Axonal Injury and Overall Tissue Loss Are Not Related in Primary Progressive Multiple Sclerosis

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Background: There is an increasing body of evidence that magnetic resonance imaging–occult tissue damage is an important component of primary progressive multiple sclerosis (PPMS) pathology. Proton magnetic resonance spectroscopy ($^1$H-MRS) can be used to measure in vivo whole-brain N-acetylaspartate (WBNA) concentrations, the decrease of whose levels is considered a marker of neuronal-axonal injury.

Objectives: To study WBNA $^1$H-MRS as a tool to provide information about irreversible brain damage in PPMS and to investigate the relationship between WBNA and other magnetic resonance imaging measures of MS disease burden, including brain atrophy.

Methods: The following magnetic resonance pulse sequences of the brain were obtained from 32 patients with PPMS and 16 age-matched healthy subjects: (1) dual-echo turbo spin-echo; (2) T1-weighted spin-echo; and (3) $^1$H-MRS to measure WBNA concentration. Brain total lesion volumes were measured. Normalized brain volumes were calculated using a fully automated technique. Absolute WBNA amounts were calculated using a phantom replacement method and were then corrected for individual subjects’ brain size.

Results: Levels of WBNA concentrations and normalized brain volumes were significantly lower in patients with PPMS (mean values, 10.2 mm and 1500.0 mL, respectively) than in healthy controls (mean values, 12.9 mm and 1585.2 mL). Both WBNA concentrations and normalized brain volumes were included as independent factors in the final model of a multivariable analysis predicting the subjects’ condition. No significant correlations were found between WBNA values and normalized brain volumes, WBNA and T2-weighted or T1-weighted lesion volumes.

Conclusions: Axonal-neuronal damage in the brain of patients with PPMS seems to occur, at least partially, independently of the burden of magnetic resonance imaging–visible lesions. Whole-brain N-acetylaspartate values and normalized brain volumes were unrelated in this cohort, thereby suggesting that $^1$H-MRS and atrophy assessment may provide in vivo complementary information about the actual extent of brain damage in PPMS.

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Compared with equally disabled patients with secondary progressive multiple sclerosis (SPMS), patients with primary progressive MS (PPMS) have fewer and smaller T2-weighted visible lesions and develop fewer "active" lesions over time despite a clear increase in disability. On the other hand, a significant brain volume reduction can be found since the early phases of PPMS, which appears to be closely related to the clinical outcome. All of this suggests that magnetic resonance imaging (MRI)–occult tissue degeneration rather than MRI-detectable inflammation might be an important aspect of PPMS pathology.

Proton MR spectroscopy ($^1$H-MRS) enables us to quantify axonal injury through the measurements of changes in the signal intensity of N-acetylaspartate (NAA), a metabolite localized almost exclusively to neurons and neuronal processes. Despite the relatively small sample sizes and the across-study differences in patients’ clinical characteristics and $^1$H-MRS acquisition and postprocessing, previous localized $^1$H-MRS studies have consistently shown reduced NAA concentrations in voxels with variable amounts of lesional brain tissue and normal-appearing white matter (NAWM). However, current $^1$H-MRS is not without important limitations. First, it is restricted to relatively small volumes of interest, which...
typically represent less than 100 mL of the brain. Since PPMS is likely to be a diffuse central nervous system disease, estimates of the extent of axonal pathology from such measurements must rely on the unproven assumption that changes in NAA concentrations in the volumes of interest reflect the status of the entire brain. Second, in longitudinal studies, an accurate repositioning of the volumes of interest might be challenging with available 1H-MRS techniques. Third, previous localized 1H-MRS studies of PPMS were focused exclusively on white matter changes, whereas there is increasing evidence from postmortem histopathological studies that the gray matter is not spared by MS-related damage, which, in turn, can clearly cause NAA level reductions.

These limitations have been overcome by the development of a new nonlocalized 1H-MRS acquisition sequence, which makes it possible to quantify the concentration of NAA from the whole brain (WBNAANAA). Such an approach, which can be successfully applied to illnesses characterized by a diffuse brain damage, is appealing for obtaining an overall assessment of neuronal-axonal viability in PPMS, especially in the context of natural history studies and clinical trials, where, because of the numbers of patients and MRI scans involved, it might be infeasible to obtain an overall accurate estimate of neuronal damage with other available 1H-MRS techniques. In the present study, we measured the WBNAANAA concentrations in patients with PPMS in an attempt to provide a novel approach to quantify the extent of intrinsic brain pathology in this condition. The correlations between WBNAANAA concentrations and other MRI measures of MS disease burden were also investigated.

**METHODS**

**PATIENTS**

All patients were selected from the MS population attending the Department of Neurology, San Raffaele Hospital, Milan, Italy. The disease course was classified as PP according to international criteria. Other neurologic conditions were carefully excluded by performing appropriate investigations, including cerebrospinal fluid (CSF) examination in all patients. At study enrollment, patients underwent a complete neurologic examination, with rating done using the Expanded Disability Status Scale (EDSS) scores. This was done by a single observer (P.R.), who was unaware of the MRI results, within 3 days from the MRI session.

Thirty-two patients (12 women and 20 men) were studied, of whom 28 were affected by definite PPMS and 4 by probable PPMS. All patients with probable PPMS had negative results for the CSF study and abnormal MRI findings. Twenty-seven patients with PPMS had had a spinal cord presentation at disease onset, the remaining 5 had had other unifocal (1 patient) or multifocal (4 patients) presentations, with motor (4 patients), cerebellar (2 patients), or brainstem (3 patients) disturbances. Mean age was 48.9 years (age range, 31-69 years), median disease duration was 10 years (range, 2-19 years), and median EDSS score was 6.0 (range, 3.0-8.0). Eighteen patients (56.2%) were affected by relevant locomotor disability, the median EDSS score was 6.0 (range, 3.0-8.0). Eighteen patients (56.2%) were affected by relevant locomotor disability, whereas there is increasing evidence from postmortem histopathological studies that the gray matter is not spared by MS-related damage, which, in turn, can clearly cause NAA level reductions.

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**IMAGE ACQUISITION**

Magnetic resonance imaging and 1H-MRS were performed using a 1.5-T scanner (Siemens, Erlangen, Germany). The following sequences were collected from all subjects during a single MR session: (1) dual-echo turbo spin-echo (repetition time = 3300 milliseconds; echo time = 16/98 ms; echo train length = 5; 24 contiguous, 5-mm-thick axial sections with a 256 × 256-pixel matrix; and a 250 × 250-mm² field of view); (2) T1-weighted conventional dual-echo turbo spin-echo (repetition time = 768 ms; echo time = 14 milliseconds; 24 contiguous, 5-mm-thick axial sections with a 256 × 256-pixel matrix; and a 250 × 250-mm² field of view); (3) 1H-MRS based on a 4-step cycle of nonselective 180° inversion pulses to obtain WBNAANAA measurement, following the acquisition scheme described by Gonen et al.

**IMAGE ANALYSIS**

Multiple sclerosis lesions were first identified by agreement by 2 experienced observers (M.R. and A.G.), without knowing to whom the scans belonged, on the hard copies of the first echo of the dual-echo scans and on the T1-weighted scans. The second echo of the dual-echo scans was always used to increase confidence in lesion identification. For T1-weighted scans, only areas with a signal intensity between that of the gray matter and that of the CSF and with corresponding lesions on both echoes of the dual-echo images were considered as hypointense lesions. Digital images were then transferred to a workstation (SUN Sparstation; Sun Microsystems, Mountain View, Calif) for lesion volume (LV) measurements. These were performed by a single observer, unaware of the subject's identity, using a semiautomated segmentation technique based on local thresholding and keeping the marked hard copies as a reference.

On T1-weighted images, normalized volumes of the whole of the brain parenchyma were measured using a fully automated method, the cross-sectional version of the Structural Image Evaluation of Normalized Atrophy software (SIENAX; FMRIB Analysis Group, Oxford, United Kingdom). First, SIENAX uses a brain extraction tool method to extract the brain and skull from MRIs. A tissue segmentation program is then used to segment the extracted brain image into brain tissue, CSF, and background, yielding an estimate of total brain tissue volume. 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 tissue volume for a subject is then multiplied by the normalization factor to yield the normalized brain parenchymal volume (NBV).

These 1H-MRS data from each subject and from the reference phantom were transferred to the workstation and processed offline with our custom software (IDL; Research System Inc, Boulder, Colo). The NAA concentration peak area was transferred to the workstation and processed offline with our custom software (IDL; Research System Inc, Boulder, Colo). The NAA concentration peak area was integrated by 2 operators unaware of the subjects' identity.

It was converted into an absolute amount (in millimoles) by

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scaling against the area of the signal from the reference phantom that contained 13-mm NAA (5 mM) in water. To correct for the considerable natural interindividual brain size variations, the absolute NAA amount from each individual was divided by the brain volume. For this purpose, absolute rather than normalized brain volumes were needed. Brain volumes were measured using a seed-growing technique based on signal intensity thresholding, as extensively described elsewhere. This yielded an absolute WBNAA concentration, in millimoles, which can be compared cross-sectionally.

### STATISTICAL ANALYSIS

Group comparisons were assessed using the Mann-Whitney test. Univariate correlations were assessed using the Spearman rank correlation coefficient. A univariate logistic regression analysis, where NBV and WBNAA were the independent variables, was used to identify the strongest predictors of the study subjects’ condition (patient with PPMS vs healthy control).

### RESULTS

No MRI abnormalities were seen on the scans of healthy controls. The Table summarizes MRI and ¹H-MRS findings in the 2 groups of study subjects. Normalized brain parenchymal volume and WBNAA concentrations were significantly lower in patients with PPMS than in healthy controls (P = .001 for both comparisons). Normalized brain parenchymal volume was significantly lower in patients with relevant locomotor disability than in those without (mean values, 1479.0 and 1527.0 mL, respectively; P = .03). Whole-brain N-acetylaspartate concentration was also found to be lower in patients with PPMS than in healthy controls (mean values, 9.9 and 10.6 mm, respectively), but this difference did not reach statistical significance (P = .22).

In patients with PPMS, the correlation between NBV and WBNAA concentration was not significant (r = 0.12, P = .52). Normalized brain parenchymal volume was significantly correlated with the patient’s age (r = 0.52, P = .002), T2-weighted LV (r = 0.52, P = .002), and T1-weighted LV (r = 0.48, P = .006) but not with the patient’s disease duration (r = 0.12, P = .50) or EDSS score (r = 0.20, P = .27). There were no significant correlations between WBNAA concentration and the patient’s age (r = 0.22, P = .22), disease duration (r = 0.13, P = .50), EDSS score (r = 0.19, P = .30), T2-weighted LV (r = 0.30, P = .09) or T1-weighted LV (r = 0.32, P = .08).

Both WBNAA and NBV were included as independent factors in the final multivariable model predicting the subjects’ condition. Odd ratios were 0.987 (95% confidence interval [CI], 0.976-0.997) for NBV and 0.674 (95% CI, 0.494-0.919) for WBNAA (P = .01).

### COMMENT

Several quantitative MRI-based studies indicate that one of the most important aspects of brain damage in PPMS is the occurrence of tissue disruption outside focal, T2-weighted visible lesions, that is, in the NAWM and gray (NAGM) matter. With the present study, we questioned measurement of WBNAA concentrations as a technique to quantify the presence and severity of axonal-neuronal dysfunction in the brain of patients with PPMS. Brain volume and MRI-visible lesion load were also measured, to investigate whether concentrations of WBNAA may provide additional information to that given by conventional MRI-derived measures of disease burden and irreversible brain tissue loss in PPMS.

Patients with PPMS enrolled in this study were consecutively recruited from those attending our MS clinic. On average, the resulting sample was characterized by a severe clinical impairment, but, according to natural history surveys, this fits with the expected PPMS clinical outcome after a 10-year disease duration. However, patients with a progressive spinal cord syndrome as the presenting symptom were more frequent in our sample than in large-scale, population-based studies, and this has to be considered when interpreting the observed correlations between their clinical and brain MRI aspects.

The main finding of the present study is that WBNAA ¹H-MRS is sensitive to the presence of axonal-neuronal damage in the brain of patients with PPMS. In subjects with PPMS, we found an average 24% reduction in the WBNAA concentration when compared with the mean value of age-matched healthy controls. The ability of measurements of WBNAA concentrations to detect widespread axonal damage has been reported by several cross-sectional studies of patients with established relapsing-remitting (RR) MS and at the earliest clinical stage of the disease. The magnitude of WBNAA decrease in patients with RRMS does not seem to be correlated with the severity of neurologic disability, but possible different dynamics of WBNAA changes during the course of the disease have been suggested, speculating that patients with a greater decline in WBNAA concentrations and a shorter disease duration might have a poorer prognosis. When compared with the results of previous studies, our findings do not reveal a greater reduction of WBNAA concentrations in PPMS than that found in RRMS. Admittedly, however, this might be due to a “ceiling” effect, limiting the potential of WBNAA concentration as a marker of disease progression in the more advanced and disabling phases of MS. The concomitant occurrence of brain volume reduction and loss of axons or neurons may limit our accuracy in measuring the actual decrease of WBNAA concentrations, since this...
latter quantity has to be expressed as a normalized ratio over the patients’ brain size.

Whole-brain N-acetylaspartate \(^1\)H-MRS findings in this sample of patients with PPMS are also consistent with the results of other quantitative MRI-based studies, which were conducted with different techniques, including single-voxel \(^1\)H-MRS,\(^2\)\(^8\) magnetization transfer transfer\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\) and diffusion tensor MRI,\(^2\)\(^2\)\(^2\) and indicate that “MRI-occult” brain damage might play an important role in the pathobiology of PPMS. The absence of a significant relationship between WBNAA concentration and T1-hypointense lesion load supports the hypothesis that NAWM and NAGM axonal damage in PPMS may not merely depend on the degeneration of fibers passing through discrete white matter lesions with marked tissue disruption.\(^2\)\(^9\) This suggests that a more subtle but widespread pathology might be the hallmark of this condition. Clearly, it remains to be established whether the magnitude of the correlation between WBNAA concentration changes and conventional MRI metrics is different in the early stages of PPMS, where MRI-detectable inflammation seems to be higher than later on in the course of the disease.\(^3\)\(^0\)

In this study, the severity of brain atrophy was not correlated with WBNAA concentration. Contrary to what was observed for WBNAA concentrations, NBV values were found to be significantly correlated with the extent of MRI-visible lesion load, which explained about 20% of brain volume variability. However, since the magnitude of the relationship between WBNAA concentration and MRI-visible lesion burden was close to statistical significance, a type II error, owing to the relatively small sample size, might, at least partially, explain this finding. All of this suggests that, when assessing the severity of brain damage in PPMS, measurements of WBNAA concentration and NBV might provide complementary pieces of information, as also indicated by the results of the multivariable analysis, where both these measures were retained as independent predictors of the study subjects’ condition. A recent postmortem study of the spinal cord\(^3\)\(^1\) has highlighted that measures of atrophy may underestimate the magnitude of actual axonal loss in MS. On the other hand, an extensive loss of myelin might lead to brain volume reduction without decreasing NAA levels. It is, therefore, conceivable that, by measuring both NBV and WBNAA levels, we may overcome the limitations of these metrics when considered in isolation and obtain a comprehensive in vivo estimation of the extent of irreversible brain damage in PPMS. Moreover, it is tempting to speculate that there might exist 2 patterns leading to irreversible tissue loss in PPMS. In one of them, demyelination would be predominant\(^3\)\(^2\) and might then cause axonal injury through the loss of trophic support or the increased liability to degeneration of demyelinated axons. In the other, neuronal-axonal injury might represent the main pathological feature since the initial step of this disease. Further longitudinal and multiparametric MRI studies are now warranted to investigate this intriguing hypothesis.

Consistently with previous studies of RRMS,\(^2\)\(^9\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\) we also did not find a significant relationship between WBNAA concentration and patients’ neurologic impairment. Given the clinical characteristics of the present study’s patient’s sample, the lack of significant correlations between EDSS scores and NBV or WBNAA values might be, at least partially, explained by a spinal cord involvement. The results of previous magnetization transfer\(^2\)\(^2\)\(^2\)\(^2\) and diffusion tensor\(^2\)\(^5\) MRI studies of the cervical cord in progressive MS support this interpretation. In the magnetization transfer MRI study,\(^2\) only a composite MRI score including cervical cord cross-sectional area and magnetization transfer ratio histogram peak height was significantly correlated with the EDSS score of patients with PPMS. Another, but not mutually exclusive, explanation is the role that might be played by cortical reorganization in limiting the effect of MS injury on the severity of neurologic impairment. This phenomenon has been disclosed by functional MRI studies of PPMS\(^3\)\(^9\) where the patterns of cortical activations and the severity of structural changes in the NAWM/NAGM were strongly correlated. The interpatient variability in recruiting functionally related cortical areas, in the presence of similar amounts of subcortical tissue damage, might, therefore, contribute to lessening the clinical-MRI correlation seen in these patients.

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