability and the NAA : Cr ratio was limited by our study design, which selected patients having a relatively narrow range of disability near the mid-range of the EDSS scale, where the rate of change is relatively slow. This was particularly true for the secondary progressive patients. Nevertheless, a small but significant correlation ($P < 0.005$) between clinical disability (measured by EDSS) and the NAA : Cr ratio over the period of study was found for the relapsing/remitting patients (Table 5). No meaningful correlation between the NAA : Cr ratio and disability was found for the secondary progressive patients.

We hypothesized that there might be a difference in the strength of the correlation between clinical disability and the NAA : Cr ratio for voxels within lesions compared with the NAA : Cr ratio for voxels in normal-appearing white matter. By classifying voxels in the spectroscopic images as being associated with either lesion or normal-appearing white matter, we were able to test these relationships. Applying the unbalanced repeated-measures models (Jennrich and Schluchter, 1986), a significant correlation between NAA : Cr ratios and clinical disability was only found for changes in voxels within the normal-appearing white matter ($P < 0.001$).

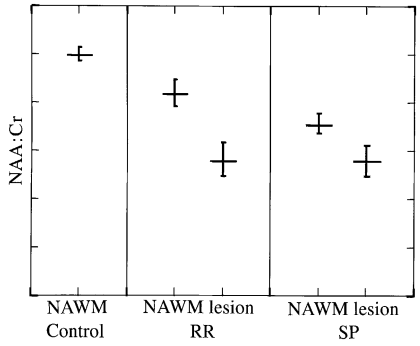

Fig. 2 Relative NAA : Cr ratios for white matter of normal control subjects and from patients with multiple sclerosis, within normal-appearing white matter (NAWM) or T_2-weighted MRI lesions. Estimated standard errors are shown. Data are from time point 1. RR = relapsing/remitting; SP = secondary progressive.

Discussion

Imaging axonal damage in normal-appearing white matter

We have summarized our results in Fig. 2. Although the largest decreases in the NAA : Cr ratio were found in lesions, both subgroups of patients also had substantial decreases in the NAA : Cr ratio in the normal-appearing white matter implying that axonal dysfunction or damage extends into

these regions. (In this study, there are gaps of 0.5 mm between the 5.5-mm MRI slices.) It is possible that multiple sclerosis lesions in the MRI gaps may contribute to the observed difference in NAA : Cr ratios between normal-appearing white matter in multiple sclerosis patients and the white matter of normal control subjects. However, this effect should be very small. Based on the relative volume of normal-appearing white matter and multiple sclerosis lesions in the imaged white matter and the measured average reduction in the NAA : Cr ratio in individual multiple sclerosis lesions, we estimate that multiple sclerosis lesions in the MRI gaps should reduce the NAA : Cr ratio in imaged normal-appearing white matter by 0.5% at most.) We conclude from this that a normal appearance on MRI may conceal potentially important pathological changes, a finding consistent with changes in quantitative T_2 and magnetization transfer ratio in the normal-appearing white matter of patients with multiple sclerosis (Tofts *et al*., 1990; Armspach *et al*., 1991; Hiehle *et al*., 1994). A quantitative subgroup comparison showed that there was a significantly lower NAA : Cr ratio in normal-appearing white matter of the secondary progressive patients, suggesting a greater accumulated load of axonal pathology in the secondary progressive subgroup, despite the lower total T_2-weighted lesion volume relative to the relapsing/remitting subgroup. It is intriguing to consider whether this difference in the aggregate axonal damage in the normal-appearing white matter may account for the differences in clinical course for patients in the two groups.

Although the NAA : Cr ratio is lower in lesions than in normal-appearing white matter, the magnitude of the effect of reduced NAA : Cr ratios within lesions on the overall brain NAA : Cr ratio must be small because of the relatively small volume of lesion compared with the volume of normal-appearing white matter in the brain of these patients (e.g. an average lesion volume of 42.7 cm^3 in relapsing/remitting patients represents $<5\%$ of the total brain white matter volume). The maximum decrease in the NAA : Cr ratio observed in voxels within lesions is ~50% of the ratio in white matter of normal control subjects (Matthews *et al*., 1991; Arnold *et al*., 1992). This is similar to quantitative histological estimates of total axonal volume loss in chronic multiple sclerosis plaques (Lumsden, 1970). Even though the changes are smaller in magnitude, the more widespread changes in the NAA : Cr ratio in the normal-appearing white matter must dominate NAA : Cr ratios measured from large volumes containing predominantly normal-appearing white matter. Thus, the greatest contribution to net axonal damage in the brain of multiple sclerosis patients is not reflected with conventional imaging other than, perhaps, as atrophy (Loseff *et al*., 1996).

Our results also imply that the differences between pathological changes in the normal-appearing white matter of relapsing/remitting and secondary progressive patients (rather than in the lesions) therefore must account for our previous observation of differences in the ratio of (Lesion Volume)/(NAA : Cr) from a single voxel MRS comparison of the two groups (Matthews *et al*., 1996).

The pathological basis of imaging changes

A number of pathological processes may contribute to the observed decreases in the NAA : Cr ratio. Decreases in NAA may result from loss of axonal volume, either from thinning of axons or decreases in number (Arnold *et al*., 1990; Larsson *et al*., 1991; Husted *et al*., 1994; De Stefano *et al*., 1995*b*). Also, neuronal metabolic dysfunction may lead to decreases in NAA (Matthews *et al*., 1995). Previous work suggests that changes in metabolite relaxation times do not have a significant effect on the observed NAA : Cr ratios (Matthews *et al*., 1991), but this effect of changes in relaxation times has not been studied in detail. In addition, increases in Cr could decrease the observed NAA : Cr ratio. A recent post-mortem study found decreases in total Cr in and near multiple sclerosis plaques (Davies *et al*., 1995) and normal Cr in normal-appearing white matter remote from plaques. Indirect evidence of increases of Cr in normal-appearing white matter *in vivo* have been reported (Husted *et al*., 1994; Pan *et al*., 1996). The discrepancy between the post-mortem chemical studies and the *in vivo* MRS could arise in part from changes in Cr relaxation times with multiple sclerosis (Haughton *et al*., 1992), also with patient heterogeneity, systematic errors in concentration calculations or sampling differences. The assumption that the NAA concentration in the cortex of multiple sclerosis patients is unchanged by disease, that was used by Pan *et al.* (1996) in order to estimate relative Cr concentrations, is questionable given our evidence and that of previous studies (Davies *et al*., 1995) for diffuse axonal damage.

The widespread decreases in the NAA : Cr ratio in the normal-appearing white matter may be a marker of axonal changes extending from sites of focal damage in lesions (either those visible on the T_2-weighted image *or* 'microscopic' lesions scattered diffusely through the normal-appearing white matter). However, although microscopic lesions have been reported to account for a large percentage of the total number of lesions (Lumsden, 1970), their total volume relative to that of the normal non-lesion white matter seems unlikely to be sufficient to account for changes in the overall brain NAA : Cr ratio of the magnitude observed. Given the observed ratios of NAA : Cr that we have measured for macroscopic lesions, if diffuse axonal loss and damage were *not* present in normal-appearing white matter, lesions (either visible or microscopic) would need to occupy almost two-thirds of the normal-appearing white matter to account for the NAA : Cr ratio in the normal-appearing white matter reported here. We therefore believe that the observed decreases in the NAA : Cr ratio in the normal-appearing white matter reflect either the consequences of axonal degeneration extending from focal lesions or of axonal pathology in normal-appearing white matter that is independent of focal inflammatory demyelinating lesions.

Axonal damage, disability and clinical course

Although the mean EDSS did not change significantly in either the relapsing/remitting or secondary progressive subgroups over the 30 months of the study, a correlation between the changes in the EDSS and NAA : Cr ratios was found in the relapsing/remitting subgroup. This probably reflects the greater disease activity in this subgroup and correlated fluctuations in the EDSS and NAA : Cr ratios; relapsing/remitting patients showed a larger variation in EDSS over the course of the study. The lack of fluctuations in EDSS in the secondary progressive group, the fact that disease activity was less in this group, the relatively narrow range of EDSS, and the small number of patients all combined could probably explain the fact that changes in the EDSS and the NAA : Cr ratio were not correlated in the secondary progressive subgroup. Another possible contributing factor was the already more severe damage in the white matter of this group. If the rate of new damage to axons is proportional to the density of existing axons, then new inflammatory foci may lead to less further axonal damage in later stages of the disease when there are fewer axons in close proximity to new inflammatory foci. Finally, the failure to observe changes in the secondary progressive group could simply reflect insufficient sensitivity of the spectroscopic method. As the dynamic range of changes in the NAA : Cr ratio for the normal-appearing white matter is smaller for the secondary progressive subgroup than for the relapsing/remitting subgroup, a larger relative change in the secondary progressive subgroup may be needed to reach significance.

The concept that axonal damage or loss may be a major cause of functional impairment is supported by the correlation between decreases in the NAA : Cr ratio in the normal-appearing white matter and changes in disability in the relapsing/remitting subgroup. This concept is attractive for several reasons, among them: (i) animal models showing that demyelination alone need not lead to chronic functional impairment (Rasminsky 1984); (ii) a dissociation of the time course of electrophysiological changes associated with demyelination and functional recovery, suggesting that factors other than demyelination can be directly responsible for the functional impairment (MacDonald and Sears, 1970); and (iii) the strong correlation between functional recovery and increases in NAA : Cr ratios in isolated, large, acute demyelinating lesions (De Stefano *et al*., 1995*a*).

Although the evidence from the present study suggests strongly that pathological changes in the normal-appearing white matter may be related to changes in clinical disability, there are potential limitations to our interpretation of the data. Partial volume effects and possible minor mis-registration errors between serial scans lower the statistical power of comparison of changes of in the NAA : Cr ratio in lesions either with time or between patient subgroups. Because the volume of normal-appearing white matter is so much larger, it is much less sensitive to these effects.

Could the differences in accumulated damage in the normal-appearing white matter account for the differences in the clinical courses of our patients (relapsing/remitting versus secondary progressive)? A number of lines of evidence suggest that functional plasticity in the cortex may be an important mechanism of recovery after focal brain injuries (Weiller *et al*., 1992). Functional reorganization might be expected to become less robust with progression of diffuse neuronal and axonal dysfunction or loss. The availability of functional MRI offers new opportunities for testing the possible role of functional reorganization in remissions and the potential loss of cortical plasticity with progression of multiple sclerosis.

Conclusion

The results of this study support the hypothesis that axonal damage may be an important cause of chronic disability in multiple sclerosis. For the patients studied here, the extent of axonal damage, as assessed by decreased NAA : Cr ratios, showed a stronger correlation with clinical disability than did lesion volume. At entry into the study, evidence of axonal damage was greater in the patient subgroup with greater disability, whereas lesion volume was greater in the subgroup with lower disability. Over the 30 months of the study, the most striking correlated changes were between EDSS and NAA in the normal-appearing white matter of the relapsing/remitting patients. This suggests that diffuse axonal damage or dysfunction extending outside of lesions identified by conventional imaging may make a major contribution to chronic disability.

Our study illustrates how general linear models can be applied to the analysis of spatially resolved time course data to integrate quantitative information available from both different imaging modalities and from clinical assessments. Our approach is readily generalizable to include additional imaging or other data. We believe that a more specific definition of lesion pathology and its spatial distribution will lead to improved understanding of the relationship between multiple sclerosis lesions defined by MRI and clinical disability.

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