High Levels of Perivascular Inflammation and Active Demyelinating Lesions at Time of Death Associated with Rapidly Progressive Multiple Sclerosis Disease Course: A Retrospective Postmortem Cohort Study

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Objective: Analysis of postmortem multiple sclerosis (MS) tissues combined with in vivo disease milestones suggests that whereas perivascular white matter infiltrates are associated with demyelinating activity in the initial stages, leptomeningeal immune cell infiltration, enriched in B cells, and associated cortical lesions contribute to disease progression. We systematically examine the association of inflammatory features and white matter demyelination at postmortem 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 was associated with increased prevalence and higher levels of PVI (both $p < 0.0001$). Shorter time from onset of progression to wheelchair use was associated with higher prevalence of ALs (odds ratio [OR] = 0.921, 95% confidence interval [CI] = 0.858–0.989, $p = 0.0230$) and higher level of PVI (OR = 0.932, 95% CI = 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.

ANN NEUROL 2024;00:1–14

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.[1–3] In individuals, the manifestations are highly variable but are characterized by two dominant clinical events: relapses, that is, transient periods of neurological deterioration with variable recovery; and progression, characterized by the gradual accumulation of disability that leads to the major personal and societal cost of MS.[4] Relapses have proven amenable to therapy, but progression has thus far been resistant to treatment, leading to a major unmet need.[5] Thus, despite extensive pathological evidence for inflammation in progressive MS,[1,6] no immunomodulatory treatment that has been shown to suppress relapses and gadolinium magnetic resonance imaging (MRI) activity has had a major impact on the progressive course.[7,8] 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.[9–13] Furthermore, although MS is a highly heterogeneous disease, characterized by large interindividual 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.[14–16]

The UK MS Society Tissue Bank (UKMSTB) postmortem 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 standardized 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 gray matter (GM) demyelination and immune-pathological cell and molecular alterations that in turn may lead to more rapid and severe disease progression.[17–20]

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 postmortem tissue in well-characterized cases: active and demyelinating/early active lesions (ALs)[1,21,22] and the perivenular infiltrates.[1,6,16] ALs are classified by the presence of inflammation with evidence of recent myelin breakdown indicating that they have been present for only 3 months.[21] Perivenular inflammation (PVI) is an infiltration of lymphocytes into the venule outside the blood–brain barrier producing thickening of the venule wall.[6,16,23] 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, 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 postmortem tissue.

Materials and 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 summarized by a neurologist prior to the neuropathological analysis.[1] 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 were examined by a neuropathologist according to standardized criteria (BrainNet Europe: https://www.imperial.ac.uk/medicine/multiple-sclerosis-and-parkinsons-tissue-bank/).

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 summarized by a neurologist blinded to the neuropathological findings to determine the key clinical milestones above, but also the number of relapses in the first 2 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 primary progressive MS (PPMS) and secondary progressive MS (SPMS) cases in the UK.[1] None was receiving disease-modifying treatments in the period prior to death or at the time of death.

All demographic and clinical characteristics of the cohort are described in Table 1 and Supplementary Table S1. All donor brains had been examined for the presence of early ALs and PVI (graded 0–5), as this formed the focus of this investigation.[1] PVI 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 (87/269) previously investigated for the presence of meningeal inflammation and associated subpial cortical demyelination.[18]

Neuropathology Examination

Donor brain and spinal cord were examined by a neuropathologist according to standardized criteria (BrainNet Europe:

TABLE 1. Demographic and Clinical Characteristics of MS Cases

Parameter	Total MS	Female	Male	SPMS	PPMS
Patients, n	269	174	95	251 (F 166/M 85)	18 (F 8/M 10)
Disease duration, yr, mean ± SD	30.4 ± 12.0	30.6 ± 11.9	29.9 ± 12.2	30.7 ± 11.7	26.0 ± 15.2
Age at onset, yr, mean ± SD	31.5 ± 9.9	32.6 ± 10.1	29.3 ± 9.2	31.0 ± 9.7	38.2 ± 11.1
Relapses in first 2 years, n, 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 2 years, n (%)	76/260 (29.2)	52/169 (30.8)	24/91 (26.4)	74/242 (30.6)	2/18 (11.1)
Age at death, yr, mean ± SD	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, yr, mean ± SD	12.2 ± 9.4	11.8 ± 8.4	13.0 ± 10.9	13.1 ± 9.1	—
Time from progression to death, yr, mean ± SD	18.1 ± 9.2	18.8 ± 9.0	16.9 ± 9.4	17.6 ± 8.3	26 ± 15.2
Time from progression to wheelchair, yr, mean ± SD	6.8 ± 6.0	6.8 ± 5.7	6.7 ± 6.4	6.1 ± 4.9	14 ± 10.5
MS progressive in last illness, n (%)	109/239 (45.6)	67/153 (43.8)	42/86 (48.8)	103/224 (46.0)	6/15 (40.0)

F = female; M = male; MS = multiple sclerosis; PPMS = primary progressive MS; SD = standard deviation; SPMS = secondary progressive MS.

https://www.imperial.ac.uk/medicine/multiple-sclerosis-and-parkinsons-tissue-bank/). Tissue blocks (2cm × 2cm × 1cm) 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 focused on the standardized set of paraffin-embedded blocks that are prepared for diagnostic confirmation. Paraffin serial sections (7μm) from each block were stained with hematoxylin–eosin, Luxol fast blue (LFB)/periodic acid–Schiff (PAS), and LFB/major histocompatibility complex class II (MHCII) antigen, with additional histochemical and immunohistochemical stains performed when required for diagnosis.[1] (Supplementary Table 2) The classification of plaques used at the UKMSTB was proposed by Professor I. Allen (http://www.ICDNS.org) and refers 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,[1] similar to what has recently been described as "active and demyelinating lesions."[22]

PVI assessment was a semiquantitative 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.[1] PVI assessment (n = 265; Fig 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 higher (see Fig 1).

For each examined MS cases, the presence of ALs (n = 267) and 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

To characterize the inflammatory cell populations present in the perivenular infiltrates associated with ongoing 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 1cm rostral to the temporal pole), thalamus, primary visual (striate) cortex (sampled 1.5cm 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-lymphocyte markers, respectively (Supplementary Table S2) following the procedures previously described,[17,18] and digitalized images from entire slices were acquired.

In NAWM, CD3+ and CD20+ cells were quantified from up to 4 vessels (veins) in cross-section, presenting with a thin tunica media and with a total area (vessel and perivascular space) >0.002mm². Two randomly noncontinuous selected fields
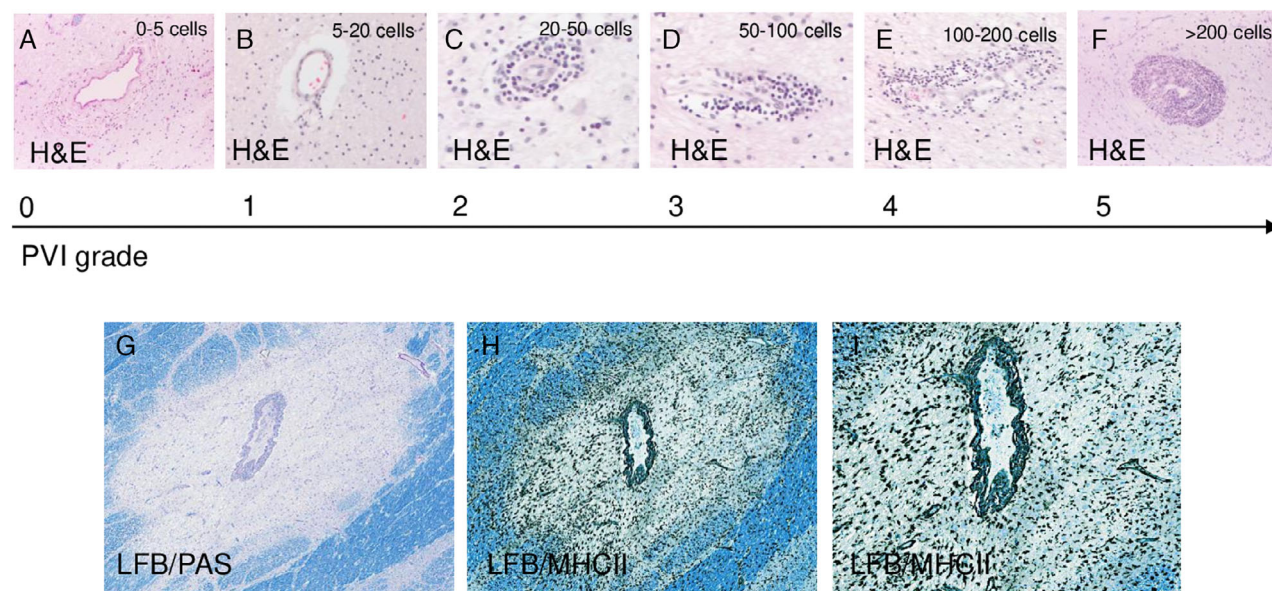
FIGURE 1: Assessment of perivenular inflammation (PVI) and active lesions (ALs) in progressive multiple sclerosis. (A–F) Semiquantitative evaluation of PVI, ranging from 0 to 5, calculated accordingly to the number of cells stained by hematoxylin–eosin (H&E) detected within the perivenular space. (G–I) ALs were defined by Luxol fast blue (LFB)/periodic acid–Schiff (PAS) histology (G) and LFB/major histocompatibility complex class II (MHCII) immunostaining (H, I), showing membranous debris and the elevated density of MHCII+ cells around PVI and throughout the lesion core. Original magnification, A–H: ×100, I: ×200.

per each block (areas of each field of 4mm^2) were examined. Fields in the 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) >0.003mm^2. For each examined MS case, the number of perivenular CD3+ and CD20+ cells was calculated in 4 vessels with perivascular space >0.003mm^2.

Evaluation of Meningeal Inflammation

Eighty-seven of 269 evaluated cases had previously been extensively investigated for the presence of meningeal inflammation and lymphoidlike structures.[18] 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 lymphoidlike organization 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 Ig-A, -G, -M+ plasmablasts/plasma cells.[18,24]

Image Acquisition and Analysis

Tissue sections were analysed on a Nikon E1000M microscope using brightfield imaging (Nikon Instruments, Tokyo, Japan) 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 Technologies (Vista, CA) AT2 Scan Digital Whole Slide Scanner (20× magnification). Image files were handled using QuPath.[25] 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 (SDs) in the case of continuous variables and by frequencies and percentages in the case of categorical variables. Logistic regression models were used with stepwise variable selection to model the probabilities of early ALs and PVI. Factors within the models included the time interval from birth to onset, onset to progression and from progression to death, gender, >2 relapses in the first 2 years after onset, MS being progressive in last illness, a high grade of PVI (none/minimal [0–1] vs significant presence [2–5]), and the presence of early active plaques found in the standardized 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 at onset ≤ 20 years; 20 < age at onset ≤ 30 years; 30 < age at onset ≤ 40 years; 40 < age at 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-squared test was used to compare the relationship between PVI and early ALs (Supplementary table 3).

Results of cell count analysis were presented as scatter dot plots with a line at the mean or as box-and-whiskers plots showing minimum to maximum values, interquartile range, and group medians. Two-group comparisons were performed using the Mann–Whitney U test or Wilcoxon matched pairs test, whereas 3 or more groups were compared by nonparametric 1-way analysis of variance (Kruskal–Wallis test), using Dunn multiple comparisons post-test. Correlations were tested by Spearman analysis. Due to the exploratory nature of this study, p values and confidence intervals (CIs) were not corrected for multiple testing; 2-sided p values < 0.05 were considered statistically significant. All computations were carried out using SAS version 9.4, Stata version 13.0, and GraphPad Prism version 7.0 software Boston, MA.

Results

Demographic and Clinical Characteristics
In this investigation, we selected patients from a 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. A total of 174 (64.7%) were females; no significant differences in clinical milestones were noted according to gender. A progressive disease course from onset (PPMS) was found in 18 cases. PPMS cases had a greater age at MS onset ($p = 0.008$), longer 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 (see 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. A total of 54.8% (136/248) had at least one AL, and 61.3% (152/248) had at least one late/chronic AL (LA/CAL); 91.5% (227/248) and 53.2% (132/248) of cases had inactive or shadow plaques, respectively (Fig 2).

In the total cases, high PVI was detected in 52% of cases (n = 137), and at least one AL (see Fig 2A) was present in 22% of the cases (n = 59; Table 2). No significant differences in the incidence of ALs 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; see 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] vs low PVI 10% [11/115], $p < 0.001$; see Fig 2E). ALs were spatially associated with the presence of a PVI. LAs/CALs were also significantly increased in high PVI compared to low PVI cases (high PVI 84% [112/133] vs low 35% [40/115], $p < 0.001$; see Fig 2F).

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

PVI and ALs Are Associated with a More Severe MS Course
A high PVI grade corresponded to a more severe disease course (Table 3, Supplementary Table S3). 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 was also associated with a more severe disease course through all the disease phases (see Table 3). Patients with both ALs and high PVI (n = 48) had the youngest age at onset (29.1 ± 9.8 years) and at death (50.5 ± 9.6 years), with a mean time from onset to progression of 10 ± 7.1 years and from progression to death of 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 PVI and ALs at Postmortem Is Associated with Shorter Time from Progression to Death
After applying the stepwise logistic regression model, the probability of high PVI was most increased in patients with a shorter time from progression to death (odds ratio [OR] = 0.915, 95% CI = 0.884–0.946, $p < 0.001$), a shorter time from MS onset to progression (OR = 0.922, 95% CI = 0.891–0.955, $p < 0.001$), and disease onset at a younger age (OR = 0.938, 95% CI = 0.908–0.968, $p < 0.001$; Fig 3). A similar association was found regarding ALs (age at onset: OR = 0.943, 95% CI = 0.908–0.980, $p = 0.003$; time from MS onset to progression: OR = 0.935, 95% CI = 0.897–0.975, $p = 0.0018$; time from progression to death: OR = 0.868, 95% CI = 0.824–0.913, $p < 0.001$; see Fig 3).
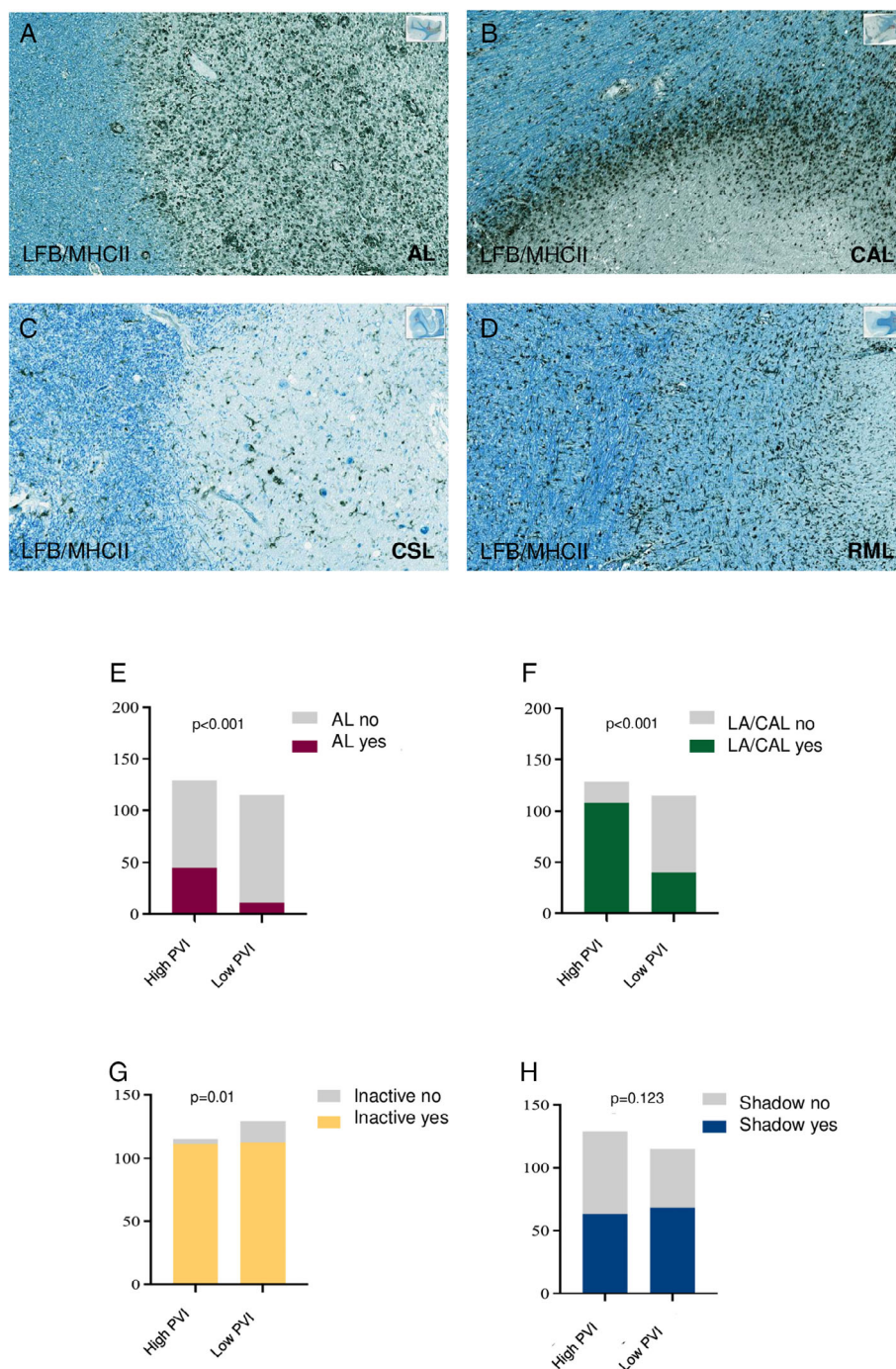
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FIGURE 2: (A–D) Immunostaining for major histocompatibility complex class II (MHCII) microglia/macrophages combined with Luxol fast blue (LFB) myelin staining for the detection of active (A; AL), chronic active (B; CAL), inactive/chronic silent (C; CSL), and remyelinating shadow lesions (D; RML). (E–H) Cases rated as "high" perivenular inflammation (PVI) were more likely to present with at least one AL or late active lesion (LA)/CAL, and less likely to harbor inactive lesions and shadow plaques. Original magnification, A–D: ×100.

After adding LAs/CALs to the stepwise logistic regression model, ALs were 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 and age at death is shown in Figure 3.

Age at onset (OR = 0.909, 95% CI = 0.877–0.943, $p < 0.001$), time from onset to progression (OR = 0.930, 95% CI = 0.896–0.965, $p < 0.001$), and time from progression to death (OR = 0.934, 95% CI = 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 ALs ($p = 0.648$). The association between

TABLE 2. Pathological Findings at the Routine UK MS Society Tissue Bank Assessment: EALs and PVI Grades in the Whole Population and according to Gender and MS Type

Finding	Total MS	Female	Male	SPMS	PPMS
EAIs, n (%)	59/267 (22.1)	39/173 (22.5)	20/94 (21.3)	57/249 (22.9)	2/18 (11.1)
High PVI, n (%)	137/265 (51.7)	85/173 (49.1)	52/92 (56.5)	126/247 (51.0)	11/18 (61.1)
Grade of PVI, n (%)					
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)

EAL = early active lesion; MS = multiple sclerosis; PPMS = primary progressive MS; PVI = perivenular inflammation; SPMS = secondary progressive MS.

TABLE 3. Demographic and Clinical Characteristics according to Degree of PVI (high/low) and Presence/Absence of EALs

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

EAL = early active lesion; MS = multiple sclerosis; PVI = perivenular inflammation; SD = standard deviation.

ALs and high PVI and MS progression in terminal illness was then explained through age at onset, time from onset to progression, and time from progression to death.

PVI and ALs Are Associated 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 ALs (OR = 0.921, 95% CI = 0.858–0.989, $p = 0.0230$) as well as a higher level of PVI (OR = 0.932, 95% CI = 0.886–0.981, $p = 0.0071$) at

postmortem, adjusted for age at onset and time from onset to progression, which were also found to be statistically significant (see Fig 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 and CD20+ B lymphocytes was detected in those cases defined as having high PVI in the semiquantitative analysis in both white matter lesions and NAWM (Fig 4).
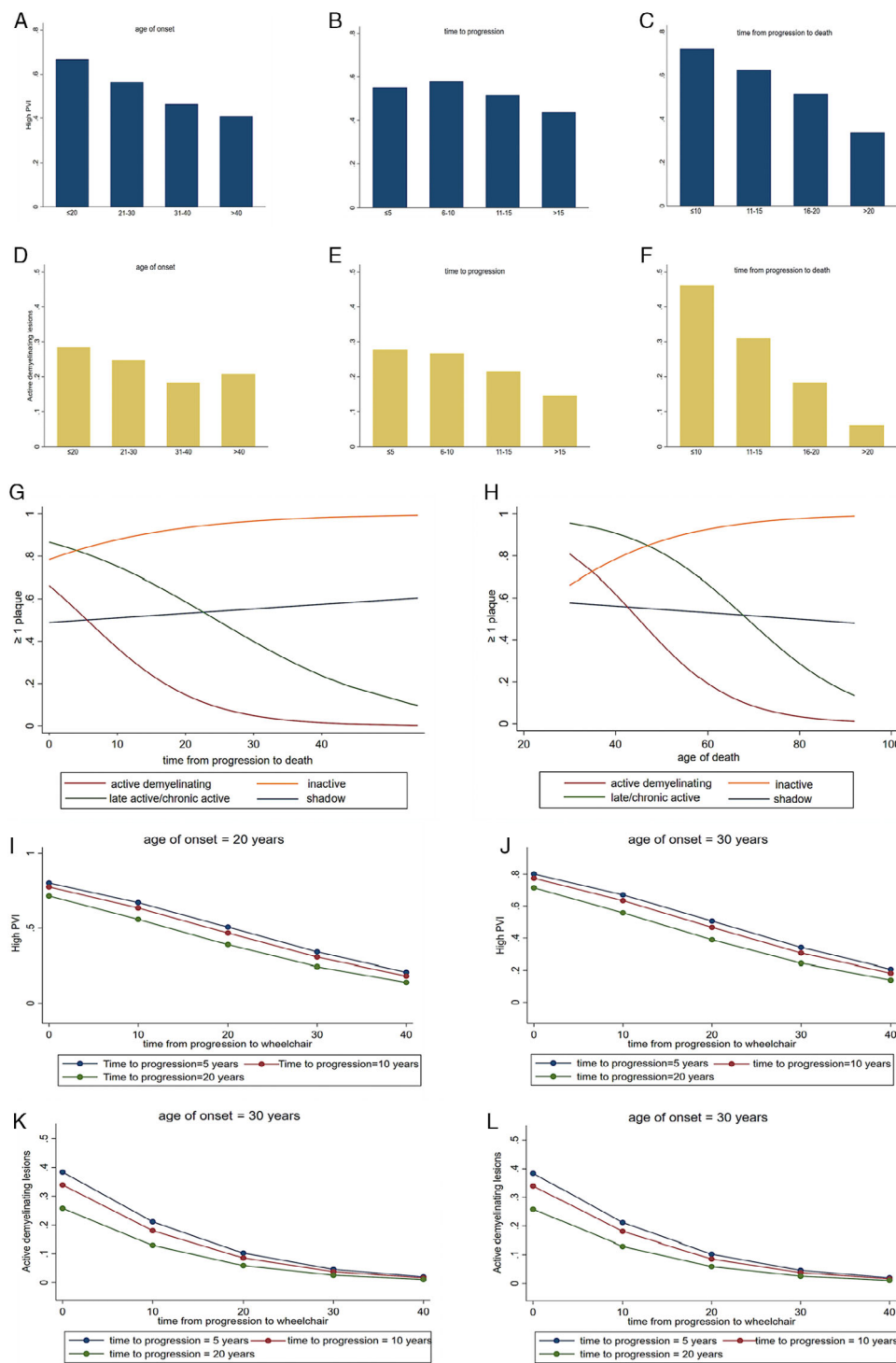
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FIGURE 3: Increased probability of perivenular inflammation (PVI) and active lesions at postmortem is associated with a younger onset and more rapidly evolving disease course. (A–F) Probability of high PVI (A–C) and active lesions (Als; D–F) depending respectively on age at multiple sclerosis onset (A, D), time from onset to progression (B, E), and time from progression to death (C, F). PVI and ALs were associated with younger age at onset, shorter time from disease onset to progression, and shorter time from progression to death. (G, H) Logistic regression models estimating proportion of patients with at least one of each type of lesion (active lesion (Al), Chronic active lesion (CA), Chronic inactive lesion (CI), or Shadow plaque (SP)) according to time from progression to death (G) and age at death (H). (I–L) The probabilities of high PVI (I–J) and ALs (K–L) according to the time from progression to requiring a wheelchair, adjusted for age at onset (20 years [I, K] and 30 years [J, L]) and time from onset to progression (5, 10, or 20 years). (I) The chance of having high PVI at postmortem rises to approximately 80% in a person with an age at onset of 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 early active lesions rises to >40%. All ages are given in years.
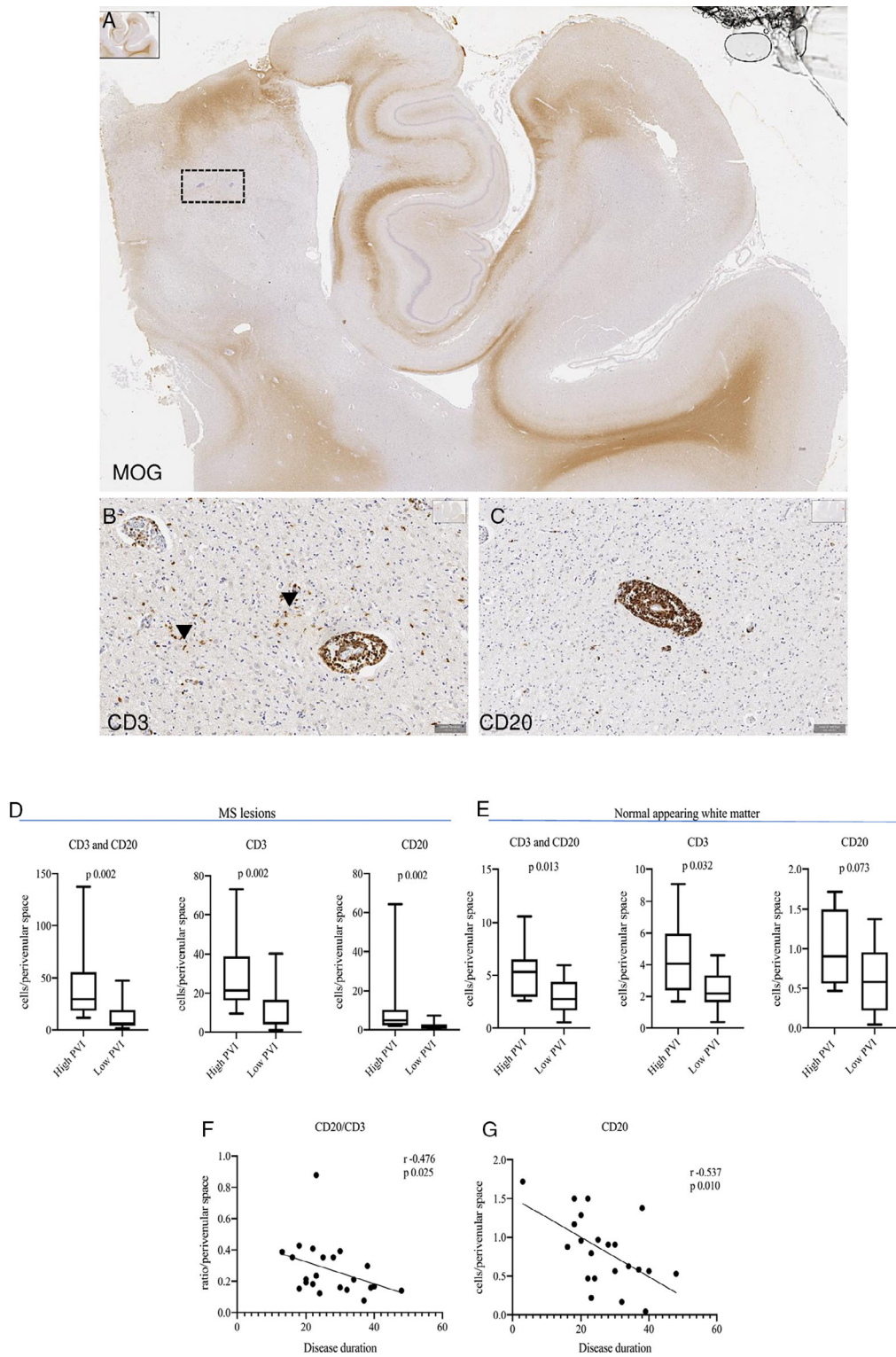
FIGURE 4: Perivenular infiltrates of B lymphocytes are associated with greater demyelination and a more rapidly evolving disease. (A–C) Substantial demyelination (A) was observed in cases with an elevated density of perivenular CD3+ T lymphocytes (B) and CD20+ B lymphocytes (C) in multiple sclerosis (MS) cases defined as having high perivenular inflammation (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 with and in close proximity to perivascular infiltrates. (D, E) 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 normal-appearing white matter (E) in comparison to low PVI cases. (F, G) 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: ×10, B: ×200. MOG = myelin oligodendrocyte glycoprotein.
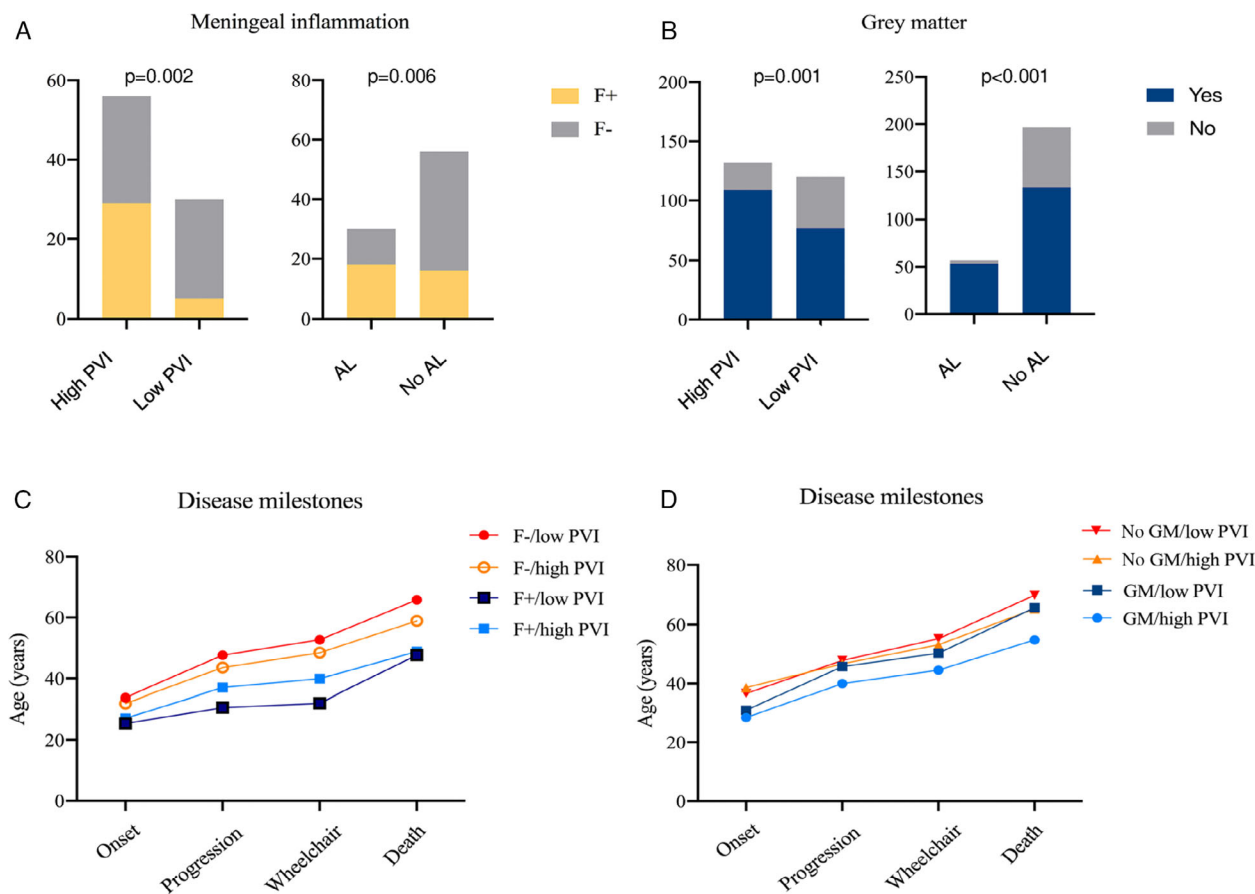
FIGURE 5: Investigating the inter-relationship between perivenular and meningeal inflammation. (A, B) Association between perivenular inflammation (PVI) status and meningeal inflammation (A) and gray matter (GM) pathology (B), revealing that a greater proportion of PVI high cases are also characterized as harboring ectopic folliclelike structures (F+; A). (B) High PVI cases were also likely to present high cortical GM lesion load. **(C, D)** Disease course (mean age is 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 folliclelike 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 greater age at progression, at wheelchair use, and at death (*p* < 0.001). **(D)** The study cohort was separated according to presence or absence 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). Multiple sclerosis 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 versus the GM/high PVI group (Kruskal–Wallis test and Dunn multiple comparison post-test were used). AL = active lesion.

Lesions (n = 127) from cases with high PVI had increased total lymphocyte number (mean ± SD = 40.9 ± 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). ALs (n = 12, including both ALs and LAs/CALs) 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 lesions (n = 19). No substantial difference in parenchymal lymphocyte frequency was observed in association with the presence of ALs. The presence of parenchymal lymphocytes, mainly CD3+ T cells and very rarely CD20+ B cells, was rare and heterogeneous, as 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.

The presence of ALs was evident in the majority of the high PVI cases analyzed in detail (11 high PVI and 11 low PVI), with 10 ALs 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 ALs (2 ALs) compared to 11 inactive lesions in the same blocks.

Cases with high PVI were also confirmed to be harboring 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 (see Fig 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, $p = 0.285$).

An increased B/T ratio inside the lesions was associated with a reduced disease duration ($r - 0.476$, $p = 0.025$; see Fig 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 ($r - 0.443$, $p = 0.039$; see Fig 4G).

Perivenular and Meningeal Inflammation Are Associated in Progressive MS

Among 87 cases previously evaluated for the presence and extent of meningeal inflammation,[17,18] 34 (42.5%) had been defined as having tertiary lymphoidlike structures, also named folliclelike 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 ALs (n = 18/34, 52.9%, $p = 0.006$; Fig 5A,C). Seventeen of the 18 subjects with ALs 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 ALs (53/186, 28.5%, $p < 0.001$). Both meningeal inflammation and GM pathology significantly contributed to a severe disease course through all phases (see Fig 5B,D).

Discussion

Understanding the role of central nervous system (CNS) inflammation in progressive disease is a key issue in MS.[6,10,16,26] Several neuropathological studies have demonstrated ongoing inflammation compartmentalized within the CNS in progressive MS,[6,10,11,16,18,26] suggesting its role in driving disease progression.[9] 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 postmortem tissue. The UKMSTB dataset's particular strength is that it has a large number of subjects with confirmed MS and a wide spectrum of outcomes together with consistent and systematic postmortem information. A particular issue with the 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 standardized assessment (http://www.ICDNS.org). Although 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 are subject to ascertainment bias, as the study was retrospective and based on available clinical records and notably these records did not contain MRI. 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.[1] One of the limitations of the study could therefore be 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 ALs and substantial PVI in the postmortem 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,[16,27] strongly suggest that PVI and the demyelinating lesion activity are 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,[28,29] 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.[29,30] 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.[3]

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 3 months as a plaque develops from an early active to an inactive plaque, converging into a final common pathway that is probably mainly linked to accumulated neuroaxonal degeneration.[22,31] We herein refer 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 (ie, MOG+, CNP+ or MAG+) prevented us from better defining (ie, early vs late and active vs demyelinating lesions) the earliest stage of plaque formation.[14,21,22] 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 is associated with disability progression.[16,32,33] A high percentage of our cases showed chronic plaque activity, herein defined as late active/chronic active,[1] somehow corresponding to active and postdemyelinating and mixed active/inactive.[22,26]

The occurrence of an AL 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 AL presence at postmortem is increased to nearly 50% if the progressive phase lasts <10 years; this probability does not increase further if MS is progressive in the terminal phase. These data imply the importance of a prolonged period of inflammatory activity in the disease and therefore support the use of anti-inflammatory therapeutic strategies also late during the disease progression.

At the same time, high levels of PVI are also associated with a shorter progressive period, and the chance of their presence is approximately 75% in those with a progressive phase of <10 years; again, this is not increased further if MS is progressive in the terminal phase. All together, these data suggest that when ALs and high levels of PVI 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[6] 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.[11] However, in addition, focal T2 MRI lesions combined with relapses have been shown to possibly explain later Expanded Disability Status Scale progression.[34,35] However, the exact load and effect of underlining neuropathological damage in the disease outcome and whether it is visible using current conventional imaging techniques are not known. The inflammation seen at time of death is almost certainly compartmentalized behind the blood–brain barrier and so will not be detectable as acute changes.[36,37] Only recent advanced imaging methodologies have enabled detecting more precisely the inflammatory lesion stages in in vivo MS patients.[13,29,38] In addition, it should be mentioned that the high inflammatory activity might also interfere with (delay and/or halt) remyelinating and repair mechanisms.[39]

Both early active and increased PVI were found in a subset of MS patients who also had a high level of meningeal infiltrates, corroborating the hypothesis that a generally higher inflammatory activity in the CNS/cerebrospinal fluid space characterizes an MS subgroup with more rapid progression.[17,18,40,41] The close, anatomical and functional, association between blood–brain barrier and the subarachnoid space[42] is further supported by the finding that B-cell clonality has been demonstrated between cells present in the meningeal infiltrates and in perivascular cuffs.[43] B cells are relatively predominant in the perivascular cuffs of ALs[37] and meningeal lymphoidlike infiltrates,[17,18,40,44] suggesting their key inflammatory role in MS progression.[45,46] The ALs and perivenular infiltrates we have seen are associated in particular with the preponderance of B lymphocytes, characteristically found in meningeal infiltrates.[17,18,44] 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 proinflammatory and regulatory molecules and by their antigen-presenting function.[47–49] 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 a specific phenotype of noncirculating tissue-resident memory CD8+ T cells[27] and specific B subsets, as well as the colabeling with vascular markers, might help to better understand the precise spatial and mechanistic features of perivascular inflammation in the pathology of white matter 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 blood–brain barrier. PVI could therefore be considered as a potential relevant surrogate marker of lesion activity that, if validated and assessed in early disease stages, might help to discriminate MS patients with higher lesion and disease activity who will benefit from early and more potent anti-inflammatory treatment. This would require an early identification of disability accumulation,[50,51] which could be improved with the use of molecular and imaging biomarkers to quantify the intrathecal inflammatory processes underpinning progressive MS,[40,52,53] aiming to capture the window of opportunity for a targeted anti-inflammatory approach.[54,55] In such a context, then, an immunosuppressant approach aiming to reduce disease activity in the early stages would have a fundamental role.[56] 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 ALs and focal PVI within the white matter at postmortem 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.

Acknowledgments

Author Contributions

R.N., R.M., R.R., and T.F. contributed to the conception and design of the study. R.N., R.M., D.M., O.H., and F.R. contributed to the acquisition and analysis of data. All authors contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

Data Availability

Data used for this article are available upon reasonable request.

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