

Demyelination versus remyelination in progressive multiple sclerosis

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The causes of incomplete remyelination in progressive multiple sclerosis are unknown, as are the pathological correlates of the different clinical characteristics of patients with primary and secondary progressive disease. We analysed brains and spinal cords from 51 patients with progressive multiple sclerosis by planimetry. Thirteen patients with primary progressive disease were compared with 34 with secondary progressive disease. In patients with secondary progressive multiple sclerosis, we found larger brain plaques, more demyelination in total and higher brain loads of active demyelination compared with patients with primary progressive disease. In addition, the brain density of plaques with high-grade inflammation and active demyelination was highest in secondary progressive multiple sclerosis and remained ~18% higher than in primary progressive multiple sclerosis after adjustments for other plaque types and plaque number ($P < 0.05$). Conversely, the proportion of remyelinated shadow plaques ($P < 0.05$) and the overall remyelination capacity ($P < 0.01$) per brain were higher in primary, compared with secondary, progressive multiple sclerosis. By contrast, there were no group differences in the brain load or frequency of low-grade inflammatory plaques with slowly expanding demyelination. Spinal cord lesion loads and remyelination capacity were also comparable in the two patient groups. Remyelinated areas were more vulnerable than the normal-appearing white matter to new demyelination, including active demyelination in secondary progressive multiple sclerosis. 'Recurrent' slowly expanding demyelination, affecting remyelinated areas, and the load of slowly expanding demyelination correlated with incomplete remyelination in both groups. In turn, incomplete remyelination in the spinal cord correlated with higher disease-related disability (determined retrospectively; $r = -0.53$; $P < 0.05$ for remyelination capacity versus disease severity). By contrast, such a correlation was not observed in the brain. We propose that regulatory and reparative properties could protect the white matter of the brain in patients with primary progressive multiple sclerosis. These patients may, thereby, be spared symptoms until the spinal

cord is affected. By contrast, recurrent active demyelination of repaired myelin could explain why similar symptoms often develop in consecutive relapses in relapsing-remitting/secondary progressive multiple sclerosis. Our data also indicate that slowly expanding demyelination may irreparably destroy normal and repaired myelin, supporting the concept of slowly expanding demyelination as an important pathological correlate of clinical progression.

Keywords: multiple sclerosis; chronic progressive; immune-mediated demyelination; remyelination; disability; spinal cord

Abbreviations: EDSS = Expanded Disability Status Score; MANOVA = Multivariate Analysis of Variance; MSSS = Multiple Sclerosis Severity Score; PPMS = primary progressive multiple sclerosis; RM = area of remyelination; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; WM = white matter without de- or remyelination

Introduction

Multiple sclerosis is a chronic, inflammatory demyelinating disease of the CNS (Charcot, 1880). Most patients have relapsing-remitting multiple sclerosis (RRMS) from onset. Although some patients suffer severe disease and die within a year (acute multiple sclerosis), the vast majority have chronic disease and, with time, develop secondary progressive multiple sclerosis (SPMS), characterized by gradual accumulation of irreversible impairment. Ten to fifteen percent have progression from onset, i.e. primary progressive multiple sclerosis (PPMS) (Lublin and Reingold, 1996; Miller and Leary, 2007). Although patients with PPMS typically have a later disease onset and a more equal male:female ratio, they reach major disability milestones at similar ages as patients with SPMS (Confavreux and Vukusic, 2006). By contrast, several studies indicate that patients with PPMS remain cognitively better preserved than those with SPMS (Comi *et al.*, 1995; Huijbregts *et al.*, 2004; Bergendal *et al.*, 2007). In line with these observations, patients with PPMS have lower T₂ brain lesion burdens on MRI, but similar degrees of spinal cord involvement compared with patients with SPMS (Nijeholt *et al.*, 1998; Stevenson *et al.*, 1999; Agosta *et al.*, 2007). Apart from a single study showing higher densities of inflammatory cells in selected brain plaques in SPMS compared with PPMS (Revesz *et al.*, 1994), none of neuropathological data explains the clinical differences observed between these two patient groups.

Remyelination by oligodendrocyte lineage cells can be identified pathologically by sharply demarcated areas of uniformly thin myelin sheaths (Prineas and Connell, 1979; Brück *et al.*, 2003; Patrikios *et al.*, 2006). Remyelination may, in part, underlie remission following a relapse by restoring conduction velocity (Smith *et al.*, 1979) and by protection from axonal injury (Kornek *et al.*, 2000). However, remyelination may not always be stable and recurrent demyelination of remyelinated areas has been documented in acute/RRMS (Prineas *et al.*, 1993). In addition, remyelination of >60% of the total lesion area was less common in SPMS than in RRMS brains (Patrikios *et al.*, 2006). This suggests either that new plaques remyelinate less efficiently in SPMS compared with RRMS or that acquired remyelination may be destroyed by renewed inflammatory demyelination as the disease progresses. Furthermore, the presence of slowly expanding plaques were considered a pathological correlate of progression in SPMS (Prineas *et al.*, 2001). Consistent with the hypothesis that slowly expanding demyelination might also be involved in

remyelination failure, these smouldering inflammatory plaques were frequently found in patients with progressive disease, but not in those with acute or RRMS (Frischer *et al.*, 2009).

Relapses and related disability are regarded as a consequence of focal inflammatory demyelination and axonal loss (Marburg, 1906). By contrast, the inflammatory nature of progression has been questioned by poor correlations between lesion load on MRI and irreversible disability, particularly in PPMS (Nijeholt *et al.*, 1998) and by the lack of efficacy of immunomodulatory treatment for progressive multiple sclerosis. The best correlates of chronic, irreversible disability have so far been axonal loss and atrophy in the spinal cord (Stevenson *et al.*, 1998; Bjartmar *et al.*, 2000), which may occur unrelated to demyelination (DeLuca *et al.*, 2006). However, the findings of diffuse white matter inflammation, cortical demyelination (Kutzelnigg *et al.*, 2005) and an observed correlation between inflammation and axonal injury in slowly expanding plaques (Frischer *et al.*, 2009) reinforce the concept that inflammation plays a central role in contributing to disease progression.

Using planimetric analysis of brains and spinal cords from patients with clinically well-characterized progressive multiple sclerosis, we report higher brain loads of active and total demyelination in SPMS compared with PPMS, despite similar degrees of spinal cord involvement. Conversely, we find a higher remyelination capacity in the brain in PPMS compared with SPMS. We describe that remyelinated areas may be more prone to inflammatory demyelination compared with the white matter outside plaques and report that slowly expanding demyelination correlates with incomplete remyelination in PPMS and SPMS. In turn, incomplete remyelination in the spinal cord correlates with severe disease-related disability, whereas this seems not to be the case in the brain.

Patients and methods

Sample characterization

We used paraffin-embedded archival autopsy material from 51 patients with multiple sclerosis and 12 age-matched normal controls. The multiple sclerosis sample included 14 patients with PPMS, 34 with SPMS and three patients with progressive disease, in whom sub-classification was not possible due to insufficient clinical data. We analysed tissue from patients with clinically diagnosed multiple sclerosis and at least 18 months of gradual disease progression and in whom

multiple sclerosis plaques were subsequently found during neuropathological examination. Exclusion criteria were other known CNS disease or significant pathology not attributable to multiple sclerosis. Brain tissue was available from 50 out of 51 cases. This included large hemispheric or double hemispheric tissue blocks in 15 patients. From six patients with PPMS and 19 with SPMS, a median of three spinal cord blocks from different levels could additionally be analysed (number of levels: PPMS=2–5; SPMS=1–8; not significant). Observations from the brain and spinal cord were paired, except in one patient with PPMS where only spinal cord tissue was available for analysis. The majority of sub-categorized patients (41 out of 47) died before 1982 and did not receive immunomodulatory or immunosuppressive treatment, except for two patients who received prednisone during some periods.

Retrospective clinical rating

Detailed clinical charts were available for the majority of patients. The retrospective categorization of disease course was performed blinded to the neuropathological data. All patients with more than one relapse-like episode recorded prior to progression onset were rated as SPMS. The majority had reported ophthalmological and/or pyramidal symptoms during relapses. PPMS was defined when the progressive symptoms were not preceded by relapses and dominated by progressive disturbance of gait or balance. However, one patient with one fully remitting episode of paraesthesia in both feet 6 years before progression onset was also rated as PPMS because the neurological interpretation of the episode was unclear. Consistent with PPMS (Stevenson *et al.*, 1999), brain MRI during his subsequent progressive gait disturbance showed limited lesion burden (four small periventricular T₂ lesions). However, CSF immunoglobulin G index was increased and motor evoked potentials showed prolonged central conduction (Bramow *et al.*, 2008). The PPMS group also included four patients who had a few protracted bouts of gait disturbance after progression onset, i.e. suggestive of so-called progressive relapsing multiple sclerosis. Due to variability in clinical details provided, coupled with clinical similarities among these patients (Montalban, 2004), we included these cases in the PPMS cohort.

The Expanded Disability Status Scale (EDSS; Kurtzke, 1983) was estimated by two independent observers among cases with sufficient clinical documentation on disease course in the 3 years prior to death ($n=40$). Scores <6.5 were always based on a full neurological examination. However, in a few cases where the walking distance was not clearly stated, the EDSS was estimated from the Kurtzke's Functional System Score. EDSS scores between 6.5 and 9.5 could reliably be determined, based on assessment of ambulation/self-reliance. An EDSS score of 10 was used in four patients who died in terminal-stage multiple sclerosis (previous EDSS score >8.5) from sudden aspiration pneumonia ($n=3$) or sudden pulmonary oedema without other findings at general autopsy. The Multiple Sclerosis Severity Score (MSSS), which combines the EDSS with disease duration, was assigned according to the time point of the most recent EDSS score (Roxburgh *et al.*, 2005).

Neuropathological techniques

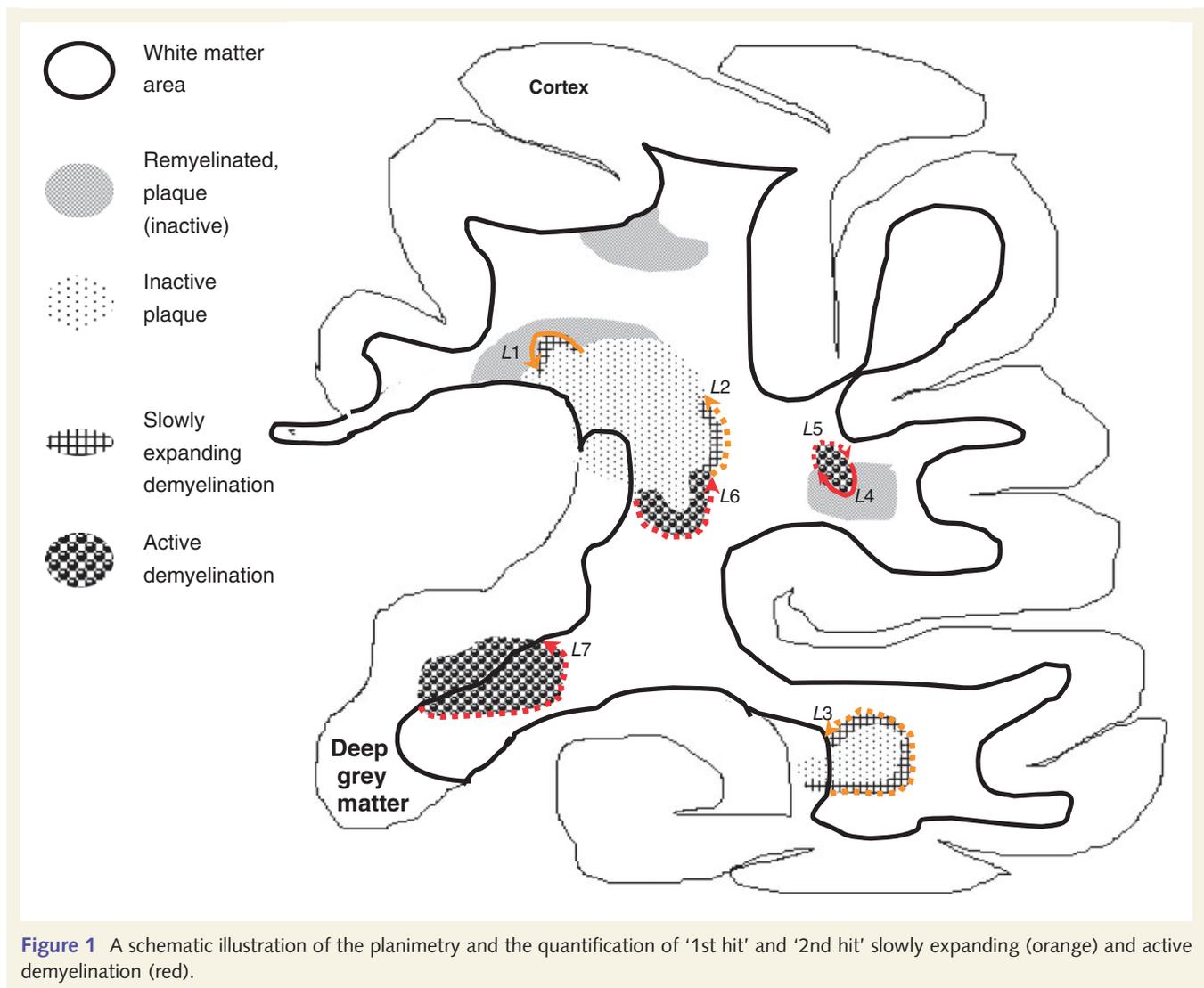
Sections that were 4–6- μ m thick were cut serially and stained with haematoxylin and eosin. Myelin was visualized with luxol fast blue-periodic acid Schiff and axons in small blocks with Bodian or Bielschowsky silver stains. The distribution of axons in different area/lesion types of the spinal cords were confirmed in nine representative patients (six SPMS, three PPMS) by staining for neurofilament

light chain (68–70 kDa, Chemicon/Millipore AB 9568, 1:1000). We stained all (including hemispheric) blocks immunohistochemically for microglia/macrophages (CD68; Dako, Denmark; M0814; diluted 1:100) and for myelin proteolipid protein (myelin; Serotec, UK; MCA839G; 1:1000). From 15 patients (3 PPMS and 12 SPMS), brains were additionally stained for T cells (CD3; LabVision, USA; RM 9107-R7; 1:1000) and B cells (CD20; LabVision, USA; MS-340-R7; 1:100) to confirm inflammation.

For immunohistochemistry, endogenous peroxidase was blocked by incubation of slides in methanol with 0.02% H₂O₂. Antigens were retrieved by steaming sections for 90 min in ethylenediaminetetraacetic acid (EDTA, 0.05 M) in 0.01 M tris(hydroxymethyl)aminomethane buffer at pH 8.5. Non-specific binding of primary antibody was blocked by a 20-min incubation of sections in foetal calf serum diluted to 10% in 0.1 M phosphate buffered saline. Primary antibodies diluted in foetal calf serum/phosphate buffered saline were then applied at 4°C overnight. Biotinylated secondary sheep anti-mouse or donkey anti-rabbit (CD3) antibodies were applied for 60 min at room temperature (GE Healthcare, UK, formerly Amersham, Sweden; RPN1001 and RPN1004; both diluted 1:200 in foetal calf serum/phosphate buffered saline). After a 60-min incubation with a complex of avidin and biotinylated horseradish peroxidase (Sigma; A3151; 1 mg/ml diluted 1:100 in foetal calf serum/phosphate buffered saline; room temperature), we visualized signals with 3,3'-diaminobenzidine-tetrahydrochloride (Fluka; 32 750; 0.5 mg/ml in 0.06 M phosphate buffered saline with 0.01% H₂O₂) for ~2 min during microscopy. Adjacent sections to which no primary antibody was applied were used as negative controls.

Plaque classification

Regarding demyelinating activity, three plaque types were distinguished. Active plaques were characterized either by the presence of numerous macrophages positive for luxol fast blue or proteolipid protein, which were distributed throughout the plaque (i.e. so-called acute active plaques), or, more often, by the presence of such macrophages concentrated at the plaque edge around an inactive centre (i.e. so-called chronic active plaques, Fig. 1). Active plaques also contained variable perivascular cuffs of mononuclear cells. Acute and chronic active plaques were grouped into a single category, as we did not observe differences in inflammatory activity or myelin breakdown. Slowly expanding plaques displayed a rim of activated microglia and at least two cells per high-power field with vacuoles positive for luxol fast blue or proteolipid protein. Inactive plaques were devoid of macrophages with such vacuoles. Remyelinated areas were determined by two independent raters, including one neuropathologist, and were characterized by sharply demarcated regions, showing uniformly thin myelin sheaths and were largely devoid of macrophages. Remyelinated areas additionally displayed relative preservation of small and large diameter axons as opposed to the more profound axonal loss and selective preservation of normally myelinated large diameter axons in areas of presumed Wallerian degeneration. This criterion has been validated by recent studies, indicating that small axons in multiple sclerosis may be particularly vulnerable (Evangelou *et al.*, 2001; DeLuca *et al.*, 2004) but that remyelination may protect axons from injury (Kornek *et al.*, 2000; Irvine and Blakemore, 2008). Areas suggestive of Wallerian degeneration were particularly often found in the spinal cord (Supplementary Fig. 1). Plaques with at least 60% of their area being remyelinated were defined as so-called shadow plaques (Patrikios *et al.*, 2006).



Strategy for quantification of demyelination and remyelination

As a first step, we determined the crude distribution of the three plaque types in the brain and spinal cord (Table 2). We then counted the numbers of each plaque type per square centimetre of brain tissue, excluding the cortex. This yielded one density value per patient for each plaque type (Table 3). In order to estimate the load of inflammatory demyelination and the remyelination capacity, we also performed a planimetric analysis across all plaques in the white matter. Identification of remyelinated white matter areas by light microscopy has been described and validated (Brück *et al.*, 2003; Patrikios *et al.*, 2006). We measured areas of inactive focal lesion (including remyelination), slowly expanding areas and active areas. By normalizing the results to the total white matter area in the sections, we obtained one load value per patient for each lesion type (Table 4). The load of combined inflammatory demyelination (slowly expanding plus active) and the total white matter lesion load were also calculated. Remyelination capacity was defined as the percentage of remyelinated area in relation to the total white matter lesion area per patient. However, lesion loads and the remyelination capacity were calculated in the brain and spinal cord, separately.

For planimetry, slides stained with luxol fast blue were placed on a light board and photographed digitally with a ruler using macro-photo equipment. Magnification of photography was chosen such that each slide was captured in a single image. Contrast and colour were adjusted automatically for red, green and blue and each slide was calibrated to a ruler. Areas were measured on screen during microscopy of adjacent slides stained for myelin, axons and microglia/macrophages as outlined above. Figure 1 shows a schematic presentation of the planimetry. In the hemispheres, we obtained a coefficient of error (SEM/mean) of <0.05 for 10 consecutive measurements of remyelinated areas $\geq 2\text{ mm}^2$ in size.

Estimation of the vulnerability to demyelination in white matter areas

We analysed whether inflammatory demyelination may affect remyelinated areas differently than the normal-appearing white matter. For reasons of accuracy (see above), we selected brains with $\geq 2\text{ mm}^2$ of coherent remyelination ($n = 43$, Table 5 and Fig. 4A–C). A demyelinating frontline was defined as the border length between areas of inflammatory demyelination (active or slowly expanding) and the surrounding white matter. We defined '1st hit' demyelination as

demyelinating frontlines affecting white matter without evidence for previous de- and remyelination (WM area), while '2nd hit' demyelination affected remyelinated areas (RM area). We measured '1st hit' and '2nd hit' frontlines displaying evidence for either slowly expanding or active demyelination and calculated the sum of the two (combined frontlines). Adjusting these data for the absolute area sizes, we derived frontline indices, estimating the vulnerability to inflammatory demyelination in remyelinated areas relative to the WM area, in fold. The estimate rests on the assumption that both '1st hit' and '2nd hit' demyelinating frontlines distribute randomly in the corresponding areas and was obtained as follows: we first defined the length ratios of '2nd hit' to '1st hit' frontlines, i.e.

$$\text{Frontline ratio (FR)} = \frac{\text{'2nd hit' frontline length at RM area}}{\text{'1st hit' frontline length at WM area}}$$

Because of skewed distributions, we subsequently log-transformed the data. Therefore, we had to add a constant (0.1 mm) to '2nd hit' frontlines in order to avoid losing observations of zero, i.e. stable remyelination. We balanced the constant added to '1st hit' frontlines in the WM area by multiplying it by the factor of area difference between the remyelinated area and the WM area, i.e. $0.1 \times (\text{WM area}/\text{RM area})$.

$$\text{FR}_{+\text{constant}} = \frac{\text{Frontline length at RM area} + 0.1 \text{ mm}}{\text{Frontline length at WM area} + [0.1 \text{ mm} \times (\text{WM area}/\text{RM area})]}$$

The $\text{FR}_{+\text{constant}}$ was then adjusted for the factor of size difference between the remyelinated area and the WM area to yield the frontline index (FI), i.e.

$$\text{FI} = \frac{\text{FR}_{+\text{constant}}}{(\text{RM area}/\text{WM area})} = \frac{\text{FR}_{+\text{constant}} \times \text{WM area}}{\text{RM area}}$$

For a calculated example of a slowly expanding frontline index (FI) based on Fig. 1, assume $L1=2$ mm, $L2=4$ mm and $L3=8$ mm, RM area = 10 mm^2 , WM area = 100 mm^2 . The crude slowly expanding frontline ratio

$$\begin{aligned} \text{FR} &= \frac{L1}{L2 + L3} \\ \text{FR} + \text{constant} &= \frac{L1 + 0.1 \text{ mm} = 2.1 \text{ mm}}{L2 + L3 + (0.1 \text{ mm} \times 100/10) = 13 \text{ mm}} \\ \text{FI} &= \frac{\text{FR} + \text{constant}}{(\text{RM area}/\text{WM area})} = \frac{2.1 \text{ mm}/13 \text{ mm}}{10 \text{ mm}^2/100 \text{ mm}^2} = \\ &1.62 \text{ and } \text{Log}_{10}(1.62) = 0.21. \end{aligned}$$

In this theoretical example, the vulnerability of the RM areas to slowly expanding demyelination is ~ 1.6 -fold higher than expected from the WM area. The white asterisk in Fig. 4 illustrates such a data point. By analogy, active and combined frontline indices were determined.

The parameter Log_{10} (combined FI) was normally distributed in both PPMS and in SPMS (Fig. 4). An index of one (zero after log-transformation) would indicate that the length of '2nd hit' frontlines was as expected from the observed length of '1st hit' frontlines. When analysing hemispheres only, the difference in overall vulnerability of remyelinated areas compared with WM areas remained at the same level as the whole group and also retained its significance when we tentatively let the constant approach zero.

To further validate this complex quantitative approach, we also counted the number of areas of '1st hit' inflammatory demyelination per cm^2 in the WM area and the number of inflammatory '2nd hit' demyelinating areas per cm^2 remyelinated area. '1st hit' and '2nd hit' areas of inflammatory demyelination were confirmed by two authors.

This approach yielded similar results as those obtained with frontline measurements, but did not allow estimation of the difference in vulnerability between RM and WM areas.

Finally, we estimated the potential importance of '2nd hit' demyelination for the remyelination capacity in the brain (Table 6 and Fig. 4D–F). The lengths of '2nd hit' frontlines impinging on remyelinated areas were expressed as proportions of the circumference length of all remyelinated areas, per brain. The resulting extent of '2nd hit' demyelination was plotted against the remyelination capacity.

Statistical analysis

Statistical Package for the Social Sciences (SPSS version 15.0; Chicago, IL, USA) was used. Due to the data reduction described above, any one patient only contributed with one degree of freedom to the analyses described below. Dichotomous variables were tested with a chi-square test. Variables that were normally distributed in both groups (ages at disease onset and death) were tested with an unpaired *t*-test, whereas variables with skewed distributions were tested with the Wilcoxon–Mann–Whitney U-test.

Regarding the hypothesized differences between PPMS and SPMS, we first tested whether the density distributions of the three plaque types (inactive, slowly expanding and active) were different using multivariate analysis of variance (MANOVA). Discriminant analysis was then performed to identify individual plaque types that discriminated between PPMS and SPMS. Positive findings were confirmed by logistical regression analysis, estimating whether the odds for SPMS changed with plaque densities. Group differences in individual plaque types were Bonferroni corrected for the three plaque types analysed. A similar strategy was used to analyse whether lesion loads differed between PPMS and SPMS. Remyelination capacity and shadow plaques were considered separately because they were pre-planned and inter-related with the individual types of plaques/lesion loads. However, differences were confirmed by equalizing other predictors that group in multiple regression analyses (Tables 3 and 4). In this way, we also obtained an estimate of the residual difference in remyelination capacity between PPMS and SPMS when other significant contributors were set equal. Further, pathologically measured predictors of remyelination capacity were assessed, controlling additionally for sampled tissue areas and other confounders, such as age. Residuals were normally distributed and variance inflation factors, testing co-linearity were <2.5 as compared with a recommended value of <5 (Field, 2005). Interrelations of variables were confirmed using non-parametric Spearman's rank correlation (Table 6).

Regarding the vulnerability in remyelinated versus WM areas, we tested frontline indices (FI) of slowly expanding, active and combined frontlines against the null-hypotheses with a one-sample *t*-test. However, the distributions of Log_{10} (slowly expanding FI) and Log_{10} (active FI) were slightly skewed in PPMS alone or in SPMS alone (Fig. 4 and Table 5).

In patients allowing paired observations from the brain and spinal cord, we assessed the Spearman's rank correlations between the MSSS and the load of white matter demyelination (excluding remyelinated areas) and between the MSSS and remyelination capacity ($n=20$). Brains were compared with spinal cords using the paired Wilcoxon signed ranks test (Fig. 3). All *P*-values are the result of two-sided testing and corrected *P*-values of <0.05 in the total collection were regarded significant. Similar trends found separately in the collections of small brain blocks and hemispheres further confirmed differences found in the total collection.

Results

Demographical and clinical characteristics of the cohort are summarized in Table 1. On average, patients with PPMS were 15 years older ($P < 0.001$) at disease onset and their disease duration was 11 years shorter ($P = 0.002$) as compared with patients having SPMS. The mean age at death was 57.2 years in patients with PPMS and 49.6 years among controls (not significant) and these groups had even sex distributions. The mean age at death in SPMS was 51.5 years and more than two-thirds were women. The median duration of the progressive phase was 8.5 years in PPMS and 13 years in SPMS (not significant). None of the 13 patients with PPMS received immunosuppressive treatment compared with 6 of the 34 patients with SPMS (not significant). The most recent EDSS and the MSSS were similar in both groups (Table 1, controls not shown).

Higher demyelinating activity of plaques, but less frequent completion of remyelination in the white matter of brains in SPMS versus PPMS

Within the brains of all patients we found 534 plaques; 54% were inactive plaques, 24% were classical active and 18% slowly expanding. Only 4% of the plaques showed areas of classical activity side by side with areas of slow expansion. Forty-four percent of plaques in the white matter demonstrated extensive regions with uniformly thin myelin sheaths and were categorized as shadow plaques. A substantial number of incompletely remyelinated plaques and some shadow plaques contained areas of ongoing demyelination with evidence for either classical active or slowly expanding demyelination into the remyelinated area (Fig. 2). In the spinal cord, 89% of plaques were inactive and only few classical active or slowly expanding lesions were seen. Also in the spinal cord, a substantial number of plaques in the white matter were shadow plaques (Table 2).

We then analysed the density distribution of plaque types (inactive, slowly expanding or active) by counting the plaque numbers per cm^2 brain tissue, excluding cortex. This yielded one density value per patient for each plaque type. Likewise, one shadow plaque percentage per patient was calculated. Overall, the relative density distribution of different plaque types was similar as described above (not shown). Comparing PPMS with SPMS, MANOVA and discriminant analysis indicated a difference in the density of active plaques. Direct comparison showed a higher density of classical active plaques in SPMS, but similar densities of slowly expanding lesions. Conversely, a higher proportion of white matter plaques were shadow plaques in PPMS compared with SPMS (Table 3). Similar results were obtained after correction for co-variables or when large hemispheric sections and small blocks were analysed separately. In addition, we calculated the mean of the areas of all plaques per patient. In the brain, the patient-specific mean plaque area was higher in SPMS compared with PPMS. These data suggest that active demyelination occurs

Table 1 Clinical and demographical characteristics of patient cohort

Group	Small brain blocks (S)		Hemispheres (H)		S + H	
	PPMS (n = 8)	SPMS (n = 26)	PPMS (n = 5)	SPMS (n = 8)	PPMS (n = 13)	SPMS (n = 34)
Age at death (years)	56.4 (3.6)	52.4 (2.2)	58.4 (7.7)	48.4 (3.1)	57.2 (3.5)	51.5 (1.8)
Age at onset (years)	45.2 (3.8)	29.5*** (1.6)	40.5 (6.6)	25.4 (3.8)	43.4 (3.4)	28.5*** (1.5)
Disease duration (years)	8.8 (5.3–23)	22.8## (8.5–44)	10.0 (7.0–37)	20.5 (11.0–37)	9.0 (5.3–37)	21.0## (8.5–44)
Progressive phase (years)	8.8 (5.3–23)	13.0 (1.5–37) n = 25	7.0 (4.5–35)	11.0 (8.0–19) n = 7	8.5 (4.5–35)	13.0 (1.5–37) n = 32
Sex (female/male)	4/4	20/6	2/3	4/4	6/7	24/10
Immunosuppressive treatment within 6 months ante-mortem	0 of 8	2 of 26	0 of 5	4 of 8 ^s	0 of 13	6 of 34
Median most recent EDSS ≤ 3 years ante-mortem	8.0 (5.0–10) n = 7	8.5 (5.0–10) n = 21	7.0 (4.0–9.5)	8.0 (6.0–10) n = 6	8.0 (4.0–10) n = 12	8.5 (5.0–10) n = 27
MSSS based on most recent EDSS	9.76 (7.66–9.99)	9.42 (3.50–9.99)	9.27 (5.64–9.98)	9.29 (5.35–9.95)	9.61 (5.64–9.99)	9.42 (3.50–9.99)
Time from EDSS to death (months)	12 (0–29)	6 (0–36)	2 (0–9)	1 (0–3)	7 (0–29)	2 (0–36)

Values represent either mean and standard error of the mean or median and range. Degrees of freedom = $n_{\text{PPMS}} + n_{\text{SPMS}} - 1$. Two-sided statistics (SPMS versus PPMS): ^s $P < 0.10$ (Chi-square test), ^{##} $P < 0.01$ (Wilcoxon–Mann–Whitney U-test), ^{***} $P < 0.001$ (Unpaired t-test).

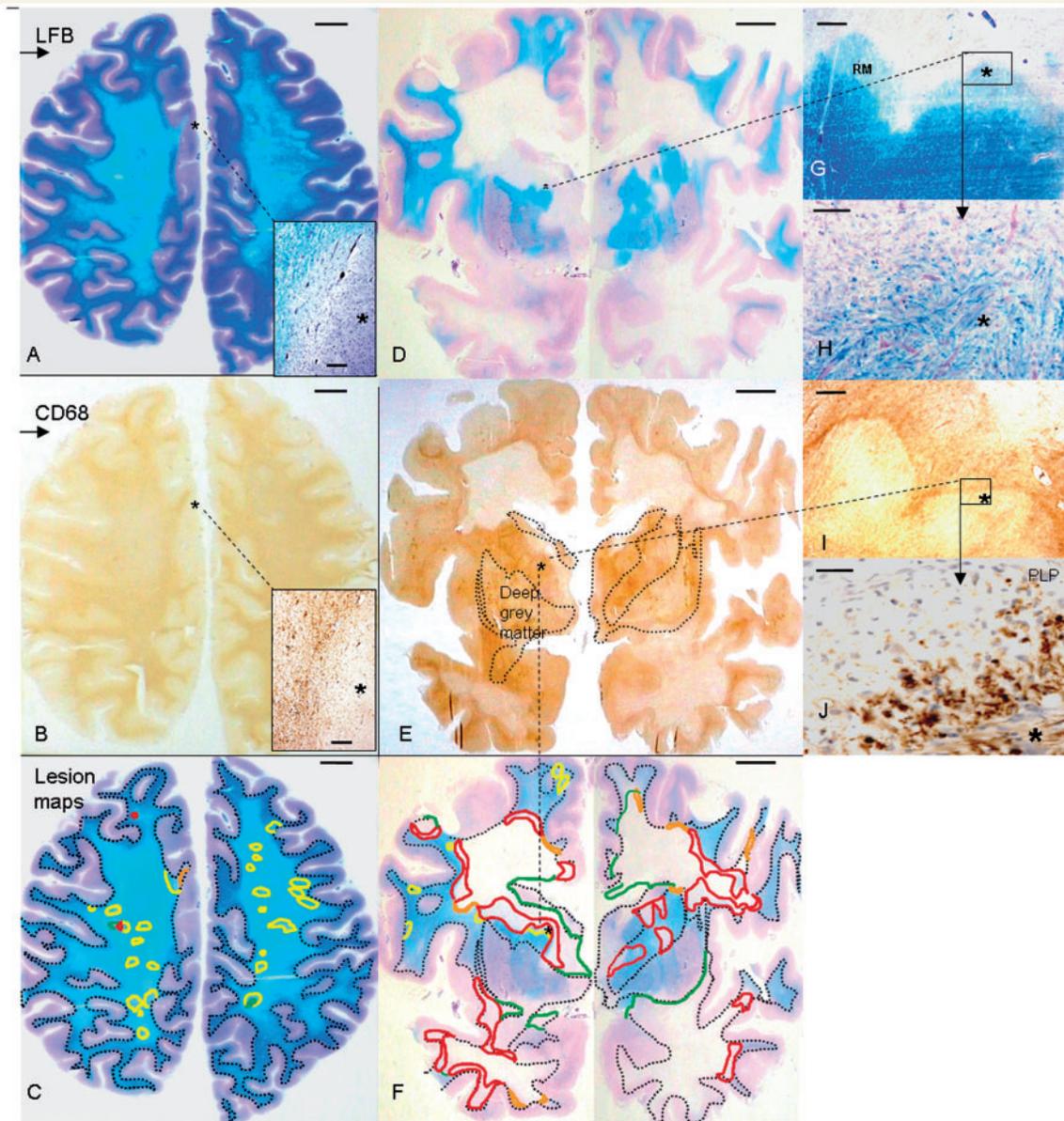


Figure 2 Characteristics of the white matter in brains from patients with PPMS (left column) and SPMS (middle column). Inserts and the right column show slowly expanding '2nd hit' and active '2nd hit' demyelination. Photos are shown from adjacent hemispheric sections stained for myelin with luxol fast blue-periodic acid Schiff (A, D, G and H), and immunohistochemically for microglia/macrophages (CD68; B, E and I). Lesion maps (C and F) were made during microscopy of slides stained additionally for proteolipid protein (J). For planimetry, cortical and deep grey matter were excluded (dotted lines in C, E and F). Asterisks represent corresponding points in each column. Rims of CD68 positive microglia and few luxol fast blue- or proteolipid protein-positive phagocytes determined areas of slowly expanding demyelination (orange lines). Confluent areas of myelin-laden CD68 positive macrophages were rated as classical active demyelination (dark red lines). Inactive borders are drawn in green or with dotted lines where they reach the cortex. Remyelinated areas (yellow lines) were sharply demarcated, displayed uniformly thin myelin stain (A) and were largely devoid of macrophages (B). (A–C) In PPMS, focal lesions were often inactive and remyelinated. Although diffuse white matter injury was often profound, remyelination was easily distinguished. Active demyelination was mostly very sparse. Inserts represent higher magnifications of the area around the asterisks and show a small rim of subcortical demyelination (A), with a corresponding rim of microglia (B), which impinges on a remyelinated area. We interpret this as so-called slowly expanding '2nd hit'. (D–F) In SPMS large, confluent lesions were frequent, which appeared to expand by active or slowly expanding demyelination at the edge. The CD68-positive areas of inflammatory demyelination are seen even at low power (E). We also found so-called acute active plaques with macrophages throughout, whereas remyelination was either incomplete at lesion edges or confined to small subcortical lesions (F). (G–J) Microphotographs of the area around the asterisk in D and E. Asterisks represent corresponding points. A remyelinated area (RM in G), itself largely devoid of macrophages (I), appears to be under renewed active demyelination by sheets of macrophages containing myelin debris positive for luxol fast blue (H) and proteolipid protein (J). We interpret this as so-called active '2nd hit' demyelination. Scale bars represent 1 cm (A–F), 1 mm (G and I), 0.5 mm (inserts), 0.1 mm (H) and 0.05 mm (J).

Table 2 Plaque numbers in the brain and the spinal cord

Plaque type	Brain	PPMS	SPMS	PP/SPMS	Spinal cord	PPMS	SPMS
No. of patients (<i>n</i>)	50	13	34	3	25	6	19
No. of plaques in white or white/deep grey matter	534	189	333	12	129	30	99
Inactive (%)	54	63	49	33	89	93	88
Slowly expanding (%)	18	21	16	25	5	0	6
Active (%)	24	14	29	25	5	7	5
Active + slowly expanding (%)	4	2	5	17	1	0	1
Shadow plaques (percentage of plaques in white matter)	44	58	38	8	38	18	44

more frequently, but that remyelination completes more rarely in the brain in SPMS compared with PPMS. Collectively, this may lead to larger brain plaques in the white/deep grey matter in SPMS (Table 3).

To further confirm our results in the brain, we analysed by planimetry the lesion areas (inactive, slowly expanding or active) relative to the total white matter area in the sections, obtaining one load value per patient for each lesion type. Overall, the relative load distribution of different lesion types resembled the distribution of plaque types described above (not shown). Regarding lesional activity and remyelination in PPMS and SPMS, the planimetric data also confirmed our results on plaque densities. MANOVA and discriminant analyses indicated a difference in the load of active demyelination. We found the highest load of active demyelination in SPMS and the difference compared with PPMS retained significance after correction for co-variables (Table 4). By contrast, there was no difference in the load of slowly expanding demyelination after correction.

We also determined areas of remyelination (incomplete or in shadow plaques) relative to the total white matter lesion area, obtaining one value for remyelination capacity per patient. In line with our observations for shadow plaques, we found a higher remyelination capacity in PPMS compared with SPMS. This finding was also confirmed in multivariate analysis (see below) and in hemispheres and small brain blocks, separately. In addition, the planimetric comparison revealed that the total white matter lesion load was lower in PPMS than in SPMS (Table 4).

In the brains from the subset of patients who also provided spinal cord tissue (5 with PPMS versus 19 with SPMS), we again found the highest load of inflammatory demyelination (slowly expanding + classical active) and the lowest remyelination capacity in SPMS. By contrast, in the spinal cord, inflammatory demyelination and remyelination capacity did not differ between the groups of patients (Fig. 3). However, a trend appeared that the average individual spinal cord plaque (per patient) had grown larger in PPMS compared with SPMS ($P < 0.1$; $df = 23$; data not shown), despite similar spinal cord plaque loads. Together, these data may indicate that the white matter of brains in PPMS is less prone to inflammatory lesion growth, allowing for more complete and more persistent remyelination as compared with SPMS. By contrast, this may not be the case in the spinal cord.

Remyelinated areas are prone to '2nd hit' demyelination

To explain the difference in remyelination between SPMS and PPMS, we analysed whether new inflammatory demyelination may affect remyelinated areas differently than the normal-appearing white matter. We defined '1st hit' demyelination as inflammatory demyelination affecting white matter without evidence for previous plaques (WM area), while '2nd hit' demyelination targeted remyelinated areas. From these data we calculated a frontline index (FI) as outlined in the 'Patients and methods' section. When inflammatory demyelination affects the WM and remyelinated areas in the same way, FI is 0 (Figs 1 and 4A–C). Among all patients with $>2 \text{ mm}^2$ of coherent remyelination in the brain ($n = 43$; 12 PPMS; 21 SPMS), the length of all '2nd hit' demyelinating frontlines (classical active + slowly expanding) were ~ 3 -fold longer than expected from the length of all '1st hit' frontlines (Fig. 4A–C and Table 5). The increased vulnerability of remyelinated tissue over the WM area was mainly caused by slowly expanding demyelination, and less by classical active demyelination (Table 5). Furthermore, '2nd hit' demyelination was predominantly seen in SPMS, and less in PPMS. The results may indicate that remyelinated areas in brains with multiple sclerosis are more prone to slow lesion expansion than white matter without previous plaques.

Slowly expanding demyelination correlates with incomplete remyelination

The results above may indicate that more complete remyelination in PPMS compared with SPMS could be, at least in part, due to less '2nd hit' demyelination. To test this further, we analysed the proportion of the total circumference of remyelinated areas of $>2 \text{ mm}^2$ in size, which showed inflammatory demyelination. The result, i.e. the extent of '2nd hit' demyelination, was plotted against the remyelination capacity. We found a highly significant negative correlation between the extent of '2nd hit' demyelination and completion of remyelination for slowly expanding frontlines, but not for classical active frontlines (Fig. 4D–F and Table 6). Multiple regression analysis revealed that slowly expanding demyelination was a correlate of incomplete remyelination after correction for co-variables and parameters of sampling. Each doubling

Table 3 Plaque densities, plaque area and shadow plaque frequency in progressive multiple sclerosis

Group	Small brain blocks (S)			Hemispheres (H)			S + H		
	PPMS (n = 8)	SPMS (n = 26)	Control (n = 12)	PPMS (n = 5)	SPMS (n = 8)	PPMS (n = 13)	SPMS (n = 8)	PPMS (n = 13)	SPMS (n = 34)
Blocks/hemispheres (No.; median, range)	2 (1–6)	2 (1–9)	3 (1–7)	4 (2–6)	3 (1–6)	2 (1–8)	2 (1–9)	2 (1–8)	2 (1–9)
Plaques in white/deep grey matter (No.)	3 (2–25)	4 (1–19)	0*** (0–0)	19 (17–53)	16 (7–68)	4 (2–53)	7 (1–68)	4 (2–53)	7 (1–68)
Total plaque density (No./cm ²)	0.42 (0.31–1.65)	0.66 (0.21–2.94)	0*** (0–0)	0.50 (0.34–0.59)	0.46 (0.32–1.68)	0.44 (0.31–1.65)	0.57 (0.21–2.94)	0.44 (0.31–1.65)	0.57 (0.21–2.94)
Inactive plaques (No./cm ²)	0.23 (0.00–0.61)	0.20 (0.00–1.54)	0*** (0–0)	0.33 (0.20–0.49)	0.24 (0.14–1.16)	0.27 (0.00–0.61)	0.21 (0.00–1.54)	0.27 (0.00–0.61)	0.21 (0.00–1.54)
Slowly expanding (SE) plaques (No./cm ²)	0.0 (0.00–0.46)	0.13# (0.00–2.31)	0.0 (0.00–0.93)	0.04 (0.04–0.16)	0.03 (0.00–0.17)	0.10 (0.00–1.05)	0.14** ^a (0.00–2.31)	0.00 (0.00–0.46)	0.14** ^a (0.00–2.31)
Active plaques (No./cm ²)	0.25 (0.00–1.52)	0.38 (0.00–2.31)	0.38 (0.00–2.31)	0.13 (0.04–0.24)	0.21 (0.03–0.52)	0.14 (0.00–1.52)	0.37* (0.00–2.31)	0.14 (0.00–1.52)	0.37* (0.00–2.31)
Plaque area (mm ² ; median and range of Patient-specific means)	17.5 (2.2–144.5)	36.0 (2.6–151.8)	17.5 (2.2–151.8)	9.1 (6.1–19.2)	22.3# (6.9–182.6)	16.6 (2.2–144.5)	33.9* (2.6–182.6)	16.6 (2.2–144.5)	33.9* (2.6–182.6)
Shadow plaques (patient-specific percentage of white matter plaques)	25 (0–50)	0 (0–50)	0 (0–50)	67 (30–88)	35 (13–81)	50 (0–88)	15* ^{a,b} (0–81)	50 (0–88)	15* ^{a,b} (0–81)

Values represent medians (ranges). Two-sided statistics, PP/SPMS versus controls: *** $P < 0.001$ (Wilcoxon–Mann–Whitney U-test, MWU). PPMS versus SPMS (df = patient n – 1), Bonferroni corrected for individual plaque types in the total cohort # $P < 0.10$; * $P < 0.05$ (MWU).

a Multiple linear regression analysis. The density of active plaques in SPMS was 18% higher compared with PPMS after correction for the densities of inactive plaques and slowly expanding plaques and for the absolute number of plaques sampled (95% confidence interval = 2–36%, $P = 0.029$, adjusted model $R^2 = 0.14$, $P = 0.033$). Neither slowly expanding nor inactive plaques contributed significantly to the model. Similar estimates were obtained after additional correction for hemispheric versus small block sampling (wider CI).

b Multiple linear regression analysis. The shadow plaque proportion was, on average, 16% points lower in SPMS compared with PPMS after correction as above. (CI = –28 to –3 %, $P = 0.015$, adjusted model $R^2 = 0.53$, $P < 0.001$). a + b: D.f. = 47 minus number of variables included in the model.

Table 4 Lesion planimetry in progressive multiple sclerosis

Group	Small brain blocks (S)		Hemispheres (H)		S + H	
	PPMS (n = 8)	SPMS (n = 26)	PPMS (n = 5)	SPMS (n = 8)	PPMS (n = 13)	SPMS (n = 34)
White matter area (WMA, cm ²)	7.0 (2.9–9.1)	7.5 (2.6–18.4)	42.6 (20.6–98.1)	31.4 (11.6–50.0)	8.9 (2.9–98.1)	9.1 (2.6–50.0)
Load of white matter lesion (WML, % of WMA)	10.2 (1.6–44.7)	23.5# (6.9–51.0)	5.0 (1.5–5.2)	21.9# (2.4–67.4)	5.9 (1.5–44.7)	23.1*** ^{§§} (2.4–67.4)
Inactive focal lesion load	10.2 (0.7–43.3)	19.8 (4.8–47.7)	4.3 (1.2–5.2)	19.0 (1.7–41.6)	5.8 (0.7–43.3)	19.8* (1.7–47.7)
Slowly expanding demyelination load	0.1 (0.0–2.9)	0.7 (0.0–4.1)	0.1 (0.0–0.7)	0.6 (0.0–2.6)	0.1 (0.0–2.9)	0.7 (0.0–4.1)
Active demyelination load	0.0 (0.0–1.5)	0.44 (0.0–16.6)	0.1 (0.0–0.4)	1.9** (0.5–31.9)	0.0 (0.0–1.5)	0.9* [§] (0.0–31.9)
Combined inflammatory demyelination load	0.3 (0.0–4.4)	1.6* (0.0–16.6)	0.3 (0.0–0.8)	2.9** (0.5–32.8)	0.3 (0.0–4.4)	2.1*** [§] (0.0–32.8)
Remyelination capacity (% of WML area)	31.9 (0.0–67.8)	14.4* (0.0–61.6)	85.8 (41.7–94.9)	4.3** (1.7–61.9)	47.0 (0.0–94.4)	11.1*** [§] (0.0–61.8)

Values represent medians (ranges). Two-sided statistics PPMS versus SPMS (d.f. = patient n – 1), Bonferroni corrected for loads of three individual lesion types in the total cohort. # $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Wilcoxon–Mann–Whitney U-test).

To obtain corrected estimates of the load differences (PPMS vs. SPMS) we used multiple linear regression analyses. The load difference in combined inflammatory demyelination was corrected for inactive focal lesion load and for WMA, while the difference in WML load was corrected for WMA. The remyelination capacity difference was corrected for the loads of inactive focal lesion and combined inflammatory demyelination and for the absolute areas of remyelination and WMA sampled.

The loads of active demyelination, combined inflammatory demyelination, and WML were higher in SPMS than in PPMS. The load of active demyelination was 1.9 fold higher in SPMS vs. PPMS when other lesion types and WMA were set equal (95% CI = 1.1 to 3.45, $P = 0.034$). In the hemispheres alone, the corresponding estimate was 2.9 fold higher in SPMS ($P < 0.01$). Conversely, the remyelination capacity was higher in PPMS compared to SPMS (see also Table 6).

Multiple regression analysis: two-sided t-test for β of change from PPMS to SPMS $\neq 0$: $^{\#}P < 0.05$, $^{\$}P < 0.01$. D.f. = 47 minus number of variables in the model.

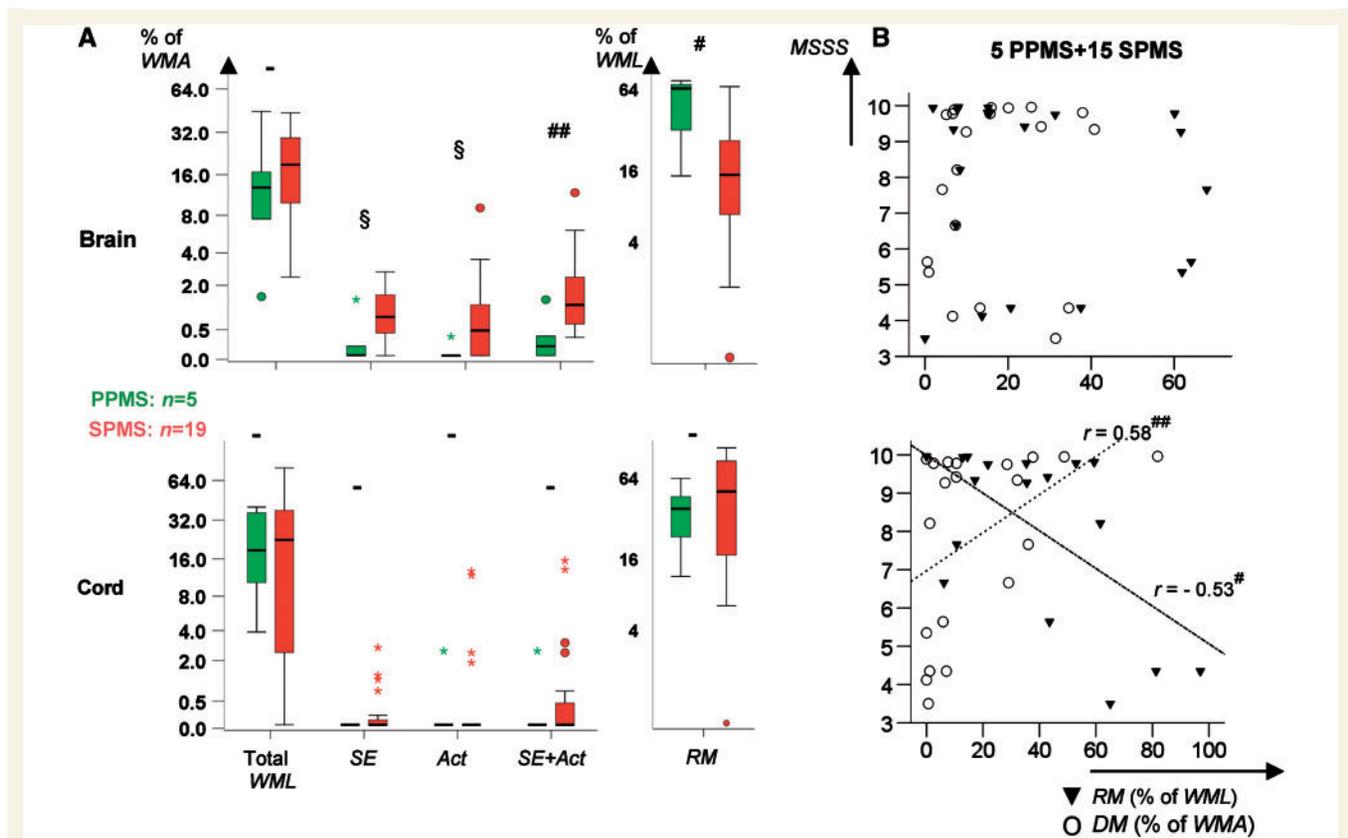


Figure 3 Extent of white matter demyelination and remyelination for the subgroup with PPMS (green, $n = 5$) and SPMS (red, $n = 19$) from whom tissue was available from both brain (upper panels) and spinal cord (lower panels). (A) Planimetry was used to calculate the loads of slowly expanding demyelination (SE), active demyelination (Act), the summed load of the two (SE + Act) and the total load of white matter lesion (WML). Lesion loads were calculated as lesion areas relative to the total brain or spinal cord white matter area (WMA) per patient. Remyelination capacity (RM) was defined as the remyelinated percentage of the total white matter lesion area per patient. In brain, the load of combined inflammatory demyelination was higher in SPMS than PPMS, whereas the remyelination capacity was conversely highest in PPMS (upper panel). By contrast, there were no differences between the two groups in the spinal cord (lower panel). In SPMS, a paired analysis showed less slowly expanding demyelination ($P < 0.01$) and higher remyelination capacity ($P < 0.05$) in the spinal cord than in the brain. This may indicate an association between slowly expanding demyelination and incomplete remyelination in SPMS. Boxes represent interquartile ranges with medians, whiskers ranges, circles observations outside 1.5 times the interquartile range from box edges. Two-sided statistics: $P > 0.10$; $0.1 > P > 0.05$; $^{\#}P < 0.05$; $^{##}P < 0.01$ (5 PPMS versus 19 SPMS; Wilcoxon–Mann–Whitney U-test). (B) The Multiple Sclerosis Severity Score (MSSS) was calculated in patients with information on disability within 3 years before death ($n = 20$; 5 PPMS, 15 SPMS) and plotted against the load of demyelinated lesion (DM = WML without remyelination) and the remyelination capacity. In the spinal cord, white matter demyelination correlated positively with the MSSS, whereas the remyelination capacity correlated negatively with the MSSS. By contrast in the brain, no such correlations were found. Two-sided statistics: $^{\#}P < 0.05$; $^{##}P < 0.01$ (Spearman's rho, degrees of freedom = 19).

in the extent of slowly expanding '2nd hit' demyelination correlated with a decrease in remyelination capacity of 5.9% points [i.e. less complete remyelination relative to the white matter lesion area, 95% confidence interval (CI) -9.5 to -2.2 % points, $P < 0.01$]. Furthermore, the extent of slowly expanding '2nd hit' demyelination and the overall load of slowly expanding demyelination correlated with incomplete remyelination independently in PPMS and in SPMS. In SPMS, the inactive lesion load and the load of active demyelination also correlated with incomplete remyelination after correction. However, '2nd hit' demyelination alone or combined with lesion loads could not explain the entire difference in remyelination between PPMS and SPMS (Table 6).

Chronic demyelination and remyelination in the spinal cord are oppositely associated with the disease severity

We also assessed the extent to which the MSSS correlated with the demyelinated area load and the remyelination capacity in the brain and in the spinal cord white matter. We selected patients in whom we had samples from both the brain and spinal cord and had detailed clinical information within 36 months prior to death ($n = 20$; 5 PPMS; 15 SPMS). The demyelinated area load (load of white matter lesion without remyelination) and the remyelination

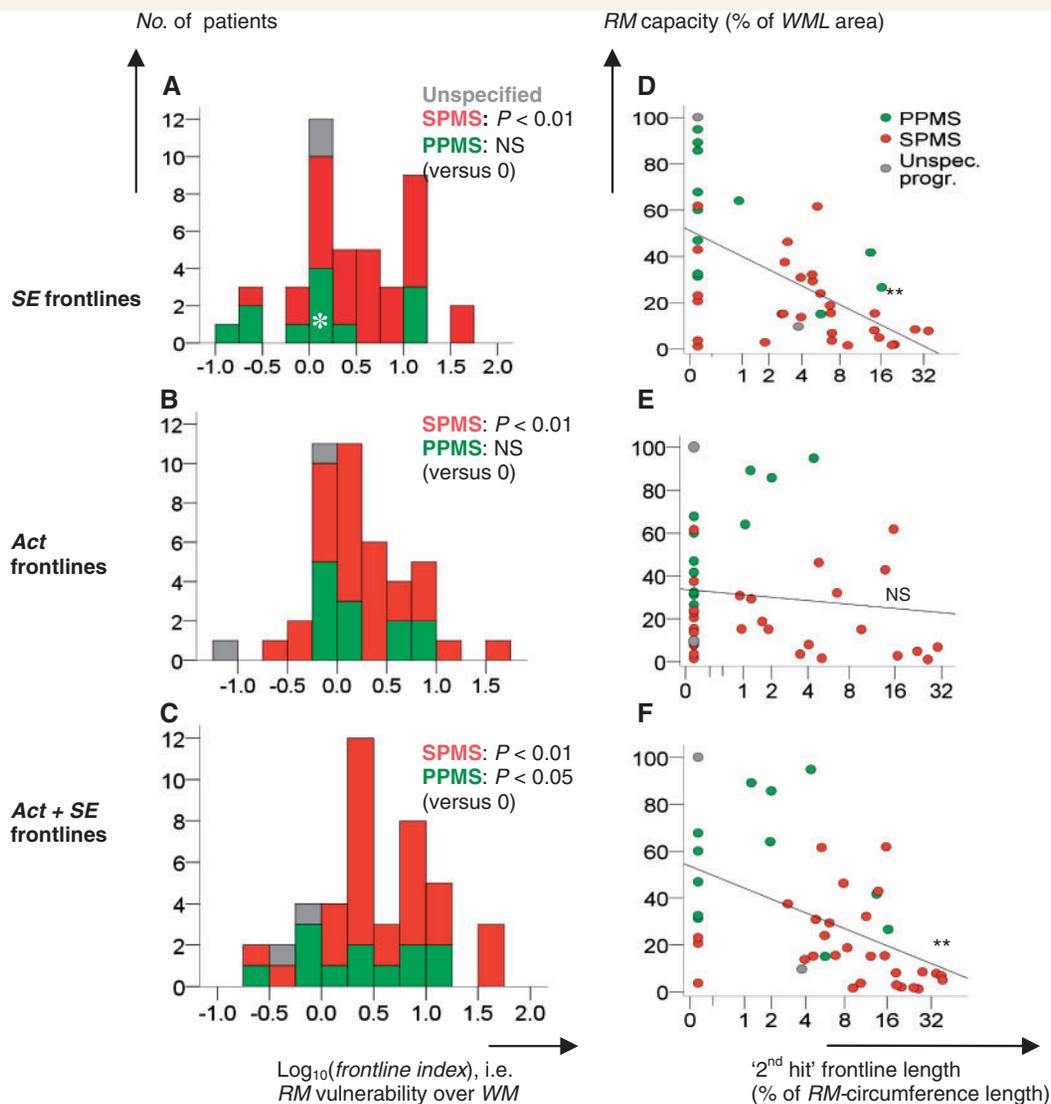


Figure 4 Remyelinated brain areas are more likely to be affected by ongoing demyelination than expected from observations in the white matter without evidence for previous de- and remyelination (WM). We defined '1st hit' demyelination as frontlines of slowly expanding (SE) or active (Act) demyelination affecting the white matter area, while '2nd hit' frontlines targeted remyelinated areas. The sums of slowly expanding and active frontlines combined (SE + Act) were calculated for '1st hit' and '2nd hit' frontlines. Correlations between '2nd hit' demyelination and remyelination capacity are shown in D, E and F. Remyelination (RM) capacity was defined as the remyelinated percentage of the total white matter lesion (WML) area per patient. (A–C): Brains with at least 2 mm² coherently remyelinated area ($n = 43$) were selected and quantified for '1st hit' and '2nd hit' frontlines. By normalizing the ratio of '2nd hit'/'1st hit' frontlines to the sizes of the affected areas, we defined frontline indices, which measure the vulnerability of remyelinated areas over WM in fold. A frontline index of 1 (0 after log transformation) indicates that the length of '2nd hit' frontline(s) affecting remyelinated areas was as expected from the observed length of '1st hit' frontlines affecting WM. Remyelinated areas in SPMS (red bars) were more affected by '2nd hit' slowly expanding (A) and by '2nd hit' active (B) demyelination than expected from the corresponding '1st hit' frontlines affecting WM areas. By contrast, this was not the case in PPMS (green bars). However combined '2nd hit' demyelination affected remyelinated areas more than expected from WM in both PPMS and SPMS (C). See Table 5 for estimates. The white asterisk (A) represents the calculated theoretical example from Fig. 1 (see Patients and methods). (D–F) The extent of '2nd hit' demyelination was defined as the proportion of the circumference of remyelinated areas affected by slowly expanding (D), active (E) or combined frontlines (F) per patient. Slowly expanding and combined '2nd hit' demyelination correlates with incomplete remyelination, whereas this is not the case for active '2nd hit' demyelination. This may indicate that slowly expanding demyelination irreparably destroys repaired myelin. Two-sided statistics (A, B and C): P -values shown in figure (one-sample t -test). Two-sided statistics (D, E and F): ** $P < 0.01$, $r = -0.56$ (D) and $r = -0.58$ (F); NS = $P > 0.1$. (Spearman's rank correlation, degrees of freedom = 42).

Table 5 Excess vulnerability of remyelinated areas over normal-appearing white matter areas

	PPMS (n = 12)	SPMS (n = 29)	PP/SPMS (n = 43)
Mean slowly expanding frontline index (FI) (95% CI)	1.38 (0.49–3.89)	3.70*** (2.31–5.95)	2.65*** (1.72–4.08)
Mean active FI (95% CI)	1.65 (0.93–2.94)	1.83** (1.20–2.78)	1.64** (1.16–2.30)
Mean FI of slowly expanding + active frontlines (95% CI)	2.34* (1.01–5.45)	3.89*** (2.41–6.27)	3.12*** (2.09–4.45)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus 1 (two-sided, one sample *t*-test, $df = n - 1$).

capacity were plotted against the MSSS (Fig. 3B). In the spinal cord, demyelination correlated positively ($r = 0.58$, $P < 0.01$) and remyelination capacity negatively ($r = -0.53$, $P < 0.05$) with MSSS. There were also trends to similar correlations with the EDSS. By contrast, neither demyelination nor remyelination capacity in the brain showed any correlation with the disease severity, emphasizing that the upper part of the disability scales predominantly reflects spinal cord damage. Furthermore, the data indicate that demyelination in the spinal cord may contribute to the accumulation of irreversible motor disability.

Discussion

It is currently unresolved why remyelination largely fails in multiple sclerosis, and in particular in SPMS (Patrikios *et al.*, 2006). Here we show in an extensive quantitative study that slowly expanding demyelination of remyelinated areas is a correlate of incomplete remyelination in patients with progressive disease. Besides this result, our study clarifies a number of other points relevant to the pathology and pathogenesis of progressive multiple sclerosis.

Firstly, we demonstrate a lower overall lesion load and more complete remyelination in the brain in PPMS compared with SPMS. As cognitive dysfunction in multiple sclerosis is generally regarded as subcortical, we speculate that sparing of the white matter in brains from patients with PPMS could, in part, explain their relatively preserved cognitive function compared with patients with SPMS (Comi *et al.*, 1995; Huijbregts *et al.*, 2004; Bergendal *et al.*, 2007). By contrast, the spinal cord white matter appears not to be relatively spared in PPMS, and individual spinal cord plaques may even grow larger in PPMS than in SPMS. These data are in line with comparable MRI findings (Nijeholt *et al.*, 1998; Stevenson *et al.*, 1999; Agosta *et al.*, 2007) and with the faster accrual of physical disability from disease onset in PPMS compared with SPMS (Confavreux and Vukusic, 2006). However, one recent pathological study showed less white matter demyelination in the cervical cords in PPMS compared with SPMS (Tallantyre *et al.*, 2009). This discrepancy is probably explained by different procedures of sampling and tissue analysis. While we included several spinal cord levels and remyelination in our plaque/lesion analysis, Tallantyre *et al.* (2009) found evidence for higher vulnerability of spinal cord axons in PPMS compared with SPMS. Such a difference could contribute to the 'catch-up' in physical disability in PPMS to similar levels as in SPMS at comparable ages, despite shorter disease duration. Larger individual plaques and more vulnerable axons in the spinal cord in PPMS compared with SPMS could therefore explain the previous findings of axonal loss and atrophy of the spinal cord in PPMS (Miller and

Leary, 2007), despite sparing of the white matter of the brain (as shown in this study). These factors may, at least in part, account for the shorter life span from disease onset among patients with PPMS. Importantly, however, the extent of brainstem involvement remains unknown.

The overall load of inflammatory demyelination in the brain was reduced in PPMS compared with SPMS, also in those patients in whom no difference could be detected in the spinal cord. Therefore, the differences in active demyelination and total lesion load in the brain are unlikely to be explained solely by different disease durations or other clinical/demographic imbalances in the groups. To further address potential patient selection bias, we matched the survival of our cohort with that of the population of Danish patients with multiple sclerosis by means of data from the Danish Multiple Sclerosis Registry, which contains data from all diagnosed patients in Denmark. We identified a group of 22 patients (5 PPMS and 17 SPMS) who died between 1963 and 1975 and whose survival was similar to the survival of Danish patients with multiple sclerosis during this period (54.8 years). We infer limited or no selection bias in this group. By contrast, the mean survival among patients in our study who died after 1975 was 51.6 years, i.e. ~10 years shorter than the Danish population of the time. When we restricted our analysis to the putatively more unbiased group collected before 1976, the results resembled those obtained in the whole collection with respect to white matter lesion load, active demyelination, combined inflammatory demyelination and remyelination capacity. Survival and the last recorded EDSS and MSSS were comparable between patients with PPMS and SPMS regardless of the period of collection. Together, it is unlikely that patient selection bias accounts for the pathological differences between PPMS and SPMS.

Secondly, we found all types of plaques in both PPMS and in SPMS and the densities of all plaque types together were similar. Therefore, our data do not support the notion of fundamentally different disease mechanisms. Rather, they may reflect differential responses to common mechanisms of demyelination in the white matter of the brain. While we found evidence for equally frequent episodes of demyelination with or without remyelination (i.e. equal densities of all plaque types together), we found a lower density and load of active demyelination, lower total lesion load and more complete remyelination in PPMS. Furthermore, the brain plaques were, on average, smaller in PPMS than in SPMS. This may indicate that a more efficient regulation and repair of the inflammatory disease process contribute to sparing of the white matter of the brain in PPMS compared with SPMS. Although speculative, a more efficient regulation of the inflammatory process could arise from different antigen presentation in PPMS since human leucocyte antigen DR-DQ haplotype frequencies differed between

Table 6 Correlates of incomplete remyelination

Group Model Co-variable change	PPMS (n = 13)		SPMS (n = 34)		PP/SPMS (n = 50)	
	Multipel linear Corrected β (95% CI) Model variables Adjusted R^2					
Age (1-year increase, n = 50)	0.8 (-0.9 to 1.0) R, S, I, C 0.68 [§]	0.1 (-0.4 to 0.6) R, S, I, C 0.55 ^{§§§}	0.9** (0.3–1.5)		0.2 (-0.2 to 0.8) Co, R, S, I, C 0.66 ^{§§§}	
Doubling inactive focal lesion load	-2.1 (-12.2 to 8.0) R, S, C 0.72 ^{§§}	-4.7* (-8.8 to -0.6) R, C 0.55 ^{§§§}	-12.0*** (-16.9 to -7.2)		-8.6*** (-12.4 to -4.7) Co, R, C ^a 0.67 ^{§§§}	
Doubling slowly expanding demyelination	-24.0* (-43.7 to -4.2) R, S, AC 0.75 ^{§§}	-9.0** (-14.8 to -3.1) R, AC 0.54 ^{§§§}	-18.0** (-27.8 to -8.1)		-9.4* (-17.3 to -1.6) Co, R, I, AC ^a 0.61 ^{§§§}	
Doubling active demyelination	-1.5 (-28.2 to 25.2) R, S, SE 0.75 ^{§§}	-3.7* (-6.7 to -0.6) R, SE 0.54 ^{§§§}	-7.2* (-12.7 to -1.7)		-4.5* (-8.5 to -0.5) Co, R, I, SE ^a 0.61 ^{§§§}	
Doubling combined inflammatory demyelination	-18.8* (-36.3 to -1.4) R, S, I 0.72 ^{§§}	-4.0* (-7.5 to -0.4) R, I 0.55 ^{§§§}	-11.0*** (-16.2 to -5.7)		-5.7** (-9.8 to -1.5) Co, R, I ^a 0.67 ^{§§§}	
Doubling slowly expanding '2nd hit' frontlines (% of RM circumference length) n = 43; 12 PPMS, 29 SPMS, 2 progressive	-7.5* (-14.6 to -0.3) R, S, ac 0.73 ^{§§}	-3.8* (-7.4 to -0.2) R, ac 0.37 ^{§§}	-9.6%*** (-13.8 to -5.3)		-5.9** (-9.5 to -2.2) A, Co, R, S, I, C, ac 0.59 ^{§§§}	
Doubling active '2nd hit' frontlines n = 43	3.7 (-27.1 to 34.4) R, S, se 0.73 ^{§§}	-1.9 (-5.0 to 1.3) R, se 0.37 ^{§§}	-2.1 (-7.4 to 3.3) 0		-0.7 (-5.9 to 4.5) A, Co, R, S, I, C, se 0.59 ^{§§§}	
Doubling combined '2nd hit' frontlines	-7.9* (-14.7 to -1.1) R, S 0.72 ^{§§}	-2.8 (-6.5 to 1.2) R 0.31 ^{§§§}	-8.0** (-12.4 to -3.6) 0.23		-4.3* (-8.5 to -0.2) A, Co, R, S, I, C 0.55 ^{§§§}	
Change from PPMS to SPMS (13 PPMS versus 34 SPMS)			-33.9*** (-47.8 to -19.9) 0.33 ^{§§§}		-16.3** (-26.4 to -6.2) A, R, S, I, C, (C) 0.75 ^{§§§}	

In each regression analysis, upper values represent mean change in remyelination capacity in % points of white matter lesion area (CI) with change in predicting variables (see left column). Lower values in each analysis represent adjusted R^2 , which is a measure of the predictive power of the model.

Two-sided t-test for $\beta \neq 0$: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Two-sided F-test for adjusted $R^2 \neq 0$: [§] $P < 0.05$; ^{§§} $P < 0.01$; ^{§§§} $P < 0.001$. Patient n minus number of model variables. Clinical variables: A = age; Co = disease course (PPMS, SPMS or progressive MS with unknown onset). Variables of sampling: R = absolute remyelinated area sampled; S = hemispheres versus small blocks. Pathological predictors: I = inactive focal lesion load; SE = slowly expanding demyelination load; AC = active demyelination load; C = combined inflammatory demyelination (SE + AC); se = slowly expanding '2nd hit' frontlines; c = combined '2nd hit' frontlines (se + ac).

a: A and S did not improve the model. They were therefore excluded.

(c) Similar estimate of remyelination capacity difference in 12 PPMS versus 29 SPMS with data for c and after adjusting additionally for c.

patients with RRMS/SPMS and PPMS (Olerup *et al.*, 1989; Hillert *et al.*, 1992). However, unknown genetic differences unrelated to inflammatory demyelination could additionally explain the difference in remyelination between patients with PPMS and SPMS because this difference persisted after correction for inflammatory demyelination, including '2nd hit' demyelination. For instance, allelic variants in growth-related genes have been suggested to influence remyelination in mice (Suwansrinon *et al.*, 2009).

It remains obscure why the spinal cord is as equally involved in PPMS as in SPMS, despite sparing of the white matter in the brain in PPMS. In addition to potential blood–brain barrier differences between the brain and the spinal cord (Juhler *et al.*, 1984; Silwedel and Forster, 2006), the spinal cord white matter is, by anatomy, more directly exposed to inflammatory cells in the cerebrospinal fluid (Monson *et al.*, 2005) than white matter in the brain, with the exception of the periventricular regions. A connection between demyelination and the cerebrospinal fluid in PPMS is also supported by the previous paired observation of equal levels of cortical demyelination in patients with PPMS and SPMS, but a trend of sparing of the white matter in brains with PPMS as compared with SPMS (Kutzelnigg *et al.*, 2005), similar to this study. Thus we speculate that in the spinal cord, putative regenerative/repairative properties of the CNS white matter in PPMS could be offset by chronic inflammation in the cerebrospinal fluid, possibly driven by B cells (Sellebjerg *et al.*, 2009).

Thirdly, the positive correlation between spinal cord demyelination and the MSSS and the negative correlation between spinal cord remyelination capacity and the MSSS represents the first direct link between the demyelination–remyelination balance and the severity of clinical disease. By contrast, MRI studies have only detected weak correlations between spinal cord T₂ lesion load and the EDSS (Stevenson *et al.*, 1999). However, the EDSS does not consider the disease duration required to develop a given disability level. Inability to distinguish demyelinated from remyelinated and white matter from grey matter lesions may also explain the weak cross-sectional correlations between the EDSS and focal lesion load as seen on MRI. Our data are consistent with the high weight of motor function in the upper part of the EDSS and the high condensation of motor fibres in the spinal cord, although we did not separate extents of demyelination in different tracts.

Lastly, although the vulnerability of remyelinated areas to active demyelination was increased in SPMS, the extent of active '2nd hit' demyelination did not correlate with remyelination capacity. Also, the load of active demyelination correlated only weakly with incomplete remyelination, whereas the load of inactive lesion correlated more strongly with incomplete remyelination. These data are in line with animal experiments of remyelination that led to the so-called Chari–Blakemore hypothesis of temporal mismatch (Chari and Blakemore, 2002), stating that oligodendrocyte lineage cells may migrate, differentiate and remyelinate only in areas with a sufficient drive from inflammatory mediators and that demyelination of large areas may uncouple remyelinating cells from these mediators. Smaller brain plaques in PPMS may allow for cells of the oligodendrocyte lineage to migrate throughout the plaque during inflammation and to establish remyelination. The potential importance of inflammation for remyelination to succeed was also emphasized by showing signs of complete

maturation of remyelinating cells only in active, but not in inactive plaques (Kuhlmann *et al.*, 2008). The perspective that transplanted oligodendrocyte lineage cells also need inflammatory stimuli to complete remyelination was recently shown in dysmyelinated rat spinal cord (Foote and Blakemore, 2005). However, the increased vulnerability of remyelinated areas to active demyelination in SPMS could, at least in part, explain why similar symptoms often develop in consecutive relapsing–remitting episodes (Deen *et al.*, 2008). Additionally, many relapses in RRMS/SPMS, although most likely arising from active demyelination, do not remit fully (Lublin *et al.*, 2003). Pathologically, this may be reflected in our data by the weak negative correlation between the load of active demyelination and permanent remyelination. Others have found evidence that each bout of active demyelination may lead to acute axonal injury (Kornek *et al.*, 2000) and evidence for long-lasting axonal injury whether seen in relapsing–remitting or in progressive multiple sclerosis (Frischer *et al.*, 2009). Supplementary Fig. 2 may also reflect a potential loss of conductive tissue from recurrent active demyelination, even if yet again remyelinated. Thus, we propose that a first episode of demyelination and remyelination renders the area vulnerable to new waves of active demyelination that, even if again remyelinated, may cause stepwise progressive loss of conductive tissue and function, if occurring in a non-redundant area.

It is unknown why remyelination predominantly fails in SPMS compared with PPMS (as shown in this study) and RRMS (Patrikios *et al.*, 2006). SPMS is distinguished pathologically from RRMS by the presence of slowly expanding demyelination (Frischer *et al.*, 2009) and possibly by more rare active demyelination (Molyneux *et al.*, 2000). Therefore, we investigated whether slowly expanding and active demyelination showed a predilection for remyelinated areas over white matter without evidence for previous de- and remyelination. Our observations indicate that slowly expanding demyelination may have a predilection for remyelinated areas in SPMS, but not necessarily in PPMS. However, slowly expanding '2nd hit' demyelinating frontlines correlated with incomplete remyelination in both PPMS and in SPMS, whereas active '2nd hit' frontlines did not. The load of slowly expanding demyelination also correlated more strongly with incomplete remyelination than did the load of active demyelination. These observations suggest involvement of slowly expanding demyelination in remyelination failure and that recurrence of slowly expanding demyelination could destroy remyelinated areas in an irreparable manner as opposed to recurrence of active demyelination. However, in SPMS, high loads of active demyelination may not be fully repaired as reflected by a weak correlation with incomplete remyelination. According to the Chari–Blakemore hypothesis, such a negative correlation could arise if the inflammatory reaction in actively demyelinating areas in SPMS was not always sufficient to attract and mature remyelinating cells. This is in line with previous observations of lower densities of adaptive immune cells in active plaques in progressive multiple sclerosis as compared with acute/relapsing–remitting disease (Frischer *et al.*, 2009). Furthermore, the inactive lesion load also correlated with incomplete remyelination in SPMS, possibly reflecting a limited migration ability of remyelinating cells, but possibly

also reflecting accumulated irreparable slowly expanding and active demyelination. In addition to the abovementioned factors influencing remyelination capacity, plaques located in the subcortical white matter seem to remyelinate more efficiently than plaques located in the periventricular white matter (Goldschmidt *et al.*, 2009). However, we did not separate the plaques according to these locations. Therefore, it remains unknown to what extent different plaque locations may account for the different remyelination capacity between PPMS and SPMS.

We found slowly expanding demyelination in amounts that were comparable in PPMS and SPMS and that correlated with incomplete remyelination. In turn, incomplete remyelination in the spinal cord correlated with disease severity. Although not a direct indication, these observations are consistent with the concept of slowly expanding demyelination as a pathological correlate of clinically progressive multiple sclerosis (Prineas *et al.*, 2001). However, active demyelination could additionally determine the clinical outcome during progressive disease. The presence of either active or slowly expanding demyelination has previously been associated with shorter survival as compared with pathologically inactive disease (Frischer *et al.*, 2009).

In conclusion, we confirm that the white matter of the brain is more severely affected in SPMS than in PPMS, but we do not find pathological suggestions of fundamentally different disease mechanisms between patients with PPMS versus SPMS. Rather, our findings suggest that regulatory and reparative properties could protect the white matter of the brain in PPMS. Patients with PPMS could thereby remain spared of symptoms until the spinal cord is affected. By contrast, remyelinated areas in SPMS are prone to recurrent active demyelination, possibly explaining the frequent recurrence of similar symptoms in consecutive relapsing-remitting episodes. We find that incomplete remyelination in the spinal cord correlates with more severe disease and that slowly expanding demyelination may irreparably destroy existing remyelination in the brain. Together, our data are consistent with a hypothesis of focal plaque formation by slowly expanding inflammatory demyelination as a correlate of clinical progression.

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Supplementary material

Supplementary material is available at *Brain* online.

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