Heterogeneity of white matter hyperintensities in Alzheimer’s disease: post-mortem quantitative MRI and neuropathology

A. A. Gouw,1,2 A. Seewann,2,6 H. Vrenken,1,3 W. M. van der Flier,1,2 J. M. Rozemuller,4 F. Barkhof,1,5 P. Scheltens,1,2 and J. J. G. Geurts1,4,5

1Alzheimer Center, 2Department of Neurology, 3Department of Physics and Medical Technology, 4Department of Pathology, 5Department of Radiology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands and 6Department of Neurology and MRI Institute, Medical University Graz, Austria

Correspondence to: A. A. Gouw, Department of Neurology, Alzheimer Center and Image Analysis Center, Vrije Universiteit Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands
E-mail: aa.gouw@vumc.nl

White matter hyperintensities (WMH) are frequently seen on T2-weighted MRI scans of elderly subjects with and without Alzheimer’s disease. WMH are only weakly and inconsistently associated with cognitive decline, which may be explained by heterogeneity of the underlying neuropathological substrates. The use of quantitative MRI could increase specificity for these neuropathological changes. We assessed whether post-mortem quantitative MRI is able to reflect differences in neuropathological correlates of WMH in tissue samples obtained post-mortem from Alzheimer’s disease patients and from non-demented elderly.

Thirty-three formalin-fixed, coronal brain slices from 11 Alzheimer’s disease patients (mean age: 83 ± 10 years, eight females) and 15 slices from seven non-demented controls (mean age: 78 ± 10 years, four females) with WMH were scanned at 1.5 T using qualitative (fluid-attenuated inversion recovery, FLAIR) and quantitative MRI (diffusion tensor imaging (DTI) including estimation of apparent diffusion coefficient (ADC) and fractional anisotropy (FA), and T1-relaxation time mapping based on flip-angle array). A total of 104 regions of interest were defined on FLAIR images in WMH and normal appearing white matter (NAWM). Neuropathological examination included (semi-)quantitative assessment of axonal density (Bodian), myelin density (LFB), astroglia (GFAP) and microglia (HLA-DR). Patient groups (Alzheimer’s disease versus controls) and tissue types (WMH versus NAWM) were compared with respect to QMRI and neuropathological measures. Overall, Alzheimer’s disease patients had significantly lower FA ($P < 0.01$) and higher T1-values than controls ($P = 0.04$). WMH showed lower FA ($P < 0.01$) and higher T1-values ($P < 0.001$) than NAWM in both patient groups. A significant interaction between patient group and tissue type was found for the T1 measurements, indicating that the difference in T1-relaxation time between NAWM and WMH was larger in Alzheimer’s disease patients than in non-demented controls. All neuropathological measures showed differences between WMH and NAWM, although the difference in microglial activation was specific for Alzheimer’s disease. Multivariate regression models revealed that in Alzheimer’s disease, axonal density was an independent determinant of FA, whereas T1 was independently determined by axonal and myelin density and microglial activation. Quantitative MRI techniques reveal differences in WMH between Alzheimer’s disease and non-demented elderly, and are able to reflect the severity of the neuropathological changes involved.

**Keywords:** age-related white matter hyperintensities; post-mortem MRI; Alzheimer’s disease; neuropathological characteristics

**Abbreviations:** 3D-FLAIR = 3D-fluid-attenuated inversion recovery; ADC = apparent diffusion coefficient; CERAD = Consortium to establish a Registry for Alzheimer’s disease; DTI = diffusion tensor imaging; FA = fractional anisotropy; FLASH = fast low-angle shot; GFAP = glial fibrillary acidic protein; HE = haematoxylin–eosin; LFB = Luxol Fast Blue; NAWM = normal appearing white matter; NBB = Netherlands Brain Bank; PBS = phosphate-buffered saline; QMRI = quantitative MRI techniques; ROIs = regions of interest; T2SE = T2-weighted spin-echo; WM = white matter; WMH = white matter hyperintensities

© The Author (2008). Published by Oxford University Press on behalf of the Guarantors of Brain. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
Introduction

White matter hyperintensities (WMH) form an important expression of small vessel disease on MRI and are observed in a significant proportion of demented and non-demented elderly subjects (De Leeuw et al., 2001). WMH have been distinguished by location into periventricular WMH, which are directly adjacent to the lateral ventricles, and deep WMH, which are located in the subcortical white matter (WM). Furthermore, the severity of deep WMH is generally scored into punctate, early confluent and confluent WMH (Fazekas et al., 1987). In healthy elderly, WMH are mostly regarded as a normal aging phenomenon, but especially confluent WMH have been associated with loss of specific cognitive functions, such as psychomotor speed (De Groot et al., 2000). However, the associations are only weak and inconsistently found across MRI studies (O’Brien et al., 2002; Schmidt et al., 2005; van der Flier et al., 2005).

MRI studies have shown that WMH are prevalent in patients with Alzheimer’s disease (Scheltens et al., 1992; Barber et al., 1999). Although Alzheimer’s disease is considered to be a mainly cortical dementia with senile plaques and neurofibrillary tangles in the grey matter of the brain (Braak and Braak, 1991), cerebrovascular pathology often coexists in brains of Alzheimer’s disease patients (Smith et al., 2000, MRC CFAS, 2001). The clinical significance of WMH in Alzheimer’s disease patients is insufficiently known. Some authors reported that WMH have an additive effect on cognitive decline in dementia, whereas others could not confirm this association (Stout et al., 1996; Hirono et al., 2000; Mungas et al., 2001).

A possible explanation for the inconsistent and weak associations between WMH and clinical symptoms in demented and non-demented elderly would be heterogeneity of the neuropathological substrates underlying WMH (Scheltens et al., 1992). Although with the abovementioned classification system an attempt was made to specify WMH on T2-weighted images, these MRI sequences are generally not sufficient specific for the demonstration of underlying pathological changes in the composition of the brain. It is well conceivable that the high signal intensity on T2-based images (e.g. fluid-attenuated inversion recovery, FLAIR) actually reflects a spectrum of neuropathological substrates or tissue damage with varying severity. Whether this is the case for Alzheimer’s disease patients and non-demented subjects with WMH, can be investigated by post-mortem MRI scanning and direct matching of the MRI hyperintensities to pathological stainings (Geurts et al., 2005). Previous post-mortem MRI—pathology correlation studies in Alzheimer’s disease or in non-demented elderly showed that the pathological correlates of WMH include myelin and axonal loss, astrogliosis, reduction of oligodendrocytes, mild microglial activation and dilated perivascular spaces to variable degrees providing support for the notion of heterogeneity in WMH pathology (Fazekas et al., 1991, 1993; Scheltens et al., 1995). Recent studies provided detailed immunohistochemical characterization of different expressions of WMH including associations with specific hypoxia markers, altered fluid dynamics or discontinuity of the ependymal lining (Bronge et al., 2002; Fernando et al., 2006; Simpson et al., 2007a).

From a clinical standpoint, investigations with sufficient pathological specificity are needed in vivo to be able to assess the clinical impact of WMH. In vivo studies using more advanced quantitative MRI techniques (QMRI), such as diffusion tensor imaging (DTI) and T1-relaxation time measurements, claim that these techniques are more specific to the presence of structural brain damage than the current gold standard for the detection of WMH (conventional T2-weighted MRI) (Jones et al., 1999; Pierpaoli et al., 2001; Shenkin et al., 2005). DTI quantifies the extent of diffusivity of water molecules as well as tissue anisotropy, which is the spatial restriction of water movements in certain directions (Basser et al., 1994). Normal WM, for example, is highly anisotropic because diffusion is more readily directed along the long axis of fibre bundles whereas the perpendicular movement of water molecules is relatively restricted (Basser and Pierpaoli, 1996; Pierpaoli et al., 2001). T1-mapping that determines tissue-specific T1-relaxation times, may reflect pathological processes related to intraparenchymal changes in water content such as oedema, widening of the extracellular space, subtle blood–brain barrier leakage or glial proliferation (Vrenken et al., 2006a). These in vivo QMRI techniques are promising to further improve understanding of WMH in aging. However, the neuropathological substrates that define changes in QMRI parameters in WMH are still unknown (Bronge et al., 2002; Fernando et al., 2004).

In this study, we therefore investigated whether post-mortem QMRI reflects heterogeneity in basic neuropathological hallmarks of WMH, i.e. whether QMRI shows differences between WMH of Alzheimer’s disease patients and of non-demented controls. Also, we aimed to identify which of these neuropathological substrates most strongly determines the possible changes in QMRI parameters of white matter.

Methods

Patients

Eleven consecutive brain specimens of patients older than 70 years with a clinical diagnosis of Alzheimer’s disease were prospectively selected from the Netherlands Brain Bank (NBB). Controls were selected from the VU University Medical Center (i.e. hospitalized patients at the VU University Medical Center who died during the admission). Controls were defined as non-demented subjects according to clinical records. Patients and controls were only
Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Post-mortem delay</th>
<th>Cause of death</th>
<th>Number of slices (left/right)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>93</td>
<td>F</td>
<td>6 h 45 min</td>
<td>Cerebral infarction</td>
<td>3 (0/3)</td>
</tr>
<tr>
<td>2</td>
<td>88</td>
<td>F</td>
<td>4 h 35 min</td>
<td>Cachexia and dehydration</td>
<td>3 (1/2)</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>M</td>
<td>5 h 25 min</td>
<td>Dehydration</td>
<td>2 (0/2)</td>
</tr>
<tr>
<td>4</td>
<td>73</td>
<td>M</td>
<td>6 h 15 min</td>
<td>Sudden death</td>
<td>3 (0/3)</td>
</tr>
<tr>
<td>5</td>
<td>77</td>
<td>F</td>
<td>3 h 05 min</td>
<td>Dehydration, mamma carcinoma</td>
<td>2 (0/2)</td>
</tr>
<tr>
<td>6</td>
<td>99</td>
<td>F</td>
<td>5 h 10 min</td>
<td>Dehydration</td>
<td>3 (0/3)</td>
</tr>
<tr>
<td>7</td>
<td>75</td>
<td>F</td>
<td>15 h</td>
<td>Cardiac arrest</td>
<td>4 (0/4)</td>
</tr>
<tr>
<td>8</td>
<td>86</td>
<td>F</td>
<td>5 h 55 min</td>
<td>Cachexia and dehydration</td>
<td>4 (0/4)</td>
</tr>
<tr>
<td>9</td>
<td>70</td>
<td>M</td>
<td>4 h 50 min</td>
<td>Pneumonia</td>
<td>2 (0/2)</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>F</td>
<td>5 h 35 min</td>
<td>Colon carcinoma, dehydration</td>
<td>3 (0/3)</td>
</tr>
<tr>
<td>11</td>
<td>86</td>
<td>F</td>
<td>5 h 05 min</td>
<td>Pneumonia</td>
<td>4 (0/4)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>98</td>
<td>M</td>
<td>10 h 46 min</td>
<td>Unknown</td>
<td>2 (0/2)</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>F</td>
<td>&lt;24 h</td>
<td>Myocardial infarction</td>
<td>2 (2/0)</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>F</td>
<td>&lt;24 h</td>
<td>Ruptured abdominal aorta</td>
<td>3 (1/2)</td>
</tr>
<tr>
<td>4</td>
<td>83</td>
<td>F</td>
<td>3 h 20 min</td>
<td>Euthanasia</td>
<td>1 (1/0)</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>M</td>
<td>&lt;24 h</td>
<td>Myocardial infarction</td>
<td>3 (0/3)</td>
</tr>
<tr>
<td>6</td>
<td>79</td>
<td>F</td>
<td>&lt;24 h</td>
<td>Bronchopneumonia</td>
<td>1 (0/1)</td>
</tr>
<tr>
<td>7</td>
<td>71</td>
<td>M</td>
<td>&lt;24 h</td>
<td>Myocardial infarction</td>
<td>3 (2/1)</td>
</tr>
</tbody>
</table>

Post-mortem MRI protocol

Post-mortem MRI scanning was performed using a 1.5-T MRI scanner (Vision, Siemens AG, Erlangen, Germany). The brain slices were separately placed in a purpose-built perspex multi-slice holder that exactly fits into the head-coil of the MRI system. The MRI protocol that was optimized for fixed brain slices included:

- Qualitative MRI sequences:
  1. Dual-echo T2-weighted spin-echo (T2SE) images—TR/TE/NEX: 2755 ms/45 ms and 90 ms/2; field of view: 80 × 128 mm; matrix size: 160 × 256; slice thickness: 3 mm; acquisition time: 7 min 25 s.
  2. 3D-FLAIR images—TR/TE/TI/NEX: 6500 ms/120 ms/2200 ms/1; 8 partitions per slab; partition thickness: 1.25 mm; field of view: 125 × 200 mm; matrix size: 160 × 256; acquisition time: 7 min 54 s.

- Quantitative MRI sequences:
  1. T1-relaxation time measurements: 3D fast low-angle shot (FLASH), TR/TE/TI/NEX: 15 ms/4 ms/2; partition thickness: 3 mm; field of view: 80 × 128 mm; matrix size: 80 × 128, with flip angles of 2°, 5°, 10°, 15°, 20°, and 25°, respectively. The T1-relaxation times (T1) were then calculated for each pixel through a non-linear least-squares fit (Vrenken et al., 2006b).
  2. DTI: diffusion-weighted single shot STEAM sequence, TR/TE/TI/NEX: 6000 ms/65 ms/8; slice thickness: 8 mm; field of view: 80 × 128 mm; matrix size: 40 × 64; flip angle: 11°. Diffusion encoding gradients were applied in six non-collinear directions: (0, 1, 1), (0, 1, −1), (1, 0, 1), (1, 0, −1), (1, 1, 0), and (1, −1, 0), given in the gradient coordinate system (x, y, z), using a b-value of 750 s/mm² (Vrenken et al., 2006c). For each slice, one image without use of a diffusion gradient (b=0) was also acquired. Pixelwise maps of the apparent diffusion coefficient (ADC) and fractional anisotropy (FA) maps were calculated. ADC reflects the magnitude of water diffusivity, whereas the FA indicates spatial directionality of water diffusivity. DTI measurements had to be excluded in seven brain slices due to image artifacts and were therefore available for 41 brain slices.
Definition of WMH and selection of regions of interest (ROIs)

The scans and quantitative maps were displayed on a Sun workstation (Sun Microsystems, Mountainview, CA, USA) using an in-house developed image viewer (Show_Images) for analyses. Rectangular regions of interest (ROIs) were defined on 3D-FLAIR images in a consensus meeting of two experienced raters. The ROIs (mean size: 9.7 ± 3.0 mm² and minimum size: 4.9 mm²) were drawn within areas of WMH and normal appearing white matter (NAWM). Care was taken not to include the borders of the area of WMH or NAWM in the ROIs to avoid miscategorization. WMH included extensive periventricular WMH and (beginning) confluent areas of deep WMH, according to the widely used Fazekas scale (Fazekas et al., 1987). Rimlike periventricular WMH or small punctate deep WMH were not included, because it is more difficult to match these small areas exactly with (immuno-) histochemical stainings (Fazekas et al., 1991). NAWM was defined as white matter that showed no visually appreciable signal intensity on T2-weighted or 3D-FLAIR images. Only scans of brain slices with at least one WMH ROI were included for analyses. Forty-five ROIs in WMH and 30 ROIs in NAWM were defined in 33 brain slices of Alzheimer’s disease patients; 16 WMH and 13 NAWM ROIs were defined in 15 slices of controls (totalling 104 ROIs).

Neuropathology and matching procedure

As part of routine pathological description of each brain specimen, Braak staging was performed for confirmation of the clinical diagnosis of Alzheimer’s disease and controls (Braak et al., 1991). Furthermore, evaluation of the agonal state was performed on haematoxylin–eosin (HE) stainings by assessment of red (hypoxic) neurons and cortical pericellular oedema in the frontal, temporal and occipital lobes (graded as absent/mild/moderate/severe). Hemispheric brain slices were paraffin-embedded and subsequently cut until halfway to reveal the center of the imaged plane on which the ROIs were defined. This way, the brain slices could be reliably matched to the MR images. Serial 8-μm thick sections were then cut, mounted onto glass slides and stained with HE, Luxol Fast Blue/Cresyl Violet (LFB) and Bodian silver to assess general tissue morphology, myelin density, and axonal density, respectively. Standard immunohistochemical stainings were performed on adjacent sections, which were mounted on poly-l-lysine-coated glass slides for microglial activation with HLA-DR antibodies and for astrogliosis with antibodies directed against glial fibrillary acidic protein (GFAP). Endogenous peroxidase activity was blocked by incubating the sections in methanol with 0.3% H2O2 for 30 min. The sections were rinsed for 30 min with 0.01 M phosphate-buffered saline (PBS; pH 7.4). For HLA-DR staining, sections were pretreated by heating in a microwave oven (750 W) at 100°C for 10 min in a citrate buffer (0.01 M, pH 6.0), cooling down to room temperature and rinsing with PBS. Primary antibodies (HLA-DR, clone LN3: mouse antibody, dilution 1:50, gift from Dr. J.H. Hilgers, Department of Obstetrics and Gynaecology, VUMc); GFAP: rabbit polyclonal antibody, dilution 1:2000, gift from Dr Hilgers) were diluted in PBS containing 1% bovine serum albumin and were incubated overnight at 4°C. Primary antibodies were omitted for negative controls. After rinsing, immunolabeling with primary antibodies was detected with the EnVision-HRP complex (DakoCytomation, Glostrup, Denmark). Sections were lightly counterstained with haematoxylin and mounted with Depex (BDH, Poole, UK).

The FLAIR images, on which the ROIs were defined, were carefully matched to the corresponding QMRI maps and to the hemispheric tissue sections. The WMH and NAWM ROIs were then copied onto the corresponding areas in the QMRI maps and tissue sections by visual inspection using cortical anatomy and WM abnormalities as landmarks (Geurts et al., 2005). A representative matching of post-mortem MRI to histopathology is illustrated in Fig. 1.

All measurements were performed within the ROIs and were assessed blinded to clinical, pathological and MRI information. Myelin and axonal densities were quantified by assessing light transmittance of digital images of the LFB and Bodian Silver stainings (TmLFB and TmBodian, respectively), using the software programme ImageJ version 1.37 (freely downloadable from rsb.info.nih.gov). Images were converted into 8-bit grey scale images and the mean light transmittance within the ROIs was measured (arbitrary units, ranging from 0 to 255). High values (increased light transmittance) correspond to low staining intensity. Severity of microglial activation was scored on HLA-DR stained sections using templates (Fig. 2A): (0) no increase of microglia, only perivascular macrophages are stained; (1) mild microglial activation: slight increase of activated microglial cells; (2) moderate increase of activated microglial cells; (3) severe microglial activation with severe increase of activated microglial cells. Furthermore, the most predominant microglial phenotype was assessed: predominantly microglial cells or predominantly macrophages (Simson et al., 2007b). Severity of astrogliosis was assessed using templates on the GFAP-immunostained sections according to the following semi-quantitative score (range 0–3, see Fig. 2B): (0) normal: normal cell bodies with visible ramifications and low staining of glial processes; (1) mild reactive astroglisis: slight enlargement of cell bodies and slightly increased staining of glial processes; (2) moderate reactive astroglisis: significant enlargement of cell bodies, ramifications of glial processes not visible due to increased staining; (3) severe reactive astroglisis: gemistocytic appearance of cell bodies and dense staining of glial processes.

Statistical analysis

Data analysis was performed by using SPSS version 12.0.1 for Windows (SPSS, Inc., Chicago, IL, USA). Patient demographics were compared between groups using Student’s t-tests and Chi-squared tests, where appropriate. Neuropathological and quantitative MRI measures were compared between patient groups (Alzheimer’s disease and non-demented controls) and tissue types (WMH and NAWM). Quantitative variables of the QMRI (T1, FA and ADC) and neuropathological (TmLFB and TmBodian) measures were compared using linear mixed model analyses, accounting for the nested design where several brain slices were selected from each subject and one or more ROIs were drawn on each brain slice. For these analyses, the quantitative neuropathological and MRI measures were entered as the dependent variables, while the patient groups (Alzheimer’s disease versus controls) and tissue types (WMH versus NAWM) were the independent variables, and variables for both patient and slice were entered as repeated measures. Semi-quantitative neuropathological scorings (HLA-DR scores and GFAP scores) were evaluated between groups (NAWM controls, WMH controls, NAWM Alzheimer’s disease patients, and WMH Alzheimer’s disease patients) using Kruskal–Wallis tests.
and post hoc Mann–Whitney U-tests. To illustrate relationships between neuropathological measures mutually and with QMRI variables, univariate associations were assessed using Pearson’s correlation coefficients. Subsequently, to assess which neuropathological measures determined the QMRI parameters in Alzheimer’s disease patients, linear mixed model analyses were used. The QMRI parameters were the dependent variables (T1, FA and ADC) and the neuropathological measures (TmLFB, TmBodian, HLA-DR score and GFAP-score) the independent variables, whereas variables for both patient and slice were entered as repeated measures. Standardized $\beta$s are reported. First, all analyses were performed for each neuropathological measure separately, corrected for age, gender and fixation time. Subsequently, a stepwise backward model was performed, in which first all neuropathological measures and covariates (age, gender, fixation time) were entered, and the neuropathological measures with the highest $P$-value were subsequently excluded until only significant predictors remained in the model.

**Results**

**Cases**

Thirty-three formalin-fixed, coronal hemispheric brain slices from 11 Alzheimer’s disease patients (mean age: 83 ± 10 years,
eight females, mean Braak stage: 5, post-mortem delay (h:min): 6:09/3:05) and 15 hemispheric slices from seven controls (mean age: 78 ± 10 years, four females, mean Braak stage: 1, post-mortem delay: 5 ± 24 h) were selected. The groups were age- and gender-matched. At slice level, the fixation time for T1-relaxation time measurements and neuropathological measures were comparable [fixation time (months): 2.6 ± 1.2 for controls versus 2.3 ± 0.9 for Alzheimer’s disease patients]. The fixation time for DTI scanning, however, was slightly longer in brain slices of control subjects than Alzheimer’s disease patients, because some slices were rescanned due to initial imaging artifacts [fixation time (months): 4.4 ± 0.4 for controls versus 3.1 ± 1.1 for Alzheimer’s disease patients, P < 0.001].

Quantitative MRI
To differentiate tissue characteristics of WMH and NAWM between Alzheimer’s disease patients and controls, the results of the QMRI parameters are listed per patient group and tissue type in Table 2 and illustrated as boxplots in Fig. 3. Linear mixed models showed main effects for tissue type, as more axonal loss (TmBodian) and myelin loss (TmLFB) were found in WMH compared to NAWM of both patient groups together. However, for axonal loss (TmBodian) and myelin loss (TmLFB), there was no main effect for patient group and there were no significant interaction terms. Higher values of GFAP-scores were observed in WMH compared to NAWM of both patient groups together, indicating more astrogliosis. No differences between Alzheimer’s disease subjects and controls and no significant interaction between patient group and tissue type exist for severity of astrogliosis. Alzheimer’s disease patients showed more severe microglial activation than controls, as represented by higher HLA-DR scores. Moreover, more microglial activation (HLA-DR scores) was observed in the WMH than in the NAWM of Alzheimer’s disease patients, whereas this effect was not found in controls. Furthermore, a higher proportion of macrophages as the most predominant HLA-DR positive cell type was found in Alzheimer’s disease subjects compared to controls [N(%): 31(43%) for Alzheimer’s disease versus 1(4%) for controls, P < 0.001]. Moreover, more macrophages were

Neuropathology
Group differences of the neuropathological characteristics are given in Table 2 and are illustrated as bargraphs and boxplots in Fig. 4. Linear mixed models showed main effects for tissue type, as more axonal loss (TmBodian) and myelin loss (TmLFB) were found in WMH compared to NAWM of both patient groups together. However, for axonal loss (TmBodian) and myelin loss (TmLFB), there was no main effect for patient group and there were no significant interaction terms. Higher values of GFAP-scores were observed in WMH compared to NAWM of both patient groups together, indicating more astrogliosis. No differences between Alzheimer’s disease subjects and controls and no significant interaction between patient group and tissue type exist for severity of astrogliosis. Alzheimer’s disease patients showed more severe microglial activation than controls, as represented by higher HLA-DR scores. Moreover, more microglial activation (HLA-DR scores) was observed in the WMH than in the NAWM of Alzheimer’s disease patients, whereas this effect was not found in controls. Furthermore, a higher proportion of macrophages as the most predominant HLA-DR positive cell type was found in Alzheimer’s disease subjects compared to controls [N(%): 31(43%) for Alzheimer’s disease versus 1(4%) for controls, P < 0.001]. Moreover, more macrophages were
Table 2 Quantitative MRI and neuropathological characteristics per patient group and tissue type

<table>
<thead>
<tr>
<th></th>
<th>Controls NAWM</th>
<th>Controls WMH</th>
<th>AD patients NAWM</th>
<th>AD patients WMH</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>QMRI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_1$ (ms)</td>
<td>285 ± 74</td>
<td>398 ± 98</td>
<td>318 ± 47</td>
<td>464 ± 83</td>
<td>0.04</td>
</tr>
<tr>
<td>FA</td>
<td>0.69 ± 0.07</td>
<td>0.57 ± 0.07</td>
<td>0.61 ± 0.11</td>
<td>0.44 ± 0.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ADC (μm²/s)</td>
<td>189 ± 60</td>
<td>203 ± 28</td>
<td>233 ± 59</td>
<td>239 ± 50</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Neuropathology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.98</td>
</tr>
<tr>
<td>TmBodian</td>
<td>147 ± 11</td>
<td>167 ± 15</td>
<td>146 ± 16</td>
<td>161 ± 15</td>
<td>0.38</td>
</tr>
<tr>
<td>TmLFB</td>
<td>126 ± 16</td>
<td>138 ± 15</td>
<td>116 ± 15</td>
<td>139 ± 20</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>GFAP-score</strong></td>
<td>1 (0–2)</td>
<td>2 (1–3)**</td>
<td>1 (0–3)</td>
<td>2 (0–3)**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>HLA-DR score</strong></td>
<td>0 (0–2)</td>
<td>0 (0–1)</td>
<td>1 (0–3)</td>
<td>2 (0–3)*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AD = Alzheimer’s disease; WMH = white matter hyperintensities; NAWM = normal appearing white matter; QMRI = quantitative MRI; FA = fractional anisotropy; ADC = apparent diffusion coefficient; TmBodian = light transmittance Bodian staining, inversely related to axonal density; TmLFB = light transmittance LFB staining, inversely related to myelin density; GFAP-score = severity of astrogliosis, range (0–3); HLA-DR score = severity of microglial activation, range (0–3).

*Values are means (SD); differences between patient groups (AD versus controls), tissue types (WMH versus NAWM) and the interaction between patient group and tissue type were tested using linear mixed models.

Post hoc Mann–Whitney U-tests were used: †$P < 0.001$ for total score AD versus controls; ‡$P < 0.05$, §§$P < 0.01$ and §§§$P < 0.001$ for WMH versus NAWM within patient groups.

Discussion

We found that QMRI techniques reveal differences between WMH of Alzheimer’s disease patients and WMH of controls and reflect the severity of underlying neuropathological changes.

QMRI increases specificity for neuropathological changes within WMH

We hypothesized that the inconsistent and weak relationships between the presence and severity of WMH on conventional $T_2$-weighted MRI and clinical symptoms in both demented and non-demented elderly subjects may be explained by heterogeneity in the neuropathological substrates of WMH. Several post-mortem MRI studies have demonstrated that pathological substrates of WMH are heterogeneous. Punctate WMH corresponded to mild perivascular tissue damage around lipohyalinotic arterioles with large perivascular spaces, atrophic neuropil and slight axonal loss (Fazekas et al., 1991). Confluent areas of WMH, however, reflect severe incomplete ischemic damage with

present in the WMH than in the NAWM of the Alzheimer’s disease group [$N(%)$: 24(55%) in WMH versus 7 (25%) in NAWM, $P = 0.014$], whereas the proportion of macrophages was comparable in both tissue types of controls. As expected, axonal loss (TmBodian) and myelin loss (TmLFB) were partially interrelated (Pearson’s $r = 0.46$, $P < 0.001$). Furthermore, severity of astrogliosis (GFAP-scores) correlated with axonal and myelin loss (Pearson’s $r = 0.25$, $P = 0.047$ for astrogliosis—myelin loss; $r = 0.26$, $P = 0.039$ for astrogliosis—axonal loss), whereas microglial activation (HLA-DR score) was not correlated with any other pathological measure (data not shown).
extensive axonal and myelin loss, reactive astrogliosis and lipohyalinotic arterioles, partly developing towards complete infarctions (Fazekas et al., 1993). More recently, detailed immunohistochemical characterization of WMH in a large prospective neuropathological study (MRC CFAS, 2001) has contributed substantially to our understanding of the pathology and pathogenetic mechanisms of WMH, and has yet again confirmed the heterogeneous nature of WMH histopathology. Differences in microglial responses and the expression of hypoxia-related markers suggest that in the development of a proportion of WMH a hypoxic environment is involved, whereas other WMH are related more to immune activation resulting from disintegration of the ependyma or blood–brain barrier dysfunction (Fernando et al., 2006; Simpson et al., 2007a).

In this study, we focused on QMRI techniques in an attempt to increase MRI specificity for WMH. It has been reasoned, though not verified, that QMRI measurements reflect histopathological changes in aging and dementia (Fernando et al., 2004). Only one previous post-mortem DTI study in demented subjects demonstrated that DTI is feasible in the post-mortem setting and correlated with myelin loss using one conventional myelin stain in two Alzheimer’s disease patients (Englund et al., 2004). In the current study, we demonstrated that, in Alzheimer’s disease, FA reflects the extent of astrogliosis and of myelin and axonal loss, and that the strongest predictor of the FA is axonal loss, whereas the relationships with myelin loss and astrogliosis are largely determined by their correlation with axonal density. \(T_1\)-relaxation times independently reflected microglial activation, axonal loss and myelin loss. The FA was therefore more specific than \(T_1\)-relaxation time measurements for the severity of axonal loss, whereas \(T_1\)-relaxation time reflected a larger array of pathology.

**QMRI demonstrates that WMH tissue change is more severe in Alzheimer’s disease**

Our data confirm the in vivo literature claiming that QMRI techniques show differences between WMH and NAWM (O’Sullivan et al., 2001; Shenkin et al., 2005). We further showed that Alzheimer’s disease patients had lower FA values and longer \(T_1\)-relaxation times than controls. Additionally, we have found that QMRI parameters show differences between WMH of Alzheimer’s disease patients and WMH of controls as the difference in \(T_1\)-relaxation time between WMH and NAWM was larger in Alzheimer’s disease patients than in controls. The \(T_1\)-relaxation time difference between patient groups may be a reflection of the difference in microglia/macrophage activation, as we also found that WMH in Alzheimer’s disease patients had more severe microglial activation than NAWM, whereas in controls no difference was found. These findings are consistent with our correlation data showing that the degree of microglial activation was an independent predictor of \(T_1\)-relaxation time. It is known that microglial activation...
plays a crucial role in Alzheimer’s disease as microglial cells become overactivated when they are engaged in the clearance of amyloid-β (Block et al., 2007). Alternatively, amyloid-β may directly recruit and activate microglial cells as suggested by studies of congophilic angiopathy (Eikelenboom et al., 2008). Microglial activation may subsequently lead to neuronal loss and to damage in the microvasculature (Block et al., 2007). We postulate that the T1-relaxation time prolongation in WMH of Alzheimer’s disease may be an expression of a diffuse inflammatory reaction due to microglial activation and accompanying increase of interstitial fluid due to blood–brain barrier leakage and vascular damage (Vrenken et al., 2006a). More extensive immunohistochemical stainings for inflammatory markers and leakage of blood–brain barrier proteins such as fibrinogen or collagen should be performed to further investigate this hypothesis (Vos et al., 2005). One previous study of the MRC CFAS could not confirm the difference in microglial activation between demented and subjects when using CD68-stainings (Fernando et al., 2006). The use of different antibodies for microglial staining could explain this difference (Block et al., 2007). An alternative explanation is the difference in study design. Our case–control design may reflect two ends of a spectrum and is therefore more likely to demonstrate actual differences, whereas the MRC CFAS reflects an epidemiologically based community sample. One could argue that several other factors such as agonal state or concomitant disease could also play a role, though our brain specimens were carefully controlled for other dementias and Braak stage. Moreover, comparable results were yielded when all analyses were repeated with and without subjects with a severe grade of hypoxic neurons (three Alzheimer’s disease subjects and one control subject; data not shown), which renders it highly likely that our findings are truly disease-specific tissue changes. Other neuropathological differences in WMH such as extensive denudation of the ventricular ependyma, gliosis and more severe axonal loss in Alzheimer’s disease patients as compared to controls were also reported (Scheltens et al., 1995). On the other hand, a recent study using markers for hypoxic injury concluded that WMH in demented and non-demented subjects are comparable and form part of a pathological continuum common to the aged brain (Fernando et al., 2006). In our study using basic measures for neuropathology, we found differences in inflammatory responses and in QMRI parameters that reflect underlying tissue changes between Alzheimer’s disease patients and controls.
Fig. 5  Relationships between quantitative MRI parameters $[T_1$-relaxation time (ms; first column), FA (second column) and ADC ($\mu m^2/s$; third column)] and neuropathological measures $[Tm_{Bodian}$ (higher values represent more axonal loss; first row), $Tm_{LFB}$ (higher values represent more myelin loss; second row), GFAP-score (astroglisis; third row) and HLA-DR score (microglial activation; fourth row)] are illustrated in scatterplots. The coloured dots correspond to white matter hyperintensities (WMH), whereas the open dots represent NAWM. Univariate associations are assessed using Pearson’s correlation coefficient. The scatterplots show that WMH and NAWM have partly overlapping distributions, implying a gradual difference between these tissue types.
Table 3  Neuropathological determinants of QMRI parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>0.72***</td>
<td>0.47***</td>
</tr>
<tr>
<td>TmBodian</td>
<td>0.35**</td>
<td>0.29**</td>
</tr>
<tr>
<td>TmLFB</td>
<td>0.32**</td>
<td>0.37**</td>
</tr>
<tr>
<td>GFAP-score</td>
<td>0.37**</td>
<td>0.35***</td>
</tr>
<tr>
<td>HLA-DR score</td>
<td>0.37**</td>
<td>0.35***</td>
</tr>
<tr>
<td>FA</td>
<td>-0.62***</td>
<td>-0.62***</td>
</tr>
<tr>
<td>TmBodian</td>
<td>0.13</td>
<td>0.002</td>
</tr>
<tr>
<td>TmLFB</td>
<td>0.08</td>
<td>-0.18</td>
</tr>
<tr>
<td>GFAP-score</td>
<td>0.08</td>
<td>-0.18</td>
</tr>
<tr>
<td>HLA-DR score</td>
<td>0.08</td>
<td>-0.18</td>
</tr>
</tbody>
</table>

Values are standardized βs, estimated with linear mixed models. The dependent variables were T1 (T1-relaxation time), FA (fractional anisotropy) and ADC (apparent diffusion coefficient) and the neuropathological measures were the independent variables. TmBodian = light transmittance Bodian staining, inversely related to axonal density; TmLFB = light transmittance LFB staining, inversely related to myelin density. Model I: each neuropathological measure was separately entered, adjusted for age, gender and fixation time. Model 2: stepwise backward model: first the covariates (age, gender and fixation time) were entered. Subsequently the neuropathological measures were included in a stepwise backward manner.

**P < 0.01; ***P < 0.001.

Periventricular versus deep WMH

Previous literature reported that PVL and DWMH areas differ with respect to several pathological measures (Fazekas et al., 1993; Fernando et al., 2006; Simpson et al., 2007b). We have performed comparison of QMRI and neuropathological measures between DWMH and PVL in our study (data not shown), but we did not find consistent differences and therefore pooled them into 'WMH' for further analyses. An explanation for the fact that we did not find differences between PVL and DWMH may lie in our definition of WMH. We only included WMH when significant PVL and/or (beginning) confluent DWMH were present. Whereas rimlike PVL or smooth halos and DWMH have been found to have different pathological correlates, we are probably comparing irregular large PVL with confluent DWMH, that have been described to be pathologically comparable (Fazekas et al., 1993).

Normal appearing white matter

We have predominantly focused on changes that are visible on T2-weighted MRI, but it may be possible that pathological changes in the NAWM, i.e. WM that appears normal on MRI, also contribute to the clinical correlates of WMH (Bronge et al., 2002; O’Sullivan et al., 2004).

Although our study could not adequately assess pathological and QMRI characteristics of NAWM, as this would have necessitated inclusion of WM from non-diseased control brains, our data suggest that a fundamental segregation between WMH and NAWM could not be made on the basis of histopathology and QMRI data. We postulate that a dichotomization of the white matter into WMH and NAWM may be arbitrary and even inappropriate, as our correlation data between QMRI and histopathological measures suggest that a continuum may exist from neuropathologically normal to abnormal white matter. These changes could therefore also partly explain the weakness of the clinico-radiological correlation. Our findings should be reproduced or confirmed in studies that also include NAWM of non-lesional brains as this tissue may be pathologically different from NAWM of lesional brains and could therefore be separate from the suggested continuum in pathological changes.

Methodological considerations and recommendations for future study

In our study, the ADC was not correlated to any of the neuropathological measures and was not able to reveal differences between patient groups or tissue types. Possibly, the use of formalin-fixed brain specimens influenced the ADC, as death and fixation changes the cellular structure by dehydration, change in temperature, failure of energy dependent ion transport, lactate acidosis and formation of cross links. It has been shown that diffusivity, which largely depends on the free mobility of water molecules, decreases after death and declines further with longer fixation times (Pfefferbaum et al., 2004; Schmierer et al., 2008). On the contrary, FA was found not to be influenced by formalin fixation (Schmierer et al., 2008). Formalin fixation also shortens relaxation times, and post-mortem MRI values are therefore not directly comparable to in vivo MRI values. However, translation to clinically relevant values could still be achieved using formalin-fixed material (Schmierer et al., 2008). As it has been described that relaxation times stabilize after 3–4 weeks of fixation (Pfefferbaum et al., 2004), each brain specimen in this study was fixed for at least 4 weeks to minimize the variability of fixation effects within the study group. Additionally, linear mixed model analyses were used to correct for any remaining effects of fixation time. Furthermore, post-mortem delay until tissue fixation could be an other factor that causes structural damage due to autolysis and influences the brain’s diffusion properties (D’Arceuil and de Crespigny, 2007). Future analyses should therefore consider sampling fresh, unfixed tissue with a short post-mortem delay when studying post-mortem MRI characteristics of ageing and dementia. In addition, as the resolution of our FA and ADC maps were lower than the FLAIR and T1, which may have rendered it more difficult to match the ROIs with the stained sections. To avoid possible misclassification, we used FLAIR images...
with higher resolution for the definition of the ROIs and copied the ROIs to the QMRI-maps. We further took care to have at least a rim of WMH around each WMH ROI and to draw NAWM ROIs in areas at a distance of signal hyperintensities.

**Understanding the clinical impact of WMH**

DTI and \( T_1 \)-relaxation time measurements were shown to be more specific than conventional \( T_2 \)-weighted MRI, as these techniques revealed differences between WMH of demented and WMH of non-demented subjects, and reflected the severity of the underlying neuropathological changes. Our findings should be reproduced in the in vivo setting, in order to determine the clinical correlates of WMH changes measured with QMRI in demented and non-demented subjects (O’Sullivan et al., 2004; Shenkin et al., 2005). Hence, these studies should lead to a better understanding of the clinical impact of WMH in demented and non-demented elderly.

**Acknowledgements**

We would like to acknowledge M. Cornella, M. Jacobs, A. Sanders and E. van Haastert for their help with (immuno)histochemical stainings and Dr P.J.W. Pouwels for her assistance with the development of the MRI protocol. J.J.G.G. and H.V. are partly financially supported by the Dutch MS Research Foundation through a program grant (Grant no 05-358c).

**References**


