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Quantifying multiple sclerosis pathology in *post mortem* spinal cord using MRI

K. Schmierer [a,b,*], A. McDowell [c], N. Petrova [a], D. Carassiti [a], D.L. Thomas [d], M.E. Miquel [e]

[a] Queen Mary University of London, Barts and The London School of Medicine & Dentistry, Blizard Institute (Neuroscience), London, UK
[b] Barts Health NHS Trust, Clinical Board Medicine (Neuroscience), The Royal London Hospital, London, UK
[c] UCL Great Ormond Street Institute of Child Health, Developmental Imaging and Biophysics Section, London, UK
[d] UCL Institute of Neurology, Leonard Wolfson Experimental Neurology Centre, Department of Brain Repair and Rehabilitation, Queen Square, London, UK
[e] Barts Health NHS Trust, Clinical Physics, London, UK

A B S T R A C T

Multiple sclerosis (MS) is a common inflammatory, demyelinating and degenerative disease of the central nervous system. The majority of people with MS present with symptoms due to spinal cord damage, and in more advanced MS a clinical syndrome resembling that of progressive myelopathy is not uncommon. Significant efforts have been undertaken to predict MS-related disability based on short-term observations, for example, the spinal cord cross-sectional area measured using MRI. The histo-pathological correlates of spinal cord MRI changes in MS are incompletely understood, however a surge of interest in tissue microstructure has recently led to new approaches to improve the precision with which MRI indices relate to underlying tissue features, such as myelin content, neurite density and orientation, among others. Quantitative MRI techniques including T_1 and T_2, magnetisation transfer (MT) and a number of diffusion-derived indices have all been successfully applied to *post mortem* MS spinal cord. Combining advanced quantification of histological features with quantitative - particularly diffusion-based - MRI techniques provide a new platform for high-quality MR/pathology data generation. To more accurately quantify grey matter pathology in the MS spinal cord, a key driver of physical disability in advanced MS, remains an important challenge of microstructural imaging.

Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating and degenerative disease of the central nervous system (CNS). In the Northern hemisphere, MS is the most common non-traumatic cause of chronic disability in young adults (Mackenzie et al., 2014), and there is currently no cure. Evidence suggests MS is a complex condition associated with a number of immune-associated genes and environmental elements which contribute to the risk of developing the immune response that in turn leads to the extensive CNS tissue damage characteristic of the disease (Compston and Coles, 2008; Ramagopalan et al., 2010; Lemus et al., 2018).

For decades, though largely supported by circumstantial evidence based on animal models (Baker and Amor, 2014), MS has been considered to be a T cell-mediated autoimmune disease (Martin et al., 2016). However, the recent success of B cell targeted therapies (Montalban et al., 2017; Hauser et al., 2017; Hawker et al., 2009), and new insights into the effects on MS disease activity of B cell, particularly $CD19^+/CD27^+$ memory B cell, depletion have fundamentally shifted our understanding of MS pathophysiology and created a unifying concept consistent with therapeutic, histopathological and aetiological aspects of MS (Baker et al., 2017).

The spinal cord is heavily involved in MS. Most people with MS (pwMS) present with symptoms indicative of cord damage (Katz Sand and Ilana, 2015). Spinal cord lesions characteristic of demyelination predict (i) development of MS in people with radiologically isolated (i.e. asymptomatic) and (ii) clinically isolated syndromes (CIS) of demyelination (Okuda et al., 2011) (Arrambide et al., 2017), as well as (iii) poor disability outcomes in people with CIS (Brownlee et al., 2017) (Arrambide et al., 2017). At more advanced stages the clinical syndrome of pwMS often resembles that of progressive myelopathy (Kremenchutzky et al., 2006; McDonald and Compston, 2006; Kearney et al., 2015; Giovannoni et al., 2017/2).

Given the importance of spinal cord pathology for chronic disease deterioration, and the emphasis on ambulation of the most commonly used clinical assessment tool, the expanded disability status scale (EDSS) (Kurtzke, 1983), significant efforts have been undertaken to predict MS-related disability based on short-term observations such as the spinal cord cross-sectional area (CSA) measured *in vivo* using MRI (Losseff et al., 1996).

However, whilst several teams have reported on the histological substrates of MRI in MS *post mortem* brain (Newcombe et al., 1991) (Bö

* Corresponding author. Blizard Institute (Neuroscience), Queen Mary University of London, 4 Newark Street, London E1 2AT, UK.
E-mail address: k.schmierer@qmul.ac.uk (K. Schmierer).

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et al., 2004) (Moore and Laule, 2012), relatively few studies have applied this approach to explore microstructural changes in the spinal cord (Nijeholt et al., 2001; Bergers et al., 2002a,b; Mottershead et al., 2003). The paucity of such studies is unlikely to be due to a lesser importance of the spinal cord pathology for the disability pwMS develop, but rather to the difficulties associated with obtaining good quality human *post mortem* MS spinal cord (Petrova et al., 2017), which requires extensive use of autopsy chisel and saw to remove the cord tissue intact ("Dissection: Deep Back & Spinal Cord - Anatomy Guy" 2010).

Combining MRI with histology measures enables investigation of fundamental associations between tissue compartments and structure with clinical relevance, such as the grey and white matter (Schlaeger et al., 2014), and related cell types affected by disease, for example the degree of tissue loss measured using volumetric MRI (Losseff et al., 1996), and the microscopic changes underlying this loss. Evidence suggests the assumption that results obtained using brain samples are directly applicable to the spinal cord can be misleading (McDowell et al., 2014). Here, we therefore review *post mortem* MRI studies of the MS spinal cord with reference to *in vivo* MRI, and to histology studies without MRI correlation, as appropriate.

MS lesion detection and grey-white matter contrast

The first reported study of an MS spinal cord combining MRI with histopathology was the case of a 37 year old woman who died with severe MS-related disability (Nagao et al., 1994). *Post mortem* axial T_2-weighted (T_2W) spin echo MRI was acquired at 1.5T. Though not evident from the report, the published figures suggest scans of the cord and brainstem were acquired on the excised tissue. Areas of high signal intensity in the cervical proportion were demonstrated and on visual inspection broadly match the histopathological sections stained for myelin thereby confirming that high signal on T_2W MRI may correspond to areas of demyelination.

In 2001, Nijeholt and co-workers examined a series of 19 formalin-fixed *post mortem* MS spinal cords donated by pwMS who had all had a deteriorating ("progressive") disease course, along with three cords from control subjects. A total of n = 59 spinal cord specimens were examined using proton density weighted (PDW) MRI optimised at two different field strengths (1T and 4.7T) followed by correlation with histology in 10 MS cases (Nijeholt et al., 2001). On visual inspection of both imaging techniques (MRI, histology) in parallel, all histologically abnormal areas corresponded to hyperintensities on MRI at both field strengths (an example for 1T is shown in Fig. 1). However, scans acquired at 4.7T enabled distinction between clearly demarcated lesions and rather diffuse changes, possibly reflecting Wallerian degeneration remote from the acute axonal transection within lesions (Trapp et al., 1998; Nijeholt et al., 2001; Dziedzic et al., 2010).

The usefulness of PDW images to investigate *post mortem* tissue had previously been examined in the brain with a focus on the distinction between grey and white matter. Blamire and co-workers measured the grey/white matter contrast on PD weighted MRI of three human *post mortem* samples imaged at 2T (Blamire et al., 1999). Samples were examined in an unfixed condition, and then weekly for five weeks following formalin fixation. Regions of interest were placed in the thalamus, white matter tracts, and the neocortex. The grey/white matter ratio dropped rapidly with the largest change during week 1 reaching a plateau by week 5. By the time Blamire and colleagues published their findings, accurately separating grey and white matter using MRI had become an important challenge following the histological re-discovery of widespread demyelination of not only the white but also the grey matter in MS (Kidd et al., 1999). Whilst both the study by Blamire, et al. and Kidd, et al. were undertaken on brain samples, they evidently informed subsequent work on the spinal cord (McDowell et al., 2014).

Albeit incompletely understood, the effect of fixation on MR signal and contrast is not negligible. The fixative formaldehyde solutions used appear to react with functional groups of macromolecules forming crosslinks. These intra- and intermolecular crosslinks result in altered tissue characteristics, which can be described as a gel that retains the cellular constituents in their topographic relationships. Physical distortion of tissue as a result of fixation may occur, however, and shrinkage by up to 19% using 10% formalin solution has been reported (Schmierer et al., 2008).

Studies on MS brain tissue exploring the quantitative effects of fixation on MR indices, including relaxation times, magnetisation transfer

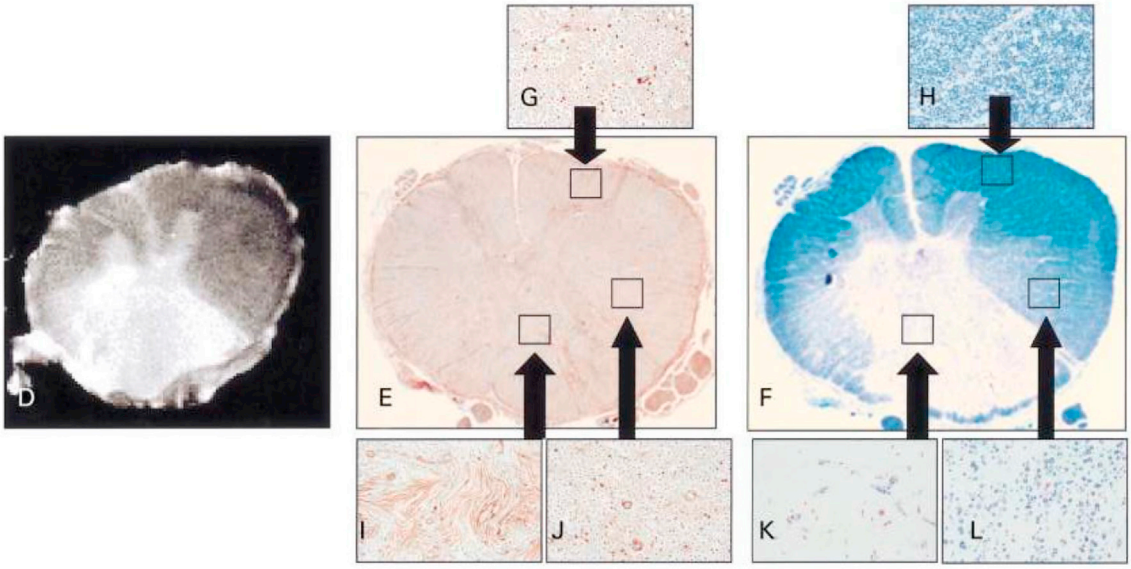

Fig. 1. MRI at 1T, and corresponding histological sections, of an MS cervical spinal cord. The patient died at the age of 56 years with a history of progressive MS for 12 years. **D**. PDW image (3 mm slice, 1 mm resolution, 10 min s acquisition) and corresponding histology sections stained for axons (Bodian stain) **E, G, I, J** and myelin (luxol fast blue) **F, H, K, L**. Histology shows areas of complete demyelination **K** corresponding to high signal on MRI **D**. The convoluted fibres in **I** correspond to significant gliosis. Areas of non-lesional white matter (NLWM; **G & H)** can be separated from areas with a lesser degree of demyelination (**J & L**) corresponding with less pronounced high signal on MRI. Axons in NLWM appear more preserved than in areas of demyelination (**J versus G**). Reproduced from (Nijeholt et al., 2001) with permission.

(MT) and diffusion reported that, except for fractional anisotropy, formalin fixation significantly impacted upon all MR indices beyond *post mortem* changes *per se* (Schmierer et al., 2008, 2010). Whilst these changes did not significantly alter the contrast between white matter lesions and non-lesional white matter (NLWM), a study using 7T MRI reported a decrease of the difference in T_1 (and hence contrast) between NLWM and grey matter, as well as between grey matter and grey matter lesions (Bainbridge et al., 2004).

The first correlative MR/pathology study focusing on lesion pathology in the spinal cord grey matter was reported by Gilmore and co-workers (Gilmore et al., 2009a,b). Using a 4.7T system, PDW images were acquired of formalin-fixed spinal cord samples of 11 pwMS and two controls obtained from the cervical and upper thoracic level. After MRI, specimens were dissected and immuno-stained for myelin basic protein. A total of n = 40 'white matter only' lesions, 55 mixed white/grey matter lesions, and a single 'grey matter only' lesion were detected on PDW scans. Separating the white and grey matter proportions of the mixed lesions, the authors reported that 87% of histologically confirmed areas of white matter demyelination and 73% of histologically confirmed areas of grey matter demyelination were detected on MRI, a significantly better result compared to the brain where the yield of detected lesions in the neocortex remains rather more challenging (Geurts et al., 2011). Gilmore, et al. identified two main reasons for this difference in detectability between cortical and spinal cord grey matter demyelination: (i) rather more pronounced partial volume effects in the neocortex and (ii) significantly more straightforward registration between histopathological sections and corresponding MRI in spinal cord samples (Gilmore et al., 2009a,b). Interestingly, the previous study by Mottershead and co-workers applying quantitative MRI (magnetisation transfer (MT), diffusion) to four *post mortem* spinal cord samples (three MS, one control) using a 7T small bore system did not include assessment and/or discussion of grey matter pathology, despite grey matter demyelination being clearly observable in the presented images (Mottershead et al., 2003).

Although inflammation and demyelination are important aspects throughout the course of MS, degeneration and loss of chronically demyelinated axons has been considered the major pathological correlate of chronic disease deterioration (Bjartmar et al., 2000), possibly, at least in part, independent of the inflammatory 'penumbra' of MS lesions (DeLuca et al., 2006; Dziedzic et al., 2010). Whilst in their 2001 study, Nijeholt and co-workers showed that high signal intensity on T_2W MRI closely maps onto the extent of demyelination (Nijeholt et al., 2001), in a subsequent study the same group also concluded that axonal loss in the MS spinal cord may be relatively independent of demyelination (Bergers et al., 2002a,b). This conclusion was based on an MRI/pathology study focusing on the association between axonal damage and signal abnormalities on T_2W high-resolution MRI. Axial scans of *post mortem* spinal cord specimens were obtained at 4.7 T from nine pwMS and four controls, and signal intensity scored as normal appearing cord tissue (NACT), mildly increased (signal intensity lower than grey, however above NACT), or high (above grey matter intensity). Histological sections were stained for neurofilaments and myelin. Results show that (i) in pwMS, areas with high signal correlate with the lowest axonal density, highest axonal irregularity and largest axonal diameter compared to NACT and (ii) compared to controls, axonal density was lower and axons of larger diameter in NACT. Whilst there clearly appeared to be some degree of association, the authors concluded the degree of signal abnormality on high resolution T_2W MRI is not a strong predictor of axonal loss (Bergers et al., 2002a,b). Crucially, their study was based on cross-sectional analysis of specimens without reporting on remote effects of lesions on axonal preservation and loss. As recently shown, ruling out lesions above and below the index lesion is key to drawing robust conclusions about the effect of lesions on axonal loss (Petrova et al., 2017).

Correlates of spinal cord volume changes

Irrespective of the abovementioned methodological concerns, limitations in (i) the association between demyelinating lesions and axonal loss and (ii) lesion detection in the MS spinal cord due to, among others, flow and motion artefacts highlighted the need for alternative indices with potential to be more robust predictors of axonal damage and loss. Since the early study by Losseff and coworkers over 20 years ago (Losseff et al., 1996) numerous clinical studies confirmed association between reduction of the spinal cord cross-sectional area (CSA) and disability and - by inference - axonal degeneration and loss (Losseff et al., 1996; Kearney et al., 2015; Aymerich et al., 2017) in pwMS, and CSA loss has been applied as an outcome in a small number of clinical trials (Kapoor et al., 2010; Rice et al., 2015).

A number of methods have been used to measure CSA including semi-automated edge finding (Lin et al., 2003), edge detection with partial volume corrections (Tench et al., 2005), voxelwise mapping (Rocca et al., 2013), an active surface model (Kearney et al., 2014) and semi-automated cord volume estimation techniques (Lukas et al., 2015). Spinal cord MRI is less commonly applied than brain imaging. This is likely due to the inherent technical challenges in imaging this small, mobile structure. The spinal cord CSA *in vivo* is approximately 90 mm^2 at the C_2 level, and the cord is surrounded by proportionately more bone, fat and cerebrospinal fluid than the brain. Moreover, spinal cord MRI can be affected by motion artefacts including breathing, cardiac motion, CSF pulsation and bulk participant motion (Kearney et al., 2015).

The relationship between CSA and nerve fibre loss had never been systematically investigated using *post mortem* spinal cord tissue. Given some of the reported associations between CSA and disability have been rather moderate (Schlaeger et al., 2014; Aymerich et al., 2017) there was a need for pathological validation of the tissue changes underlying CSA shrinkage and - by inference - disability.

Following preliminary work by Bjartmar and co-workers (Bjartmar et al., 2000), a recent study extensively sampled spinal cords of 13 pwMS with a mean disease duration of 29 years, and five healthy controls to quantify axonal density and its association with demyelination and CSA (Petrova et al., 2017). 396 tissue blocks were embedded in paraffin and immuno-stained for myelin basic protein and phosphorylated neurofilaments. Reduction in CSA was detected at all (cervical, thoracic and lumbar) levels ranging between 19% and 24%. White (19–24%) and grey (17–21%) matter atrophy contributed equally across levels. Axonal density in MS was lower by 57–62% across all levels. Demyelination affected 24–48% of the grey matter and 11–13% of the white matter, with no significant differences across levels. Disease duration was associated with reduced axonal density, however not with any area index, including the CSA (Bjartmar et al., 2000; Petrova et al., 2017). By carefully excluding lesions in adjacent areas above and below focal index lesions, significant association emerged between focal demyelination and loss of axons: axonal density within and below cortico-spinal tract lesions was lower by 51% and 42%, respectively, compared to the density measured above lesions ($p < 0.001$) (Petrova et al., 2017) (Fig. 2).

If CSA does not correlate with axonal density, other factors need to be considered. The expected degree of atrophy based on axonal loss could be offset by "space filling" tissue components such as oedema (unlikely in *post mortem* tissue), inflammation (typically not observed to any significant degree) and particularly gliosis, given it counteracts the space reducing effect of nerve fibre loss (Bjartmar et al., 2000; Hampton David et al., 2013). As suggested by the contribution of grey matter area reduction to the overall loss of CSA, it is unlikely that tract systems are exclusively contributing to CSA changes. Neuronal shrinkage, loss of neurite orientation dispersion (Grussu et al., 2017) (see NODDI method, described below) and a degree of spinal cord neuronal loss are further candidates potentially contributing to both chronic disability and CSA loss (Gilmore et al., 2009a,b; Schirmer et al., 2009). Finally, preliminary results indicate loss of synaptophysin affecting both the non-lesional as well as lesional spinal cord grey matter. Depending on the index used, this loss exceeded 90% compared to controls, and correlated moderately with grey matter area (Petrova et al., 2016).

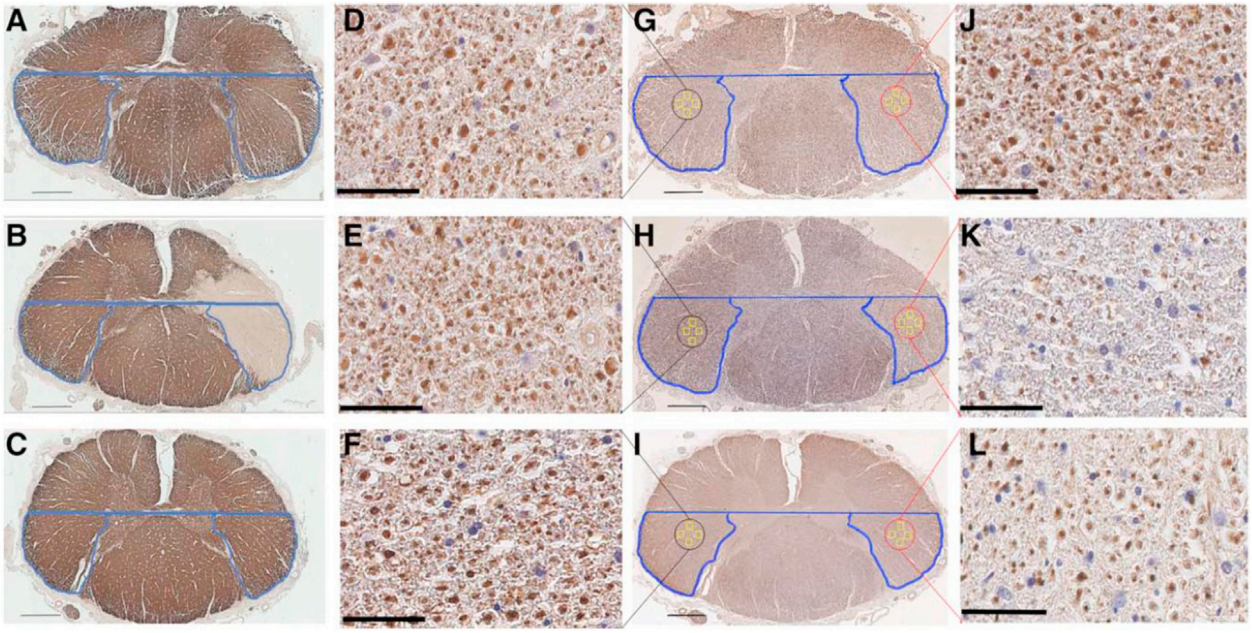

Fig. 2. Focal demyelination and axonal loss in the spinal cord cortico-spinal tract (CST). Myelin basic protein (MBP) stained sections showing the non-lesional CSTs on the blocks directly above **A** and below **C** the isolated lesion, as well as the demyelinated CST on the level of the plaque **B**. Sequential sections immunostained with SMI-31 (for axons) are shown for the three levels in G to I, respectively, in low magnification. Red circles show the CST boundaries on the homotopic, ipsilateral side of the lesion, while black circles show the CST boundaries on the contralateral, functionally identical, side of the lesion. The four yellow squares in each CST represent the four counting fields cast in each CST as described in the methods and Fig. 2, where axonal density was counted. Scale bar: 1 mm. Examples of SMI stained images from one of the four counting fields on the contralateral side of the lesion are shown on high magnification in **D, E** and **F** for each of the three regions. **K** shows one example counting field from the lesional CST, while **J** and **L** correspond to the counting field examples directly above and below the lesional CST respectively, on the ipsilateral side of the lesion. Scale bar: 50 μm. Reproduced from (Petrova et al., 2017) with permission.

Quantifying microstructure in the *post mortem* MS spinal cord using MRI and histology

Due to the nonspecific nature of PD and T_2 weighted MRI for the pathological substrates of MS, and the above described limitations of volumetric/area measurements, a range of quantitative MR techniques including MT (Lema et al., 2017), diffusion (Cohen et al., 2017; Stikov et al., 2015) and spectroscopic metabolite concentration (though not in *post mortem* samples) have been explored to improve depiction and quantification of MS-related microstructural changes in the spinal cord (Gass et al., 2015).

MT is the process by which macromolecules and closely associated "bound water" molecules cross-relax with "free water" protons. Changes in the underlying macromolecular microstructure can be inferred and quantified from changes in MT (Tozer et al., 2003). Several indices of MT have been investigated quite comprehensively in *post mortem* MS brain (van Waesberghe et al., 1999; Barkhof et al., 2003; Schmierer et al., 2004; Schmierer, et al., 2007a,b), and appeared most strongly influenced by myelin content, though axonal damage and loss (Petzold, et al., 2011), and free water content due to inflammation and oedema may also play a role (Vavasour et al., 2011).

Two *post mortem* studies explored the potential of MT in the spinal cord. In 2004, Bot and co-workers examined changes in T_1, T_2, MT ratio (MTR) and CSA in *post mortem* cervical cord samples of 11 pwMS and two controls (Bot et al., 2004). Formalin fixed specimens were examined at 4.7T, and the tissue subsequently processed and stained for myelin (Klüver) and axons (phosphorylated neurofilaments). The quantitative MR indices obtained revealed a 30% increase of T_1 in MS specimens ($p < 0.001$), a mean 13% increase of T_2 ($p < 0.001$), and MS specimens showed, on average, lower MTR with a reduction of 10.5% compared with MTR in control specimens ($p < 0.001$). With increasing demyelination in the MS cases, T_1 and T_2 relaxation times increased and MTR values decreased ($p < 0.001$ for all); with decreasing cord area, a steady decrease in MTR values was measured. A decreasing number of axons was associated with an increase in T_1 and T_2, and a decrease in MTR ($p < 0.01$). These findings were all in line with expectation, except that T_2, rather than MTR, was found to be the strongest predictor of myelin content (Bot et al., 2004). Effects of sample selection and tissue fixation may have impacted on this result since the magnitude of change is quite different between T_2 and both MTR and (inversely related) T_1, at least in *post mortem* MS brain (Schmierer et al., 2008). Nevertheless, the study by Bot and co-workers can be considered a landmark for its comprehensive approach to validation of multiple quantitative MRI techniques in one reasonably large sample.

The only other MR/pathology study of the MS spinal cord to investigate MTR, among other quantitative indices, was reported by Mottershead and coworkers in 2003 (Mottershead et al., 2003). In this study, three unfixed MS spinal cord specimens alongside specimens from the cervical, thoracic and lumbar cord of one control were collected. Quantitative MR indices obtained include T_1, T_2, MTR and diffusion; experiments were undertaken on a small bore 7T system providing up to 117 mm^2 *in plane* resolution thereby allowing for excellent anatomical detail (Fig. 3). After scanning, tissue was fixed and processed for embedding in paraffin, and sections stained for axons and myelin. The authors reported moderate to strong association between (i) myelin content and axonal density and (ii) MTR, T_1 and PD ($p < 0.001$ in all cases). All indices correlated less strongly with T_2, and there was moderate correlation of cellularity with T_1 ($p = 0.001$). There was also strong association between myelin content and axonal density ($r = 0.67$, $p < 0.0001$). Given tissue used in this study had not been formalin or otherwise fixed (Schmierer et al., 2008), MR indices remained closer to *in vivo* values (Bainbridge et al., 2004).

The study by Mottershead, et al. also reported diffusion MRI data. Images were obtained at two different diffusion gradient strengths, and the diffusion standard deviation index (SDI), a measure of anisotropy, was calculated. Moderate correlation was detected between SDI and

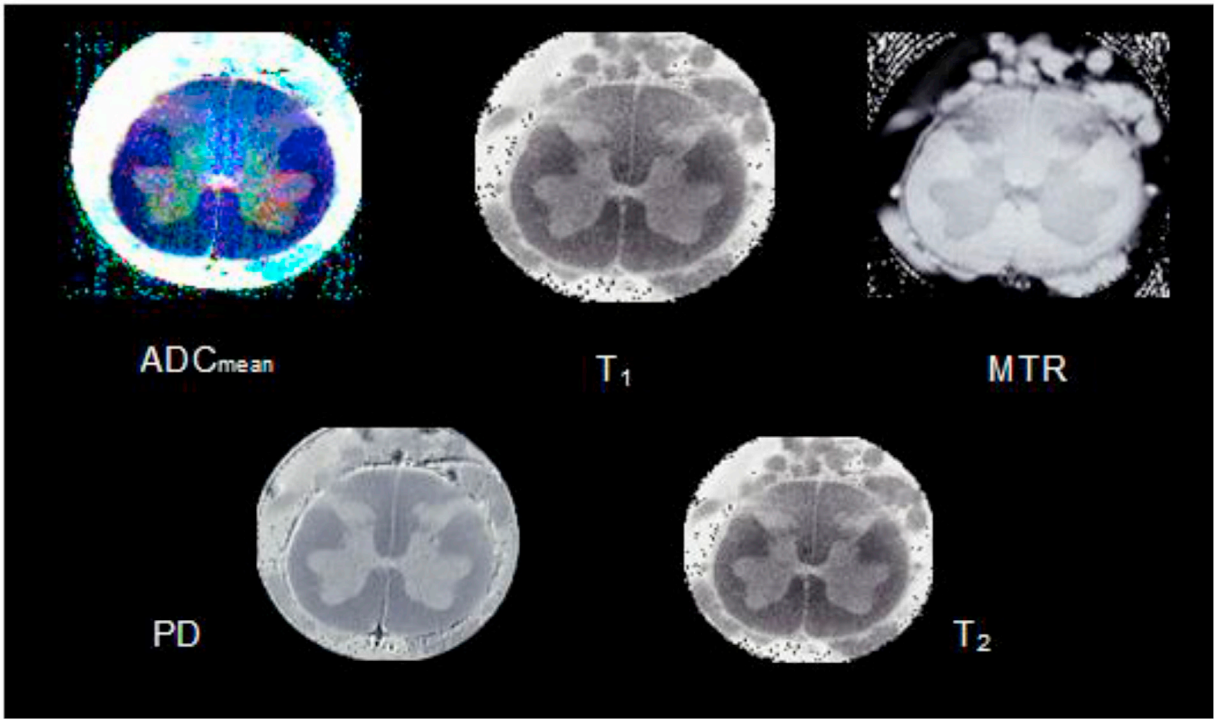

Fig. 3. MRI measures in lumbar spinal cord of a healthy subject (ADC mean = mean apparent diffusion coefficient). Reproduced from (Mottershead et al., 2003) with permission.

axonal count ($r = 0.61$, $p < 0.001$) as well as myelin content ($r = 0.51$, $p < 0.001$). The apparent lack of specificity of diffusion indices for either of these two tissue features (myelin, axons) in *post mortem* MS tissue was subsequently also reported using diffusion tensor imaging (DTI) on a clinical 1.5T MR system. Testing the hypothesis that mean (MD), axial (D_{ax}) and radial diffusivity (D_{rad}), and fractional anisotropy (FA), enable distinction between demyelination and axonal loss, our studies reported in 2007 and 2008 of DTI revealed essentially "negative" results: D_{ax}, D_{rad},and FA all turned out to be more strongly associated with demyelination rather than axonal loss (Schmierer et al., 2007b, 2008). However, these experiments were undertaken at relatively low field strength using brain (rather than spinal cord) samples, and a limited number of diffusion directions thereby potentially reducing the extraction of sufficient directional information.

Klawiter and co-workers applied DTI to *post mortem* MS spinal cord from nine pwMS and five control subjects using a 4.7T system (Klawiter et al., 2011). They placed regions of interest in areas semi-quantitatively graded as normally myelinated, mildly (<50%) and moderate-severely (>50%) demyelinated. Increasing D_{rad} values were associated with the degree of demyelination but so was the extent of axonal loss, whilst D_{ax}, radial diffusivity and relative anisotropy did not predict axonal density *in isolation*. Analysis of myelin and axonal count simultaneously indicated that both tissue features contributed independently to changes in radial diffusivity, relative anisotropy and MD.

Whilst much expectation had been pinned on single diffusion tensor models, the above evidence suggests these models do not allow accurate discrimination between key microstructural components of tissue damage in MS (demyelination and axonal loss, as described, but also inflammation and oedema). The combination of multiple diffusion tensors may account better for the different diffusion properties of tissue components. This has recently been been addressed through introduction of diffusion basis spectrum imaging (DBSI) (Wang et al., 2011). DBSI models myelinated and unmyelinated axons as anisotropic diffusion tensors, and cells and oedema/extracellular space as isotropic diffusion tensors. Quantitative histological analysis of *post mortem* MS cervical spinal cord specimens (n = 3) suggested that DBSI-determined indices of cellularity, axon and myelin acquired on a small bore 4.7T magnet are closely associated with those pathologies identified and quantified by conventional histology (Wang et al., 2015).

Another promising diffusion-based approach to increase specificity for the MS damage encountered by tissue components is neurite orientation dispersion and density imaging (NODDI) (Zhang et al., 2012; Jespersen et al., 2012). NODDI combines a three-compartment tissue model with a multi-shell high-angular-resolution diffusion imaging protocol. An index of orientation dispersion is defined to characterize angular variation of neurites. After some promising *in vivo* work in the spinal cord (Grussu et al., 2017; By et al., 2017), NODDI has recently undergone preliminary *post mortem* validation using spinal cord samples of two people with MS, and two controls (Grussu et al., 2017). Particularly strong association was detected between a quantitative histology index called "circular variance" (CV) (Grussu et al., 2016) and the NODDI derived variable "orientation dispersion index" (ODI), suggesting the latter may serve as a non-invasive biomarker of the former. CV and ODI also showed a corresponding pattern of grey-white matter contrast. Of interest, their histological index of "neuronal element density" (axons, dendrites, neuronal cell bodies) failed to match the quality of CV and ODI in terms of grey-white matter contrast, and did not correlate as well with any NODDI index, presumably owing to the variation in baseline neurite density and myelin content. Comparison with more conventional DTI metrics such as MD, FA, D_{ax} and D_{rad} underpinned that NODDI may enable more precise estimates of the microstructural complexity of dendrites and axons (Grussu et al., 2017). A further aspect highlighted in this recent work is the use of several histological (immuno) staining techniques, such as glial fibrillary acidic protein (for gliosis) and ionized calcium-binding adapter molecule 1 antigen (for microglia) to more comprehensively explore relationships between specific tissue changes and NODDI indices rather than solely focussing on myelin and neurites (Grussu et al., 2017).

Building on prior validation studies in MS brain tissue (Moore et al., 2000; Laule et al., 2006), McDowell et al. recently explored the potential of multi-component relaxometry in the spinal cord to quantify myelin, particularly in the grey matter (McDowell et al., 2016). The technique

exploits the difference in relaxation properties between free, intracellular, and interlayer (between myelin bilayers) water to measure the signal ratio of myelin water to total water, also known as "myelin water fraction" (MWF). This work followed the report of a single case in which a Carr Purcell Meiboom Gill sequence used at 7T to estimate MWF (Laule et al., 2016). Results were in line with significant variability in grey and white matter myelin content in non-lesional cord areas, and a relatively distinct separation between lesions and non-lesional grey matter. The range of observed MWF data suggests short T_2 may be a useful tool, possibly as an addition to a spinal cord toolbox (De Leener et al., 2017) to quantify grey matter myelin *in vivo*, though this will require confirmation in a larger sample size.

The current and the future: improving quantification, registration, and data sharing

The pathology of MS is complex, and since its aetio-pathogenesis and evolution on the microstructural level remain incompletely understood, it is not surprising that attempts at MRI quantification of specific tissue features (myelin, axons, microglial activation, gliosis, etc) are challenging. For example, until very recently, key MR/pathology studies of the spinal cord made hardly any reference to spinal cord grey matter which, as we become ever more aware, is significantly involved in the disease process (Gilmore et al., 2009a,b; Grussu et al., 2016; Petrova et al., 2017). Whilst a bias towards long spinal cord tracts has obvious advantages since their largely longitudinal directionality lends itself to modelling "MRI histology" in the spinal cord white matter (Stikov et al., 2015; Campbell et al., 2017), it is important to bear in mind the spinal cord is in itself a functional network with millions of perpendicular connections (Bourane et al., 2015) that may be damaged in MS, with likely significant impact on function (Bourane et al., 2015; Petrova et al., 2016; Grussu et al., 2016).

When assessing the relationship between two sets of techniques, MRI and pathology, the quality of correlative data is obviously influenced by the quality of indices derived from both these two "toolboxes". Even the selection of *post mortem* specimens can vary, as it may be guided either by MRI (Bergers et al., 2002a,b; Gilmore et al., 2009a,b), visual inspection (Mottershead et al., 2003), or both. A perhaps less appreciated aspect with potential to confound the specificity of new MRI techniques is the lack of standardised methods to spatially register spinal cord MRI with histology. In *post mortem* studies of the MS brain, this issue has been recognized for some time (Moore et al., 2000). Various techniques were subsequently developed to standardise registration, including use of a stereotaxic frame (Schmierer et al., 2003), imaging the unfixed brain *in situ* with subsequent rescanning of the fixed tissue and use of customised cutting panels (Bö et al., 2004; Fisher et al., 2007), and most recently, the introduction of individually manufactured cutting panels using 3D printing technology (Luciano et al., 2016). Whilst compared to the brain the spinal cord appears a less challenging structure to match MRI with histology, the recently published systematic framework for histological quantification (Grussu et al., 2016) combined with landmark-guided co-registration (Grussu et al., 2017) provides a new level of accuracy and reproducibility for correlative MRI/pathology studies of *post mortem* spinal cord.

It is also worth noting that histological quantification in *post mortem* MS studies has made significant progress over recent years, away from the use of largely qualitative indices (Bergers et al., 2002a,b), to optical density (transmittance) (Schmierer et al., 2004), stereology (Carassiti et al., 2017), and orientation dispersion (Grussu et al., 2016) techniques.

Quantitative histology facilitated a more rigorous use of regression analysis (Petrova et al., 2017) and multivariate directional statistics (Grussu et al., 2016). This is of significance given the challenging nature of *post mortem* data with often limited sample sizes and a number of confounding factors such as demographics, access to clinical information, or duration of fixation. Moreover, "within" and "between" sample measurements need to be considered when different sections or slices of the same specimen are included in the analysis.

Diffusion models such as DBSI (Wang et al., 2011, 2015) and NODDI may offer advantages in tissue specificity as outlined above, in the case of NODDI notably including plausible indices for assessment of the spinal cord grey matter (Zhang et al., 2012; Grussu et al., 2015, 2017). Provided preliminary data can be confirmed, and reproducibility further improved (Grussu et al., 2015; Duval et al., 2017), NODDI may offer a significant step forward in the quest for accurate *in vivo* assessment of spinal cord pathology in MS, potentially in combination with other techniques, such as MT or multi-component relaxometry (Duval et al., 2017).

Given the importance of immune-mediated demyelination for the degree of axonal loss even at later stages of the disease (Montalban et al., 2017; Petrova et al., 2017), there remains a need to further optimise techniques for detection of lesions across the entire spinal cord, to serve as outcome indices in trials, and to establish treatment efficacy in the clinical management of pwMS (Giovannoni et al., 2015). High-field MRI ($\geq$3 T) combined with contrast techniques such as phase sensitive inversion recovery (PSIR) appear to provide additional sensitivity (Kearney et al., 2015), though validation of PSIR may not be feasible using fixed *post mortem* spinal cord due to the drastic shortening of T_1 with fixation (McDowell et al., 2014).

Several initiatives to improve standardisation of spinal cord MRI analysis and data sharing, such as the StructureTensorToolbox (Grussu, 2017/2) an the Spinal Cord Toolbox (De Leener et al., 2017) are likely to facilitate MR/pathology studies thereby enabling more rapid validation of new techniques in the future.

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