CHAPTER 4.2

The substrate of increased cortical FA in MS: a 7T post mortem MRI and histopathology study

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ABSTRACT

Background: Using diffusion tensor imaging (DTI), it was previously found that demyelinated gray matter (GM) lesions have increased fractional anisotropy (FA) when compared to normal-appearing GM (NAGM) in multiple sclerosis (MS). The biological substrate underlying this FA change is so far unclear; both neurodegenerative changes and microglial activation have been proposed as causal contributors.

Objective: To test the proposed hypothesis that microglia are responsible for increased FA in cortical GM lesions.

Methods: We investigated post-mortem cortical DTI changes in hemispheric, coronally cut sections and investigated the underlying histopathology using immunohistochemistry.

Results: Microglia did not differ between GM lesions and NAGM. However, cell density was increased in GM lesions compared to NAGM (309.67 ±SD 124.44 vs. 249.95 ±SD 56.75, p=0.002).

Conclusion: FA increase was not due to lesional and non-lesional differences in microglia activation. We found an increase in cellular density without a notable difference in cellular size, i.e. tissue compaction, as a possible alternative explanation.
INTRODUCTION

Cortical gray matter (GM) pathology is common and extensive in multiple sclerosis (MS), especially in the progressive phase of the disease. Demyelinated cortical lesions are related to both physical and cognitive disability, but are difficult to visualize using conventional magnetic resonance imaging (MRI) techniques. Even when using more advanced techniques, such as double inversion recovery (DIR) or phase-sensitive inversion recovery (PSIR), or when imaging at (ultra) higher field strengths, the study of cortical GM pathology remains challenging. Quantitative MRI techniques such as diffusion tensor imaging (DTI), has the potential to provide more details regarding the microstructural environment of the GM and its pathology. A few in vivo studies have investigated fractional anisotropy (FA) and mean diffusivity (MD) differences in the cortex of MS patients compared to healthy controls. Results of these studies were somewhat contradictory; FA in cortical normal-appearing GM (NAGM; as defined by conventional MRI) either decreased or increased, while MD either increased or showed no difference compared to controls. The reason for these contradictory results in NAGM could be the considerable heterogeneity in terms of missed cortical lesions in the ‘NAGM’ areas. So far, only three DTI studies looked specifically at FA and MD differences in cortical lesions compared to NAGM, or compared to similar regions in control cortex. The FA of cortical GM lesions consistently increased in all three studies, while MD measures either increased, decreased or showed no difference. The aforementioned studies explain the observed FA increase by neuronal, dendritic and synaptic loss on the one hand and local activation of microglia that may align anisotropically on the other. In several other post mortem studies, however, it was already shown that microglial numbers in MS cortex are not importantly increased compared to white matter. In the current comparative post-mortem MRI and histopathology study, we tested the proposed hypothesis that microglia are responsible for increased FA in cortical GM lesions.

MATERIALS AND METHODS

Patients and Autopsy

Coronally cut, 10-mm thick full-hemispheric brain slices of fourteen patients with histopathologically confirmed MS were selected and formalin-fixed after rapid autopsy (mean postmortem delay 5 hours 37 minutes). Seven females and seven males (age at death 68.0 ± 13.0 years) were included with a mean disease duration of 27.5 ± 12.2 years. Demographic and neuropathological details of the donors can be found in table 1. Prior to death, all donors were registered at the Netherlands Brain Bank, Amsterdam, the Netherlands. All donors gave 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 imager (Bruker BioSpin MRI GmbH, Ettingen, Germany) and a vendor provided 8.5 cm diameter RF transmitter/receiver coil. Each formalin-fixed brain slice was placed into a rectangular plastic tissue container and immersed in 10% buffered formalin. 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, number of excitations (NEX) = 6, FOV = 100 x 80 mm$^2$, image matrix = 1000 x 80, and slice thickness 1 mm.

(ii) Diffusion Tensor Imaging (DTI) with TR = 5000, TE = 55, FOV = 100 x 80 mm$^2$, image matrix = 50 x 40, and voxel size = 2 x 2 x 1 mm$^3$, using a twice-refocused spin-echo diffusion technique with 30 different gradient-encoding directions and 3 b-values (0, 1500, and 3000 s/mm$^2$).

Table 1 | Demographic and neuropathology data of subjects

<table>
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<th>MS case</th>
<th>Sex</th>
<th>Age (in yrs)</th>
<th>PMD (in h:min)</th>
<th>DD (in yrs)</th>
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<tr>
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<td>81</td>
<td>6:30</td>
<td>21</td>
<td>heart failure</td>
</tr>
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</table>

Mean ± SD  68.0 ± 13.0  5:37 ± 2:05  27.5 ± 12.2

PMD, post-mortem delay (h:min); DD, disease duration in years since diagnosis; COD, cause of death; n/a, unavailable/unknown.

Histology

After MRI, the coronally cut full-hemispheric brain slices were cut in half to reveal the MRI imaged plane, and embedded in paraffin. 8-µm-thick sections were cut and mounted onto glass slides (Superfrost, VWR international, Leuven, Belgium). Sections were deparaffinized and rehydrated in a graded series of xylene and ethanol. Sections were stained for cell bodies
using Nissl (thionin; Thermo Fisher Scientific Inc, Waltham, MA), myelin (anti-PLP, 1:1000; Serotec, Oxford, United Kingdom) and activated microglia/macrophages (human leukocyte antigen, HLA-DR, 1:500; gift from Dr J. Hilgers, VU University Medical Center, Amsterdam, the Netherlands). For HLA-DR sections, antigen was retrieved by incubating the slides in citrate buffer (PH: 6.0) for 30 minutes at 95 °C. After primary antibody incubation sections were rinsed and incubated with EnVision horseradish peroxidase complex (DAKO, Glostrup, Denmark) for 30 min at room temperature. Finally, sections were rinsed, and peroxidase reaction was developed with 3,3’ diaminobenzidine-tetrahydrochloride dihydrate (DAB, DAKO, Glostrup, Denmark) as chromogen.

**Scoring and Matching**

Histopathological scoring of cortical lesions on hemispheric tissue sections was performed by LJ, blinded to clinical and MRI information. Lesions were defined as areas of complete demyelination (lack of PLP). Scored sections were subsequently matched to the corresponding T2-w MRI planes (with TE=19.1), using as many cortical anatomical landmarks as possible. Lesions were drawn as regions of interests (ROIs) on the MR images using the Medical Image Processing, Analysis and Visualization (MIPAV, Centre for Information Technology, National Institutes of Health, Bethesda, MD, USA). In addition, ROIs of NAGM were drawn on the MRI images, in the full width of the cortex, matching their respective histological sections to assure correct classification.

**Data Processing**

DTI images were processed with in-house Matlab (MathWorks, Natick, Massachusetts) scripts. Subsequently, B0, FA and MD maps were co-registered with the T2-w images using FLIRT (part of the FMRIB Software Library (FSL) Version 5.0. Subsequently, regions of interest (ROIs) from T2-w images were co-registered onto the B0, FA and MD maps and average FA and MD was calculated for each ROI with FSL. All co-registered images and ROIs were manually checked to confirm that there were no errors in co-registration or overlap with artifacts.

**Analysis of Histopathological Data**

Morphometric analyses were done using a Leica DM / RBE photomicroscope with a Xillix MicrolImager digital camera (1280x1024 pixels) attached to an MCID Elite image analysis system (Imaging Research Inc, Ontario, Canada). Images were taken at 50x magnification from the entire cortex (from pia to WM/GM border) in area's containing cortical lesions and in NAGM. The cortical lesions and NAGM were selected based on MRI and PLP-stainings (see paragraph Scoring and Matching). The exposure conditions were kept constant during digitization.

Within every ROI, the cortex was measured at six different points, starting at the pial surface and ending at the WM/GM border. Three measurements of 88,725.66 µm² were done in the superficial layers and three in the deeper layers. These six measurements were subsequently averaged and used in the statistical analysis. In Nissl stained sections, the cell density and averaged cell size was measured using MCID in-house developed segmentation scripts. The segmentation scripts construct a measuring template, which is used to measure cells in the original non-modified images. Subsequently, cell density was corrected for the thickness of
cortex, which is substantially influenced by cutting direction during sectioning and autopsy. The correction was done as follows: The mean cell density per ROI was divided by the thickness of the cortex measured in that ROI using a Nissl stained section (see figure 1).

HLA-DR positive microglia/macrophages were visually quantified on a scale from 0 to 4 (0 – no activated microglia/macrophages, 4 – numerous activated microglia/macrophages).

**Figure 1** | Cell density measurement. Cell density was measured at 6 points within the ROI. Subsequently, the average of those 6 measurements was divided by the thickness (T) of the cortex.

**Statistical Analysis**

Descriptive and statistical analysis was performed using IBM SPSS 20.0 for windows (SPSS, Inc., Chicago, IL). FA and MD histograms and Shapiro-Wilk tests revealed evidence for non-normality. Therefore, for the DTI analysis between GM lesions and NAGM, we used a Mann-Whitney Test for differences between GM lesions and NAGM. For the histopathological analysis, cell density and cell size showed normal histograms and Shapiro-Wilk tests, therefore analysis between GM lesions and NAGM was performed with Student’s t-test. Differences were considered significant if p < 0.05.
**Results**

After histopathological inspection of PLP stained sections, the fifteen brain slices contained a total of 53 cortical GM lesions. In addition, 43 areas of histopathologically verified NAGM were selected. For DTI analysis, 5 lesions and 4 areas of NAGM were excluded due to signal inhomogeneities. This resulted in a final dataset of 48 cortical GM lesions (5 type I lesions, 4 type II lesions, 15 type III lesions and 24 type IV lesions as described by Bo et al. [13]) and 39 areas of NAGM.

**MRI Differences in FA and MD**

ROI analysis of FA and MD maps showed that GM lesions have a significantly higher FA than NAGM (0.087 ±SD 0.040 vs 0.066 ±SD 0.021, p = 0.02). There were no significant differences in MD (0.653 ±SD 0.218 vs 0.610 ±SD 0.210, p = 0.348). These results are visualized in figure 2.

![Figure 2](image2.png)

**Histopathological Differences that could account for Increase in FA**

To investigate the possible underlying histopathology of this increase in FA, sections of the brain slices were subsequently stained for presence of microglia/macrophages (HLA-DR) and neuronal cell bodies (Nissl). Due to tissue disintegration during the (immuno)histochemical staining procedure, the final dataset consisted of 29 lesions and 20 areas of NAGM for HLA-DR and 26 lesions and 24 areas of NAGM for Nissl histopathological analysis.

Only very few microglia were observed in the brain sections. We found no difference in microglia/macrophages between GM lesions and NAGM (GM lesions: 0.069 ±SD 0.26, NAGM: 0.200 ±SD 0.41; χ²(1) = 1.89, p = .210). However, corrected for cortical thickness, GM lesions had significantly higher densities of Nissl-stained cells per mm² cortex (309.67 ±SD 124.44) than NAGM (249.95 ±SD 56.75, p=0.002), whereas cellular size remained similar (GM lesions: 68.06 ±SD 19.05, NAGM: 78.38 ±SD 20.75, p=0.98). This is shown in figure 3.
DISCUSSION

In our post-mortem brain samples, we confirmed the in vivo reported increase of FA in GM lesions compared to NAGM, while no changes in MD were found. Subsequent histological analysis suggested that this FA increase was not due to lesional and non-lesional differences in microglia activation. In an exploration, we found an increase in cellular density without a notable difference in cellular size, i.e. due to tissue compaction, as a possible alternative explanation.

Previous biopsy studies have found an increase in activated microglia of early, atypically presenting MS cases. Also, a subset of cortical lesions in longstanding MS patients with more active inflammatory profiles showed more prominent microglia activation. Generally, however, late-stage MS patients seen at autopsy are characterized by a lack of inflammation in NAGM and GM lesions, with very few (activated) microglia present in the cortex. In line with these studies, our post-mortem sample of chronic MS patients showed very few microglia/macrophages in cortical GM, though they still showed an increase in FA.

A recent post-mortem MRI study by our group has shown that neuronal size, neuronal density and axonal density jointly and predominantly explain cortical volume loss as measured with MRI in longstanding MS. Therefore, aside from microglia, we looked at neuronal density and size to possibly explain the FA increase in GM lesions. Although our findings suggest that cell size and density may play a role in FA increase, it would be interesting to investigate whether there is a more direct relationship between neurodegeneration and increase in FA. This can be achieved by including a DTI sequence to the post-mortem in situ MRI and subsequently analyzing the tissue histopathologically.

There are several limitations that need to be considered. For this dataset, it was unfortunately not possible to use an extensive list of histopathological staining to analyze other factors possibly influencing FA. In future studies, other possibilities such as remyelination should also be
considered as possible factors influencing cortical FA and MD. Recruitment of oligodendrocyte precursors (OPC) is crucial to the remyelination process and OPCs are increased in cortical lesions; especially in early MS patients. However, it is less likely that an increase in OPCs explain an increase in lesional FA, as OPCs should not necessarily align anisotropically. Lastly, a combination of differences in field strengths, sequence parameters, tissue fixation and processing tend to decrease FA and MD values in the post mortem setting. This should be kept in mind when comparing between post mortem and in vivo studies.

In conclusion, FA increase in MS cortical lesions is not likely explained by morphological changes following microglia activation as suggested in the literature. An alternative proposed in this study is tissue compaction resulting from ongoing neurodegeneration. Further exploration is now required; specific cellular components (synaptic, dendritic, axonal, somatic, glial) should be further scrutinized in relation to measured FA changes. This will increase our understanding of the biological substrates of MRI measures that can subsequently be used as prognostic and treatment monitoring tools in MS.

ACKNOWLEDGEMENT

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ETHICAL STANDARD

Prior to death, all donors gave written informed consent for the use of their tissue and medical records for research purposes (the Netherlands Brain Bank, Amsterdam, the Netherlands). Ethics approval was obtained from the institutional ethics review board.
REFERENCES


