Investigation of magnetization transfer ratio-derived pial and subpial abnormalities in the multiple sclerosis spinal cord

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Neuropathological studies in multiple sclerosis have suggested that meningeal inflammation in the brain may be linked to disease progression. Inflammation in the spinal cord meninges has been associated with axonal loss, a pathological substrate for disability. Quantitative magnetic resonance imaging facilitates the investigation of spinal cord microstructure by approximating histopathological changes. We acquired structural and quantitative imaging of the cervical spinal cord from which we calculated magnetization transfer ratio in the outer spinal cord—an area corresponding to the expected location of the pia mater and subpial region—and in spinal cord white and grey matter. We studied 26 healthy controls, 22 people with a clinically isolated syndrome, 29 with relapsing-remitting, 28 with secondary-progressive and 28 with primary-progressive multiple sclerosis. Magnetization transfer ratio values in the outermost region of the spinal cord were higher than the white matter in controls and patients: controls (51.35 ± 1.29 versus 49.87 ± 1.45, P < 0.01), clinically isolated syndrome (50.46 ± 1.39 versus 49.13 ± 1.19, P < 0.01), relapsing-remitting (48.86 ± 2.89 versus 47.44 ± 2.70, P < 0.01), secondary-progressive (46.33 ± 2.84 versus 44.75 ± 3.10, P < 0.01) and primary-progressive multiple sclerosis (46.99 ± 3.78 versus 45.62 ± 3.40, P < 0.01). In linear regression models controlling for cord area and age, higher outer spinal cord magnetization transfer ratio values were seen in controls than all patient groups: clinically isolated syndrome (coefficient = −0.32, P = 0.03), relapsing-remitting (coefficient = −0.48, P < 0.01), secondary-progressive (coefficient = −0.51, P < 0.01) and primary-progressive multiple sclerosis (coefficient = −0.38, P < 0.01). In a regression analysis correcting for age and cord area, magnetization transfer ratio values in the outer cord were lower in relapsing-remitting multiple sclerosis compared with clinically isolated syndrome (coefficient = −0.28, P = 0.02), and both primary and secondary-progressive compared to relapsing-remitting multiple sclerosis (coefficients = −0.29 and −0.24, respectively, P = 0.02 for both). In the clinically isolated syndrome and relapsing-remitting multiple sclerosis groups, outer cord magnetization transfer ratio was decreased in the absence of significant cord atrophy. In a multivariate regression analysis an independent association was seen between outer cord magnetization transfer ratio and cord atrophy (coefficient = 0.40, P < 0.01). Our in vivo imaging observations suggest that abnormalities in a region involving the pia mater and subpial cord occur early in the course of multiple sclerosis and are more marked in those with a progressive course.

Keywords: multiple sclerosis; spinal cord; MRI; MTR; magnetization transfer ratio
Introduction

Multiple sclerosis is an inflammatory disorder of the CNS, and is a common cause of disability among young adults (Confavreux and Vukusic, 2006). Although the exact causative mechanisms are unclear, it has been suggested from the findings in neuropathological studies that meningeal inflammation may play a role in the development of earlier disability in progressive forms of the disease (Magliozzi et al., 2007; Howell et al., 2011; Choi et al., 2012). It has also been reported that B cell follicles (as well as T cells) are present in the meninges of post-mortem brain specimens (Serafini et al., 2004; Lovato et al., 2011). However, Frischer et al. (2009) reported that B cells may only be found in the progressive forms of the disease. Neuropathology studies have also suggested that cortical demyelination may be a characteristic finding in multiple sclerosis (Dawson, 1916; Brownwell and Hughes, 1962). Another post-mortem study has suggested that cortical demyelination is more commonly found in progressive multiple sclerosis and inflammation is more diffuse, when compared directly to the relapsing-remitting form of the disease (Kutzelnigg et al., 2005).

Taking into account the above observations, a hypothesis has arisen that the pathological processes of meningeal inflammation and cortical demyelination may be interconnected and in turn play a role in the development of a progressive disease course. Magliozzi et al. (2010) reported the co-localization of areas of meningeal inflammation (composed of B cells and dendritic cells) and cortical demyelination. Furthermore, extensive subpial demyelination has been recorded in the cortex in multiple sclerosis (Bø et al., 2003; Bø et al., 2007). It has been proposed that cortical demyelination (especially subpial) may occur in the presence of meningeal inflammation due to the release of inflammatory cytokines in the subarachnoid space (Brown and Sawchenko, 2007; Ransohoff, 2009). In progressive forms of multiple sclerosis, where there seems to be long standing inflammation, this may create a milieu, which favours further retention of inflammatory cells (Krumholz et al., 2005; Meini et al., 2008). This retention of inflammatory cells in multiple sclerosis may result in the activation of microglia and subsequently demyelination (Magliozzi et al., 2010).

It has been reported that in relapsing-remitting multiple sclerosis, meningeal inflammation may be present, with inflammation involving T cells rather than B cells (Frischer et al., 2009). As neuropathology studies are predominantly composed of cases of progressive multiple sclerosis, an important insight was gained into the process of meningeal inflammation in a study examining biopsies of patients with early relapsing-remitting multiple sclerosis or a clinically isolated syndrome (Lucchinetti et al., 2011). In this paper it was found that subpial cortical demyelination may be associated with meningeal inflammation.

Imaging studies have also provided a means of studying patients when they present with a clinically isolated syndrome suggestive of multiple sclerosis, which is the earliest clinical manifestation of the condition in most cases. The use of a double inversion recovery sequence has demonstrated the presence of cortical lesions in patients with clinically isolated syndrome (Calabrese et al., 2007). Furthermore, a decrease in magnetization transfer ratio (MTR) of the cortex, which might reflect demyelination (Chen et al., 2013), has also been observed in subjects with clinically isolated syndrome (Audoin et al., 2005; Fernando et al., 2005). These imaging observations also suggest that cortical demyelination occurs early in the disease course.

The meninges of the spinal cord have also been reported to be involved in multiple sclerosis (Androdias et al., 2010; Deluca et al., 2013), with the inflammatory cells predominantly composed of T cells in one study (Androdias et al., 2010). Meningeal inflammation in the spinal cord has been related to axonal loss (Androdias et al., 2010; Deluca et al., 2013). As axonal loss has been hypothesized to be the pathological substrate for disability (Ganter et al., 1999; Evangelou et al., 2000; Lovas et al., 2000; Schirmer et al., 2011), and because much of the physical disability from multiple sclerosis arises from spinal cord involvement, a greater understanding of meningeal abnormalities in the spinal cord, and their relationship to disease course, would seem to be relevant.

However, to date the effect of multiple sclerosis on the spinal cord meninges has not been investigated in vivo, due to image resolution constraints and other technical challenges associated with MRI protocol optimization in the spinal cord (Dietrich et al., 2008; Stroman et al., 2014). MTR imaging in the spinal cord has been used for some time (Mezzapesa et al., 2004; Charil et al., 2006). The MTR of a tissue is related to its macromolecular structure and an important contribution in CNS tissue comes from myelin (Dousset et al., 1992). Recent MTR imaging studies in the spinal cord have provided insights into the mechanisms of disability in multiple sclerosis (Zackowski et al., 2009; Oh et al., 2013). With the implementation of higher resolution structural and MTR acquisitions in the spinal cord than those previously acquired (Yiannakas et al., 2012), the potential to investigate the effects of multiple sclerosis on the outer region of the spinal cord, that would be expected to include the pia mater of the meninges, could provide further insights into the pathophysiology of multiple sclerosis.

The aims of this paper are threefold:

(i) To characterize the outermost region of the spinal cord, which is expected to include contributions from the pia mater and subpial region of the spinal cord, using high in-plane resolution, magnetization transfer-weighted images to measure the MTR in healthy control subjects and in patients with multiple sclerosis or a clinically isolated syndrome;

(ii) To compare the outer spinal cord MTR measures of people with a clinically isolated syndrome or multiple sclerosis with those of healthy controls; and

Abbreviations: EDSS = Expanded Disability Status Scale; FFE = fast field echo; MTR = magnetization transfer ratio; PASAT = Paced Auditory Serial Addition Test.
To compare outer spinal cord MTR findings seen in different clinical subgroups: clinically isolated syndrome, relapsing-remitting, primary- and secondary-progressive multiple sclerosis, and explore the relationship of outer cord MTR with measures of both spinal cord atrophy and physical disability.

Materials and methods

Subjects

We recruited subjects with no prior neurological diseases (n = 26) and patients with either clinically isolated syndrome (n = 22) or multiple sclerosis: relapsing-remitting (n = 29), secondary-progressive (n = 28) and primary-progressive (n = 28). Multiple sclerosis was diagnosed using the 2010 McDonald criteria (Polman et al., 2011). The clinically isolated syndrome cohort was recruited following a single clinical episode consistent with demyelination and at least one lesion on a T₂-weighted axial brain scan. The multiple sclerosis subgroups were classified using the Lublin-Reingold criteria (1996). Informed written consent was obtained from each participant before inclusion in the study.

All patients with clinically isolated syndrome or multiple sclerosis had Expanded Disability Status Scale (EDSS; Kurtzke, 1983) determined by a neurostatus certified assessor as well as multiple sclerosis functional composite (Fischer et al., 1999). Subsequently we calculated Z-scores for the 25-foot Timed Walk Test, 9-Hole Peg Test and 3 s Paced Auditory Serial Addition Test B (PASAT). Z-scores were calculated from the database (Fischer et al., 1999). We also recorded American Spinal Injury Association (ASIA) motor (m) and sensory (s) scores (Maynard et al., 1997) for all subjects with multiple sclerosis and clinically isolated syndrome. Assessment of physical function was performed immediately before the MRI.

None of the subjects had experienced a relapse or received a course of corticosteroids within 1 month before imaging.

Magnetic resonance imaging protocol

Subjects were scanned at 3 T using a Philips Achieva MRI system with radiofrequency (rf) multi-transmit technology (Philips Healthcare). A 16-channel receive-only neurovascular coil was used for spinal cord scanning and brain scanning was performed using the product of corticosteroids within 1 month before imaging.

MTR maps were reviewed again by the same reader to confirm that no artefacts were introduced during the registration step. We extracted three slices centred at the C2–3 intervertebral disc from the volumetric 3D-FFE image and then created two regions of interest: (i) spinal cord outline using an active surface model (Horsfield et al., 2010); and (ii) spinal cord grey matter using a semi-automated fuzzy connector method (Udupa and Samarasekera, 1996) (Fig. 1E and F). Using these two regions of interest we created a mask of the cord outline and grey matter from the MTR map. We then converted both masks to binary format using FSL tools (http://www.fmrib.ox.ac.uk/fsl/). Subsequently we created an MTR-map in the 3D-FFE space (Fig. 1C and D) (Yiannakas et al., 2012). All MTR maps were reviewed again by the same reader to confirm that no artefacts were introduced during the registration step.

We extracted three slices centred at the C2–3 intervertebral disc from the volumetric 3D-FFE image and then created two regions of interest: (i) spinal cord outline using an active surface model (Horsfield et al., 2010); and (ii) spinal cord grey matter using a semi-automated fuzzy connector method (Udupa and Samarasekera, 1996) (Fig. 1E and F). Using these two regions of interest we created a mask of the cord outline and grey matter from the MTR map. We then converted both masks to binary format using FSL tools (http://www.fmrib.ox.ac.uk/fsl/). This process enabled us to subtract the binary grey matter mask from the binary cord outline resulting in two masks: (i) spinal cord white matter MTR mask; and (ii) spinal cord grey matter MTR mask. We recorded the mean value of the spinal grey matter MTR mask for each participant.

We then further analysed the white matter MTR mask by eroding the image using iterations based on 4-connected neighbours written in Matlab 2012a (Mathworks). We discarded the outermost row of pixels of the image to avoid contamination by CSF. As the image was acquired at 0.75 × 0.75 mm² resolution and subsequently reconstructed to 0.5 × 0.5 mm², we considered the next voxel layer to be also contaminated by CSF due to the voxels’ interpolation and consequently discarded this voxel too. Therefore, the next voxel layer was designated as being the outermost voxel layer of the spinal cord and we recorded the mean MTR values of the voxels in this layer across the three selected slices for each subject. The classification of peripheral voxels in the image is illustrated in Fig. 2 and on MRI in Fig. 3; we...
considered this layer likely to include both pia mater and subpial spinal cord tissue. Following removal of the three outermost voxels we recorded the mean value from the spinal cord white matter-MTR mask for each subject and designated this as the white matter-MTR value. Finally, we recorded the mean grey matter MTR value across the three slices.

**Spinal cord area**

We measured spinal cord mean cross-sectional area from the 3D-PSIR image using the active surface model (Horsfield et al., 2010). To achieve this we extracted five 3-mm thick slices centred at C2–C3 and then recorded the area of each of the five slices (Kearney et al., 2014). Spinal cord area was normalized by the number of slices used i.e. we calculated the mean area of the five slices for each participant. This method of normalization was chosen as a previously published report has demonstrated that normalization of spinal cord volume by

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**Figure 1** (A) Axial magnetization transfer-off image (resolution $0.5 \times 0.5 \times 5\text{mm}^3$) and (B) axial magnetization transfer-on image (resolution $0.5 \times 0.5 \times 5\text{mm}^3$) before registration to the 3D-FFE image to create the MTR map. (C) Axial 3D-FFE image (resolution $0.5 \times 0.5 \times 5\text{mm}^3$) through the C2–3 intervertebral disc. (D) Following independent linear registration of the magnetization transfer-on and magnetization transfer-off to the FFE the MTR map is created in this space (resolution $0.5 \times 0.5 \times 5\text{mm}^3$). (E) Axial 3D-FFE image demonstrating the spinal cord outline created using the active surface model. (F) Axial 3D-FFE demonstrating grey matter region of interest outlined using the fuzzy connector.

**Figure 2** Graphical representation of voxel layers analysed in the outermost region of the spinal cord.
Brain parenchymal fraction and T2 lesion volume

Lesions on each subject’s 3D T1-weighted volume were marked and filled with values consistent with normal-appearing white matter signal intensity to prevent misclassification of tissue during segmentation (Chard et al., 2010). Segmentation of the lesion-filled image was then performed using SPM8 (statistical parametric mapping; Wellcome Trust centre for NeuroImaging, UCL Institute of Neurology, London). The brain parenchymal fraction (the sum of white and grey matter relative to total intracranial volume) was then recorded for each subject.

We outlined T2 hyperintense lesions in all participants (clinically isolated syndrome or multiple sclerosis) using the semi-automated edge finding tool in JIM 6 on the axially acquired T2-weighted images. We then recorded the volume of T2-weighted lesions in millilitres.

Statistical analysis

SPSS 21 (IBM) was used for statistical analysis.

Comparisons of magnetic resonance imaging measures in controls and patient subgroups

In healthy controls we evaluated the differences in mean MTR values between: (i) outer spinal cord and spinal cord white matter; (ii) outer spinal cord and spinal cord grey matter; and (iii) spinal cord white matter with grey matter, using a paired samples t-test. Subsequently we used the same tests in patients with clinically isolated syndrome and each subgroup of multiple sclerosis separately. We also tested differences in cord area and brain parenchymal fraction between patients with multiple sclerosis and clinically isolated syndrome and control subjects using an independent samples t-test.

To explore the hypothesis that MTR abnormalities in each tissue component of the spinal cord (outer spinal cord, white matter and grey matter) may differ between clinically isolated syndrome or each subtype of multiple sclerosis patients and controls, we constructed a linear regression model, with the case (i.e. multiple sclerosis subgroup, clinically isolated syndrome and control) set as the dependent variable and MTR added as an independent variable. To compare each patient group (clinically isolated syndrome or multiple sclerosis subgroup) with controls, or with each other, a separate regression model was constructed for each comparison of MTR values (outer spinal cord, white matter and grey matter) being performed. To investigate whether differences seen in MTR values between subject groups (dependent variable) were affected by cord atrophy in the multiple sclerosis subgroups, we included spinal cord area as an independent variable in every model and also adjusted for age and gender by adding these as independent variables. Adjustments for multiple comparisons were not performed due to the exploratory nature of this study (Bender and Lange, 2001).

Relationship between cord magnetization transfer ratio measures and cord area

To investigate the relationship between spinal cord MTR and cord area we firstly calculated univariate Pearson’s correlation coefficient of the MTR of the grey matter, white matter and outer spinal cord versus cord area in all multiple sclerosis patients combined. To identify the components of the spinal cord MTR (i.e. outer spinal cord, grey and white matter) which were associated with atrophy, independently from the others and from age and gender, a multivariate linear regression model was constructed. In this model, cord area was set as the dependent variable, and MTR values from each region of the spinal cord that had a significant univariate correlation with cord area were added as independent variables, in a forward stepwise manner, to determine those with independent associations with cord area. Independent variables retained in the final model with a P-value of <0.05 were considered to be independently associated with cord area (dependent variable).

Relationship between magnetic resonance imaging and disability measures

To explore the relationship of each MRI measure analysed (spinal cord area, spinal cord MTR (outer cord, white matter and grey matter), brain parenchymal fraction and brain lesion load with disability, we firstly calculated univariate correlations between these variables, in all multiple sclerosis patients combined. We used Spearman’s rank correlation coefficient for the EDSS, as this scale is logarithmic. For
all other disability scales (multiple sclerosis functional composite Z-scores, ASIAs and ASIAM) we calculated univariate correlations with the MRI parameters using Pearson’s correlation coefficient. For univariate associations, P < 0.01 was considered significant.

To investigate the relationship between the MRI parameters analysed and physical disability, we sought to determine independent associations between these two variables. To achieve this, we constructed a multivariate linear regression model with the disability measure of interest set as the dependent variable. A separate model was constructed for each disability measure used i.e. multiple comparisons were not performed. To refine the choice of independent variables added to the model, only MRI parameters that had a significant univariate correlation with the dependent variable (i.e. the disability measure of interest), were included in the regression analysis. In each model constructed, age and gender were also added as independent variables, to correct for any influence these may have on each model constructed, and with multiple sclerosis.P

Comparison of cord area and brain volume between groups

There were no significant differences in the cord area of healthy controls with either the clinically isolated syndrome (P = 0.06) or relapsing-remitting multiple sclerosis (P = 0.18) groups. The secondary-progressive (63.5 ± 10.0 mm² versus 80.2 ± 6.8 mm², P < 0.01) and primary-progressive multiple sclerosis (68.1 ± 9.7 mm², P < 0.01) groups both had lower cord areas than healthy controls. Results for all groups are presented in Table 1.

No significant difference was seen in brain parenchymal fraction in the clinically isolated syndrome group compared to controls, but significant differences in brain parenchymal fraction were seen between controls and all subgroups of multiple sclerosis (P < 0.01), with smaller brain parenchymal fractions in the multiple sclerosis subgroups.

Comparison of outer spinal cord and spinal cord white matter magnetization transfer ratio values within each subject group

In each subject group, the MTR of the outer spinal cord was higher than the MTR of spinal cord white matter: controls (51.35 ± 1.29 versus 49.87 ± 1.45, P < 0.01), clinically isolated syndrome (50.46 ± 1.39 versus 49.13 ± 1.19, P < 0.01), relapsing-remitting multiple sclerosis (48.86 ± 2.89 versus 47.44 ± 2.70, P < 0.01), secondary-progressive multiple sclerosis

Table 1 Demographics and conventional MRI parameters in healthy controls, and patients with clinically isolated syndrome and with multiple sclerosis

<table>
<thead>
<tr>
<th></th>
<th>Controls n = 26</th>
<th>Clinically isolated syndrome n = 22</th>
<th>Relapsing-remitting multiple sclerosis n = 29</th>
<th>Secondary-progressive multiple sclerosis n = 28</th>
<th>Primary-progressive multiple sclerosis n = 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender female: male</td>
<td>18:8</td>
<td>12:10</td>
<td>20:9</td>
<td>17:11</td>
<td>12:16</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40.6 ± 10.2</td>
<td>36.2 ± 9.3</td>
<td>38.1 ± 9.5</td>
<td>51.3 ± 9.4</td>
<td>50.5 ± 9.9</td>
</tr>
<tr>
<td>Mean disease duration (years)</td>
<td>0.5 ± 0.4</td>
<td>6.1 ± 4.0</td>
<td>6.1 ± 4.0</td>
<td>20.11 ± 11.8</td>
<td>10.9 ± 7.6</td>
</tr>
<tr>
<td>Median EDSS (range)</td>
<td>1 (0–3)</td>
<td>2.5 (0–7)</td>
<td>6.5 (4–8.5)</td>
<td>6.0 (2–8)</td>
<td></td>
</tr>
<tr>
<td>Brain parenchymal fraction</td>
<td>0.824 ± 0.015</td>
<td>0.822 ± 0.013</td>
<td>0.811 ± 0.017*</td>
<td>0.788 ± 0.022*</td>
<td>0.798 ± 0.014*</td>
</tr>
<tr>
<td>T_2 lesion volume (ml)</td>
<td>80.3 ± 7.7</td>
<td>82.6 ± 7.3</td>
<td>78.1 ± 9.0</td>
<td>63.6 ± 9.8*</td>
<td>68.1 ± 9.7*</td>
</tr>
<tr>
<td>Spinal cord cross-sectional area (mm²)</td>
<td>80.3 ± 7.7</td>
<td>82.6 ± 7.3</td>
<td>78.1 ± 9.0</td>
<td>63.6 ± 9.8*</td>
<td>68.1 ± 9.7*</td>
</tr>
</tbody>
</table>

*Significant differences between MRI parameters from controls (i.e. P < 0.01).
(46.33 ± 2.84 versus 44.75 ± 3.10, \( P < 0.01 \)), and primary-progressive multiple sclerosis (46.99 ± 3.78 versus 45.62 ± 3.40, \( P < 0.01 \)) (Table 2).

**Comparison of spinal cord white matter and grey matter magnetization transfer ratio values within each subject group**

In each subject group, the spinal cord white matter had higher MTR values than spinal cord grey matter (Table 2).

**Outer spinal cord magnetization transfer ratio in controls versus patient subgroups**

The outer spinal cord MTR values were higher in controls than all patient groups: controls versus clinically isolated syndrome (coefficient = −0.32, \( P = 0.03 \), 95% confidence interval (CI) −0.22 to −0.01), controls versus relapsing-remitting multiple sclerosis (coefficient = −0.48, \( P < 0.01 \), 95% CI −0.28 to 0.09), controls versus secondary-progressive multiple sclerosis (coefficient = −0.51, \( P < 0.01 \), 95% CI −0.31 to −0.15), controls versus primary-progressive multiple sclerosis (coefficient = −0.38, \( P < 0.01 \), 95% CI −0.36 to −0.06). Cord area was not a significant covariate in any of the models used (Table 3).

**Spinal cord white matter and grey matter magnetization transfer ratio in controls versus patient subgroups**

The MTR of spinal cord white matter was higher in controls than in all patient groups: controls versus CIS (\( P = 0.03 \)), controls versus multiple sclerosis (\( P < 0.01 \)). The MTR of spinal cord grey matter was not different between controls and the group with clinically isolated syndrome but was significantly higher in controls than in all three multiple sclerosis subgroups (\( P < 0.01 \)) (Table 3).

**Outer spinal cord magnetization transfer ratio: comparison between patient subgroups**

The outer spinal cord MTR was lower in relapsing-remitting multiple sclerosis than clinically isolated syndrome (coefficient = −0.28, \( P = 0.02 \), 95% CI −0.10 to −0.01). Both secondary-progressive (coefficient = −0.24, \( P = 0.02 \), 95% CI −0.07 to −0.01) and primary-progressive multiple sclerosis (coefficient = −0.29, \( P = 0.02 \), 95% CI −0.16 to −0.01) had lower outer spinal cord MTR values compared to relapsing-remitting multiple sclerosis. No significant difference was found between secondary- and primary-progressive multiple sclerosis (Table 3).

**Univariate correlations and association between magnetization transfer ratio measures and cord area in all multiple sclerosis patients combined**

The following MRI parameters were correlated with cord area: outer spinal cord MTR (\( r = 0.39, P < 0.01 \)), MTR white matter (\( r = 0.36, P < 0.01 \)), MTR grey matter (\( r = 0.36, P < 0.01 \)) and brain parenchymal fraction (\( r = 0.27, P = 0.01 \)). However, outer spinal cord MTR (coefficient = 0.40, \( P < 0.01 \), 95% CI 0.63 to 1.88) alone was found to be independently associated with cord area; no independent associations were seen between grey or white matter MTR and cord area.

**Univariate correlations between magnetic resonance imaging parameters and disability in all multiple sclerosis patients combined**

Significant univariate correlations between MRI parameters and disability are presented in Table 4. Although significant correlations were seen between outer spinal cord MTR and each disability measure used (apart from PASAT z-score), stronger correlations were seen between cord area and disability measures reflecting

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**Table 2** Mean MTR values (±SD) of spinal cord region subtypes (outer cord, white matter, grey matter) in the control group and in each patient group

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CIS</th>
<th>CIS (excluding two cases of myelitis)</th>
<th>RRMS</th>
<th>SPMS</th>
<th>PPMS</th>
</tr>
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<tbody>
<tr>
<td>Outer spinal cord MTR</td>
<td></td>
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<td></td>
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<tr>
<td>Comparison between outer spinal cord and white matter MTR values</td>
<td>51.35 ± 1.29</td>
<td>50.46 ± 1.39</td>
<td>49.9 ± 1.40</td>
<td>48.86 ± 2.89</td>
<td>46.33 ± 2.84</td>
<td>46.99 ± 3.78</td>
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<td></td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>MTR spinal cord white matter</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comparison between spinal cord white and grey matter MTR values</td>
<td>49.87 ± 1.45</td>
<td>49.13 ± 1.19</td>
<td>49.21 ± 1.31</td>
<td>47.44 ± 2.70</td>
<td>44.75 ± 3.10</td>
<td>45.62 ± 3.4</td>
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<tr>
<td></td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
<td>( p = 0.02 )</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>MTR spinal cord grey matter</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>48.23 ± 1.76</td>
<td>47.72 ± 1.23</td>
<td>47.8 ± 1.30</td>
<td>46.6 ± 2.43</td>
<td>43.88 ± 2.62</td>
<td>44.88 ± 3.09</td>
</tr>
</tbody>
</table>

CIS = clinically isolated syndrome; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary-progressive multiple sclerosis; PPMS = primary-progressive multiple sclerosis. The clinically isolated syndrome cohort is presented with and without the two cases of myelitis included.
motor function. Brain $T_2$ lesion volume was not found to be correlated with any disability measures except for PASAT z-score (Table 4).

**Table 3 Comparison of MTR values between control groups and in each patient group.**

<table>
<thead>
<tr>
<th>MTR value</th>
<th>Comparison groups</th>
<th>SE</th>
<th>Coefficient</th>
<th>Significance (P-value)</th>
<th>95% CI (lower, upper)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer spinal cord MTR</td>
<td>CIS/control</td>
<td>0.05</td>
<td>−0.32</td>
<td>0.03</td>
<td>−0.22, −0.01</td>
</tr>
<tr>
<td></td>
<td>RRMS/control</td>
<td>0.05</td>
<td>−0.48</td>
<td>&lt;0.01</td>
<td>−0.28, −0.09</td>
</tr>
<tr>
<td></td>
<td>SPMS control</td>
<td>0.04</td>
<td>−0.51</td>
<td>&lt;0.01</td>
<td>−0.31, −0.15</td>
</tr>
<tr>
<td></td>
<td>PPMS/control</td>
<td>0.07</td>
<td>−0.38</td>
<td>&lt;0.01</td>
<td>−0.36, −0.06</td>
</tr>
<tr>
<td></td>
<td>CIS RRMS</td>
<td>0.02</td>
<td>−0.28</td>
<td>0.02</td>
<td>−0.10, −0.01</td>
</tr>
<tr>
<td></td>
<td>RRMS/SPMS</td>
<td>0.02</td>
<td>−0.24</td>
<td>0.02</td>
<td>−0.07, −0.01</td>
</tr>
<tr>
<td></td>
<td>RRMS/PPMS</td>
<td>0.04</td>
<td>−0.29</td>
<td>0.02</td>
<td>−0.16, −0.01</td>
</tr>
<tr>
<td></td>
<td>SPMS PPMS</td>
<td>0.02</td>
<td>−0.02</td>
<td>0.89</td>
<td>−0.05, 0.04</td>
</tr>
<tr>
<td>MTR white matter</td>
<td>CIS/control</td>
<td>0.39</td>
<td>−0.34</td>
<td>0.03</td>
<td>−1.69, −1.05</td>
</tr>
<tr>
<td></td>
<td>RRMS/control</td>
<td>0.30</td>
<td>−0.56</td>
<td>&lt;0.01</td>
<td>−1.95, −0.76</td>
</tr>
<tr>
<td></td>
<td>SPMS/control</td>
<td>0.23</td>
<td>−0.98</td>
<td>&lt;0.01</td>
<td>−2.74, −1.80</td>
</tr>
<tr>
<td></td>
<td>PPMS/control</td>
<td>0.22</td>
<td>−0.80</td>
<td>&lt;0.01</td>
<td>−1.79, −0.92</td>
</tr>
<tr>
<td>MTR grey matter</td>
<td>CIS/control</td>
<td>0.42</td>
<td>−0.17</td>
<td>0.26</td>
<td>−1.3, 0.37</td>
</tr>
<tr>
<td></td>
<td>RRMS/control</td>
<td>0.29</td>
<td>−0.36</td>
<td>&lt;0.01</td>
<td>−1.38, −0.21</td>
</tr>
<tr>
<td></td>
<td>SPMS/control</td>
<td>0.20</td>
<td>−0.74</td>
<td>&lt;0.01</td>
<td>−1.84, −1.05</td>
</tr>
<tr>
<td></td>
<td>PPMS/control</td>
<td>0.19</td>
<td>−0.75</td>
<td>&lt;0.01</td>
<td>−1.50, −0.73</td>
</tr>
</tbody>
</table>

CIS = clinically isolated syndrome; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary-progressive multiple sclerosis; PPMS = primary-progressive multiple sclerosis.

A linear regression model was used adjusted for age, gender and cord area (SE = standard error).

**Table 4 R-values for disability measures versus MRI parameter.**

<table>
<thead>
<tr>
<th>MRI parameter</th>
<th>Disability measure</th>
<th>EDSS*</th>
<th>ASIAm</th>
<th>ASIAs</th>
<th>Z-score PASAT</th>
<th>Z-score 9-Hole Peg Test</th>
<th>Z-score 25-foot Timed Walk Test</th>
<th>Z-score multiple sclerosis functional composite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer spinal cord MTR</td>
<td>−0.41</td>
<td>0.38</td>
<td>0.30</td>
<td>0.03</td>
<td>0.41</td>
<td>0.36</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>MTR spinal cord white matter</td>
<td>−0.32</td>
<td>0.23</td>
<td>0.30</td>
<td>0.01</td>
<td>0.42</td>
<td>0.40</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>MTR spinal cord grey matter</td>
<td>−0.34</td>
<td>0.27</td>
<td>0.29</td>
<td>0.05</td>
<td>0.38</td>
<td>0.37</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Spinal cord area</td>
<td>−0.60</td>
<td>0.49</td>
<td>0.39</td>
<td>0.14</td>
<td>0.48</td>
<td>0.38</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Brain parenchymal fraction</td>
<td>−0.40</td>
<td>0.22</td>
<td>0.06</td>
<td>0.35</td>
<td>0.48</td>
<td>0.29</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>$T_2$ lesion volume</td>
<td>0.19</td>
<td>−0.04</td>
<td>0.17</td>
<td>−0.36</td>
<td>−0.29</td>
<td>−0.08</td>
<td>−0.29</td>
<td></td>
</tr>
</tbody>
</table>

Significant ($P < 0.01$) univariate correlations between MRI parameters and disability measure in all patients with multiple sclerosis combined are indicated in bold font (*Spearman’s coefficient used for EDSS and Pearson’s coefficient for all other disability measures).

Independent associations between magnetic resonance imaging parameters and disability in all multiple sclerosis patients combined

Significant independent associations between MRI variables and disability measures are summarized in Table 5. The 9-Hole Peg Test z-score was associated with MTR in the outer spinal cord. Tests of motor function (EDSS and ASIAM) were associated with cord area. The 25-foot Timed Walk Test z-score was associated with spinal cord white matter MTR in multiple sclerosis. The PASAT z-score was associated with both brain $T_2$ lesion volume and brain parenchymal fraction.

Quality control

Our study procedures aimed to minimize the risk of imaging artefacts compromising the study findings. These included a neck immobilization procedure to prevent motion artefact, as already described in the ‘Materials and methods’ section. Further information is provided in online Supplementary material of how effects of potential partial volume, motion and other magnetic resonance-related artefacts were mitigated (see Supplementary Figs 1–6 and Supplementary Tables 1a–c and 2a and b).
Table 5 Summary of MRI parameters significantly associated with disability measures from linear regression models using disability as dependent variable

<table>
<thead>
<tr>
<th>Disability Measure</th>
<th>MRI parameter associated with disability</th>
<th>SE</th>
<th>Coefficient</th>
<th>Significance (P-value)</th>
<th>95% CI (lower, upper)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDSS</td>
<td>Spinal cord area</td>
<td>0.02</td>
<td>-0.48</td>
<td>&lt;0.01</td>
<td>-0.13, -0.06</td>
</tr>
<tr>
<td></td>
<td>Brain parenchymal fraction</td>
<td>9.45</td>
<td>-0.29</td>
<td>&lt;0.01</td>
<td>-48.78, -11.17</td>
</tr>
<tr>
<td>ASIAm</td>
<td>Spinal cord area</td>
<td>0.13</td>
<td>0.42</td>
<td>&lt;0.01</td>
<td>0.32, 0.85</td>
</tr>
<tr>
<td>ASIAS</td>
<td>Spinal cord area</td>
<td>0.08</td>
<td>0.26</td>
<td>0.03</td>
<td>0.02, 0.34</td>
</tr>
<tr>
<td>Z-score PASAT</td>
<td>Brain parenchymal fraction</td>
<td>8.42</td>
<td>0.25</td>
<td>0.02</td>
<td>2.82, 36.32</td>
</tr>
<tr>
<td></td>
<td>T2 lesion volume</td>
<td>0.01</td>
<td>-0.26</td>
<td>0.02</td>
<td>-0.04, -0.004</td>
</tr>
<tr>
<td>Z-score 9-Hole Peg Test</td>
<td>Outer spinal cord MTR</td>
<td>0.04</td>
<td>0.27</td>
<td>&lt;0.01</td>
<td>0.04, 0.19</td>
</tr>
<tr>
<td></td>
<td>Brain parenchymal fraction</td>
<td>0.41</td>
<td>0.41</td>
<td>&lt;0.01</td>
<td>16.44, 14.99</td>
</tr>
<tr>
<td>Z-score 25-foot Timed Walk Test</td>
<td>MTR white matter</td>
<td>0.15</td>
<td>0.36</td>
<td>&lt;0.01</td>
<td>0.30, 0.89</td>
</tr>
<tr>
<td>Z-score multiple sclerosis functional composite</td>
<td>MTR white matter</td>
<td>0.07</td>
<td>0.30</td>
<td>&lt;0.01</td>
<td>0.07, 0.36</td>
</tr>
<tr>
<td></td>
<td>Spinal cord area</td>
<td>0.023</td>
<td>0.22</td>
<td>0.04</td>
<td>0.001, 0.09</td>
</tr>
</tbody>
</table>

Discussion

There are a number of novel findings in this study. First, we measured in vivo a quantitative imaging metric (MTR) in the outer spinal cord—a region likely to include the pia mater and subpial spinal cord. Second, in healthy control subjects, the outer cord MTR was higher than cord white matter MTR. Third, there was a reduction in outer cord MTR at an early stage of multiple sclerosis, and in the absence of significant cord atrophy, as reflected by the findings in clinically isolated syndrome and relapsing-remitting multiple sclerosis subgroups. Fourth, outer cord MTR abnormality was significantly and independently correlated with cord atrophy and was also greater in progressive multiple sclerosis subgroups when compared with relapsing-remitting multiple sclerosis. Finally, there were several independent associations between spinal cord MRI metrics and disability measures. These findings are discussed in turn.

The outer spinal cord magnetization transfer ratio measure and what it reflects anatomically

We quantified MTR in a voxel layer in the expected location of the pia mater and subpial region of the spinal cord. As the spinal cord is surrounded by CSF the outer voxels of the cord are susceptible to partial volume effects (Tench et al., 2005). However, by excluding the most peripheral voxel layers we are confident that this influence was negligible as the outer cord MTR values were higher than the remaining (deeper) spinal cord tissues, which will not be affected by CSF.

The pia mater is composed of two layers in the spinal cord; the intima pia and epipial layer, of which the epipial layer (not present over the cerebral hemispheres) is the thicker of the two due to its composition of connective tissue (Millen and Woollam, 1961). The combined thickness of these layers was 0.2 mm in a post-mortem study of the thoracic spinal cord with an increase in higher segments of the cord (Reina et al., 2004). The cord samples in this study were fixed with formaldehyde, and a reduction in tissue size of up to 19% can occur using a 10% formalin solution (Mouritzen Dam, 1979). It therefore seems likely that a significant proportion of the outer cord voxel layer of the upper cervical cord region that we studied (which has an in-plane resolution of 0.5 mm) will contain pial meningeal tissue, although it is also likely to include subpial spinal cord white matter.

Higher outer than white matter spinal cord magnetization transfer ratio in healthy control subjects

A question arising is why the MTR in the outer cord region should be higher than that of normally myelinated cord white matter. One possible explanation is that there is a tissue component other than myelin in the outer cord that has a relatively high MTR. In this regard, both the pial and subpial tissues in the spinal cord contain collagenous fibres (Reina et al., 2004) and in vitro data obtained in phantoms have shown that higher MTR is correlated with higher collagen concentration (Laurent et al., 2001). It seems therefore possible that collagen contributes to the higher outer cord MTR.

Outer spinal cord magnetization transfer ratio abnormalities in clinically isolated syndrome and relapsing-remitting multiple sclerosis in the absence of cord atrophy

The finding of reduced outer cord MTR without atrophy in the clinically isolated syndrome and relapsing-remitting multiple sclerosis groups supports the robustness of the outer cord measure: had partial volume effects of CSF caused a decrease in outer cord MTR, an abnormality would not be seen when there was no difference in cord area between patient and control groups.

In the clinically isolated syndrome cohort, 15 fulfilled the criteria for dissemination in space and 18 of 22 participants had at least one asymptomatic T2-weighted brain lesion evident on their brain
MRI scan and are therefore at higher risk of conversion to multiple sclerosis (Fisniku et al., 2008). Seven of this group had asymptomatic spinal cord lesions, the presence of which also increases the risk for conversion to multiple sclerosis (Sombekke et al., 2013). The abnormalities detected in the outermost spinal cord in clinically isolated syndrome compared to control subjects indicates that changes occur at a very early stage in relapse-onset multiple sclerosis.

The relapsing-remitting multiple sclerosis cohort were also at a relatively early stage of disease, had little disability and no cord atrophy. As cord atrophy is related to axonal loss in neuropathology studies (McGavern et al., 2000; DeLuca et al., 2004; Evangelou et al., 2005), the decrease in MTR in the outer spinal cord seen at this stage of multiple sclerosis may be occurring in the absence of—and by implication preceding—significant axonal loss.

The pathological basis of the reduced outer cord MTR warrants further consideration. In an inflammatory animal model of multiple sclerosis (experimental allergic encephalomyelitis) areas of oedema, signifying inflammation, exhibited a mildly decreased MTR in the absence of demyelination (Dousset et al., 1992). A decrease in MTR also occurs with inflammation in spinal cord experimental allergic encephalomyelitis (Cook et al., 2004). A post-mortem study in multiple sclerosis has demonstrated reduced MTR corresponding with an increased number of inflammatory T cells (Moll et al., 2009). Other post-mortem studies in multiple sclerosis also report a reduction in MTR in regions of demyelination in the cerebral cortex (Schmierer et al., 2010; Chen et al., 2013), brain white matter (Schmierer et al., 2004) and spinal cord (Bot et al., 2004). As the outer spinal cord voxel layer in this study is likely to contain both the pia mater and subpial white matter tissue, a combination of inflammation in the former and demyelination in the latter may be responsible for the decrease seen in multiple sclerosis.

Although the limits of image resolution prevent a more specific interpretation of the MTR decrease that we saw in outer spinal cord region, it may nevertheless reflect a distinct pathogenic process in so far as co-localized subpial demyelination may occur secondary to meningeal inflammation. These pathological changes have been previously associated in brain biopsies of patients with early multiple sclerosis, where meningeal inflammation was found to have a 90% probability to be topographically associated with subpial demyelination (Lucchinetti et al., 2011). Further pathological studies will be needed to determine whether such changes are topographically related in the spinal cord.

Association of outer spinal cord magnetization transfer ratio with cord atrophy and progressive multiple sclerosis

In this study, outer spinal cord MTR was independently associated with cord atrophy. In a previous study of inflammation in the spinal cord meninges there was an association seen between meningeal inflammation and diffuse axonal loss in the spinal cord parenchyma (Androdias et al., 2010). In a similar study by DeLuca et al. (2013) it was found that small peripheral axons are preferentially lost. In these studies inflammatory cells and mediators were demonstrated to be present in the cord meninges. Thus, in both of these pathology studies, meningeal inflammation and axonal loss were evident. It is likely that axonal loss, which can be profound in multiple sclerosis spinal cord (Ganter et al., 1999; Lovas et al., 2000), is the major substrate of spinal cord atrophy. Thus, the link we observed between outer cord MTR and cord atrophy would appear concordant with pathological association of meningeal inflammation and axonal loss in the cord. In contrast, the lack of independent association between the inner spinal cord (grey and white matter) MTR (implying demyelination) and atrophy (consistent with neuroaxonal loss) is consistent with a dissociation between these pathological processes, in line with previous pathological reports in the spinal cord (DeLuca et al., 2006) and brain (Wegner et al., 2006).

Although at present it is not known how meningeal inflammation might be associated with cord pathology, including axonal loss, a possible anatomical connection may be via the epipial layer of the spinal cord, which contains branches of blood vessels that penetrate the spinal cord (Millen and Woollam, 1961). Furthermore, spinal cord lesions tend to occur around small veins (Oppenheimer et al., 1978), and therefore the small epipial vessels could potentially provide a route of entry for inflammatory cells from the meninges into the cord parenchyma.

The comparison of clinical subgroups showed that greater outer cord MTR abnormality in both primary and secondary multiple sclerosis groups compared with relapsing-remitting multiple sclerosis (Tables 2 and 3). In the comparison of the two progressive subtypes of multiple sclerosis no significant differences were found. These results suggest that the outer spinal cord (and by implication pial and/or subpial) abnormalities are greater in the progressive stage of multiple sclerosis.

The findings are consistent with previous neuropathology studies in the brain that have linked both meningeal inflammation and subpial demyelination with the progressive stage of multiple sclerosis (Magliozzi et al., 2007). There can be extensive cortical subpial demyelination in progressive multiple sclerosis (Peterson et al., 2001; Bö et al., 2003; Kutzelnigg et al., 2005; Vercellino et al., 2005; Wenger et al., 2006). Although cortical subpial lesions are rarely visible on MRI (Geurts et al., 2005), post-mortem study has identified that regions of cortical demyelination have a reduced MTR (Chen et al., 2013), and a recent in vivo study reported a reduced MTR of the outer cortex in multiple sclerosis that was most marked in those with a secondary progressive course (Samson et al., 2014). These observations support use of MTR to reflect subpial demyelination in the cortex and suggest that subpial demyelination could contribute to the lower MTR we observed in the outermost region of the spinal cord.

Associations between spinal cord magnetic resonance imaging metrics and disability measures

Although outer spinal cord MTR had a univariate correlation with each disability measure used in this study, in a regression analysis it was found only to be associated with the 9-Hole Peg Test.
z-score. This limited independent association with function may reflect the small region of spinal cord included in the outer voxel layer; the purpose in studying the outer cord MTR was to investigate for abnormalities that reflect a process of pathogenic importance (i.e. meningeal and subpial pathology) and not for an association with disability.

Spinal cord white matter-MTR was independently associated with 25-foot Timed Walk Test and multiple sclerosis functional composite z-scores; this relationship plausibly reflects pathology in functionally important motor and sensory pathways of the spinal cord. Our results also confirm an earlier finding of a univariate correlation between spinal cord grey matter MTR and EDSS (Agosta et al., 2007), although grey matter MTR was not independently associated with EDSS in the subsequent regression analysis. Amongst the several MRI metrics studied, the measure of spinal cord cross-sectional area (atrophy) had a generally stronger univariate correlation with each of the disability measures used. Furthermore, cord area was found to be independently associated with a number of measures of disability. The strong relationship between cord atrophy and disability may be due to this measure reflecting axonal loss, thought to be the pathological substrate for disability (Evangetou et al., 2000; Lovas et al., 2000; Schirmer et al., 2011). A biologically coherent finding was that the PASAT z-score (a measure of cognition) was associated only with the two brain MRI metrics (brain parenchymal fraction and T2 lesion volume) and with none of the spinal cord measures.

**Limitations and future directions**

Limitations should be noted when considering the findings in this study. Firstly, we only studied the upper cervical spinal cord. However, at this level, a robust registration technique could be used and segmentation of the cord into grey matter and white matter regions was possible. Furthermore, spinal cord involvement by multiple sclerosis is most common in the cervical cord (Oppenheimer et al., 1978), and we believe that our approach was the most appropriate within the technical constraints of spinal cord MRI.

Second, this study was cross-sectional in nature and future longitudinal studies will be needed to elucidate outer spinal cord MTR changes over time and its relationship with evolution of disability and cord atrophy.

Third, the in-plane resolution of the axial images was constrained by time limitations necessitated in a clinical study. Future studies of the spinal cord at 7 T field strength (Zhao et al., 2013) may provide higher resolution images within an acceptable time frame.

Finally, our in vivo imaging study does not include MTR findings for histopathologically confirmed pia mater and subpial cord. To our knowledge no such findings have been published to date, either in ex vivo multiple sclerosis spinal cord or in animal models of the disease. A post-mortem MRI-histopathology correlation study is needed to consolidate the findings of our in vivo study.

In conclusion we have found MTR abnormalities in an area corresponding to the expected location of the pia mater and subpial region in the outer cervical spinal cord. We have found that these outer spinal cord abnormalities occur early in the course of multiple sclerosis before significant cord atrophy and that a greater reduction in MTR values is seen in progressive multiple sclerosis.

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**Supplementary material**

Supplementary material is available at Brain online.

**References**


