1H-MRSI evidence for cortical gray matter pathology that is independent of cerebral white matter lesion load in patients with secondary progressive multiple sclerosis

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A B S T R A C T

We examined: (i) neuro-axonal disturbance (as indicated by 1H-MRSI NA/Cr values) in the cortical grey matter (cGM) of 10 untreated patients with relapsing-remitting (RR) and 10 with secondary-progressive (SP) multiple sclerosis (MS), and (ii) the relationships between cGM-NA/Cr values and the degree of EDSS-measured clinical disability and cerebral white-matter (WM) lesion load (LL) in these patients. Whereas mean and median cGM-NA/Cr values in our RR group were similar to those in 18 age-matched normal controls (NC), large statistically-significant decreases (between 14.3% and 18.5%) were found in our SP group relative to both our RR and NC groups. When data from all patients was combined, we found: (i) a large negative correlation between EDSS scores and cGM-NA/Cr values ($r = -0.55$); and (ii) a larger negative correlation of cGM-NA/Cr values with cerebral T1-hypointense WM-LL ($T1-LL$, $r = -0.73$) than with cerebral T2-hyperintense-LL ($T2-LL$, $r = -0.63$). Importantly, (i) correlations of WM-LL with cGM-NA/Cr were larger in the RR group than in the SP group ($T1-LL$: $r = -0.79$ vs. $-0.54$; $T2-LL$: $r = -0.63$ vs. $-0.51$), and (ii) cerebral WM-LL values could not fully account for the extent of the decrease in mean cGM-NA/Cr that was seen in our SP group relative to our NC group. Our observations are consistent with the possibilities that: (i) in patients with RR-MS, 1H-MRSI-measured cGM neuro-axonal disturbances are strongly related to the effects of axonal transection that are associated with cerebral WM lesions; and (ii) in patients with SP-MS, such cGM neuro-axonal disturbances are more severe and are associated with a more-widespread degenerative process (which probably includes a considerable degree of cortical demyelination). © 2009 Elsevier B.V. All rights reserved.

1. Introduction

Pathology of the cortical grey matter (cGM) in patients with multiple sclerosis (MS) has long been recognized on detailed histopathological examination [1]. Nevertheless, MS has traditionally been considered a demyelinating disease of the white matter (WM) of the central nervous system because, until recently, cGM pathology has routinely been underestimated by standard techniques. For example, cGM lesions are not evident on conventional post-mortem examinations; [2] this is because: (i) cortical myelin is not readily apparent on routine histological staining with Luxol fast blue; and (ii) cortical lesions are not hypercellular and, therefore, are not obvious on hematoxylin-eosin-stained sections. Furthermore, the majority of cGM lesions are also not evident on conventional magnetic resonance imaging (MRI) examinations, [3–5] even at higher fields; [6] this is because: (i) cGM lesions are often either small or thin, which make them subject to partial volume effects on MRI; and (ii) they are associated with much less inflammation and demyelination than is typical of WM lesions [7,8] and, as a result, they are associated with very little contrast on conventional T2-weighted or T1-weighted MRI. Within the last decade, however, advances in the neurohistopathological and neuroimaging analysis of tissue from patients with MS have fostered a renewed appreciation for the importance of cGM pathology in this disease [9–16].

1.1. Histopathological studies of cGM lesions in patients with MS

A number of post-mortem and ex-vivo histopathological studies have now described and quantified cGM lesions in the brains of patients with MS [1,3,17,18,20,22]. The prevalence of such cGM lesions can be inferred from the results of the study by Peterson et al. [7] in which as many as 112 cortical lesions were identified by an immunocytochemical analysis in 110 tissue blocks from 50 MS patients. Importantly, in this study, cGM lesions showed evidence of: (i) demyelination; (ii) axonal and dendritic transection; and (iii) neuronal apoptosis, particularly in neurons whose axons showed demyelination. Furthermore, the elegant work of Kutzelnigg et al. [18] has further illustrated the prevalence of cGM pathology in MS and has suggested that cortical demyelination is much more prominent in the ultimate, progressive phase of MS than in the initial, relapsing-remitting phase of the disease.

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1.2. \(^1\text{H-MRS} \) studies of neuro-axonal integrity in patients with MS

As we have reviewed elsewhere, \([25]\) proton magnetic resonance spectroscopy (\(^1\text{H-MRS}\)) and spectroscopic imaging (\(^1\text{H-MRSI}\)) studies of patients with MS have been important in detecting and quantifying neuro-axonal injury in vivo. This is done based on the signal intensity of the neuronal marker compound, N-acetylaspartate (NAA), which is localized within neurons and neuronal processes \([26,27]\). Although NAA has been detected in other cells in vitro, particularly O2A progenitor cells, \([28,29]\) this phenomenon does not appear to be relevant in vivo; indeed, complete degeneration of rat optic nerve following transection has been demonstrated to be associated with complete loss of NAA, despite the continued presence of proliferating oligodendroglial cells \([30]\). As a result, the amount of \(^1\text{H-MRS(I)}\)-measured NAA or, more commonly, the amount of total N-acetyl (NA) groups that resonate at 2.0 ppm on the \(^1\text{H-MR}\) spectrum (of which NAA is the major component in the adult brain), has been used as a non-invasive in vivo indicator of neuro-axonal integrity \([25]\).

Whereas absolute and semi-absolute in vivo quantification of \(^1\text{H-MRS(I)}\)-measured NA is now common practice, we and others prefer the use of the total amount of \(^1\text{H-MRS(I)}\)-measured creatine and phosphocreatine (Cr) in the same voxel as an internal standard \([25]\). Importantly, such a “ratio” approach corrects for many of the sources of variability that can affect estimates of absolute concentration. For example, because Cr is present in virtually all types of brain tissue but is not present in the cerebrospinal fluid, NA/Cr ratios are not sensitive to the effects of brain atrophy and sulcal enlargement — effects that have been shown to be present even in the early, relapsing–remitting stage of MS \([31]\) and that can potentially confound the results of absolute or semi-absolute methods of quantitation. Of course, this normalization-to-Cr approach is only reliable if the within-voxel Cr concentration being measured is unaffected by the within-voxel pathology of the tissue that is being studied; however, as our recent meta-analysis of published data suggests, this seems to be true for normal-appearing cGM in patients with MS \([25]\).

\(^1\text{H-MRSI}\) studies have consistently found decreased NA/Cr values in both the lesional and normal-appearing WM of patients with MS \([32–34]\). Although a small number of studies have found values of \(^1\text{H-MRS(I)}\)-measured NA to be statistically unaffected in the cGM of patients with MS \([35–37]\) the majority of such studies have provided evidence for decreased cGM NA: \([38–44]\) and NA/Cr \([45–47]\). This is true for: \(i\) patients with clinically-isolated syndromes indicative of MS (CIS); \([47]\) \(ii\) patients in the early, relapsing–remitting stage of the disease; \([38,46,44]\) and \(iii\) patients with more-severe, progressive MS \([40–43]\). Importantly, in each of the studies that explicitly compared groups of patients with relapsing–remitting MS to those with progressive MS and found a significant decrease in cGM NA, this decrease was far greater in the group of patients with progressive MS \([40,41,43]\). This finding seems to be consistent with both: \(i\) the histopathological findings of Kutzelnigg et al. \([18]\) regarding more-severe cortical demyelination in the progressive phase of MS; and \(ii\) the histopathological findings of Peterson et al. \([7]\) which suggest that such cGM demyelination is associated with neuronal disturbance in the cortex.

1.3. cGM pathology secondary to WM lesions in patients with MS

In addition to the different types of cGM lesions that have been described histopathologically, \([7]\) cGM that is not directly affected by macroscopic lesions in patients with MS may also be affected indirectly by neuronal and dendritic changes that are secondary to axonal injury within WM lesions \([48,30]\). For example, WM lesions in patients with MS are often associated with the transection of axons \([48,30]\) and the subsequent Wallerian degeneration of the distal portion of these axons. Because many of these axons synapse on cortical dendrites and cell bodies, loss of these inputs (and the trophic factors transmitted at the synapses) can also lead to anterograde dendritic and neuronal pathology \([49,50]\). Furthermore, proximal axons that are still connected to cell bodies can undergo retrograde degeneration, \([51]\) which can eventually result in the death of the neuronal cell by apoptosis \([52]\).

1.4. The relationship of cGM pathology to cerebral WM lesion-load and clinical status in patients with MS

A number of recent studies have examined the relationship between cerebral WM lesions and cGM pathology in patients with MS. For example, in a very small study published in 2007, Bo et al. \([21]\) compared three patients with “extensive subpial cGM demyelination” (ESD) to three patients with “minimal subpial cGM demyelination” and found that the ESD group had a greater percent cerebral-WM-LL (5.3\% vs. 2.7\%); this difference did not reach statistical significance, however, which may have been due to the low power associated with the very small sample size. With regards to the relationship between cerebral WM lesions and \(^1\text{H-MRS(I)}\) measures of cGM pathology, Van Au Duong et al. \([47]\) studied cGM-NA/Cr values and cerebral WM inflammation in patients with CIS and found a significantly-lower mean NA/Cr value in the cGM of those 15 patients who had gadolinium-enhancing cerebral WM lesions than in the 25 patients who did not have any such lesions (and whose mean cGM-NA/Cr value did not differ from that of their normal controls). On the other hand, a number of \(^1\text{H-MRS(I)}\) studies of cGM neuro-axonal integrity in patients with MS have failed to find statistically-significant correlations between cerebral WM lesion-load (WM-LL) and cGM-NA \([38,40,37,44]\) or between cerebral WM-LL and cGM-NA/Cr \([47]\). Similarly, whereas some studies have found statistically-significant correlations between cGM-NA and clinical status \([42]\) and between cGM-NA/Cr and memory impairment in patients with MS, \([46]\) other studies have found to statistically-significant correlations between cGM-NA and clinical disability in such patients \([38,39,40,41,37]\). It should be noted, however, that all of these studies had relatively-low sample sizes and some of them had a very limited range of cerebral WM-LL values \([38,47]\) or scores on Kurtzke’s Expanded Disability Status Scale \([53]\) (EDSS) \([38]\) – either of which would make it very difficult to find statistically-significant correlations. Thus, to the best of our knowledge, the relationship of \(^1\text{H-MRS(I)}\) measures of cGM neuro-axonal integrity to cerebral WM-LL and clinical status in patients with MS has not yet been definitively established.

In the present study, we: \(i\) used conventional MRI to quantify cerebral WM-LL values in 10 patients with relapsing–remitting (RR) MS and 10 patients with secondary-progressive (SP) MS, \(ii\) used \(^1\text{H-MRSI}\) NA/Cr values to estimate in vivo the degree of neuro-axonal disturbance in the cGM of these same two groups of patients relative to a group of 18 age-matched normal controls, and \(iii\) examined the relationships between our patients’ mean cGM-NA/Cr values and both their EDSS scores and their cerebral WM-LL values. This was done both for our patients’ cerebral WM-LL of: \(i\) hyperintensities on T\(_2\)-weighted MRI (T\(_2\)-LL); \([54]\) and \(ii\) hypointensities on T\(_1\)-weighted MRI (T\(_1\)-LL), which are more specific markers of tissue destruction and axonal loss in patients with MS, and which correlate better with clinical disability than do T\(_2\)-LL values \([55]\).

2. Methods

2.1. Subjects

\(^1\text{H-MRSI}\) and conventional MRI data were obtained from 10 untreated patients with RR-MS (7 females, 3 males) and 10 untreated patients with SP-MS (5 females, 5 males). These were all patients who were followed in the MS Clinic at the Montreal Neurological Institute and Hospital (MNI/H). Levels of clinical disability in these patients were assessed using Kurtzke’s EDSS \([53]\). Similar data were collected...
in 18 normal control (NC) volunteers (9 females, 9 males) of similar age. The Ethics Committee of the MNI/H approved the study, and informed consent was obtained from all subjects.

2.2. MRI and $^1$H-MRSI

For each subject, combined MRI and $^1$H-MRSI imaging of the brain was obtained in a single session using a Philips Gyroscan ACS II scanner operating at 1.5 T (Philips Medical Systems, Best, The Netherlands). A transverse dual-echo, turbo spin-echo sequence [repetition time (TR) = 2075 ms; first echo time (TE) = 32 ms, second TE = 90 ms; 256 × 256 matrix, 1 signal average, 250-mm field-of-view], which yielded both proton-density-weighted (PD) and T2-weighted images with 50 contiguous 3-mm slices, was acquired parallel to the line connecting the anterior and posterior commissures (AC–PC line). This was followed by a matching T1-weighted fast field-echo sequence (TR = 35 ms, TE = 10 ms).

These conventional MR images were used to position a $^1$H-MRSI volume of interest (VOI) of approximately $90 \times 90 \times 20 \text{ mm}^3$ that included the corpus callosum and the adjacent WM. Using a PRESS sequence (TR = 2000 ms, TE = 272 ms), $^1$H-MRSI images were acquired parallel to the AC–PC line (32 × 32 phase-encodes, 250 × 250 mm field-of-view, 20-mm thick slab) [56]. To suppress the intense water resonance, frequency-selective excitation pulses were placed at the beginning of the $^1$H-MRSI sequence [57]. In order to allow for correction of B$_0$ inhomogeneity during post-processing, a quick $^1$H-MRSI without water suppression was also acquired (TR = 850 ms; TE = 272 ms; 16 × 16 phase encodes; 250 × 250 mm field-of-view, 1 signal average). Post-processing of the raw $^1$H-MRSI data was performed as described previously [58]. The nominal in-plane voxel size was approximately $8 \times 8 \text{ mm}^2$, which yielded a spatial resolution (FWHM) of the point-spread function of approximately $12 \times 12 \text{ mm}^2$ after filtering.

2.3. Brain segmentation and quantification of cerebral WM-LL values

All MR images were pre-processed using N3 in order to correct for any bias field artefacts [59]. The corrected T$_1$-weighted images were then co-registered with the corrected PD- and T$_2$-weighted images using mincmarc [60]. In the patients, the resulting images were then segmented automatically using a locally-developed, multi-spectral, Bayesian technique (Simon J. Francis, MNI): individual lesion voxels were automatically classified as to whether or not they were considered to be: (i) part of a lesion that is hyperintense on PD- and T$_2$-weighted imaging and/or, (ii) part of a lesion that is hypointense on T$_1$-weighted imaging. Results of the automatic lesion classification were reviewed by trained observers and a local-thresholding technique was used in order to make any necessary corrections. Total cerebral WM-LL values (i.e., the total volume of the voxels considered to contain such lesional tissue) were computed separately for each patient’s: (i) lesions on PD and T$_2$-weighted imaging (T2-LL), and (ii) lesions on T$_1$-weighted imaging (T1-LL).

2.4. cGM voxel selection and quantification of mean NA/Cr values

As shown in Fig. 1, locally-developed software that allows for coordinated MRI and $^1$H-MRSI examination (AVIS, Samson B. Antel, MNI) was used to manually select, for each subject, voxels of cGM that were located within the mesial posterior-parietal cortex from similar horizontal sections of the $^1$H-MRSI VOI (Technical limitations associated with our $^1$H-MRSI technique make it difficult, and less reliable, to obtain data from superficial cGM). In order to minimize signal contributions from non-cGM tissue, only those voxels in which cGM extended beyond the voxel-of-interest halfway or more into adjacent $^1$H-MRSI voxels were selected for analysis. The nominal voxel size for our $^1$H-MRSI acquisition was 7.8 mm. Thus, we required a 4-mm or greater margin of cGM visible on MRI all around each candidate voxel in order for it to be chosen as a “cGM voxel”. This was done in order to exclude voxels with signal contributions from white matter not just from within the nominal voxel, but within the larger region specified by the point-spread function of our acquisition and post-processing (FWHM = 12-mm). Furthermore, partial volume with cerebrospinal fluid (CSF) was controlled for by the use of ratios of NA to Cr, since the signal reduction due to CSF would affect both metabolite signals equally.

Using AVIS, metabolite resonance intensities of NA within each $^1$H-MRSI voxel were determined automatically from peak areas relative to a spline-corrected baseline; these were expressed as ratios relative to the resonance intensity of Cr measured the same way within the same voxel. For each subject, a mean cGM-NA/Cr value was computed across all voxels that were selected to fulfill the criteria described above. Data from either one ($n=10$) or two ($n=8$) such cGM voxels were averaged for each of the NC subjects [median = 1.00, mean = 1.44, standard deviation (SD) = 0.51]; data from either one ($n=6$), two ($n=2$), or six ($n=1$) such cGM voxels were averaged for each of the subjects with RR-MS [median = 100, mean = 170, SD = 1.57]; and data from either one ($n=8$) or two ($n=2$) such cGM voxels were averaged for each of the subjects with SP-MS [median = 100, mean = 1.20, SD = 0.42]. The groups did not differ in terms of their mean number of cGM voxels sampled ($F_{2,15}=0.78$, $p=0.467$), and the mean cGM-NA/Cr values observed did not differ depending on the number of cGM voxels that were sampled ($F_{2,15}=1.70$, $p=0.197$).

2.5. Statistical analysis

Differences between the NC subjects and the two patient groups in terms of their ages and their cGM-NA/Cr values were assessed using a
Table 1
Descriptive and inferential statistics regarding demographic and clinical data at the time of scanning in our 18 normal control (NC) subjects, 10 patients with relapsing–remitting multiple sclerosis (RR), and 10 patients with secondary-progressive multiple sclerosis (SP).

<table>
<thead>
<tr>
<th>Group</th>
<th>Median</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (in years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>35.15</td>
<td>22.10 to 55.60</td>
<td>35.49</td>
<td>8.53</td>
</tr>
<tr>
<td>RR</td>
<td>37.20</td>
<td>30.60 to 53.40</td>
<td>38.68</td>
<td>7.64</td>
</tr>
<tr>
<td>SP</td>
<td>38.25</td>
<td>27.00 to 56.70</td>
<td>38.90</td>
<td>8.36</td>
</tr>
</tbody>
</table>

\(\chi^2(2) = 1.12, p = 0.570; F(2,35) = 0.76, r^2 = 0.041, p = 0.476\)

| Disease duration (in years) |        |        |       |      |
| RR    | 8.58   | 5.72 to 16.28 | 9.51  | 3.96  |
| SP    | 13.64  | 9.55 to 36.98 | 16.3  | 8.15  |

\(U = 18.00, \chi^2(1) = 5.85, p = 0.016; F(1,18) = 5.62, r^2 = 0.238, p = 0.029\)

| EDSS scores |        |        |       |      |
| RR    | 3.25   | 1.50 to 6.50 | 3.35  | 1.67  |
| SP    | 6.75   | 3.50 to 9.00 | 6.90  | 1.70  |

\(U = 7.50, \chi^2(1) = 10.39, p = 0.001; F(2,36) = 22.27, r^2 = 0.553, p = 0.0002\)

Series of non-parametric and parametric one-way analyses of variance (ANOVA) followed, when statistically-significant, by post-hoc Tukey’s Honestly Significant Difference (HSD) parametric tests and Mann-Whitney \(U\) non-parametric tests. Differences between the two patient groups in terms of their disease durations, EDSS scores, and cerebral WM-LL values were assessed in the same way.

A series of Pearson Product-Moment correlations was used to examine the linear-fit bivariate relationships between individuals’ demographic, clinical, and neuroimaging measures. These correlations were performed separately for: (i) the NC subjects, (ii) the patients with RR-MS, (iii) the patients with SP-MS, and (iv) all of the patients combined (MS, \(n = 20\)).

When deemed appropriate, one-way analysis of covariance (ANCOVA) was used in order to examine group differences between the two patient-groups’ mean cGM-NA/Cr values while controlling for their clinical, demographic, and cerebral WM-LL data.

Because of the skewed nature of the cerebral WM-LL data, all of the patients’ T2-LL and T1-LL values underwent a log\(_{10}\) transformation prior to undergoing the previously-mentioned parametric statistical analyses. Please note that two of the patients in the RR-MS group were found to have a T1-LL of 0; for the purposes of logging and parametric statistical-analysis, each of these subjects was assigned a T1-LL of 0.1 cm\(^3\).

All of the statistical and graphical analyses included in the present study were performed using SYSTAT for Windows, version 10.2. For each of the analyses included herein, a \(p\)-value of less than 0.05 was considered to be statistically significant. Other than the use of Tukey’s HSD tests following a significant ANOVA, no corrections were made for multiple comparisons; in order for the reader to be able to better judge the statistical significance and the clinical meaningfulness of these analyses, we show all of the observed test statistics (i.e., \(r\), \(F\), \(U\), and \(r^2\) values) as well as the associated degrees of freedom and uncorrected \(p\)-values.

3. Results

3.1. Group differences in demographic and clinical data

Descriptive and inferential statistics regarding our subjects’ demographic and clinical data are presented in Table 1. At the time of scanning: (i) the NC group and the two MS patient groups did not differ in terms of their mean or median age, (ii) the group of patients with RR-MS had shorter mean and median disease duration than did the group of patients with SP-MS, and (iii) the group of patients with RR-MS also had a lower mean and median EDSS scores than did the group of patients with SP-MS. As shown in Table 1, these differences were all statistically significant.

3.2. Group differences in cerebral WM-LL values and cGM-NA/Cr values

Descriptive and inferential statistics regarding our patient’s cerebral WM-LL values and all of our subjects’ mean cGM-NA/Cr values are presented in Fig. 2. The group of patients with RR-MS had lower mean and median T2-LL values than did the group of patients with SP-MS, but neither of these differences were statistically significant (most likely due to insufficient power resulting from a combination of the small sample size and the large variance in these data). The group of patients with RR-MS also had lower mean and median T1-LL values than did the group of patients...
patients with SP-MS; once again, these differences were not statistically
significant (this time, however, there was a statistical trend suggesting
greater mean and median T1-LL values in patients with SP-MS).

As shown in Fig. 2, there was virtually no difference between
either the mean (Tukey’s HSD p-value = 0.999) or median (Mann–
Whitney U = 97.5, p-value = 0.72) cGM-NA/Cr values in the group of
patients with RR-MS relative to that in the group of NC subjects. There
was, however, a statistically-significant decrease in both the mean
(−14.3%; Tukey’s HSD p-value = 0.004) and the median
(−18.5%; Mann–Whitney U = 147, p-value = 0.006) cGM-NA/Cr
values of the group of patients with SP-MS relative to those in
the group of NC subjects. Similarly, there was a statistically-significant
decrease in both the mean (−14.3%, Tukey’s HSD p-value = 0.01) and
the median (−15.0%; Mann–Whitney U = 81, p-value = 0.02)
cGM-NA/Cr values of the group of patients with SP-MS relative to
that in the group of patients with RR-MS.

3.3. Relationship between cGM-NA/Cr values and clinical and demographic
data

3.3.1. Correlational analyses

Scatterplots showing the least-squares, linear-fit relationships
between individuals’ mean cGM-NA/Cr values and their demo-
graphic and clinical data are presented in the top row of Fig. 3. There
was no statistically-significant linear relationship between age and
mean cGM-NA/Cr values in any of the patient groups studied. There
was also no such relationship between disease duration and mean
cGM-NA/Cr values (although there was a trend towards one in the
combined MS group: r = −0.422, p = 0.064). Finally, there was a
large, [61] statistically-significant negative correlation between EDSS
scores and mean cGM-NA/Cr values, but only when data from the
two patient groups were combined (r = −0.550, p = 0.012);
furthermore, despite the small sample size, this correlation was
statistically significant (r = −0.459, p = 0.048) even after excluding
the case with RR-MS that had the very high cGM-NA/Cr value (3.02)
and very low EDSS value (1.5).

3.3.2. ANCOVAs

When we examined the impact of subjects’ demographic or
clinical data at the time of scanning as covariates in a series of one-
way ANCOVAs examining the effect of group on mean cGM-NA/Cr
values, none were found to be signi
cificant [(i) age (F1, 34 = 0.26, p = 0.615), (ii) disease duration
(F1, 17 = 0.81, p = 0.380), and (iii) EDSS
(F1, 17 = 1.21, p = 0.286)]. As a result, such ANCOVAs were not
deeoned to be justified.

![Fig. 3. Scatterplots showing the linear-fit relationships between the mean NA/Cr values within the selected cortical grey matter (cGM) voxels of each individual and: (i) their demographic and clinical data (top row), or (ii) their total cerebral white-matter lesion-loads visible on T2- and T1-weighted imaging (T2-LL and T1-LL, bottom row). These plots show the individual points and the least-squares, linear-fit smoothers for data from the 18 normal control subjects (NC, circles, dotted lines), the 10 patients with relapsing-remitting multiple sclerosis (RR, squares, broken lines), and the 10 patients with secondary-progressive multiple sclerosis (SP, triangles, solid lines). Results of the associated Pearson Product-Moment correlational analyses for each of the groups, as well as for all of the RR and SP patients combined (MS), are also shown (*For purposes of logging and correlational analysis, the two RR patients who had T1-LL values of 0 were each assigned a T1-LL of 0.1 cm³*).](image-url)
3.4. Relationship between cGM-NA/Cr values and cerebral WM-LL values

3.4.1. Correlational analyses

Scatterplots showing the least-squares, linear-fit relationships between individuals’ mean cGM-NA/Cr values and cerebral WM-LL values are presented in the bottom row of Fig. 3. When the patients from the two MS subgroups were studied separately, there was no statistically-significant linear relationship between mean cGM-NA/Cr values and log-T2-LL values in either group (although there was a trend towards a large, [61] negative correlation in the patients with RR-MS: \( r = -0.627, p = 0.052 \)). However, when data from these two patient groups were combined, there was a large, [61] statistically-significant negative correlation \( (r = -0.630, p = 0.003) \) with log-T2-LL values. Similarly, there was an even-larger, statistically-significant negative correlation between mean cGM-NA/Cr values and log-T1-LL values in the patients with RR-MS \( (r = -0.793, p = 0.006) \), but statistical significance was not reached in the patients with SP-MS. Once again, when data from the two patient groups were combined, there was a statistically-significant negative correlation \( (r = -0.733, p = 0.0002) \) with log-T1-LL values.

3.4.2. ANCOVAs

When we examined the use of patients’ log-T2-LL and log-T1-LL values as covariates in separate one-way ANCOVAs examining the effect of patient group on mean cGM-NA/Cr values, we found that both log-T2-LL values \( (F_{1, 17} = 8.04, p = 0.011) \) and log-T1-LL values \( (F_{1, 12.97}, p = 0.002) \) were significant covariates.

When the patients’ log-T2-LL values were included as a covariate in a one-way ANCOVA examining the effect of patient group on mean cGM-NA/Cr values, the statistically-significant difference in means between the RR-MS and SP-MS patient groups came close to disappearing \( (F_{1, 17} = 4.49, p = 0.049) \). This was due to: (i) a decrease in the RR-MS groups’ T2-LL-adjusted mean cGM-NA/Cr values (which decreased by 2.22% to an adjusted mean value of 2.38 with a 95% confidence interval (CI) of 2.23 to 2.54); and (ii) an increase in the SP-MS groups’ T2-LL-adjusted mean cGM-NA/Cr values (which increased by 2.59% to an adjusted mean value of 2.14 with a 95% CI of 1.99 to 2.30). Nevertheless, the RR-MS group’s T2-LL-adjusted mean cGM-NA/Cr value was still similar to the mean cGM-NA/Cr value of the NC group \( (\text{mean} = 2.44, 95\% \text{ CI} = 2.34 \text{ to } 2.54) \). Furthermore, the SP-MS group’s T2-LL-adjusted mean cGM-NA/Cr value was still decreased relative to the NC group \( (i.e., \text{the } 95\% \text{ CIs associated with these two groups’ mean cGM-NA/Cr values did not overlap}) \). Importantly, after adjusting for our patients’ T2-LL values, MS group \( (i.e., \text{RR-MS vs. SP-MS}) \) was, on its own, able to account for 52.3% of the remaining variance in mean cGM-NA/Cr values \( (\text{as opposed to only } 29.7\% \text{ prior to such an adjustment}) \).

The patients’ log-T1-LL values were also included as a covariate in a separate one-way ANCOVA examining the effect of patient group on mean cGM-NA/Cr values. This time, the previously-significant group-mean difference between the RR-MS and SP-MS patient groups did indeed disappear \( (F_{1, 17} = 2.73, p = 0.117) \). This was due to: (i) a further decrease in the RR-MS groups’ T1-LL-adjusted mean cGM-NA/Cr values \( (\text{which now decreased by } 3.48\% \text{ to an adjusted mean value of } 2.35 \text{ with a } 95\% \text{ CI of } 2.21 \text{ to } 2.50) \); and (ii) a further increase in the SP-MS groups’ T1-LL-adjusted mean cGM-NA/Cr values \( (\text{which now increased by } 4.06\% \text{ to an adjusted mean value of } 2.17 \text{ with a } 95\% \text{ CI of } 2.03 \text{ to } 2.32) \). Nevertheless, the RR-MS group’s T1-LL-adjusted mean cGM-NA/Cr value was still similar to that of the NC group. Furthermore, the SP-MS group’s T1-LL-adjusted mean cGM-NA/Cr value was still decreased relative to that of the NC group. Importantly, after adjusting for our patients’ T1-LL values, MS group was now, on its own, able to account for 60.1% of the remaining variance in mean cGM-NA/Cr values \( (\text{and adding their T2-LL as an additional covariate did not increase the amount of variance accounted for}) \).

4. Discussion

4.1. Summary

This study set out to determine: (i) the degree of neuro-axonal disturbance (as indicated by 1H-MRSI-measured NA/Cr values) that was present in the cGM of untreated patients with either RR-MS or SP-MS, and (ii) the relationship between any such cGM neuronal disturbance and the degree of clinical disability and cerebral WM-LL in these patients. We found only negligible, non-significant differences in the mean and median cGM-NA/Cr values of our 10 RR-MS patients relative to those that were observed in our 18 age-matched NC subjects. On the other hand, we found large, [61] statistically-significant decreases in the mean and median cGM-NA/Cr values of our 10 SP-MS patients relative to what was observed in our RR-MS and NC groups. Thus, we found 1H-MRSI evidence for a cGM neuro-axonal disturbance in our SP-MS group, but not in our RR-MS group. Furthermore, when data from all of our patients was combined, we found a large, [61] statistically-significant negative correlation between EDSS-measured clinical disability and cGM-NA/Cr values (with both sets of data having a large range in both patient groups). Similarly, in our combined patients, we found evidence for a large, [61] statistically-significant negative-correlation between cGM-NA/Cr values and cerebral WM-LL values (which also had a large range in both patient groups). Importantly, despite this strong relationship, cerebral WM-LL values could not fully account for the extent of the decrease that was seen in our SP-MS group’s mean cGM-NA/Cr value relative to that seen in our NC group.

4.2. cGM lesions were not detected, but were most likely there

Despite the presence of lesions in the cerebral WM of the patients included in the present study, cortical and juxtacortical lesions were not observed within the vicinity of their 1H-MRSI voxels on any of the PD-, T2-, or T1-weighted MR images that were acquired. Nevertheless, it is now well known that cGM lesions: (i) do not show much contrast on conventional MRI sequences such as the ones that we used; [3–5] and (ii) are actually very common in patients with MS, [7,18,20,23] particularly in those patients with SP-MS. [23]. Accordingly, it is possible that cGM lesions were indeed present in our patients – particularly in those with SP-MS – and could have contributed to the decreased cGM-NA/Cr values observed in our SP-MS group.

4.3. Relation of our observations to the existing literature

Evidence collected from both neuropathological [1,3,17,78,4,18–24] and 1H-MRSI(1) [38–42,46,43,44,47] studies of the brains of patients with MS have implicated cGM pathology in the pathophysiology of MS. In the present study, we found 1H-MRSI-based evidence of neuro-axonal disturbance in the cGM of our patients with SP-MS, but not in those with RR-MS. This finding contrasts with those of some other investigators who had previously provided 1H-MRSI(1) evidence of neuro-axonal disturbance in the cGM of patients with RR-MS [38,46,44]. This discrepancy, however, might be related to differences in, for example: (i) the particular 1H-MRSI(1) acquisition, quantitation, and analysis methods used; (ii) the particular samples of NC subjects and patients with MS that were studied, (iii) the sample sizes included, and (iv) the particular statistical analyses performed. Regardless of the explanation, it is important to remember that our findings agree with those of all previous studies that have directly compared groups of patients with RR-MS to those with SP-MS and found significant effects on cGM-NA/Cr values [40,41,43]. Significantly-decreased cGM-NA/Cr values in patients with SP-MS relative to those with RR-MS might reflect the fact that, for example: (i) cGM changes that are secondary to anterograde or retrograde axonal degeneration might be more prevalent and/or more severe in patients with SP-MS; and/or (ii) an accumulation of cGM...
lesions might also be more prevalent and/or more severe in patients with SP-MS, which is consistent with the histopathological findings of Kutzelnigg et al. [18].

In the present study, we found a large, [61] statistically-significant correlation between $^{1}H$-MRSI-measured cGM-NA/Cr and EDSS-measured clinical disability in our combined group of patients: a finding that seems to be consistent with those of two recent studies, [42,46] but seemingly at odds with those of a number of other studies that have failed to find statistically-significant correlations between cGM-NA and clinical disability in such patients [38,39.36.40.41,37]. However, as noted in the Introduction, all of these other studies had relatively-low sample sizes, and one of them had a limited range of EDSS values, [38] either of which would make it very difficult to find statistically-significant correlations. For example, in 2003, Adalsteinsen et al. [41] reported a Spearman rank-order correlation of $-0.53$ between EDSS scores and cGM-NA values (both of which had a relatively-large range in values) in their sample of 10 patients with MS; however, this seemingly-large correlation did not reach statistical significance (which was reported as $p<0.06$, one-tailed) in this small sample.

Finally, in the present study, we also found that $^{1}H$-MRSI-measured cGM neuro-axonal integrity was strongly related to cerebral WM-LL in our patients: a finding that is consistent with the recent, preliminary findings of Bo et al. [21]. Again, at first glance, our findings seem to disagree with a number of other recent studies that failed to find a statistically-significant linear relationship between cerebral WM-LL and cGM-NA [38,40,37,44] or between cerebral WM-LL and cGM-NA/Cr [47]. However, as noted in the Introduction, these other studies all suffered from relatively-low sample sizes and some had a limited range of cerebral WM-LL values, [38,47] thereby making it very difficult to have found a statistically-significant correlation. Furthermore, most of these studies only examined the relationship between cGM $^{1}H$-MRSI findings and cerebral T2-LL, [40,37,47] which is less specific to tissue destruction and axonal loss in patients with MS than is T1-LL [55] and, as we found in this study, the relationship with cGM-NA/Cr seems to be stronger for cerebral T1-LL than it is for cerebral T2-LL.

### 4.4. Relationship of cGM neuro-axonal disturbance to cerebral WM-LL

Overall, the degree of our patients’ cGM neuro-axonal disturbance (as indicated by decreases in their $^{1}H$-MRSI-measured mean cGM-NA/ Cr values) was significantly related to their total cerebral WM-LL values. Our patients’ mean cGM-NA/Cr values shared 39.7% of their variance in common with that in their T2-LL values, and 53.7% in common with that in their more tissue-destruction-related T1-LL values [55]. Importantly, both of these relationships seemed to be consistently stronger in our patients with RR-MS than in those with SP-MS (T2-LL: $r^{2} = 39.3\%$ vs. 26.1%; T1-LL: $r^{2} = 62.9\%$ vs. 28.9%). As reviewed in the Introduction, neuronal loss and disturbance in the cGM can occur: (i) as the direct result of lesions located within the cGM, and/or (ii) secondarily to lesions located within the WM. Accordingly, our observation of a seemingly-stronger correlation between cGM-NA/Cr values and cerebral WM-LL in patients with RR-MS is consistent with the possibility that: (i) $^{1}H$-MRSI-measured neuro-axonal disturbances in the cGM of patients with RR-MS are strongly related to the effects of axonal transection within WM lesions; but (ii) such disturbances in the cGM of patients with SP-MS (which are greater than those seen in patients with RR-MS) may reflect a more-widespread degenerative process than that seen in RR-MS [18].

In the present study, our patients’ cerebral WM-LL values were found to account for a significant portion of the cGM-NA/Cr differences that were observed between our two patient groups. Indeed, the statistically-significant difference in mean cGM-NA/Cr values that was found between our RR-MS and our SP-MS groups disappeared when T1-LL values were covaried out. One possible explanation for this finding is that neuro-axonal damage in the cGM does not occur independently of cerebral WM-LL; that is to say, cerebral WM-LL-related axonal degeneration is likely to be a main contributor to the loss of cGM-NA/Cr. It is also possible, however, that cGM disturbance in patients with SP-MS may be increased proportionately to their cerebral WM-LL because the evolution of lesions in the cGM and in cerebral WM occurs in parallel. Nevertheless, it is important to note that the reduction in our SP-MS patients’ mean cGM-NA/Cr values relative to those in our NC subjects persisted even after adjusting for their T2-LL values and/or their T1-LL values: again, this suggests that such disturbance in the cGM of patients with SP-MS reflects a degenerative process that is not limited to the pathology reflected by cerebral WM-LL [18].

Unfortunately, the limitations of the conventional MRI techniques that were used to acquire the anatomical data in the present study preclude us from making any conclusions about the relationship between neuronal disturbances in cGM (as measured by $^{1}H$-MRSI decreases in NA/Cr) and the possible, accompanying presence of demyelinating cGM lesions. Furthermore, not much is yet known about the relationship between: (i) the location, the type, and the prevalence of cGM lesions in patients with MS, and (ii) these patients’ disease duration, progression, and degree of clinical disability — relationships that are not easily studied on the basis of post-mortem materials. It is hoped that ongoing advances in MRI acquisition and analysis techniques will soon lead to methods that are capable of directly visualizing and quantifying such cGM lesions, developments that should prove to be extremely helpful in furthering our understanding of the relevance and time course of cGM pathology and pathophysiology in patients with MS.

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