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High levels of perivascular inflammation and active demyelinating lesions at time of death associated with rapidly progressive multiple sclerosis disease course: a retrospective post-mortem cohort study.

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Abstract

Objective: Analysis of post-mortem multiple sclerosis (MS) tissues combined with in-vivo disease milestones suggest that while perivascular white matter infiltrates associate with demyelinating activity in the initial stages; leptomeningeal immune cell infiltration, enriched in B cells and associated with cortical lesions, contribute to disease progression. We systematically examine the association of inflammatory features and white matter demyelination at post-mortem with clinical milestones.

Methods: In 269 MS brains; 20 sites were examined using immunohistochemistry for active lesions (ALs) and perivenular inflammation (PVI). In a subset of 22 a detailed count of CD20+ B cells and CD3+ T cells in PVIs was performed.

Results: ALs were detected in 22%, whereas high levels of PVI were detected in 52% of cases. ALs were present in 35% of cases with high levels of PVI. Shorter time from onset of progression to death associated with increased prevalence and higher levels of PVI (both $p<0.0001$). Shorter time from onset of progression to wheelchair use associated with higher prevalence of ALs (OR 0.921, 95% CI (0.858, 0.989), $p=0.0230$) and higher level of PVI (0.932, (0.886, 0.981), $p=0.0071$). High levels of PVI were associated with meningeal inflammation and increased cortical demyelination and significantly higher levels of B lymphocytes within the PVI.

Interpretation: ALs, a feature of early disease stage, persist up to death in a subgroup with high levels of PVI. These features link to a rapid progressive phase and higher levels of meningeal inflammation and B-cell infiltrates, supporting the hypothesis that chronic inflammation drives progression in MS.

Keywords: multiple sclerosis, disease progression, early active lesions, perivenular inflammation

Introduction

623 words

Inflammation is thought to be the fundamental driver of the pathology in multiple sclerosis (MS) at all stages of the disease course, giving rise to demyelination and axonal and neuronal loss and this combined pathology results in the characteristic symptoms experienced by people with MS (Reynolds et al., 2011, Kutzelnigg & Lassmann, 2014, Steinman & Zamvill, 2016). In individuals the manifestations are highly variable but are characterised by two dominant clinical events: relapses, transient periods of neurological deterioration with variable recovery; and progression, characterised by the gradual accumulation of disability that leads to the major personal and societal cost of MS (Olesen et al., 2014). Relapses have proven amenable to therapy but progression has thus far been resistant to treatment leading to a major unmet need (Humphries, 2012). Thus, despite extensive pathological evidence for inflammation in progressive MS (Reynolds et al., 2011; Frischer et al., 2009), no immunomodulatory treatment that has been shown to suppress relapses and gadolinium MRI activity has had a major impact on the progressive course (Montalban et al., 2017; Kappos et al., 2018). This may be due to the dominant type of intrathecal compartmentalized inflammation in progression being 'hidden' behind the blood brain barrier or due to alternative mechanisms, such as cortical pathology and slowly expanding lesions (Mahad et al., 2015; Lassmann et al., 2019; Monaco et al., 2020; Haider et al., 2021; Absinta et al., 2016). Furthermore, although MS is a highly heterogeneous disease, characterized by large inter-individual differences in disease course, several lines of evidence from both MRI and pathological assessment (biopsies and autopsies) indicate that the immunologic pattern of tissue pathology in MS characterizes each MS patient from the initial disease phase (Lucchinetti et al., 2000; Metz et al., 2014; Luchetti et al., 2018).

The UK MS Society Tissue Bank (UKMSTB) post-mortem MS cohort offers a unique opportunity to investigate how inflammation evident at time of death reflects the lifetime course of MS, offering pathological confirmation of MS in concert with a clinical history and standardised pathological assessment in a large community-based cohort. This resource has contributed to our understanding of the impact of meningeal B-cell inflammation on subpial grey matter (GM) demyelination and immune-pathological cell and molecular alterations that in turn may lead to a more rapid and severe disease progression (Magliozzi et al., 2007; Howell et al., 2011; Bevan et al., 2018; Picon et al, 2021).

To determine whether there are any other pathological features of inflammation, in addition to meningeal infiltration, that could be related to the timing of the progressive phase in subjects with MS, we examined two aspects of inflammation in post-mortem tissue in well characterised cases: active and demyelinating/early active lesions (Brück et al., 1995, Reynolds et al., 2011; Kuhlmann et al., 2017) and the perivenular infiltrates (Reynolds et al., 2011, Frischer et al., 2009; Luchetti et al., 2018). Active lesions (AL) are classified by the presence of inflammation with evidence of recent myelin breakdown indicating that they have been present for only 3 months (Brück et al., 1995). Perivenular inflammation is an infiltration of lymphocytes into the venule outside the blood brain barrier producing thickening of the venule wall (Charcot, 1869, Frischer et al., 2009; Luchetti et al., 2018). Both these features are distributed throughout the brain in MS, requiring widespread sampling to confirm or refute their presence. The extensive and reproducible assessment of brain tissue used in the UKMSTB allows the consistent

identification of these inflammatory processes, if present. The possible association between early active plaques, perivenular inflammation (PVI) and clinical outcome has therefore been examined in detail in a large MS cohort to determine whether variations in clinical milestones in MS were associated with the prevalence of these inflammatory lesions in post-mortem tissue.

Methods

UKMSTB cohort

The UKMSTB operates a nationwide community-based donor scheme of people, with and without MS, who register to donate their brain and spinal cord after death. After donation donor histories, where available, are summarised by a neurologist prior to the neuropathological analysis (Reynolds et al., 2011). All tissues were collected with fully informed consent via a prospective donor scheme with ethical approval by the National Research Ethics Committee (08/MRE09/31). Donor brain and spinal cord are examined by a neuropathologist according to standardised criteria (BrainNet Europe: http://www.brainet-europe.org).

This analysis focused on a group of 269 MS donors, who died between 2004 and 2012, where it was possible to clearly characterize the disease course with sufficient clinical records to provide information on the key clinical milestones: age at disease onset, an estimate of the time to onset of progression, the time at which a wheelchair was required or not and the age at death. In brief the history was summarised by a neurologist blinded to the neuropathological findings to determine the key clinical milestones above, but also the number of relapses in the first two years of disease (n=260), the time at which a wheelchair was required (n=220), and the occurrence of progression during terminal illness (n=239), defined by accumulation of disability in the last months prior to death. The examined cohort closely reflects the UK MS population and the range and ratio of PPMS and SPMS cases in the UK (Reynolds et al., 2011). None were receiving disease modifying treatments in the period prior to death and at the time of death.

All demographic and clinical characteristics of the cohort are described in Table 1. All donor brains had been examined for the presence or not of early active lesions and perivenular inflammation (graded 0-5) as this formed the focus of this investigation (Reynolds et al., 2011). Perivenular inflammation was scored as the highest degree of severity seen in this assessment. Further information available included: gender and the number and nature of clinical relapses. In addition, the examined cohort includes a subset of brains (87 out of 269) previously investigated for the presence of meningeal inflammation and associated subpial cortical demyelination (Howell et al., 2011).

Neuropathology examination

Donor brain and spinal cord were examined by a neuropathologist according to standardised criteria (BrainNet Europe: http://www.brainet-europe.org). Tissue blocks (2x2x1cm) were prepared from whole coronal slices dissected immediately on brain retrieval and fixed in 4% paraformaldehyde for a minimum of 12 hours and processed for paraffin embedding or rapid freezing. This study focussed on the standardised set of paraffin embedded blocks that are prepared for diagnostic confirmation. Paraffin serial sections (7 μm) from each block were stained with haematoxylin–eosin, Luxol Fast Blue/Periodic Acid-Schiff (LFB/PAS) and Luxol Fast Blue/Major Histocompatibility Complex class II (LFB/MHC class II) antigen, with additional histochemical and immunohistochemical stains performed when required for diagnosis (Reynolds et al., 2011). The classification of plaques used at the UKMSTB has been proposed by Professor I. Allen (http://www.ICDNS.org). and referred to the first stage of inflammation and myelin breakdown as AL, characterized by hypercellularity with microglial

activation throughout the lesion, signs of myelin phagocytosis and degradation with LFB fragments of myelin within macrophagic cells (Reynolds et al., 2011), similar to what has recently described as 'active and demyelinating lesions' (Kuhlmann et al., 2017).

PVI assessment was a semi-quantitative assessment performed by the neuropathologist independent of this work. Sampling of at least 20 tissue blocks from each of the 269 examined brains within a spectrum of lesions identified macroscopically was carried out, with grading as described by Reynolds et al (2011). PVI assessment (n=265, Figure 1) provided a grading (0-5) of the extent of PVI: a grade from 0 to 5 was evaluated according to the extent of cellular infiltration. In particular, a score of 5 corresponded to the highest degree of severity detected; a high degree of inflammation (high PVI) was defined by a grade of 2 or more (Figure 1).

For each examined MS cases, the presence of active lesions (AL, n=267), late active/chronic active, inactive, shadow plaques (n=248), as well as the involvement of GM, at the routine analysis (yes/no, n=252) was performed in the same brain tissues examined for the presence/level of PVI.

Perivascular cell count

In order to characterize the inflammatory cell populations present in the perivenular infiltrates associated with on-going demyelination, a quantitative analysis of T and B cells in the perivenular infiltrates associated with ongoing demyelination and in normal-appearing white matter (NAWM), was performed on serial paraffin sections from 22 MS cases (11 with high PVI and 11 with low PVI). Region-matched sections from each case from superior frontal gyrus (sampled 1 cm rostral to the temporal pole), thalamus, primary visual (striate) cortex (sampled 1.5 cm rostral to the occipital pole) and pons (including locus coeruleus) were immunostained with the monoclonal antibodies for myelin oligodendrocyte glycoprotein (MOG) and for CD20 and CD3 as B and T lymphocytes marker respectively (Supplementary Table 2) following the procedures previously described (Magliozzi et al., 2007; Howell et al., 2011) and digitalized images from entire slices were acquired.

In NAWM, CD3+ and CD20+ cells were quantified from up to four vessels (veins) in cross-section, presenting with a thin tunica media and with a total area (vessel and perivascular space) >0.002 mm^2. Two randomly non-continuous selected fields per each block (areas of each field of 4 mm^2) have been examined. Fields in proximity of lesions were excluded; if an infiltrate/vessel was on the edge of the selected area it was included in the analysis; if more than 4 vessels with such characteristics were present in the selected area, only the 4 vessels with more cellularity were counted. The mean number of CD3+ and CD20+ cells was calculated per each field and each case. In the lesions, infiltrates were counted in vessels (veins) in cross-section presenting with a thin tunica media and with a total area (vessel and perivascular space) >0.003 mm^2. For each examined MS case the number of perivenular CD3+ and CD20+ cells was calculated in 4 vessels with perivascular space >0.003 mm^2.

Evaluation of meningeal inflammation

Eighty-seven out of 269 evaluated cases had previously been extensively investigated for the presence of meningeal inflammation and lymphoid-like structures (Howell et al., 2011). Briefly, each case was screened on paraffin-embedded sections for the presence of B cell

aggregates, assigning an index of inflammation based on the maximum density of meningeal and/or perivascular infiltrates seen. Only tissue blocks containing substantial meningeal infiltrates with lymphoid-like organisation were processed further for anti-CD20 immunohistochemistry and characterized as follicle positive SPMS if at least one aggregate enriched of CD20+ B cells was identified in the meninges together with the presence of CD35+ follicular dendritic cells, proliferating Ki67+ CD20+ cells and IgA, -G, -M+ plasmablasts/plasma cells (Serafini et al., 2004; Howell et al., 2011).

Image acquisition and analysis

Tissue sections were analysed on a Nikon E1000M microscope using brightfield imaging (Nikon Instruments Inc.) with a digital camera (QImaging). Digitized images from entire slices of the 22 cases evaluated in the quantitative analysis (stains for CD3, CD20, MOG, LFB/MHCII, LFB/PAS) were acquired by means of an Aperio AT2 Scan Digital Whole Slide Scanner (20x magnification). Image files were handled using QuPath (Bankhead et al., 2017). All quantifications were manually performed with the observer blinded to case identification of perivascular/meningeal inflammatory status.

Statistical analysis

Demographic, clinical and neuropathological characteristics were described by means and standard deviations in case of continuous variables and by frequencies and percentages in case of categorical variables. Logistic regression models were used with stepwise variable selection to model the probabilities of early active lesions and perivenular inflammation. Factors within the models included: the time interval from birth to onset, onset to progression and progression to death, gender, >2 relapses in the first two years after onset, MS was progressive in last illness, a high grade of perivenular inflammation (none/minimal [0-1] vs. significant presence [2-5]), and the presence of early active plaques found in the standardised assessment. Where appropriate the time interval from progression to use of a wheelchair was used in place of progression to death. To calculate probabilities groups were generated for the time interval from birth to onset (age of onset≤20 years; 20<age of onset ≤30 years; 30<age of onset ≤40 years; 40<age of onset), onset to progression (time to progression≤5 years; 5<time to progression≤10 years; 10<time to progression≤15 years; 15<time to progression) and progression to death (time from progression to death≤10 years; 10<time from progression to death≤15 years; 15<time from progression to death≤20 years; 20<time from progression to death). Otherwise, the Chi-square test was used to compare the relationship between perivenular inflammation and early active lesions.

Results of cell count analysis were presented as scatter dot plots with a line at the mean or as box-and-whiskers plots showing min-to-max values, interquartile range and group medians. Two-group comparisons were performed using the Mann-Whitney U test or Wilcoxon matched pairs test, whilst three or more groups were compared by non-parametric one-way ANOVA (Kruskal-Wallis test), using Dunn's multiple comparisons post-test. Correlations were tested by Spearman analysis. Due to the exploratory nature of this study p-values and confidence intervals were not corrected for multiple testing; two-sided p-values smaller than 0.05 were

considered statistically significant. All computations were carried out using SAS version 9.4, Stata version 13.0, and GraphPad Prism Software 7.0.

Results

Demographic and clinical characteristics

In this investigation we selected patients from the MS tissue bank database who had both sufficient information from patient records to determine the key events in the disease course and a neuropathology assessment performed (n=269). Demographic and clinical characteristics of the cohort are described in Table 1. All cases had a history of progressive MS, with a mean disease duration of 30.4±12.0 years. 174 (64.7%) were females; no significant differences in clinical milestones were noted according to gender. A progressive disease course from onset (primary progressive multiple sclerosis, PPMS) was found in 18 cases. PPMS cases had a higher age of MS onset (p=0.008), time from progression to wheelchair (p=0.002) and death (p=0.008), and a lower number of relapses (p=0.01) in the first 2 years of MS (Table 1).

Substantial inflammatory activity is present at the time of death in progressive MS

We next quantified the lesion types present in the cases, in each of the 4 blocks examined. 54.8% (136/248) had at least one active lesion (AL, Figure 2A, E); 61.3% (152/248) had at least one late active/chronic active lesion (LA/CAL, Figure 2B, F). 91.5% (227/248) and 53.2% (132/248) of cases had inactive (Figure 2C, G) or shadow plaques (Figure 2D, H), respectively. In the total cases high PVI was detected in 52% of cases (n=137) and at least one AL (Fig 2A) was present in 22% of the cases (n=59, Table 2). No significant differences in the incidence of AL and high PVI were detected according to gender (p=0.304 and p=0.878, respectively) and MS type (p=0.378 and p=0.470, respectively, Table 2).

Looking at high and low PVI and the types of lesion present and their relationship to PVI, In high PVI cases ALs were significantly increased compared to low PVI cases (High PVI 35% (48/136) versus Low PVI (10% (11/115), p<0.001, Figure 2E). ALs were spatially associated with the presence of a PVI. LA/CAL were also significantly increased in high PVI compared to low PVI cases (High PVI 84% (112/133) versus low 35% (40/115), p<0.001, Figure 2F).

In contrast in high PVI cases there was a significantly lower rate of inactive lesions (high PVI 87% (116/133) versus low PVI 97% [111/115], p=0.01, Figure 2G) and a trend towards a lower number of shadow plaques (high PVI cases (48% (64/133) versus low PVI 58% [67/115], p=0.12, Figure 2H).

Perivenular inflammation and active lesions are associated with a more severe MS course

A high PVI grade corresponded to a more severe disease course (Table 3, Supplementary Table 3). Both a significantly earlier age at onset (30.0±10.2 vs 32.9±9.4 years), a shorter time from onset to progression (10.9±8.1 vs 13.6±10.5 years) and a shorter time from progression to death (15.6±8.0 vs 20.8±9.6 years), were seen in cases with high PVI compared to the low PVI group. Likewise, the presence of ALs also associated with a more severe disease course through all the disease phases (Table 3). Patients with both ALs and high PVI (n=48) had the youngest age of onset (29.1±9.8 years) and of death (50.5±9.6 years), with a mean time from onset to progression of 10±7.1 years and from progression to death 11.5±6.1 years.

Notably, disease activity in the last illness was documented in 45.6% of cases. Among patients with MS progression during their terminal illness, 60% had high PVI, compared to 46% in those not progressing from MS in their terminal illness (p=0.037). Similarly, in those with MS progression driving their terminal illness, 30% had ALs compared to 15% where MS was not relevant (p=0.008).

Increased probability of perivenular inflammation and active lesions at post-mortem is associated with shorter time from progression to death

After applying the stepwise logistic regression model, the probability for high PVI was most increased in patients with a shorter time from progression to death (OR 0.915, CI 95% [0.884-0.946], p<0.001), a shorter time from MS onset to progression (OR 0.922, CI 95% [0.891-0.955], p<0.001 and who had a disease onset at a younger age (OR 0.938, CI 95% [0.908-0.968], p<0.001; Figure 3). A similar association was noticed regarding ALs (age at onset: OR 0.943, CI 95% [0.908-0.980], p=0.003; time from MS onset to progression: OR 0.935, CI 95%[CI 0.897-0.975], p=0.0018; time from progression to death OR 0.868, CI 95% [0.824-0.913], p<0.001; Figure 3).

After adding LA/CAL to the stepwise logistic regression model AL still associated with time from progression to death (p<0.001). The estimated proportion of patients with at least one of each plaque type, including ALs, as a function of time from progression to death (Figure 3G) and age of death (Figure 3H) is shown in figure 3.

Age of onset (OR 0.909, CI 95% [0.877-0.943], p<0.001), time from onset to progression (OR 0.930, CI 95% [0.896-0.965], p<0.001), and time from progression to death (OR 0.934, CI 95% [0.904-0.966], p<0.001) were also identified as predictive factors for MS progression in terminal illness. When MS progression in terminal illness was added to the logistic regression models, the activity of disease was not found to predict independently either high PVI (p=0.458) or AL (p=0.648). The association between AL and high PVI and MS progression in terminal illness was then explained through age of onset, time from onset to progression and time from progression to death.

Perivenular inflammation and active lesions associate with a shorter time from onset of progression to wheelchair

A shorter time from onset of progression to needing a wheelchair was associated with a higher probability of the presence of AL (OR 0.921, 95% CI [0.858, 0.989], p=0.0230) as well as a higher level of perivenular inflammation (OR 0.932, 95% CI [0.886, 0.981], p=0.0071) at post-mortem, adjusted for age of onset and time from onset to progression which were also found to be statistically significant (Figure 3I-L).

Preponderance of B lymphocytes is associated with the extent of demyelination and a more severe disease course

A higher number of perivenular CD3+ T lymphocytes (Figure 4A, B) and CD20+ B lymphocytes (Figure 4C) was detected in those cases defined as having high PVI in the semi-quantitative analysis (Figure 4A), in both white matter lesions and NAWM. Lesions (n=127) from cases with high PVI had increased total lymphocyte number (mean$\pm$SD: 40.9$\pm$36.7 vs

12.2±13.5, p=0.002), B cells (11.5±18 vs 2±2.1, p=0.002) and T cells (29.4±20.7 vs 10.2±11.5; p=0.002) (Figure 4D). Active lesions (n=12, including both AL and late/chronic active ones) were characterized by an increased number of T cells (67.1±76.2 vs 11.5±15.9) and B cells (20.5±35.6 vs 2.6±4.3) in the perivenular infiltrates (p<0.001) compared to inactive ones (n=19). No substantial difference in parenchymal lymphocyte frequency was observed in association with the presence of AL. The presence of parenchymal lymphocytes, mainly CD3+ T cells and very rarely CD20+ B cells (Fig 4 C, D), was rare and heterogeneous, since it was not observed in all the examined MS cases. In particular, the presence of parenchymal infiltrating lymphocytes was mainly observed in close proximity to PVI (Fig 4 C, D).

The presence of active lesions was evident in the majority of the high PVI cases analysed in detail (11 high PVI and 11 low PVI) with 10 AL detected in high PVI compared with 8 inactive lesions in the same blocks. In contrast the 11 cases with low PVI were characterized by a lower proportion of AL (2 AL) compared to 11 inactive lesions in the same blocks.
Cases with high PVI were also confirmed to be harbouring higher levels of total (5.4±2.4 vs 3.1±1.6, p=0.013), T (4.4±2.3 vs 2.4±1.2, p=0.032) and B lymphocytes (1±0.4 vs 0.6±0.4, p=0.073) in the perivenular NAWM when compared with the low PVI group (Figure 4E). A difference in the total perivenular number of cells was found when comparing thalamus (2.7±2.1) with both prefrontal gyrus (4.9±3.1, p=0.01) and pons (4.7±2.9, p=0.003), but not with visual cortex (4.8±4.5, p=0.285) was detected.
An increased B/T ratio inside the lesions associated with a reduced disease duration (r -0.476, p=0.025, Figure 4F). Total number of cells in lesions and in the NAWM perivenular infiltrates did not correlate with the main disease milestones. When evaluating the number of CD20+ cells, a correlation was found with a shorter disease duration (r -0.537, p=0.01) and a younger age at death (r -0.443, p=0.039) (Figure 4G).

Perivenular and meningeal inflammation are associated in progressive MS

Among 87 cases previously evaluated for the presence and extent of meningeal inflammation (Magliozzi et al., 2007; Howell et al., 2011), 34 (42.5%) had been defined as having tertiary lymphoid-like structures, also named follicle-like structures. The presence of tertiary lymphoid structures in the meninges was associated with both high PVI (n=29/34, 85.3%, p=0.002) and AL (n=18/34, 52.9%, p=0.006, Figure 5A, C). Seventeen of the 18 subjects with AL also had high PVI.
Furthermore, GM damage (n=186) was associated with both a high PVI grade (109/186, 58.6%, p=0.001) and presence of AL (53/186, 28.5%, p<0.001). Both meningeal inflammation and GM pathology significantly contributed to a severe disease course through all phases (Figure 5B, D).

Discussion

Understanding the role of CNS inflammation in progressive disease is a key issue in MS (Frisher et al., 2009, 2015; Luchetti et al., 2018; Lassmann et al., 2019). Several neuropathological studies have demonstrated ongoing inflammation compartmentalized within the CNS in progressive MS (Frisher et al., 2009, 2015; Howell et al., 2011; Luchetti et al., 2018; Lassmann et al., 2019; Monaco et al., 2020), suggesting its role in driving disease progression (Mahad et al., 2015). However, anti-inflammatory strategies, despite some recent successes, have not yet had a significant impact on slowing disability progression - the dominant clinical manifestation of progressive MS. We approached this problem by examining clinical courses with a variable rate of progression and testing their association with CNS inflammation in post-mortem tissue. The UKMSTB dataset's particular strength is that it has large numbers of subjects with confirmed MS and a wide spectrum of outcomes together with consistent and systematic post-mortem information. A particular issue with histopathological study of brain tissues is the limited sampling. In the UKMSTB, both the brain and spinal cord are available, and information gathered from both areas form part of the comprehensive standardised assessment (http://www.ICDNS.org). Though this analysis was extensive, a potential source of bias could be that it was not exhaustive due to the practical constraints in processing a large cohort. In particular detailed analysis was only carried out in a subgroup of the whole population. The clinical data is subject to ascertainment bias as it was retrospective and based on available clinical records and notably these records did not contain MRI imaging. However, in the UKMSTB 92% of clinical records are of high quality and the database has previously been shown to have characteristics similar to other natural history cohorts (Reynolds et al., 2011). One of the limitations of the study could be therefore the lack of paired data reporting MRI disease activity immediately prior to death and/or at time of death.

The strong individual and spatial association found between the presence of active lesions and substantial perivenular inflammation in the post-mortem brain of SPMS patients with a shorter time from progression to death and a more aggressive disease course implies that inflammatory activity plays a key role in MS pathogenesis not only in the initial relapsing phase, but also during the disease progression, as observed at time of death. Our data, together with further studies on independent cohorts (Luchetti et al., 2018; Fransen et al., 2020), strongly suggest that perivenular inflammation and the demyelinating lesion activity is widely present even in the late stage of the disease, at least in a subgroup of MS cases characterized by rapid disease progression. These findings, together with radiological evidence (Konig et al., 2008; Metz et al., 2021), support the idea that MS heterogeneity is linked to precise patient-dependent immunopathology and may characterize individuals from the beginning of the disease, persisting during the progressive phase, nevertheless with reduced rate of lesion accumulation (Metz et al., 2021; Tobin et al., 2021). In turn, these findings may be helpful to predict the presence of markers of inflammation early in the progressive phase, prior to requiring a wheelchair, which might extend the timeframe where the inflammatory response could still be a target for therapy (Steinman & Zamvill, 2016).

Despite different terms being used to describe the stages of activity of demyelinated lesions, between authors and studies, there is an agreed sequence of events and pathological changes that evolve over three months as a plaque develops from an early active to an inactive plaque, convergence into a final common pathway that is probably mainly linked to accumulated neuro-axonal degeneration (De Groot et al., 2001; Kuhlman et al., 2017). We herein referred to 'early' demyelination according to the presence of LFB-positive myelin fragments in the cytoplasm of activated macrophages/microglia. The lack of assessment of presence of the minor myelin proteins (i.e. MOG+, CNP+ or MAG+), prevented us to better define (i.e. early vs late and active and demyelinating lesions) the earliest stage of plaque formation (Lucchinetti et al., 2000; Bruck et al., 1995; Kuhkmann et al., 2017). According to the idea that plaque composition changes over time, early active plaques, during progressive MS, leave the space to chronic inflammatory process with persistence of microglial activation with demyelination at lesion edge, whose extent associates with disability progression (Luchetti et al., 2018; Zvarwy et al., 2017; Weiner, 2008). A high percentage of our cases showed chronic plaque activity, herein defined as late active/chronic active (Reynolds et al., 2011), somehow corresponding to active and post-demyelinating and mixed active/inactive (Kuhlmann et al., 2017; Frischer et al., 2015).

The occurrence of an active lesion at time of death, strictly and spatially associated with the presence of PVI and accumulating disability, underlines the key role of persisting chronic inflammation in MS. The probability of active lesion presence at post-mortem is increased to nearly 50% if the progressive phase is less than 10 years; this probability doesn't increase further if MS is progressive in the terminal phase. These data imply the importance of prolonged period of inflammatory activity in the disease feature and therefore support the use of anti-inflammatory therapeutic strategies also late during the disease progression.

At the same time, high levels of perivenular inflammation is also associated with a shorter progressive period and the chance of their presence is about 75% in those with a progressive phase of less than 10 years and again this is not increased further if MS is progressive in the terminal phase. All together, these data suggest that when active lesions and high levels of perivenular inflammation coexist they have the greatest impact on the progressive phase, implying they are complementary inflammatory processes contributing to active disease progression.

The finding that focal white matter inflammatory tissue damage contributes to rapid progression in patients who died in early stages after disease onset (Frischer et al., 2009), challenges the idea that slow degenerative axonal loss, that is independent of inflammation, might underlie clinical progression or might act together with ongoing chronic intrathecal inflammation (Monaco et al., 2020). However, in addition, focal T2 MRI lesions combined with relapses have been shown to possibly explain later EDSS progression (Miller et al., 1988; Sormani et al., 2011). However, it is not known the exact load and effect of underlining neuropathological damage in the disease outcome and whether it is visible using current conventional imaging techniques. The inflammation seen at time of death is almost certainly compartmentalised behind the blood brain barrier and so will not be detectable as acute changes (Meinl et al., 2008;

Machado-Santos et al., 2018). Only recent advanced imaging methodologies enabled to detect more precisely the inflammatory lesion stages in in-vivo MS patients (Absinta et al., 2016; Dal Bianco et al., 2021; Metz et al., 2021). In addition, it should be mentioned that the high inflammatory activity might also interfere (delay and/or halt) with remyelinating and repair mechanisms (Plemel et al., 2017).

Both early active and increased perivenular inflammation were found in a subset of MS patients who also have a high level of meningeal infiltrates corroborating the hypothesis that a generally higher inflammatory activity in the CNS/CSF space characterizes an MS subgroup with more rapid progression (Magliozzi et al., 2007, 2018; Lucchinetti et al., 2011; Howell et al., 2011). The close, anatomical and functional, association between blood brain barrier and the subarachnoid space (Shechter et al., 2013) is further supported by finding that B-cell clonality has been demonstrated between cells present in the meningeal infiltrates and in perivascular cuffs (Lovato et al, 2011). B cells are relatively predominant in the perivascular cuffs of active lesions (Machado-Santos et al., 2018) and meningeal lymphoid-like infiltrates (Magliozzi et al, 2007, 2018; Howell et al, 2011; Haider et al., 2016), suggesting their key inflammatory role in MS progression (Comi et al, 2021; Li et al., 2018). The active lesions and perivenular infiltrates we have seen are associated in particular with the preponderance of B lymphocytes, characteristically found in meningeal infiltrates (Magliozzi et al, 2007; Howell et al, 2011; Haider et al., 2016). Accordingly, our quantitative analysis, notwithstanding the limited sample size, confirmed a possible correlation between perivascular CD20+ B lymphocytes and more severe disease course. It remains to be better elucidated whether meningeal B cell infiltration in the subarachnoid space preferentially mediates diffuse subpial cortical demyelination. Perivenular B cell infiltration could possibly contribute to the focal white matter pathology, not only through the production of immunoglobulins, but also by producing different pro-inflammatory and regulatory molecules and by their antigen presenting function (Cepok et al., 2001; Duddy et al., 2004; Lisak et al., 2012). The assessment of the exact phenotypes of all the infiltrating cells characterizing the PVI and of the scattered parenchymal lymphocytes, such as the expression of specific phenotype of non-circulating tissue resident memory CD8+ T cells (Fransen et al., 2020) and specific B subsets, as well as the co-labelling with vascular markers, might help to better understand the precise spatial and mechanistic features of perivascular inflammation in the pathology of WM lesions.

From our results the perivascular compartment emerges as one of the potential predictors of persisting lesion activity and relevant target for therapies, subject to the ability of the treatment to cross the BBB. Perivenular inflammation could therefore be considered as a potential relevant surrogate marker of lesion activity that, whether validated and assessed in early disease stages, might help to discriminate MS patients with higher lesion and disease activity that will benefit of early and more severe anti-inflammatory treatment. This would require an early identification of disability accumulation (Katz Sand et al., 2014; USF MS-EPIC Team 2019) which could be improved with the use of molecular and imaging biomarkers to quantify the intrathecal inflammatory processes underpinning progressive MS (Matthews, 2019; Dal-Bianco et al., 2017; Magliozzi et al., 2018) aiming to capture the window of opportunity for a targeted

anti-inflammatory approach (Sorensen et al., 2020; Rotstein and Montalban 2019). In such a context, then, an immunosuppressant approach aiming to reduce disease activity in the early stages would have fundamental role (Amato et al., 2020). This population did not receive highly active disease modifying therapies thus we are not able to determine how this could affect outcome.

Conclusions

High levels of both active lesions and focal perivenular inflammation within the white matter at post-mortem are associated with rapid disease evolution from onset and to the terminal stages. Associated diffuse and/or organized leptomeningeal inflammation, relevant in subpial cortical pathology, contributes to widespread inflammatory damage in a subset of patients. These pathological features are associated with a more rapid worsening after the onset of progression, but before a wheelchair is required, widening the potential use of an anti-inflammatory approach to halt or delay disease activity in progressive MS.

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Author Contribution

RN, RM, RR and TF contributed to the conception and design of the study; RN, RM, DM, OW, FR contributed to the acquisition and analysis of data; RN, RM, DM, OW, FR, PM, RR and TF contributed to drafting the text and preparing the figures.

Potential conflicts of interest:

The authors declare no conflicts of interest related to this study.

Data Availability Statement

Data used for this manuscript are available upon reasonable request.

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Legends

Figure 1

Assessment of perivenular inflammation (PVI) and ~~early~~ active lesions (~~E~~AL) in progressive MS. Semi-quantitative evaluation of PVI: ranging from 0 to 5 was calculated accordingly to the number of cells stained by haematoxylin-eosin (H&E) detected within the perivenular space (A-F). Active lesions (AL) were defined by LBF/PAS histology (G) and LFB/MHCII immunostaing (H-I), showing membraneous debris and the elevated density of MHC-II+ cells around PVI and throughout the lesion core. Original magnification= A-H: 100X, I: 200X.

Figure 2

Immunostaining for MHCII+ microglia/macrophages combined with LFB myelin staining for the detection of active (A, AL), chronic active (B, CAL), inactive/chronic silent (C, CSL) and remyelinating shadow lesion (D, RML). Cases rated as 'high' PVI were more likely to present with at least one AL, or LA/CAL, and less likely to harbour inactive lesions and shadow plaques (E- H). Original magnification= A-D: 100X.

Figure 3

Increased probability of perivenular inflammation and active lesions at post-mortem is associated with a younger onset and more rapidly evolving disease course. Probability of high PVI (A-C) and ALs (D-F) depending respectively on age of MS onset (A, D), time from onset to progression (B, E) and time from progression to death (C, F). PVI and ALs were associated with a younger age at onset, shorter time from disease onset to progression and a shorter time from progression to death.

Logistic regression models estimating proportion of patients with at least one of each type of lesion (Al, CA, CI or SP) according to time from progression to death (G) and age of death (H). The probabilities of high PVI (I-J) and ALs (K-L) according to the time from progression to requiring wheelchair (adjusted for age of onset (20 years (I, K) and 30 years (J, L) and time from onset to progression (5, 10 or 20 years). The chance of having high PVI at post-mortem rises to around 80% (I,) in a person with an age of onset at 20 years who reaches the progressive phase in 5 years and who then requires a wheelchair within 5 years of progression onset whereas in this scenario the chance of finding EALs rises to >40%.

Figure 4

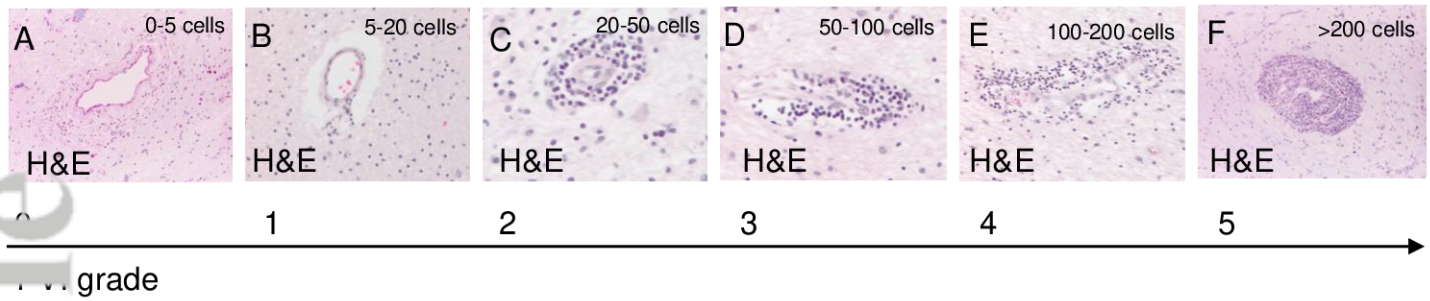
Perivenular infiltrates of B lymphocytes are associated with greater demyeliation and a more rapidly evolving disease. Substantial demyelination (A) was observed in cases with an elevated density of perivenular CD3+ T lymphocytes (B) and CD20+ B lymphocytes (C) in MS cases defined as having high PVI. Only scattered parenchymal infiltrates of CD3+ T cells (arrowheads in B), but not CD20+ B cells were seen (C), in particular always in strict association and in close proximity to perivascular infiltrates. Cell count analysis confirmed the significantly higher number of perivenular lymphocytes in those cases defined as high PVI, in both white matter lesions (D) and NAWM (E), in comparison to low PVI cases. An increased CD20/CD3 ratio (F) and density of CD20+ cells (G) negatively correlated with disease duration.

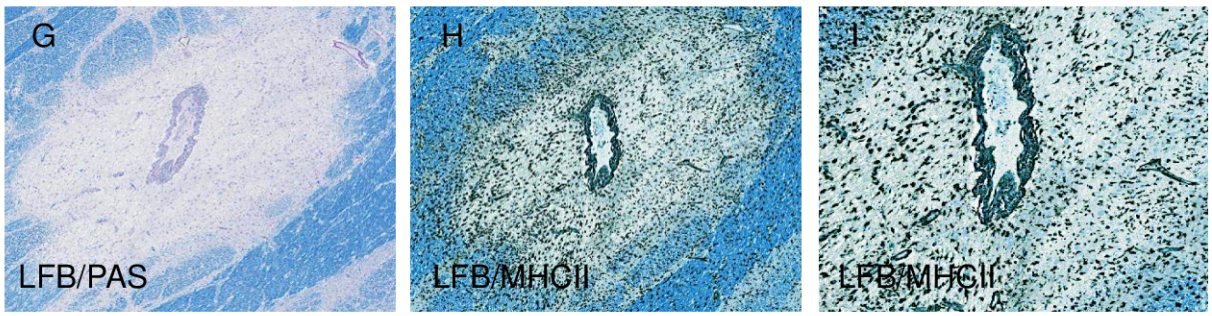
Kruskal Wallis test and Spearman correlation analysis were used. Original magnification= A: 10X; B: 200X.

Figure 5

Investigating the inter-relationship between perivenular and meningeal inflammation. Association between PVI status and meningeal inflammation (A) and GM pathology (B), revealing that a greater proportion of PVI high cases are also characterised as harbouring ectopic follicle-like structures (F+; A). High PVI cases were also likely to present high cortical grey matter lesion load (B)

Disease course (mean age are plotted) according to PVI status (high/low) and presence/absence of meningeal lymphoid structures (C) and GM pathology (D). C: The study cohort was separated according to the presence (F+) or absence (F-) of meningeal follicle-like structures and PVI status: F- and low PVI (n=27), F- and high PVI (n=25), F+ and low PVI (n=5), F+ and high PVI (n=29). Absence of both meningeal and perivenular inflammation is associated with less severe disease outcome, including higher age at progression and at wheelchair use and age at death ($p<0.001$). D: the study cohort was separated according to presence or not of GM pathology and PVI status: no GM and low PVI (n=46), no GM and high PVI (n=23), GM and low PVI (n=74), GM and high PVI (n=109). MS cases with both GM damage and high PVI had a lower age at progression and at wheelchair use ($p<0.01$) and age at death ($p<0.001$, for all groups against GM/high PVI group (Kruskal Wallis test and Dunn's multiple comparison post-test were used).





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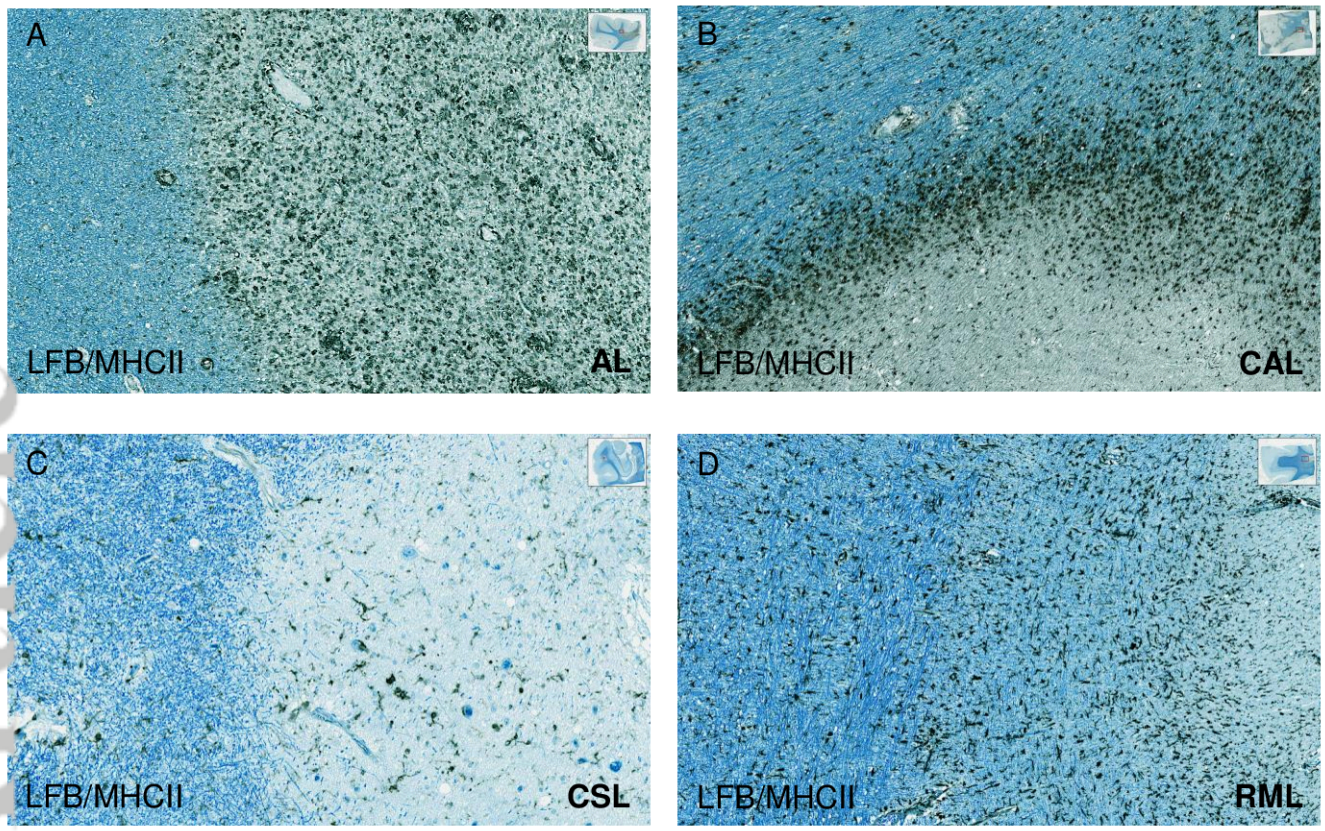

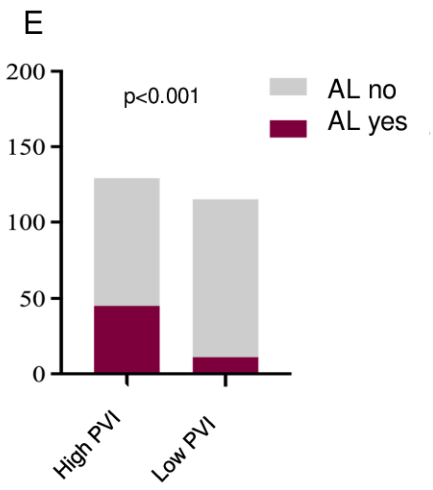

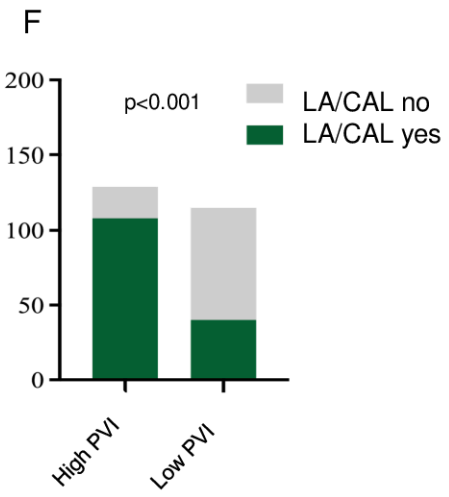

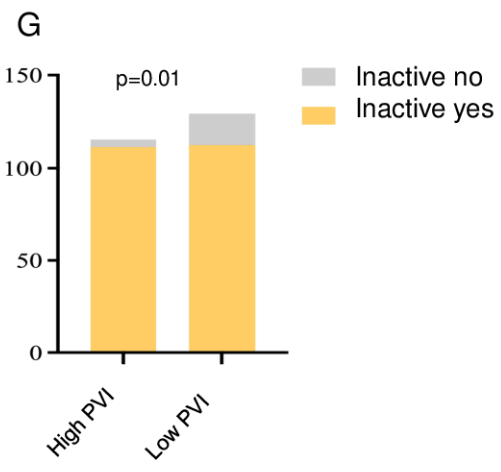

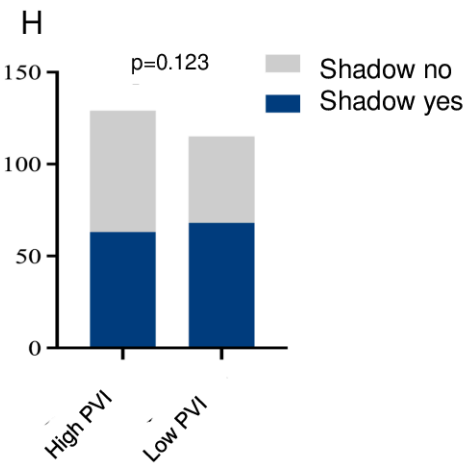

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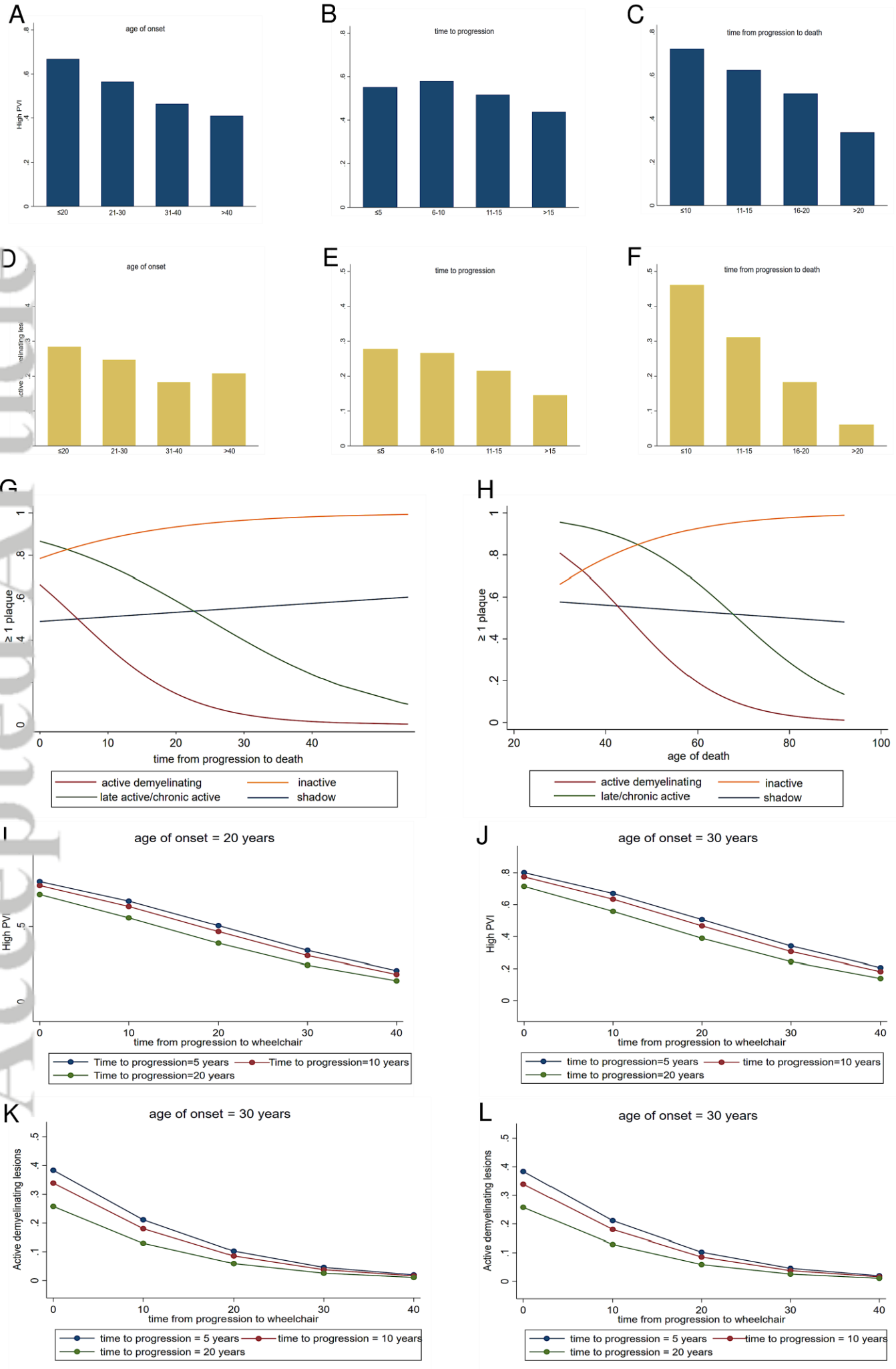

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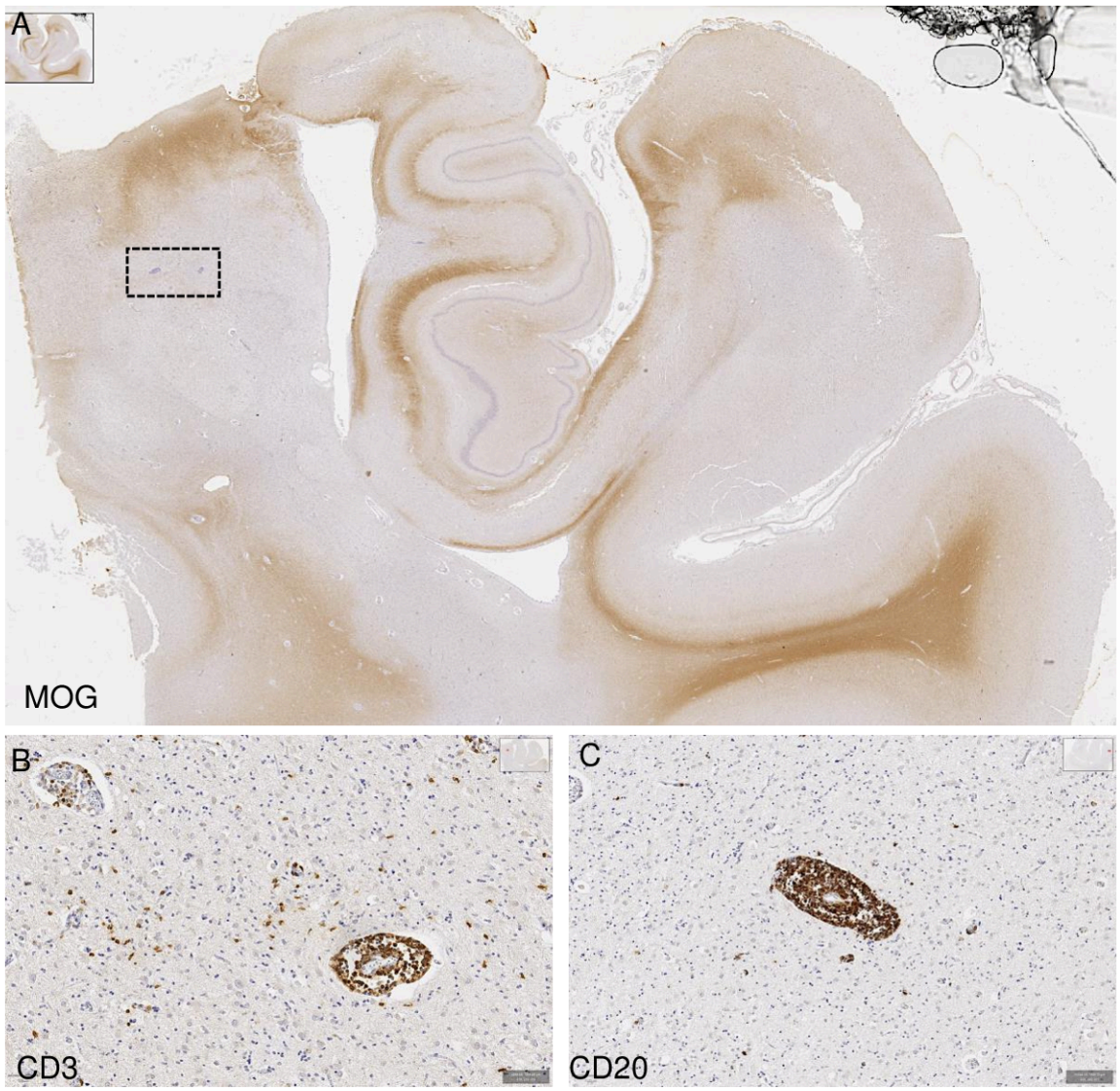

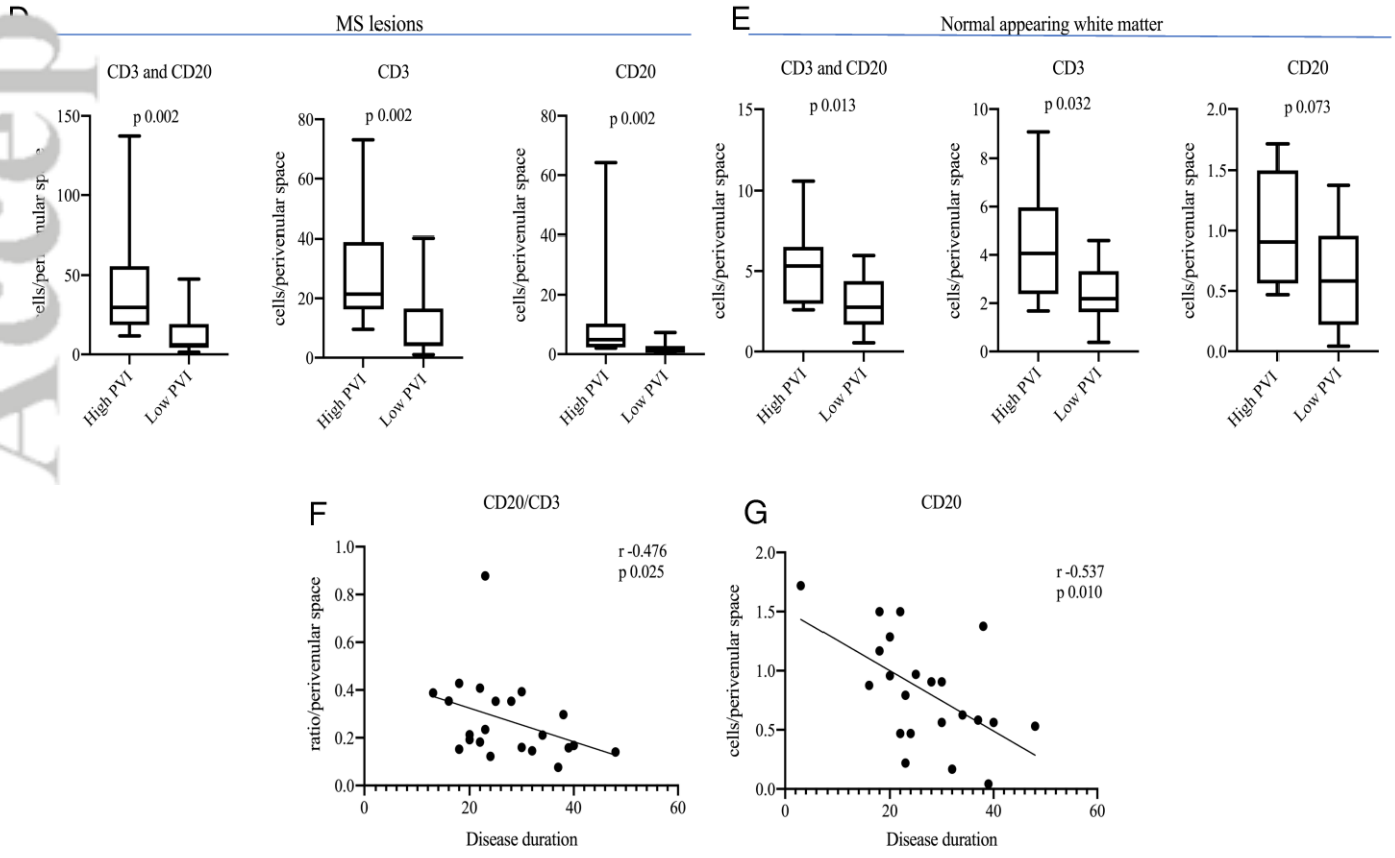

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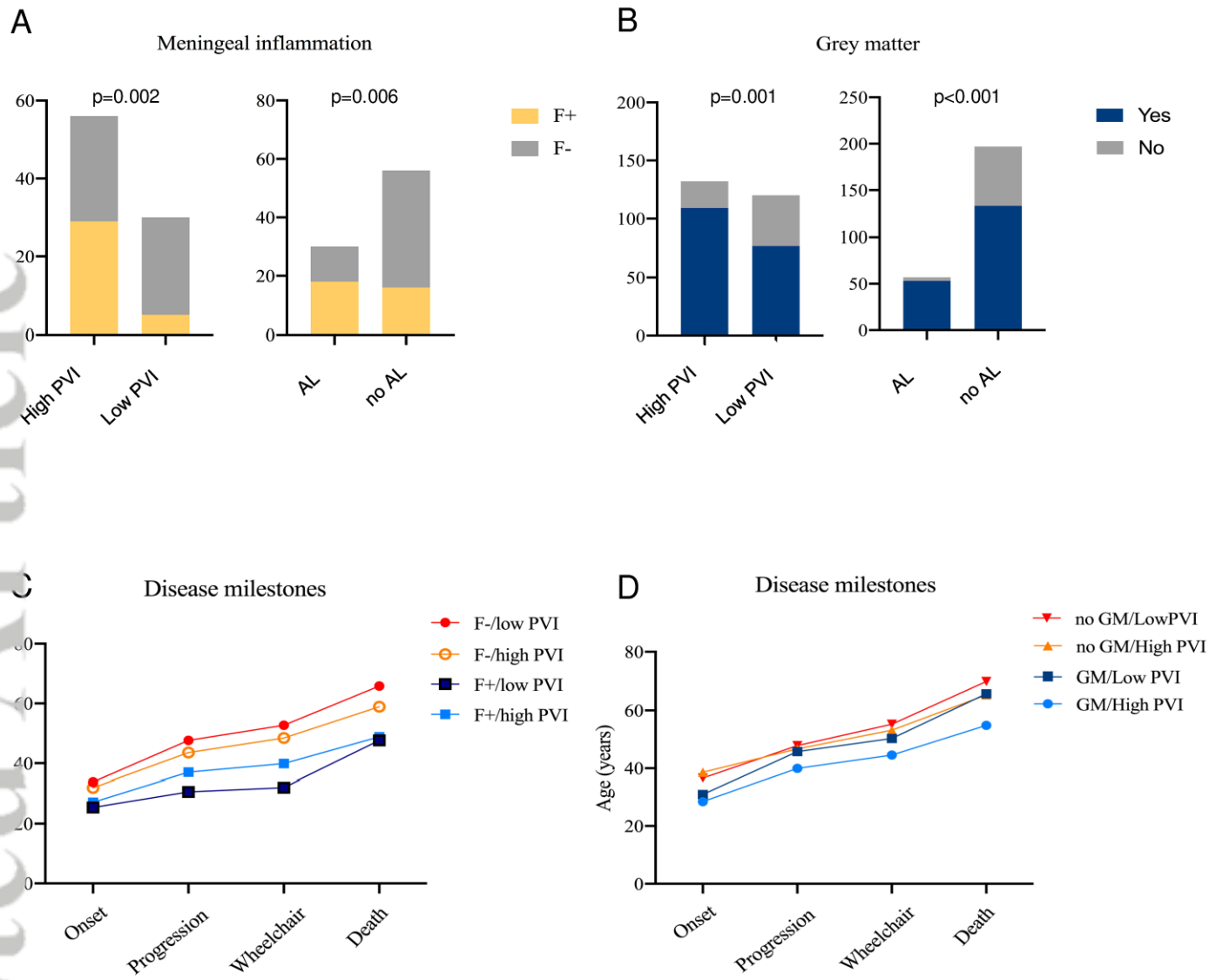

fig5.tiff

Table 1. Demographic and clinical characteristics of MS cases. *SPMS:* Secondary progressive multiple sclerosis; *PPMS:* Primary progressive multiple sclerosis; *F*: Female; *M*: Male.

Parameter	Total MS	Female	Male	SPMS	PPMS
Number of patients	269	174	95	251(F166/M 85)	18(F8/M10)
Disease duration (mean ± SD, years)	30.4 ± 12.0	30.6 ± 11.9	29.9 ± 12.2	30.7 ± 11.7	26.0 ± 15.2
Age of onset (mean ± SD, years)	31.5 ± 9.9	32.6 ± 10.1	29.3 ± 9.2	31.0 ± 9.7	38.2 ± 11.1
Number of relapses in first two years (mean ± SD)	2.1 ± 1.3	2.1 ± 1.3	2.0 ± 1.3	2.1 ± 1.3	1.4 ± 0.9
>2 relapses in first two years (frequency, %)	76/260 (29.2)	52/169 (30.8)	24/91 (26.4)	74/242 (30.6)	2/18 (11.1)
Age of death (mean ± SD, years)	61.8 ± 12.8	63.2 ± 12.8	59.2 ± 12.2	61.6 ± 12.7	64.2 ± 13.9
Time from onset to progression (mean ± SD, years)	12.2 ± 9.4	11.8 ± 8.4	13.0 ± 10.9	13.1 ± 9.1	/
Time from progression to death (mean ± SD, years)	18.1 ± 9.2	18.8 ± 9.0	16.9 ± 9.4	17.6 ± 8.3	26 ± 15.2
Time from progression to wheelchair (mean ± SD, years)	6.8 ± 6.0	6.8 ± 5.7	6.7 ± 6.4	6.1 ± 4.9	14 ± 10.5
MS progressive in last illness (frequency, %)	109/239 (45.6)	67/153 (43.8)	42/86 (48.8)	103/224 (46.0)	6/15 (40.0)

Table 2. Pathological findings at the routine UKMSTB assessment: EALs and PVI grades in the whole population and accordingly to gender and MS type.

	Total MS	Female	Male	SPMS	PPMS
Early Active Lesions (frequency, %)	59/267 (22.1)	39/173 (22.5)	20/94 (21.3)	57/249 (22.9)	2/18 (11.1)
High PVI (frequency, %)	137/265 (51.7)	85/173 (49.1)	52/92 (56.5)	126/247 (51.0)	11/18 (61.1)
Grade of PVI (frequency, %) 0	30/265 (11.3)	23/173 (13.3)	7/92 (7.6)	29/247 (11.7)	1/18 (5.6)
1	98/265 (37.0)	65/173 (37.6)	33/92 (35.9)	92/247 (37.2)	6/18 (33.3)
2	69/265 (26.0)	41/173 (23.7)	28/92 (30.4)	63/247 (25.5)	6/18 (33.3)
3	47/265 (17.7)	28/173 (16.2)	19/92 (20.7)	43/247 (17.4)	4/18 (22.2)
4	20/265 (7.5)	16/173 (9.2)	4/92 (4.3)	19/247(7.7)	1/18 (5.6)
5	1/265 (0.4)	0/173 (0)	1/92 (1.1)	1/247 (0.4)	0/18 (0)

Table 3. Demographic and clinical characteristics according to degree of PVI (high/low) and presence/absence of EAL.

Parameter	High PVI	Low PVI	EALs	No EALs
Age of onset (mean ± SD, years)	30.0 ± 10.2	32.9 ± 9.4	29.7 ± 9.6	31.9 ± 10
Disease duration (mean ± SD, years)	26.5 ± 11.0	34.3 ± 11.8	22.8 ± 10.9	32.6 ± 11.4
Number of relapses in first two years of MS (mean ± SD)	2.1 ± 1.4	2.1 ± 1.2	2.2 ± 1.4	2.1 ± 1.3
>2 relapses in first two years of MS (frequency, %)	42/132 (31.8)	33/124 (26.6)	23/58 (39.7)	52/200 (26.0)
Age of death (mean ± SD, years)	56.4 ± 11.6	67.2 ± 11.5	52.5 ± 10.6	64.5 ± 12.1
Time from onset to progression (mean ± SD, years)	10.9 ± 8.1	13.6 ± 10.5	10.5 ± 8.0	12.7 ± 9.7
Time from progression to death (mean ± SD, years)	15.6 ± 8.0	20.8 ± 9.6	12.4 ± 6.5	19.8 ± 9.1
Time from progression to wheelchair (mean ± SD, years)	5.8 ± 5.2	8.0 ± 6.7	5.0 ± 4.2	7.3 ± 6.3
MS progressive in last illness (frequency, %)	65/123 (52.8)	44/112 (39.3)	32/52 (61.5)	76/186 (40.9)