Brain intra- and extracellular sodium concentration in multiple sclerosis: a 7 T MRI study

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Intra-axonal accumulation of sodium ions is one of the key mechanisms of delayed neuro-axonal degeneration that contributes to disability accrual in multiple sclerosis. In vivo sodium magnetic resonance imaging studies have demonstrated an increase of brain total sodium concentration in patients with multiple sclerosis, especially in patients with greater disability. However, total sodium concentration is a weighted average of intra- and extra-cellular sodium concentration whose changes reflect different tissue pathophysiological processes. The in vivo, non-invasive measurement of intracellular sodium concentration is quite challenging and the few applications in patients with neurological diseases are limited to case reports and qualitative assessments. In the present study we provide first evidence of the feasibility of triple quantum filtered $^{23}$Na magnetic resonance imaging at 7 T, and provide in vivo quantification of global and regional brain intra- and extra-cellular sodium concentration in 19 relapsing-remitting multiple sclerosis patients and 17 healthy controls. Global grey matter and white matter total sodium concentration (respectively $P<0.05$ and $P<0.01$), and intracellular sodium concentration (both $P<0.001$) were higher while grey matter and white matter intracellular sodium volume fraction (indirect measure of extracellular sodium concentration) were lower (respectively $P=0.62$ and $P<0.001$) in patients compared with healthy controls. At a brain regional level, clusters of increased total sodium concentration and intracellular sodium concentration and decreased intracellular sodium volume fraction were found in several cortical, subcortical and white matter regions when patients were compared with healthy controls ($P<0.05$ family-wise error corrected for total sodium concentration, $P<0.05$ uncorrected for multiple comparisons for intracellular sodium concentration and intracellular sodium volume fraction). Measures of total sodium concentration and intracellular sodium volume fraction, but not measures of intracellular sodium concentration were correlated with T2-weighted and T1-weighted lesion volumes ($0.05<P<0.01$) and with Expanded Disability Status Scale ($P<0.05$). Thus, suggesting that while intracellular sodium volume fraction decrease could reflect expansion of extracellular space due to tissue loss, intracellular sodium concentration increase could reflect neuro-axonal metabolic dysfunction.

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Introduction

Multiple sclerosis is the most common cause of non-traumatic neurological disability in young adults and has a high socio-economic impact, which increases as disability progresses (Noseworthy et al., 2000). Although there is increasing evidence that neuro-axonal degeneration is a relevant cause of permanent disability (De Stefano et al., 1998; Miller et al., 2002; Bjartmar and Trapp, 2003; Frischer et al., 2009; Tallantyre et al., 2010; Filippi et al., 2013), the pathophysiological mechanisms underlying neuroaxonal injury or loss are poorly understood and there are no therapeutic agents with proven efficacy in preventing or slowing the progressive accumulation of disability. Several histological and experimental studies have suggested that the increase of sodium influx in demyelinated axons could be one of the key mechanisms of delayed axonal injury and that partial blockade of sodium channels protects axons from degeneration in experimental models of multiple sclerosis (Lo et al., 2003; Bechtold et al., 2006; Black et al., 2006, 2007; Waxman, 2008; Al-Izki et al., 2014).

Recent in vivo MRI studies using sodium ($^{23}$Na) imaging, have shown increased brain total sodium concentration (TSC) in patients with multiple sclerosis (Inglese et al., 2010; Zaarouei et al., 2012; Paling et al., 2013; Maarouf et al., 2014). Brain sodium concentration is increased both in lesions and normal-appearing brain tissue, especially in the advanced and progressive stages of the disease, and in patients with greater disability. TSC is a weighted average of intracellular sodium concentration (ISC ~10–15 mM) and extracellular sodium concentration (ESC ~140 mM) and its increase in multiple sclerosis can result from neuro-axonal metabolic dysfunction (Trapp and Stys, 2009), and/or from the expansion of the extracellular space secondary to neuro-axonal loss or presence of edema (Perier and Gregoire, 1965; Turski et al., 1986). However, TSC measurement does not allow discrimination between the two compartments and, therefore, the disease-related change in ISC and ESC and their relationship with structural MRI measures and clinical parameters is yet unknown.

The measurement of the MRI signal from ISC is quite challenging and can be performed in vivo with a few techniques that include the use of shift reagents, inversion recovery pulses, and multiple quantum filters. Multiple quantum filters are based on the different relaxation properties of the sodium nuclei in the extracellular and intracellular space, which generate a mono- and bi-exponential nuclear magnetic resonance signal decay, allowing the differentiation of the signal from the two compartments (Muller et al., 1987; Borthakur et al., 1999; Hancu et al., 1999). Despite its importance, in vivo measurement of ISC in humans has been hampered by technical challenges and the few examples of the application of multiple quantum filters ($^{23}$Na MRI to the human brain available in the literature are limited to case reports and qualitative results (Hancu et al., 1999; Boada et al., 2004).

Our group has recently implemented a magnetic resonance pulse sequence for the acquisition of triple-quantum filtered (TQF) $^{23}$Na MRI (Fleysher et al., 2010) and developed a non-invasive method that uses single quantum (SQ) and TQF imaging at 7 T to quantify ISC and intracellular sodium volume fraction (ISVF), an indirect measure of ESC. As the brain–sodium model considers the cell membrane a part of intracellular space, the term ISVF is synonymous of cell volume fraction (cell volume divided by tissue volume). Therefore, an ISVF reduction indicates loss of the intracellular volume and reflects an increase of the extracellular space and, as a consequence, of ESC (Fleysher et al., 2013). We obtained quantitative mapping of ISC and ISVF maps in healthy volunteers, with ISC and ISVF values in good agreement with those obtained with invasive methods and/or ex vivo studies (Fleysher et al., 2013). Ultra-high field MRI is particularly suited for the application of this method, due to the relatively low sensitivity of $^{23}$Na MRI.

The present study is the first to investigate the feasibility of ISC and ISVF measures in patients with multiple sclerosis. We sought to explore the hypothesis that while a decrease in ISVF would be associated with tissue loss as measured by conventional MRI techniques, an increase in ISC would not be associated reflecting axonal metabolic dysfunction rather than tissue destruction. Therefore, the aims of our study were to: (i) determine the feasibility of TQF $^{23}$Na MRI in patients with multiple sclerosis at 7 T; (ii) measure the global and regional brain distribution of TSC, ISC and ISVF; (iii) investigate the relationship between intra- and extracellular sodium concentration and measures of lesion and brain volume; and (iv) evaluate the clinical impact of abnormal brain sodium distribution.
Materials and methods

Subjects

Nineteen patients diagnosed with multiple sclerosis according to the McDonald criteria (McDonald et al., 2001) and presenting a relapsing-remitting (Lublin and Reingold, 1996) course were prospectively enrolled in the present study. The exclusion criteria were: (i) a current or past medical or psychiatric disorder other than multiple sclerosis; (ii) current or past substance abuse; and/or (iii) multiple sclerosis relapse or corticosteroid use in the previous 6 weeks. Disability was assessed by a single, experienced neurologist who was blind to the MRI findings, using the Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983) within 1 week of MRI. All the patients were under immunomodulatory treatment with either interferon beta-1a or glatiramer acetate. Seventeen age- and gender-matched healthy volunteers were enrolled as control group. Demographic characteristics of the study populations are presented in Table 1. Approval for this study was obtained from the local Institutional Board of Research Associates, and informed consent was obtained from all subjects before study initiation.

MRI acquisition

All subjects underwent 1H-MRI at 3 T (Siemens Medical Solutions) and SQ and TQF 23Na MRI at 7 T (MAGNETOM, Siemens Healthcare) in two separate sessions on the same day of their clinical assessment. The 3 T 1H-MRI protocol included the following sequences: (i) dual-echo turbo spin echo (repetition time = 5000 ms, echo time = 11 ms, 48 contiguous 3-mm thick axial slices); (ii) 3D T1-weighted magnetization-prepared rapid-acquisition gradient echo (repetition time = 150 ms, echo time = 2.71 ms, inversion time = 900 ms, flip angle = 12°, voxel size = 5 × 5 × 5 mm3); TQF 23Na MRI was acquired using a modified 3D gradient-echo sequence with a new 12-step phase-cycling B0-corrected TQF scheme (repetition time = 150 ms, echo time = 6.8 ms, flip angle = 90°, field of view = 240 × 240 × 240 mm3, matrix 30 × 30 × 24, voxel size 8 × 8 × 10 mm3, r1 = 6.8 ms r2 = 15 ms, two averages) (Fleysher et al., 2013). For the purposes of TSC quantification, calibration phantoms with known sodium concentrations (50 and 100 mM/l) were placed into the field of view. For TQF B0 correction, B0 maps were computed from the phase difference between two SQ images acquired over 3.6 min (repetition time = 150 ms) with echo times = 6.8 ms and 8.8 ms, respectively. The SQ image with echo time = 6.8 ms was used for TSC calculation. B1 maps were computed from the ratio of two additional SQ images acquired over 3.6 min with fractional anisotropy = 60° and 120°, repetition time = 150 ms using the double flip angle method. Using the B1 maps, B1 correction was applied to SQ and TQF images (Fleysher et al., 2013).

Image analysis

Image processing was performed off-line on a PC workstation. All images were assessed by consensus by two experienced observers who were blind to the patients’ identity and clinical status.

Lesion volume assessment

T2-hyperintense and T1-hypointense white matter lesions were identified and outlined, for each patient, respectively on the dual echo and T1-weighted images, using a semi-automated technique based on user-supervised local thresholding (Jim version 6; Xinapse Systems, http://www.xinapse.com).

Lesion probability map assessment

For the patients group, lesion probability maps were obtained using imaging analysis tools of the FMRIB Software Library (FSL 5 (www.fmrib.ox.ac.uk/fsl) as described in Rossi et al. (2012). Briefly, the procedure consisted of three steps: brain extraction, registration to standard-space and computation of the maps. First, the brain was extracted from the 3D T1-weighted and T2-weighted images using the brain extraction tool and then corrected for intensity non-uniformity (Smith, 2002). Next, a two-stage registration was performed to align the T2 lesion masks of each patient to the Montreal Neurological Imaging (MNI) 152 standard brain: (i) each lesion mask was linearly co-registered to the corresponding 3D T1-weighted brain images with FMRIB’s Linear Image Registration Tool using the transformation parameters derived by linearly registering the T2-weighted on the 3D T1-weighted image; (ii) each lesion mask previously registered on the 3D T1-weighted brain image was non-linearly registered on the standard brain template using the transformation parameters derived by non-linearly registering the 3D T1-weighted image on the standard brain template. Finally, the lesion probability maps were generated by first merging and then averaging all the standard-space lesion masks. For each map, voxel intensity

Table 1 Demographics characteristics and structural 1H-MRI parameters in patients with multiple sclerosis and in healthy control subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Multiple sclerosis</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender M:F</td>
<td>11:8</td>
<td>8:9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40.06 ± 11.27</td>
<td>46.16 ± 11.65</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>9.11 ± 7.48</td>
<td>–</td>
</tr>
<tr>
<td>Median EDSS (range)</td>
<td>2.0 (0.0–5.5)</td>
<td>–</td>
</tr>
<tr>
<td>T2 lesion volume (ml)</td>
<td>6.61 ± 8.93</td>
<td>–</td>
</tr>
<tr>
<td>T1 lesion volume (ml)</td>
<td>1.79 ± 4.84</td>
<td>–</td>
</tr>
<tr>
<td>NBV (ml)</td>
<td>1415 ± 153.84***</td>
<td>1523.24 ± 57.96</td>
</tr>
<tr>
<td>GMV (ml)</td>
<td>744.47 ± 91.13*</td>
<td>793.76 ± 55.65</td>
</tr>
<tr>
<td>WMV (ml)</td>
<td>868.68 ± 76.46</td>
<td>735.59 ± 35.96</td>
</tr>
</tbody>
</table>

EDSS = Expanded Disability Status Scale; GMV = grey matter volume; NBV = normalized brain volume; WMV = white matter volume.

**P < 0.05; ***P < 0.01.
represents the frequency of lesion occurrence in that voxel. A threshold of 50% was used to include the peaks of the lesion frequency on the lesion probability maps.

### Brain volume assessment

Normalized brain volume, grey matter and white matter volumes were computed for all subjects on the 3D $T_1$-weighted sequence using FSL’s SIENAX program (SIENAX; FMRIB Centre, Oxford, UK) as described elsewhere (Battaglini et al., 2012). To avoid tissue misclassification, $T_2$-weighted lesions were refilled with intensities matching the surrounding normal-appearing white matter (Battaglini et al., 2012).

### Sodium imaging post-processing

The computation of Na maps is based on the canonical brain–sodium tissue model (Thulborn et al., 1999; Ouwerkerk et al., 2003), which assumes that sodium is distributed between only two compartments: intracellular and extracellular (see Supplemental material for further details).

As previously described (Fleysher et al., 2013), ISC and ISVF were quantified as follows. First, SQ and TQF images were acquired and corrected for B0 and B1 inhomogeneities as described in Fleysher et al. (2010). Second, TSC maps were quantified in Image J v.1.36b on a voxel-by-voxel basis from the SQ images using a linear method, dependent on the calibration phantom, as described by Inglese et al. (2010). Third, single-quantum and triple-quantum images were combined with TSC maps using an in-house procedure developed in Matlab (Fleysher et al., 2013) and ISC and ISVF maps were computed.

The resulting concentration maps (Fig. 1) were further analysed with a global approach to measure TSC, ISC and ISVF over the entire grey and white matter tissue and with a voxel-based approach to measure TSC, ISC and ISVF at a regional level.

Test–retest variance evaluation, conducted on three patients and three controls imaged on two separate occasions, at baseline and after 1 month (range 25 to 37 days), showed a coefficient of variation smaller than 5% for ISVF measures, ranging from 6% to 5% for TSC measures and from 10% to 6% for ISC measures.

### Global analysis of sodium concentration

The analysis of the $^{23}$Na concentration maps (i.e. TSC, ISC and ISVF maps) was performed with FSL 5 (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki). The TSC maps were co-registered to the corresponding $T_1$-weighted images with an affine-linear registration and the transformation matrices were saved. $T_2$-weighted images were segmented and the obtained grey and white matter masks were superimposed onto $^{23}$Na concentration maps in native $^{23}$Na-image space using the inverse transformation matrix obtained in the step above; mean values from each tissue were extracted for each subject, obtaining grey matter, white matter and cerebrospinal fluid TSC, ISC and ISVF values. A probability of 50% was considered as threshold for grey and white matter tissue type classification due to the bigger voxel sizes and hence higher mixture of tissue type.

### Sodium concentrations in $T_2$- and $T_1$-weighted lesions

Because of the limited spatial resolution of ISVF and ISC scans, only TSC was measured in $T_2$- and $T_1$-visible lesions. The assessment of sodium concentrations was performed with FSL 5 (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki). First, the TSC maps were co-registered to the $T_2$-weighted and 3D $T_1$-weighted images with an affine linear registration using correlation ratio and trilinear interpolation. The generated inverse transformation matrix was applied to the lesions masks (including only lesions with a diameter equal or higher than 5 mm) in order to transfer them from the structural images space into the sodium native space. The $T_2$- and $T_1$-weighted lesions masks in sodium space were therefore superimposed on $^{23}$Na concentration maps, obtaining mean TSC values in white matter lesions.

### Voxel-based analysis of sodium concentration

The voxel-based processing on $^{23}$Na concentration maps (i.e. TSC, ISC and ISVF maps) was performed with SPM8 (Welcome Institute, London, UK). For each patient, the $^{23}$Na concentration maps were co-registered to the 3D $T_1$-weighted image using normalized mutual information and trilinear interpolation. The 3D $T_1$-weighted scans were spatially normalized into the MNI space (VBM8); the transformation matrix between the native image space and the normalized stereotaxic MNI space with affine transformation and nonlinear warping generated during this process was applied to the co-registered $^{23}$Na image maps, to reduce inter-individual differences. The partial volume effect was minimized by subtracting the cerebrospinal fluid segmented maps from each of the $^{23}$Na concentration maps. Finally, the obtained quantitative $^{23}$Na maps were smoothed with an 8-mm full width at half maximum Gaussian kernel for statistical mapping analysis. The Talairach Daemon Atlas from Wake Forest University Pickatlas toolbox from SPM8 (http://www.nitrc.org/projects/wfu_pickatlas) was used for grey matter labeling, whereas the JHU white matter tractography atlas from FSL (http://www.fmrib.ox.ac.uk/fsl/data/atlases-descriptions.html#wm) was used for white matter labeling.

### Statistical analysis

SPSS version 20.0 (IBM) was used for all statistical computations in terms of structural and global sodium parameters. Between-group comparisons of structural MRI parameters (brain volumes and lesion volumes) were assessed with an ANCOVA test, controlling for age and gender ($P < 0.05$). Between-group comparisons of global $^{23}$Na concentrations (grey matter and white matter TSC, ISC and ISVF) were assessed with an ANCOVA test, controlling for age, gender and intra-cranial volume ($P < 0.05$). Within-group comparisons of global $^{23}$Na concentrations (grey matter and white matter TSC, ISC and ISVF) were assessed with a paired sample t-test ($P < 0.05$).

Pearson bivariate correlation was applied to evaluate the association between sodium concentrations, age and disease duration.

A voxel-based statistical mapping analysis (SPM8) was used to assess regional brain differences in sodium concentration on the TSC compartment, using the spatially normalized TSC maps, and a two-groups ANCOVA test controlling for age,
gender and intracranial volume ($P < 0.001$, corrected for family-wise error at $P < 0.05$, cluster extent 20 voxels). Then, the significant TSC clusters were extracted with MarsBaR toolbox (http://marsbar.sourceforge.net/) and used to restrict the ISC and ISVF voxel-based statistical mapping analysis, which was performed with a two-group ANOVA ($P < 0.05$, cluster extent 10 voxels, uncorrected for multiple comparisons).

Partial correlations were assessed between global and regional $^{23}$Na concentration, lesion and brain volumes, and clinical data (disease duration, EDSS score) controlling for age and gender ($P < 0.05$).

Because of the exploratory nature of this study, multiple testing correction was not performed and therefore the reported $P$-values should be interpreted as descriptive. However, all analyses were performed with established a

Figure 1 $^{23}$Na group maps. Mean TSC, ISC and ISVF maps for patients with multiple sclerosis (A, C and E, respectively) and controls (B, D and F, respectively). In both groups, TSC appears higher while ISC and ISVF are lower in grey matter than in white matter. Note that, as intracellular molar content and cell volume are equal to zero in extracellular tissue, ISVF and ISC measurements in CSF have to be considered meaningless.
priori hypotheses. Correction for family wise error was only applied to the whole brain voxel wise comparison of TSC maps to avoid a massive multiple comparison penalty.

Results

Structural imaging

T2-weighted, T1-weighted lesion volumes and brain volumes are shown in Table 1. The anatomical distribution of T2 lesions across the brain is shown in Fig. 2, superimposed on the statistical parametric maps of brain sodium concentrations in multiple sclerosis patients. The peak of lesion frequency was localized in the right anterior thalamic radiation (39%). None of the patients presented Gd-enhancing lesions. Normalized brain volume, grey matter volume and white matter volume were decreased in multiple sclerosis patients compared to healthy controls (P < 0.01, P < 0.05 and P = 0.05, respectively).

Global analysis of grey and white matter sodium concentration

Global grey matter and white matter sodium concentrations for patients and controls are shown in Table 2 and Fig. 3. In both groups, TSC was higher, and ISVF lower in grey matter than in white matter (P < 0.05) while there was no difference in terms of ISC (P > 0.1). TSC was higher in T1 lesions than in T2 lesions (respectively, 52.27 ± 23.9 and 37.54 ± 12.18 mM; P < 0.015) and both TSC in T2 lesions and T1 lesions were higher than in white matter (respectively, 37.54 ± 12.18 versus 31.38 ± 4.03 mM, P < 0.05 and 52.27 ± 23.9 versus 31.38 ± 4.03 mM, P < 0.01).

Compared to healthy controls, multiple sclerosis patients showed higher global grey matter and white matter TSC and ISC (respectively P < 0.05 and P < 0.01 for TSC; P < 0.001 for ISC) and lower global grey matter and white matter ISVF (respectively, P = 0.62 and P < 0.001).

Regional analysis of grey and white matter sodium concentration (TSC, ISC and ISVF)

Between-groups statistical mapping analyses performed on sodium maps are displayed in Fig. 2.

The voxel-based analysis of grey matter showed clusters of increased TSC values in bilateral thalamus, left caudate, right anterior cingulate gyrus (BA25 and BA32), left middle frontal gyrus (BA10), right precentral gyrus (BA6), right postcental gyrus (BA40), (P < 0.001 Ke = 20, family wise error corrected P < 0.05) (Supplementary Table 1). A voxel by voxel comparison of ISVF and ISC values within significant grey matter TSC clusters, showed lower ISVF values in portions of bilateral thalamus, right anterior cingulate gyrus (BA25 and BA32), left middle frontal gyrus (BA10), right precentral gyrus (BA6) (P < 0.05, Ke = 10, uncorrected for multiple comparisons) (Supplementary Table 2) and higher ISC values in bilateral thalamus, left middle frontal gyrus (BA10), right precentral gyrus (BA6) (P < 0.05, Ke = 10, uncorrected for multiple comparisons) (Supplementary Table 3).

The voxel-based analysis of white matter showed clusters of increased TSC values in bilateral cortico-spinal tract, bilateral anterior thalamic radiation, corpus callosum, bilateral inferior fronto-occipital fasciculus, forceps minor, uncinate fasciculus (P < 0.001 Ke = 20, family wise error P < 0.05) (Supplementary Table 1). A voxel by voxel comparison of ISVF and ISC values within significant white matter TSC clusters, showed lower ISVF values in the bilateral corticospinal tract and forceps minor (P < 0.05, Ke = 10, uncorrected for multiple comparisons) (Supplementary Table 2) and higher ISC values in left cortico-spinal tract and forceps minor (P < 0.05, Ke = 10, uncorrected for multiple comparisons) (Supplementary Table 3).

In the restricted analysis of ISC and ISVF maps in TSC significant clusters, no cluster survived the family-wise error correction (because 133 independent voxels comparisons were tested, an adjusted significance level of 0.0004 would have granted significant results at P < 0.05 after multiple comparisons); therefore, for ISC and ISVF we report uncorrected results.

No clusters of decreased TSC were identified in patients with multiple sclerosis.

Correlations among sodium concentrations, lesion and brain volumes and clinical parameters

T2 lesion volume was associated with TSC in corticospinal tract, right anterior cingulate gyrus and right precentral gyrus (r ranging from 0.47 to 0.71; 0.05 < P < 0.01); and with ISVF in right postcentral gyrus (r = −0.59, P < 0.01) and showed a trend of correlation with ISVF in corticospinal tract (r = −0.46, P = 0.06).

T1 lesion volume was associated with global grey matter ISVF (r = −0.53 P < 0.05) and with TSC in corticospinal tract, right precentral gyrus, right anterior cingulate gyrus and thalamus (r ranging from 0.50 to 0.63, 0.05 < P < 0.01); and with ISVF in corticospinal tract and anterior cingulate gyrus (respectively, r = −0.55, P < 0.05 and r = −0.70, P < 0.01).

There was no association between global and regional TSC, ISC and ISVF and measures of brain volumes except for a trend between white matter ISVF and normalized grey matter volume (r = 0.42, P = 0.08).

While in healthy controls age was correlated with white matter (r = 0.5, P < 0.05) and grey matter (r = 0.73, P < 0.01) TSC and with grey matter ISC (r = −0.50, P < 0.05), in patients age was inversely correlated with
white matter \((r = -0.68, P < 0.01)\) and grey matter \((r = -0.60, P < 0.01)\) ISC but not with ISVF and TSC values \((P > 0.1)\). Disease duration was associated with white matter TSC \((r = 0.63, P < 0.01)\), white matter ISC \((r = -0.67, P < 0.01)\) and grey matter ISC \((r = -0.57, P = 0.01)\).

Finally, an association was detected between global grey matter ISVF and EDSS \((r = -0.47, P < 0.05)\).

### Discussion

The present study confirms previous findings of increased TSC in grey matter, white matter and focal white matter lesions (Inglese et al., 2010; Zaaraoui et al., 2012; Paling et al., 2013; Maarouf et al., 2014) and provides first evidence for non-invasive brain intra- and extracellular sodium quantification in patients with multiple sclerosis.

Although TSC holds promise as a clinically meaningful marker of tissue injury, it does not allow one to discern between the ISC concentration that reflects metabolic cellular dysfunction and/or plasticity, and the ESC that reflects expansion of the extracellular space secondary to irreversible cellular damage or oedema. Herein, we used a newly developed, non-invasive method based on the combined use
Figure 3  Global grey and white matter sodium concentrations for patients and controls. Box plots displaying the 25% to 75% values (boxes) ± 95% values (whiskers), median values (horizontal lines within boxes) of mean TSC (A), ISC (B) and ISVF (C) value distribution in grey matter and white matter among healthy controls (empty box) and patients with relapsing-remitting multiple sclerosis (hatched box). GM = grey matter; WM = white matter.
of SQ and TQF $^{23}$Na to measure ISC and ESC in patients with multiple sclerosis (Fleysher et al., 2010, 2013).

There has been a significant effort over the years, to optimize the acquisition scheme and the reconstruction technique of TQF $^{23}$Na imaging and improve signal to noise ratio, spatial resolution and acquisition time (Stobbe and Beaulieu, 2005; Tanase and Boada, 2005; Fleysher et al., 2010, 2013; Matthies et al., 2010, 2013; Madelin et al., 2012, 2015; Tsang et al., 2012, 2015). To date, the application of TQF $^{23}$Na imaging in humans has been limited to a few case reports (Boada et al., 2004; Fiege et al., 2013) of patients with brain tumours that have suggested a possible role for TQF $^{23}$Na MRI to discriminate cell proliferation (tumour recurrence) from areas of tumour necrosis and oedema (Boada et al., 2004; Fiege et al., 2013). Although promising, these approaches were limited to a qualitative assessment of the pathologic tissue.

To overcome this limitation, our method combined SQ and TQF $^{23}$Na MRI, to quantify TSC and the intracellular sodium molar fraction (ISMF) and then derive ISC and the ISVF, an indirect measure of ESC (the lower the ISVF, the higher the ESC) (Fleysher et al., 2013). A previous application of this method in healthy volunteers resulted in brain ISC values between 10–15 mmol/l and an ISVF values between 85–95%, in line with previous theoretical predictions and experimental works (Fleysher et al., 2013).

**Global and regional white matter TSC, ISC and ISVF**

Global white matter TSC and ISC values were higher and ISVF values lower in patients than controls supporting the concept that TSC is not only the mere consequence of the expansion of the extracellular space secondary to demyelination and neuroaxonal loss. Similar results were found at a regional level where the voxel based analysis showed not only a quite widespread ISVF decrease within the white matter clusters of increased TSC, but also a fewer clusters of ISC increase.

As none of our patients had Gd-enhancing lesions, the decrease of ISVF (i.e. ESC increase) is likely to reflect tissue disruption, demyelination and axonal loss not only within lesions but also, to a lesser extent, within normal-appearing white matter. Due to the difference in spatial resolution between the $T_2$-weighted images and the TQF $^{23}$Na images we decided to analyse the global white matter rather than normal-appearing white matter. However, as reported in previous $^{23}$Na MRI studies of patients with multiple sclerosis (Inglese et al., 2010; Zaaraoui et al., 2012; Paling et al., 2013; Maarouf et al., 2014) and as visualized in Fig. 2, TSC, ISC and ISVF are altered not only in white matter areas occupied by lesions but also in white matter areas outside clusters of lesions. The lack of complete correspondence between $T_2$ lesion map and sites of $^{23}$Na increases on SPM maps can be explained by the low resolution of sodium maps. The averaging of lesional and non-lesional sodium concentration in each voxel most likely prevented the voxel wise statistical analysis from identifying increased sodium concentration specifically within white matter lesions.

Several factors may explain the increase of ISC. First of all, the upregulation of voltage-gated sodium channels along demyelinated axons in lesions and normal appearing white matter leads to restoration of conduction at the cost of an increased influx of intraxonal sodium ions (Waxman, 2006). Second, the disease-related mitochondrial dysfunction can lead to a reduction in energy supply and to the failure of ATP-dependent pumps (Dutta et al., 2006; Trapp and Stys, 2009) that further increase the intraxonal sodium concentration. Third, the intraxonal influx of sodium ions can occur through persistent sodium channels (Stys et al., 1993; Taylor, 1993) and glutamate receptors (Ouardouz et al., 2006) contributing to the intracellular accumulation and to the dysfunction of the Na/Ca exchanger. Finally, the well-established upregulation of sodium channels in activated microglia, macrophages and reactive astrocytes within active and chronic multiple sclerosis lesions (Craner et al., 2005; Black et al., 2010) is likely to contribute to the measured increase of ISC. All together, these pathophysiological processes can either lead to neuroaxonal death through the increase of intracellular calcium, the activation of calcium dependent proteases, and glutamate-mediated cytotoxicity (Trapp and Stys, 2009) or to a spontaneous reverse of metabolic dysfunction into a physiological condition (Nikic et al., 2011). Although it remains only a speculation due to the cross-sectional design of our study and the lack of confirmatory post-mortem data, we believe that the ISC increase in our patients with relapsing-remitting multiple sclerosis reflects neuroaxonal metabolic dysfunction rather than loss. This is supported by several findings of our study: (i) the association of conventional MRI measures of tissue destruction such as $T_1$ lesion volume and normalized grey matter volume with the decrease of ISVF but not with the increase of ISC; (ii) the correlation between the EDSS score and measures of ISVF decrease but not ISC increase; (iii) the presence of an inverse relationship of disease duration with ISC and a direct relationship with TSC and ISVF suggesting that while TSC and ISVF are expression of structural damage, which increases as the disease progresses, ISC increase could reflect a compensatory mechanism, more active in the initial stage of the disease and becoming less efficient as the disease progresses. Hence, ISC might provide information about brain areas that are metabolically dysfunctional but still able of functional compensatory mechanisms preventing structural alterations.

**Global and regional grey matter TSC, ISC and ISVF**

Global grey matter TSC and ISC values were higher in patients than controls whereas the difference in ISVF did
not reach a statistical relevant difference although the values were lower in multiple sclerosis patients. Similar to the white matter regional analysis, a decrease in ISVF was detected in almost all the cortical and deep grey matter regions that showed a TSC increase, while ISC increase was detected only in a few areas.

Our findings about the regional brain distribution of increased TSC are in agreement with those of a previous study in relapsing-remitting multiple sclerosis (Zaaraaoui et al., 2012), which showed increased TSC in areas involved in locomotor function (left caudate, right pre-motor cortex) as well as areas involved in high cognitive functions, speech, stimuli integration and emotion (thalamus, cingulate cortex, bilateral prefrontal cortex, right postcentral gyrus). The presence of cortical grey matter lesions may explain changes in both ISVF and ISC. Cortical lesions are a very frequent finding at histological examination of brain samples (Kutzelnigg et al., 2005) and a quite common finding in patients with multiple sclerosis when non-conventional MRI sequences such as double inversion and phase sensitive inversion recovery are used. Neuronal loss can be very extensive in cortical lesions and can lead to the increased TSC found in the grey matter of our patients. However, in patients with relapsing-remitting multiple sclerosis cortical lesions are less frequent than in patients with progressive multiple sclerosis and they are characterized by demyelination rather than axonal loss (Kutzelnigg et al., 2005). Thus, explaining the relevant increase of ISC in presence of a less conspicuous change in global ISVF.

Nevertheless, ISVF was decreased in grey matter clusters of increased TSC suggesting that, in addition to cortical lesions, other mechanisms can lead to neuroaxonal loss in the cortex. Indeed, it has been shown that grey matter damage occurs as a consequence of trans-synaptic degeneration of axons transected in distant white matter lesions and/or secondary to the diffuse microscopic damage occurring in normal-appearing white matter (Sailer et al., 2003; Bodini et al., 2009). Pro-inflammatory and cytotoxic mediators derived from the meninges may also have a direct pathological effect on normal-appearing, non-demyelinated grey matter as suggested by recent post-mortem and in vivo PK11195 PET studies (Maglizotti et al., 2010; Politis et al., 2012).

In addition to the redistribution of sodium channels in demyelinated cortical lesions, cortical mitochondrial dysfunction can contribute to the increase of intracellular sodium. Campbell and colleagues have demonstrated the presence of respiratory-deficient neurons with multiple mitochondrial DNA deletions or absence of catalytic subunits of complex IV in layer VI of the cortex of brain samples from patients with multiple sclerosis (Campbell et al., 2011).

Our findings in the grey matter should be interpreted with caution due to the spatial resolution of our triple-filtered $^{23}$Na scans. We tried to minimize partial volume effect by using a 0.5 probability (rather than the more commonly used 0.75) as a threshold for grey and white matter tissue type classification. However, we cannot rule out that the inclusion of voxels containing in part CSF and white matter may have biased the grey matter measures. Future improvement in MRI acquisition techniques and coil design will lead to smaller voxel sizes and more accurate measurements.

Several limitations have to be considered when interpreting our results. First, we focused on patients with relapsing-remitting multiple sclerosis and on a cross-sectional design study to determine the feasibility of our method; future longitudinal studies, designed to follow over time patients with different clinical subtypes will allow us to extend our investigation on the clinical impact of sodium concentration increase in the transitional stage between relapsing and progressive phases of the disease. Second, due to the inherently low signal of TQF $^{23}$Na, we selected larger voxel size that precluded a reliable assessment of ISVF and ISC within white matter lesions. Future implementations of our technique and the use of multichannel receive arrays for sodium imaging will improve image signal-to-noise ratio and will allow the voxel size to be reduced, thus improving the accuracy and precision of concentration quantification in white matter lesions.

Third, the application of our model in multiple sclerosis is based on the assumption that the pathology itself does not determine any substantial, persistent, alteration of $^{23}$Na relaxation times. Nonetheless, the specific effect of micro-structural alterations on $^{23}$Na relaxation times has not been ruled out and the possibility of protein induced alteration of $^{23}$Na relaxation times cannot be excluded (Bansal et al., 2000).

Fourth, as this was an exploratory study, correction for multiple comparisons was not performed; however, all analyses were performed with established a priori hypotheses. Correction for family-wise error was only applied to the whole brain voxel wise comparison of TSC maps to avoid a massive multiple comparison problem.

Finally, even if a correction for B1 inhomogeneity was applied, we cannot exclude the presence of residual radio-frequency field artefacts. In addition, possible contribution to TQF signal from the extracellular sodium could have biased the results of the measurement. Although the intracellular origin of the TQF signal is supported by studies in experimental models (Winter and Bansal, 2001), it is not possible to clarify the magnitude of the bias, if any, based on the tissue model and the acquisition used in this work. We believe, however, that as ISC and ISVF values obtained in our study (Fleysher et al., 2013) were within the expected physiological range, the possible biases remained small. Future studies comparing ours with new models and acquisition schemes (Stobbe and Beaulieu, 2005; Madelin et al., 2014) will help clarify this issue.

Although preliminary, our findings demonstrate that TQF $^{23}$Na MRI is feasible in patients with multiple sclerosis and that ISC and ISVF values can complement TSC
measures by providing information about different pathophysiological aspects of multiple sclerosis. ISVF values reflect expansion of the extracellular volume related to cellular loss and development of tissue atrophy; ISC values reflect changes in cellular metabolism related to mitochondrial and ion channels dysfunction. Abnormal cellular metabolism and ion dyshomeostasis can either lead to cellular death or can reverse to physiological conditions. Hence, ISC may be a potential tool for the selection of patients with brain neuroplastic reserve and repair capability who may benefit from preventive neuroprotective treatments before the occurrence of structural damage (Kapoor, 2006; Nikic et al., 2011; Arun et al., 2013). Future improvements in $^{23}$Na MRI acquisition (Matthies et al., 2010), reconstruction algorithms and coil design as well as the employment of alternative approaches (Madelin et al., 2014) are needed to understand the dynamics of sodium changes in the different stages of the disease and their clinical impact.

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**Supplementary material**

Supplementary material is available at Brain online.

**References**


