Evidence for widespread axonal damage at the earliest clinical stage of multiple sclerosis

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Summary
Although axonal pathology is recognized as one of the major pathological features of multiple sclerosis, it is less clear how early in its course it occurs and how it correlates with MRI-visible lesion loads. To assess this early axonal pathology, we quantified the concentration of whole-brain N-acetylaspartate (WBNAA) in a group of patients at the earliest clinical stage of the disease and compared the results with those from healthy controls. Conventional brain MRI and WBNAA using unlocalized proton magnetic resonance spectroscopy were obtained from 31 patients at presentation with clinically isolated syndromes suggestive of multiple sclerosis and paraclinical evidence of dissemination in space, and from 16 matched controls. An additional conventional MRI scan was obtained in all patients 4–6 months later to detect dissemination of lesions in time. The mean WBNAA concentration was significantly lower in patients compared with the controls ($P < 0.0001$). It was not significantly different between patients with and without enhancing lesions at the baseline MRI or between patients with and without lesion dissemination in time. No correlation was found between WBNAA concentrations and lesion volumes. Widespread axonal pathology, largely independent of MRI-visible inflammation and too extensive to be completely reversible, occurs in patients even at the earliest clinical stage of multiple sclerosis. This finding lessens the validity of the current concept that the axonal pathology of multiple sclerosis is the end-stage result of repeated inflammatory events, and argues strongly in favour of early neuroprotective intervention.

Keywords: multiple sclerosis; magnetic resonance imaging; magnetic resonance spectroscopy; whole-brain N-acetylaspartate; axonal pathology

Abbreviations: EDSS = Expanded Disability Status Scale; Gd = gadolinium; 1H-MRS = proton MR spectroscopy; MR = magnetic resonance; NAA = N-acetylaspartate; PD = proton-density; SE = spin-echo; SIENA = structural image evaluation, using normalization, of atrophy; VOI = volume of interest; WBNAA = whole-brain N-acetylaspartate

Introduction
Axonal pathology has been known to occur in multiple sclerosis from the earliest post-mortem descriptions of the disease (Charcot, 1868). Recent pathological studies have confirmed that irreversible axonal loss is a prominent feature of the disease (Ferguson et al., 1997; Trapp et al., 1998; van Waesberghel et al., 1999; Evangelou et al., 2000; Peterson et al., 2001), and the extensive in vivo application of quantitative magnetic resonance (MR) techniques has led to a general appreciation that axonal loss contributes significantly to the development of ‘fixed’ disability in multiple sclerosis patients (Davie et al., 1995; De Stefano et al., 1995, 1998; Trapp et al., 1999; Filippi, 2001). However, it is less clear how early in the course of the disease this axonal damage occurs and how it relates to the MRI-visible lesion load.

Proton magnetic resonance spectroscopy ($^1$H-MRS) enables us to quantify axonal injury and loss through the measurement of changes in the signal intensity of N-acetylaspartate (NAA) (Davie et al., 1995; De Stefano et al., 1995, 1998), a metabolite localized almost exclusively...
to neurons and neuronal processes (Simmons et al., 1991). Localized $^1$H-MRS studies have shown reduced NAA in $T_2$-visible lesions (Arnold et al., 1994; Davie et al., 1994, 1997; De Stefano et al., 1995; Narayana et al., 1998), normal appearing white matter (NAWM) (Davie et al., 1995; De Stefano et al., 1998; Fu et al., 1998; Narayana et al., 1998; Kapeller et al., 2001) and grey matter (Kapeller et al., 2001) of patients with clinically definite multiple sclerosis. Unfortunately, current $^1$H-MRS is restricted to small volumes of interest (VOI), which typically represent <100 ml of the brain. Since multiple sclerosis is a diffuse disease of the CNS (Trapp et al., 1997; De Stefano et al., 1998), estimates of the extent of axonal pathology from such measurements must rely on the unproven assumption that changes in NAA in the VOI accurately reflect the status of the entire brain. The volume restriction of localized $^1$H-MRS has recently been overcome with a new unlocalized $^1$H-MRS sequence which makes it possible to quantify the concentration of NAA from the whole brain (WBNAA) (Gonen et al., 1998, 2000), providing an overall assessment of the total load of the disease both within and outside MRI-visible lesions.

To gain insight into the nature of axonal pathology in multiple sclerosis, we quantified WBNAA from a group of patients at the earliest clinical stage of multiple sclerosis (McDonald et al., 2001) and correlated it with the MRI-visible lesion loads.

**Material and methods**

**Patients**

We studied 31 consecutive patients (23 women, eight men, mean age 28.7 years, SD 6.0 years) at presentation with clinically isolated syndromes suggestive of multiple sclerosis and paraclinical evidence of dissemination in space (McDonald et al., 2001). All patients were assessed within 3 months from the onset of the clinical symptoms (mean 26 days). The presenting symptoms were optic neuritis, brainstem syndromes and spinal cord syndromes in 10, 12 and nine patients, respectively. All alternative neurological diseases were excluded by appropriate investigations (McDonald et al., 2001). Oligoclonal bands were found in the CSF of 26 patients. Before MR acquisition, but on the same day, each patient underwent a full neurological assessment performed by a single observer who was blind to the MRI and WBNAA results. Disability was rated using the Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983), which had a median of 0.0 (range 0.0–2.0) in this cohort. None of the patients had been treated with corticosteroids within the previous month. To demonstrate the presence of new lesions, and hence the dissemination in time of the disease (McDonald et al., 2001), all patients were followed up both clinically and with a second MRI 4–6 months after the first. Sixteen healthy volunteers (12 women, four men; mean age 29.7 years; SD 4.5 years), with no history of neurological dysfunction and a normal neurological examination, served as controls. We obtained approval from The Fondazione San Raffaele Ethics Committee and written informed consent from each subject before study initiation.

**MR acquisition**

**First session**

$^1$H-MRS and MRI were performed using a 1.5 T scanner (Vision; Siemens, Erlangen, Germany). The following sequences were collected from all subjects during a single MR session: (i) WBNAA $^1$H-MRS based on the method described by Gonen et al. (2000); (ii) dual-echo turbo spin-echo (SE) [repetition time (TR) 3300 ms; first echo time (TE) 16 ms; second echo TE 98 ms; echo-train length 5; 24 contiguous 5 mm thick axial slices with a 256 $\times$ 256 matrix and a $250 \times 250$ mm$^2$ field of view]; (iii) $T_1$-weighted conventional SE (TR 768 ms, TE 14 ms, 24 contiguous 5 mm thick axial slices with a 256 $\times$ 256 matrix and a $250 \times 250$ mm$^2$ field of view) before and after the administration of 0.1 mmol/kg of gadolinium (Gd).

**Second session**

Patients were repositioned using ad hoc guidelines (Miller et al., 1991). Dual-echo turbo SE and $T_1$-weighted conventional SE before and after administration of 0.1 mmol/kg of Gd were obtained in a single session using the same acquisition procedures as those used previously.

**MRI analysis**

Dual-echo hard copies were revised in random order by two observers who did not know who the scans belonged to. Lesions were identified and marked by consensus on the proton-density (PD) and post-contrast $T_1$-weighted scans. $T_2$-weighted and precontrast $T_1$-weighted images were always used to increase confidence in lesion identification. The number and location of $T_2$-hyperintense and Gd-enhancing lesions were evaluated, and the fulfillment of MRI criteria for multiple sclerosis diagnosis was assessed (McDonald et al., 2001). Then all images were transferred to an offline workstation (Sun Sparcstation; Sun Microsystems, Mountain View, CA, USA) for lesion volume assessment by a single observer, again unaware of who the scans belonged to. This procedure used a segmentation technique based on signal intensity thresholding and characterized by high intra-rater reproducibility (Rovaris et al., 1997). In patients, changes in normalized brain volume over the follow-up period were also measured using a fully automated and accurate method, SIENA (structural image evaluation, using normalization, of atrophy) (Smith et al., 2001). SIENA performs segmentation of brain from non-brain tissue in the head, estimates the outer skull surface (for two time points), and uses these results to register the two
images while correcting for imaging geometry changes. Then
the registered segmented brain images are used to estimate
percentage changes in normalized brain volume (Smith et al.,
2001).

WBNAA \(^1\)H-MRS analysis
The \(^1\)H-MRS data from each subject and from the reference
phantom were transferred to the workstation and processed
offline with our custom software (IDL; Research Systems,
Boulder, CO, USA). The NAA peak area was integrated by
two operators unaware of the subject’s identity (Gonen et al.,
1998, 2000). It was converted into an absolute amount (in
mmol) by scaling against the area of the signal from the
reference phantom which contained 15 mmol NAA (5 mM) in
water (Gonen et al., 1998, 2000). To correct for the
considerable natural interindividual variation in brain size,
the absolute amount of NAA from each individual was
divided by their 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 described exten-
sively elsewhere (Rovaris et al., 2000). This yielded an
absolute WBNAA concentration, in mM, which could be
compared cross-sectionally (Gonen et al., 2000).

Statistical analysis
Differences in WBNAA concentrations between individual
groups of subjects were assessed using the Mann–Whitney
test. To correct for multiple comparisons, only \(P\) values <0.01
were considered significant (Bonferroni correction).
Univariate correlations were assessed using the Spearman
rank correlation coefficient.

Results
No abnormalities were seen on any of the conventional MRI
scans from the controls. By definition, all patients had
paraclinical evidence of dissemination in space; in 19 this was
demonstrated by MRI alone and in the remaining 12 by the
combination of MRI findings and the presence of oligoclonal
bands in the CSF (McDonald et al., 2001). One or more
enhancing lesions were seen in eight patients (seven had 1
and one had 7 enhancing lesions). From the clinical and MRI
follow-up data, 16 patients met McDonald’s criteria for lesion
dissemination in time (McDonald et al., 2001).

The average WBNAA concentration was 12.98 mM
(standard error 0.58 mM) in controls and 10.09 mM (standard
error 0.43 mM) in patients \((P < 0.0001)\). Specifically, the
patients showed, on average, a 22.3% decrease in NAA
concentration in the whole-brain parenchyma. No significant
difference was found \((P = 0.17)\) in average WBNAA
concentration between patients with dissemination in space
shown by MRI alone (mean 9.82 mM, standard error
0.57 mM) and those in whom a combination of MRI and
CSF findings was needed to demonstrate dissemination in
space (McDonald et al., 2001) \((mean 10.50 \text{ mM, standard}
error 0.61 \text{ mM})\). Average WBNAA concentration also did not
differ significantly \((P = 0.27)\) between patients with \((mean
9.32 \text{ mM, standard error 0.67 mM})\) and without \((mean
10.39 \text{ mM, standard error 0.53 mM})\) enhancing lesions on the
baseline MRI. No correlation was found between WBNAA
concentrations and lesion volume \((\text{WBNAA versus } \text{T}_2 \text{ lesion}
volume, \(r = 0.18); \text{WBNAA versus } \text{T}_1\text{-hypointense lesion}
volume, \(r = 0.05)\). The mean brain volume and the average
WBNAA concentrations did not differ \((P = 0.57)\) between
patients fulfilling McDonald’s criteria at follow-up \((mean
9.78 \text{ mM, standard error 0.59 mM})\) and those who did not
(mean 10.49 mM, standard error 0.62 mM). Between the first
and the second MRI sessions, a mean 0.27\% \((\text{standard error}
0.16%)\) decrease in normalized brain volume was observed in
the patients. Considering the length of the follow-up \((average
4.7 \text{ months})\), this figure corresponds to an average annualized
percentage decrease in normalized brain volume of 0.69%.

Discussion
The NAA loss indicates that axonal pathology is an early
event in the course of multiple sclerosis and fits with the
demonstration that brain atrophy occurs at a relatively fast
rate in patients with early multiple sclerosis (Rudick et al.,
1999; Brex et al., 2000). This observation is corroborated by
the finding of an average annualized percent decrease of
normalized brain volume of about 0.7%, a figure that closely
matches those reported in patients with more advanced
disease (Filippi and Grossman, 2002). Admittedly, not all the
patients in this study met McDonald’s criteria for an early
diagnosis of multiple sclerosis (McDonald et al., 2001).
Nevertheless, alternative diagnoses were carefully excluded
and all had clinical pictures suggestive of multiple sclerosis at
presentation. They were also selected for having a dissem-
ination in space at presentation, i.e. MRI and/or CSF findings
consistent with a diagnosis of multiple sclerosis (McDonald
et al., 2001). As a consequence, we believe that all of them
are at high risk of ultimately progressing to clinically definite
multiple sclerosis (Brex et al., 2002).

With this in mind, the first issue to be addressed is how
much of the observed WBNAA decrease is irreversible.
Partially reversible NAA reductions in \(\text{T}_2\)-visible lesions
(Davie et al., 1994; De Stefano et al., 1995; Narayana et al.,
1998) and NAWM (De Stefano et al., 1999) of multiple
sclerosis patients have been described and attributed to
possible recovery from sublethal axonal injury following
resolution of inflammatory changes. Consequently, the
reduced WBNAA concentrations in these patients could be
interpreted as a transient functional axonal impairment
secondary to inflammatory changes associated with the recent
clinical attack. Indeed, we found that patients with at least one
enhancing lesion, indicative of increased blood–brain barrier
permeability and associated inflammation (Katz et al., 1993),
had a lower average WBNAA than those with an inactive
MRI scan. Nevertheless, the average WBNAA reduction in the latter group of patients was still remarkable (19.9%) compared with the matched controls. Although a certain amount of inflammation is known to go undetected on conventional MRI with the standard Gd dose (Filippi et al., 1998), we believe it is unlikely that this ‘occult’ inflammatory component could lead to such a substantial WBNAA decrease. Therefore, we interpret such ‘NAA loss without MRI-visible inflammation’ as an indication that at least a considerable portion of the axonal pathology is permanent, even this early in the clinical course of the disease.

The next question relates to the relative contributions of MRI-visible lesions and occult damage in the normal-appearing tissue to the reduced WBNAA concentrations in these patients. Since average T2 lesion volume at this early stage of the disease is negligible (<1% fraction of the brain volume; on average, 2.6 versus 1165 ml) the decrease of >20% in WBNAA in the patients must be accounted for by widespread axonal damage in the NAWM alone or in association with neuronal injury in the grey matter. Our results disagree with those of a previous study (Brex et al., 1999), which was unable to detect a significant reduction in NAA concentration in the NAWM from patients with clinically isolated syndromes suggestive of multiple sclerosis. This discrepancy is likely to result from the fact that Brex et al. (1999) did not select patients for having a dissemination in space at presentation (McDonald et al., 1998), we believe it is unlikely that this ‘occult’ inflammatory component could lead to such a substantial WBNAA decrease. Therefore, we interpret such ‘NAA loss without MRI-visible inflammation’ as an indication that at least a considerable portion of the axonal pathology is permanent, even this early in the clinical course of the disease.

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In conclusion, this study suggests that significant irreversible axonal damage occurs in patients at the earliest stage of multiple sclerosis. This, together with the recognition that MRI-detectable inflammation is only indirectly linked to the neuronal/axonal damage in these patients should prompt the development and use of treatments capable of neuroprotective action.

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