CHAPTER 4
Chapter 4.1

Ultra-high field MTR and qR2* differentiates subpial cortical lesions from normal appearing gray matter in multiple sclerosis.

Jonkman, LE.¹, Fleysher, L.², Steenwijk, MD.³, ⁴, Koeleman, J.¹, de Snoo, TP.¹, Barkhof, F.³, Inglese, M.³, ⁵, ⁶, ⁷, Geurts, J.J.G.*¹

* both authors contributed equally to this work

Department of Anatomy and Neurosciences¹, Radiology and Nuclear Medicine³, Physics and Medical Technology⁴, VU University Medical Center, Amsterdam, the Netherlands. Department of Radiology², Neurology⁵ and Neuroscience⁶, Icahn School of Medicine at Mount Sinai, New York, NY, USA. Department of Neuroscience, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health (DINOGMI)⁶, University of Genoa, Genoa, Italy.

ABSTRACT

Background: Cortical gray matter (GM) demyelination is frequent and clinically relevant in MS. Quantitative MRI sequences such as magnetization transfer ratio (MTR) and quantitative R2* (qR2*) can capture pathological subtleties missed by conventional MRI sequences. Although differences in MTR and qR2* have been reported between lesional and non-lesional tissue, differences between lesion types or lesion types and myelin density matched normal appearing GM (NAGM) have not been found or investigated.

Objectives: Identify quantitative differences in histopathologically verified GM lesion types and matched NAGM at ultra-high field strength.

Methods: Using 7T post-mortem MRI, MRI lesions were marked on T2 images and co-registered to the calculated MTR and qR2* maps for further evaluation. Fifteen brain slices were collected, containing a total of 74 cortical GM lesions and 45 areas of NAGM.

Results: Intracortical lesions had lower MTR and qR2* values compared to NAGM. Type I lesions showed lower MTR than type III lesions. Type III lesions showed lower MTR than matched NAGM, and type I and IV lesions showed lower qR2* than matched NAGM.

Conclusion: qMRI at 7T can provide additional information on extent of cortical pathology, especially concerning subpial lesions. This may be relevant for monitoring disease progression and potential treatment effects.
**INTRODUCTION**

Gray matter (GM) pathology is a key component of multiple sclerosis (MS), a degenerative and inflammatory disease of the central nervous system. GM pathology is already present early on in the disease course, and is related to cognitive and physical disability.\(^1\,^2\) According to criteria proposed by Bo et al.,\(^3\) cortical lesions can be classified as follows: type I lesions involve the deeper layers of the GM as well as the adjacent WM; type II lesions are often small and confined within the cortex; type III lesions extend from the pial surface into the cortex; and type IV lesions span the entire cortex without entering the subcortical white matter. Visualizing these GM lesions with conventional magnetic resonance imaging (MRI) techniques has been challenging,\(^4\) but improvements have been made with specific sequences\(^5\,^7\) and imaging at (ultra-)high field.\(^7\,^8\) Prospectively, the majority of lesions are still missed,\(^9\,^{10}\) but retrospectively (i.e. with histopathological knowledge of lesion location), it is now possible to identify up to 93% of lesions at 7T MRI.\(^9\) At low field strengths, quantitative MRI techniques have shown to be more sensitive to pathological subtleties than conventional techniques. For instance, at 1.5 Tesla, magnetization transfer (MT) imaging can detect focal abnormalities in normal-appearing white matter (NAWM) before the appearance of lesions on conventional MRI.\(^11\) MT ratio (MTR) has also revealed abnormalities in the GM of MS patients; previous studies showed lower MTR in cortical lesions compared to normal appearing gray matter (NAGM)\(^12\,^{13}\) and baseline average GM MTR showed predictive value for patients’ worsening disability.\(^14\) In turn, quantitative R2* (=1/T2*) also revealed lower values in GM lesions than in nearby non-lesional cortex.\(^10\) Although differences in MTR and qR2* have been found between lesional and non-lesional tissue, a previous attempt to find quantitative MTR differences between type I and III lesion types did not yield any significant results, possibly due to insufficient power.\(^12\) In vivo, although the assessment of GM lesion types is challenging, it has been shown that different lesion types may exert different effects on physical or cognitive disability.\(^15\) In the current study, we aimed to compare MTR and qR2* values in histopathologically verified cortical GM lesion and NAGM at ultra-high field strength. Specifically, we looked to quantitatively distinguish different lesion types (I-IV) from each other and from myelin density matched NAGM. This would be especially interesting for the frequently occurring, but frequently missed type III lesions.\(^7\,^{9\,16\,17}\)

**MATERIALS AND METHODS**

**Patients and Autopsy**

Fifteen coronally cut, 10-mm thick full-hemispheric brain slices of 15 patients with histopathologically confirmed MS were selected after rapid autopsy (mean post-mortem delay 5 hours 56 minutes) and were formalin-fixed in 4% formalin. All brain slices were from the frontal area of the left hemisphere. Demographic and neuropathological details of the donors have been previously published.\(^18\) Briefly, the patient group consisted of seven females and eight males, mean age was 68.5 ± 12.7 years and mean disease duration 29.1 ± 13.2 years. Prior to death, all donors had registered at the Netherlands Brain Bank, Amsterdam, the Netherlands and given written informed consent for the use of their tissue and medical records for research.
purposes. Permission for performing autopsies, use of tissue and access to medical records, was granted by the institutional ethics review board.

**MR Acquisition and Analysis**

Imaging was performed using a 7.0 Tesla Bruker Biospec USR70/30 MRI scanner (Bruker BioSpin MRI GmbH, Ettlingen, Germany) and 8.5 cm diameter RF transmitter/receiver coil. Each formalin-fixed slice was placed into a rectangular plastic tissue container and immersed in 10% buffered formalin. All MRI sequences were acquired with an in-plane spatial resolution of 100μm x 100μm with a slice thickness of 1mm. The MRI protocol included:

(i) 2D multi-echo spin-echo (SE) T2-weighted image with repetition time (TR) = 4000 ms, echo times (TE) = 19.1/38.2/57.3 ms, number of averages (NEX) = 6.

(ii) 3D GRE with and without saturation pulse magnetization transfer ratio (MTR) images with TR = 40.9 ms, TE = 12.9 ms, NEX = 6, offset from water resonance = 3 KHz, MT pulse α = 850.

(iii) Multi-echo 3D GRE quantitative R2* images with TR=73.3 ms, TE= 9.89/27.46/45.03/62.60 ms, NEX = 8, α = 28.

MTR maps were calculated using the equation (M0 - Ms/M0) x 100, where M0 and Ms are the signal intensities found during non-saturated and saturated acquisition respectively. MTR values are reported in percent units (pu) as described earlier. Computation of the quantitative R2* maps was done by using least squares method to fit the logarithm of the image intensity versus TE, using Matlab (Matlab 6.1, The MathWorks Inc., Natick, MA, 2000). See figure 1 for an example of a T2-weighted image, MTR map and qR2* map with matching histology for a type I, III and IV lesion.

**Histology**

After MRI, the coronally cut full-hemispheric brain slices were cut in half to reveal the imaged plane, and embedded in paraffin. Average fixation time before embedding was 504 days (±SD 160). 8-µm-thick sections were cut and mounted onto glass slides (Superfrost, VWR international, Leuven, Belgium). Staining was performed with antibodies against proteolipid protein (PLP). PLP (Serotec, Oxford, UK) was diluted in TBS (1:500) containing 1% normal goat serum (NGS; DAKO, Glostrup, Denmark) and stored overnight at 6oC. Immunolabeling was detected by incubating the sections in biotinylated goat anti-mouse (1:400; Vector Laboratories, Burlingame, CA) and in Vectastain avidin-biotinperoxidase complex (ABC-HRP; 1:200; Vector Laboratories, Burlingame, CA) for 60 min at room temperature. Afterwards, the sections were washed in 0.05M tris-HCL (pH 7.6). Peroxidase activity was demonstrated with 0.5 mg/mL 3,3’ diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) in 0.01 mol/L tris-hydrochloride containing 0.03% H2O2 for 5 minutes, which led to a brown reaction product. Sections were counterstained with hematoxylin (Sigma, St. Louis, MO) and mounted (Depex, BDH; Poole, UK).
Figure 1 | T2-w image (left), MTR map (middle) and qR2* map (right) of a coronally cut full-hemispheric brain slice (left frontal area). The big yellow arrow depicts a type I lesion, the box contains a type II lesion, the arrowhead points toward a WML, the two small yellow arrows point to a type III lesion and the two small red arrows to a type IV lesion.
Scoring, Matching and Co-registration

Histopathological scoring of cortical lesions on hemispheric tissue sections was performed by three observers in consensus, all blinded to the clinical information. Lesions were defined as areas of complete demyelination (lack of PLP). Sections were subsequently matched to the corresponding T2-weighted MRI planes (with TE=19.1) using as many cortical anatomical landmarks as possible. Lesions were drawn as regions of interests (ROIs) on the MRI images using the Medical Image Processing, Analysis and Visualization (MIPAV, Centre for Information Technology, National Institutes of Health, Bethesda, MD, USA). For type I lesions, only the cortical part of the lesion was included, as the WM part of the lesion would skew MTR/qR2* values. In addition, ROIs of NAGM were drawn on the MR images; (i) a measure over the full width of the cortex (totalNAGM), as well as (ii) measures dividing the full width of the cortex in two approximately equal-sized areas: an area from the pial surface to approximately 50% of the cortex (lowNAGM) and an area from 50% of the cortex to the border of the white matter (highNAGM). See figure 2 for an overview. This distinction was made due to the natural variation of myelin content across cortical layers; low myelin density in the superficial layers closer to the pial surface, and higher myelin density in layers closer to the white matter. This way we could make specific and meaningful comparisons between NAGM and lesion types according to their location in the cortex. The cortical side of type I lesions are next to the WM border in the high myelin density area and will therefore be compared to highNAGM, type III lesions extent from the low myelin density pial surface and will therefore be compared to lowNAGM. Type II lesions can occur throughout the cortex and type IV lesions span the entire width of the cortex and will therefore be compared to totalNAGM. All NAGM ROIs were selected after inspection of histological sections (same sections on which GM lesions were detected).

MTR and qR2* maps were co-registered with the T2-weighted images using FLIRT (part of the FMRIB Software Library (FSL) Version 5.0.4). Subsequently, the masks were co-registered to the MTR and qR2* maps to extract mean values for each ROI. All co-registered images and masks were manually checked to confirm that there were no errors in co-registration or inclusion of MTR or qR2* artifacts.

Analysis of Data

Descriptive and statistical analysis was performed using IBM SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL). MTR and qR2* histograms and Shapiro-Wilk tests revealed no evidence for non-normality. We compared MTR and qR2* values between lesion types (I, II, III and IV) with ANCOVA and Bonferroni corrected post-hoc tests. Due to multiple lesions coming from the same subject (patient), we controlled for ‘subject’ in addition to lesion size and fixation time before embedding (in days). For comparison of lesion types to myelin density matched (low/high/total)NAGM, we did several ANCOVAs instead of T-tests so we were able to control for subject, lesion size and fixation time. We compared Type I to highNAGM, type II to totalNAGM, type III to lowNAGM and type IV to totalNAGM, please see the previous paragraph “scoring, matching and co-registration” for the rationale. Subsequently we corrected for multiple (four) comparisons. Intracortical lesions (ICL; type II, III and IV grouped together) were compared to all NAGM options (low/high/total) in an ANCOVA with Bonferroni corrected post-hoc tests, controlling for subject, lesion size...
and fixation time. Correlations between MTR and qR2* were assessed with a Pearson correlation and corrected for subject. Differences were considered significant if p < 0.05.

**Figure 2** | Boxplot with MTR values (in pu) of the different lesion types, intracortical lesions (ICL) and Normal Appearing Gray Matter (NAGM). ** p < 0.01, *** p < 0.001.

**Results**

Cortical demyelination was variable across cases, with an average area of demyelination of 13% (range: 1% - 95%) in the analyzed hemispheric slices. The fifteen brain slices contained a total of 81 lesions that were also found on MRI. Of these, 7 lesions did not correctly co-register to MTR/qR2* maps, resulting in 74 lesions. Additionally, 45 areas of NAGM were selected. For MTR analysis, four lesions had to be excluded due to signal artefacts, leaving 70 lesions (median: 3; range: 0-16 per slice) and 45 areas of NAGM (median: 3; range: 0-6 per slice) for analysis. For qR2* analysis, 10 lesions and three areas of NAGM were excluded due to image artefacts, leaving 64 lesions (median: 3; range 0-14 per slice) and 42 areas of NAGM (median: 3; range 0-6 per slice) for analysis. Distribution of lesion types and corresponding mean ± SD of MTR and qR2* for the different lesion types and (low/high/total) NAGM can be found in table 1.

There was an overall significant positive correlation between MTR and qR2* values, corrected for subject, for all lesions grouped together (r = 0.35, p < 0.01). Upon further inspection by lesion type, only lesion type III showed a significant correlation between MTR and qR2* (r = 0.42, p < 0.05). NAGM also showed a significant correlation between MTR and qR2* (r = 0.42, p < 0.01).

**Table 1** | mean ± SD of MTR and qR2* lesion size and value

<table>
<thead>
<tr>
<th></th>
<th>type I</th>
<th>type II</th>
<th>type III</th>
<th>type IV</th>
<th>ICL</th>
<th>NAGM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>7</td>
<td>30</td>
<td>25</td>
<td>62</td>
<td>45</td>
</tr>
<tr>
<td>sizea</td>
<td>6.33 ±5.68</td>
<td>1.98 ±1.02</td>
<td>11.48 ±16.62</td>
<td>39.54 ±31.23</td>
<td>21.72 ±27.23</td>
<td>23.98 ±16.03</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>7</td>
<td>27</td>
<td>24</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>sizea</td>
<td>7.35 ±6.17</td>
<td>2.08 ±0.98</td>
<td>11.31 ±16.90</td>
<td>39.83 ±31.64</td>
<td>21.99 ±27.77</td>
<td>24.86 ±15.81</td>
</tr>
<tr>
<td>qR2*</td>
<td>41.66 ±2.22</td>
<td>43.74 ±3.60</td>
<td>39.71 ±4.38</td>
<td>38.20 ±3.86</td>
<td>39.57 ±4.37</td>
<td>45.32 ±4.77</td>
</tr>
</tbody>
</table>

a in mm3

MTR= magnetization transfer ratio (values in %), qR2* (values in Hz), ICL = intracortical lesions (type II, III and IV combined), NAGM = normal appearing gray matter.
MTR

For MTR, there was a statistically significant difference between lesion types (I-IV) as determined by ANCOVA ($F(3,63) = 5.375$, $p = 0.002$). Bonferroni corrected post-hoc tests revealed that Type I lesions ($29.14 \pm SD 4.05$ pu) showed a significantly higher MTR than type III lesions ($23.40 \pm SD 3.97$ pu, $p < 0.05$).

Looking at comparisons with NAGM, there was a statistically significant difference between ICL (II-IV) and NAGM as determined by ANCOVA ($F(3,186) = 14.853$, $p < 0.001$). Bonferroni corrected post-hoc tests revealed that ICL ($24.37 \pm SD 4.30$ pu) showed a significant lower MTR than totalNAGM ($28.41 \pm SD 3.73$ pu, $p < 0.001$) and highNAGM ($29.41 \pm SD 3.95$ pu, $p < 0.001$). When comparing the different lesion types (I-IV) to location matched NAGM, type III lesions showed a significantly lower MTR than lowNAGM ($26.66 \pm SD 3.60$ pu, $p < 0.001$). These results are shown in figure 3.

![Figure 3](image)

**Figure 3** | Boxplots with $qR^2*$ values (in Hz). On the left the different lesion types, intracortical lesions (ICL) and Normal Appearing Gray Matter (NAGM). *** $p < 0.001$

$qR^2*$

For $qR^2*$, there was no statistically significant difference between lesion types (I-IV). Looking at comparisons with NAGM, there was a statistically significant difference between ICL (II-IV) and NAGM as determined by ANCOVA ($F(3,169) = 24.450$, $p < 0.001$). Bonferroni corrected post-hoc tests revealed that ICL ($39.57 \pm SD 4.37$ Hz) showed a significant lower $qR^2*$ than totalNAGM ($45.32 \pm SD 4.77$ Hz, $p < 0.001$) and highNAGM ($46.86 \pm SD 3.86$ Hz, $p < 0.001$). When comparing the different lesion types (I-IV) to location matched NAGM, type I lesions ($41.66 \pm SD 2.22$ Hz) showed a significantly lower $qR^2*$ than highNAGM ($p<0.05$). Type IV lesions ($38.20 \pm SD 3.86$) showed a significantly lower $qR^2*$ than totalNAGM ($45.32 \pm SD 4.77$ Hz, $p < 0.001$). These results are shown in figure 4. Figure 5 is an example of lesion and NAGM with MTR and $qR^2*$ values.
UHF MTR AND QR2* DIFFERENTIATES SUBPIAL CORTICAL LESIONS FROM NAGM

Figure 4 | Example of a histopathological (A) type III lesions (arrow) and corresponding T2-weighted image (B) with ROI (C), MTR map (D) and qR2* map (E). On the T2-w image a visible subpial lesion (yellow ROI) and area of NAGM (green ROI). Average MTR and qR2* values are depicted next to the ROIs.

Figure 5 | Example of a histopathological (A) type III lesions (arrow) and corresponding T2-weighted image (B) with ROI (C), MTR map (D) and qR2* map (E). On the T2-w image a visible subpial lesion (yellow ROI) and area of NAGM (red ROI). Average MTR and qR2* values are depicted next to the ROIs.

Discussion

7T quantitative MR imaging data obtained from 15 MS post-mortem samples demonstrated heterogeneity in cortical MTR and qR2* contrasts. Consistent with previous work, intracortical lesions had lower MTR and qR2* than non-lesional NAGM. More importantly, different lesion types could be quantitatively distinguished from myelin density matched NAGM; type III lesions showed lower MTR values than matched NAGM, and type I and IV lesions showed lower qR2* values than NAGM.

A previous MTR study at 1.5T found no quantitative differences between cortical lesions and NAGM. However, studies at higher field strength such as 3T MTR, 7T R2* and 9.4T MTR did find quantitative differences, indicating the necessity for higher field strength with better spatial resolution and contrast-to-noise to detect subtle differences. Nevertheless, these studies grouped lesion types together and therefore did not match for myelin density differences in NAGM.
The extent of quantitative MTR differences between GML and NAGM ranges from a 5% decrease at 3T to a 17% decrease at 9.4T. Our study found a MTR decrease of 7.4% for GML (lesion types I-IV) and 14.1% decrease for ICL (lesion types II-IV) at 7T. With regard to qR2*, at 7T a previous study (with n=2) found a decrease of approximately 30% while our study found a decrease of 9.9% for GML and 12.6% for ICL. These differences in MTR and qR2* may be caused by differences in field strength, MTR saturation pulses (power, frequency, offset), resolution, post mortem delay (PMD) and variation in sample sizes used.

The pathological substrate of cortical lesions is predominantly demyelination, but also (minor) axonal transection, microglial activation and neuronal, glial and synaptic loss. Previous combined post-mortem MRI and histopathology studies have shown that MTR changes predominantly reflect myelin and axonal density changes, whereas qR2* changes could reflect both myelin loss and iron changes. Iron is located in oligodendrocytes and myelin; a lesional area lacks myelin and therefore has less iron and less qR2* contrast. The correlation we found between MTR and qR2* can be explained by their common reflection of demyelination. However, the moderate strength of this correlation indicates that MTR and qR2*, as mentioned above, may reflect more than myelin alone and therefore has a wider scope of interpretation.

It has been observed that outer cortical qMRI (MTR and qT2*) is different in MS patients than controls, with a more pronounced MTR reduction in SPMS patients than in RRMS patients. This is in line with histopathological studies of subpial demyelination and neuronal loss being most extensive in SPMS patients. Our study looked at the capacity to distinguish with qMRI techniques and found lower mean MTR and qR2* in histopathological verified subpial lesions compared to myelin density matched NAGM, bridging the histological and in vivo findings. It also supports the notion that lower MTR in the outer cortex of patients is (at least partially) related to demyelination and could reflect the frequently overlooked subpial lesions.

Several in vivo studies have assessed the association of MTR and qR2* changes in relation to disability in MS patients, with contradictory results. One recent study found that NAGM (more than lesional) MTR was consistently associated with physical and cognitive outcome measures. However, another recent study found that cortical lesions rather than NAGM was related to physical and cognitive outcome measures. A study looking at qR2* found increased basal ganglia R2* in MS patients which was related to age, disease duration and Expanded Disability Status Scale (EDSS) score.

Although we have a larger sample size than previous studies, the number of type I and type II lesions are still limited, which may have underpowered our statistical analysis of these lesion types and warrant careful interpretation and preferably require replication in a larger sample of lesions. Furthermore, rather than an ANCOVA of individual lesions and controlling for subject, statistical analysis would ideally include a multi-level analysis controlling for nested data (lesions within patients) within the model. However, this type of analysis is unreliable with smaller sample sizes such as in the current study. Furthermore, the studied post-mortem MS population, with its long disease duration, is not representative for the whole MS population in vivo, which makes it difficult to translate results from the post-mortem to the in vivo setting.
Quantitative MRI parameters of brain tissue change after fixation and are influenced by post-mortem delay, age and duration of fixation. For MTR, formalin fixation causes cross-linking of macromolecules, thereby changing freely moving protons into restricted protons and altering the magnetization transfer between the two groups. For qR2*, formalin fixation and in turn cross-linking, reduces the T2 relaxation time. Therefore, a direct translation to the in vivo setting cannot be made. However, the direction of qMRI change in cortical lesion and NAGM remains the same from post-mortem brain slices (current study) to the post-mortem in situ and the in vivo setting.

Summarizing, our post-mortem findings suggest that at 7T, it is possible to find qMRI differences between cortical lesions and myelin density matched NAGM. More interestingly, we were able to differentiate type III subpial lesions from NAGM, indicating the added value of MTR to conventional MRI measures such as T2 and T2*, with which type III lesions are generally difficult to detect. However, to investigate the benefit of MTR/qR2* in vivo, several steps need to be taken; first with parameters similar to those used post mortem, MTR/qR2* should be compared between the post-mortem and in vivo setting. Then lesional differences may be studied in vivo and in direct relation to clinical disability. Eventually, qMRI may be used as a complementary marker for disease burden (extent of pathology), disease progression and monitoring of treatment effects.

ACKNOWLEDGEMENT

The authors thank the Netherlands Brain Bank (http://www.brainbank.nl/), VUmc pathology department and the MS-MRI autopsy team for their help in acquiring the data.

FUNDING ACKNOWLEDGEMENT

This work was supported by the Dutch MS Research Foundation [grant number 09-358b] and National MS Society USA [RG 4916A2/1] to MI and JG and Noto Foundation to MI.
REFERENCES


CHAPTER 4


