Heterogeneity of cortical lesions in multiple sclerosis
Clinical and pathologic implications

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ABSTRACT

Objective: Autopsy cases show that cortical lesions (CLs) in multiple sclerosis (MS) lack lymphocyte/macrophage influx, blood-brain barrier breakdown, and complement activation. However, some CLs were demonstrated to harbor activated microglia. Here, we assessed the clinical significance of microglia activation in CLs in a large autopsy sample, and we investigated possible interrelationships with other pathologic characteristics.

Methods: We cross-sectionally investigated the clinicopathologic characteristics of 22 patients with MS with extensive subpial demyelination (CL group) and 19 patients with MS with only little demyelination of the cerebral cortex (non-CL group).

Results: A subset of the patients in the CL group (12 patients) showed rims of activated microglia (RAM) at the border of the CLs (RAM-CL group), whereas the other 10 patients in this group did not show microglia activation (non−RAM-CL group). A subsequent comparison between groups showed that patients with MS harboring RAM-CLs were significantly younger at the time of their death (53.5 years) than patients harboring mainly non–RAM-CLs (68.7 years; $p < 0.05$) or patients without extensive numbers of CLs (66.9 years; $p < 0.01$). In addition, a significantly shorter disease duration was found for the RAM-CL group (mean 20.9 years) than for the non-CL group (mean 34.5 years; $p < 0.05$). We also found that the presence of RAM-CLs is associated with a higher number of chronic active white matter (WM) lesions (Spearman $\rho = 0.74$; $p < 0.0001$).

Conclusions: RAM-CLs were found in a subset of patients with MS who also have more active WM inflammation and a less favorable disease course. *Neurology*® 2012;79:1369-1376

GLOSSARY

ANOVA = analysis of variance; **BSA** = bovine serum albumin; **CL** = cortical lesion; **HLA** = human leukocyte antigen; **MS** = multiple sclerosis; **PBS** = phosphate-buffered saline; **PLP** = proteolipid protein; **PP** = primary progressive; **PR** = progressive relapsing; **RAM** = rim of activated microglia; **SP** = secondary progressive; **WML** = white matter lesion.

Gray matter pathology in multiple sclerosis (MS) contributes importantly to clinical disability and cognitive decline[1–6] and increases substantially with progression of disease.[7,8]

Subpial cortical lesions (CLs) are by far the most common CL type in MS brains.[9,10] What causes subpial CLs to develop is poorly understood so far, but because of their superficial localization, it has been suggested that myelinotoxic substances that are produced as a result of inflammation in the meninges might be a contributing factor.[10–12] Although significant inflammation can indeed be found in the MS meninges,[13] we were previously unable to show an association between meningeal inflammation and the presence or extent of subpial cortical demyelination in chronic MS.[13]

CLs are generally devoid of inflammation,[11,14,15] and the blood-brain barrier is intact.[16] However, it should be noted that these facts are especially true for autopsy material, because a recent biopsy study using material from patients with early MS with tumefactive lesions[17]

Table 1		Clinical data of patients with MS included in this study			
Group and patient	Age, y	Type of MS	Sex	Disease duration, y	Cause of death
Non−RAM-CL					
1	69	PP	M	34	Cardiac arrest
2	57	ND	F	25	Euthanasia
3	75	SP	F	33	Pneumonia
4	77	PP	M	27	CVA
5	71	ND	F	25	Postsurgery respiratory problems
6	55	PP	F	19	Possible CVA
7	84	ND	F	49	Euthanasia
8	70	ND	M	51	Cardiac arrest
9	70	PP	F	40	Urinary tract infection
10	59	SP	F	25	Euthanasia
RAM-CL					
1	56	SP	M	27	Pneumonia
2	66	ND	M	Unknown	Sepsis
3	47	SP	M	7	Urosepsis
4	66	SP	F	23	Unknown
5	49	SP	M	26	Pneumonia by MS
6	61	SP	M	17	Euthanasia
7	44	PP	M	16	Pneumonia
8	44	SP	M	21	General deterioration due to MS
9	57	SP	F	27	Respiratory insufficiency
10	43	PP	M	17	Pneumonia
11	68	PP	F	38	Aspiration pneumonia
12	41	SP	F	11	General deterioration due to MS
Non-CL					
1	65	PR	M	25	Urosepsis
2	66	PP	F	43	Metastasis
3	81	PP	M	59	General deterioration
4	48	PP	F	48	Euthanasia
5	49	PP	F	30	Metastasized breast carcinoma
6	73	PP	M	16	Shock
7	59	SP	M	32	Myocardial infarct
8	49	PP	F	27	Metastasis of cervical carcinoma
9	68	PP	F	43	CVA
10	77	PP	F	48	Pneumonia
11	44	PP	F	8	Decompensation
12	77	ND	F	ND	Respiratory insufficiency with aspiration pneumonia
13	63	PP	M	27	Cardiac arrest
14	76	PP	F	19	Unknown
15	66	PP	M	26	Unknown
16	57	PP	M	25	Euthanasia
17	88	PP	F	25	Exhaustion by chronic colitis
18	81	PP	F	45	Sepsis by aspiration pneumonia
19	84	PP	F	49	Unknown

Abbreviations: CL = cortical lesion; CVA = cerebrovascular accident; MS = multiple sclerosis; ND = MS subtype not determined; PP = primary progressive; PR = progressive relapsing; RAM = rim of activated microglia; SP = secondary progressive.

demonstrated that early cortical demyelination, such as white matter demyelination, may be characterized by profound T-cell infiltration, as well as the presence of foamy macrophages.[17]

Despite the reported lack of inflammation in CLs in postmortem MS tissue,[11,14–16] several pathologic studies reported activated microglia at the borders of CLs.[11,14] The clinical and pathologic significance of these activated microglia in CLs is, however, so far unclear. In this large autopsy study, clinical and pathologic profiles of patients with MS with varying extents of cortical demyelination and different types of CLs, including those with a rim of activated microglia (RAM-CLs), were investigated.

METHODS **Postmortem tissue and lesion classification.** Brain material was obtained from The Netherlands Brain Bank, Amsterdam, the Netherlands. A total of 1,036 paraffin-embedded tissue blocks from 41 patients with MS were investigated in the study. Blinded to the patients' clinical statuses, 22 patients with MS were selected for the presence of extensive subpial cortical demyelination (CL group),[18] and 19 patients with MS were selected based on a lack of extensive subpial cortical demyelination (non-CL group). The number of white matter lesions (WMLs) in the 22 patients in the CL group was determined in a total of 639 tissue blocks. In a subset of these tissue blocks, selected based on the presence of cerebral cortex (random selection), the percentage of subpial cortical demyelination was determined. A total of 102 tissue blocks, of which 19 tissue blocks were full hemispheric tissue blocks and 83 were standard-sized tissue blocks, were examined for the extent of subpial cortical demyelination. The number of WMLs of the 19 patients with MS in the non-CL group was determined in a total of 397 tissue blocks. From a subset of the 397 tissue blocks examined for the non-CL group, namely 90 tissue blocks, the percentages of subpial cortical demyelination were determined. From these 90 tissue blocks, 19 tissue blocks were full hemispheric tissue blocks and 71 were standard-sized tissue blocks. Clinical data for both groups are summarized in table 1.

The age of patients with MS at the time of death ranged from 41 to 88 years (mean ± SD 63 ± 13 years) with a mean postmortem delay of 8 hours and 38 minutes (SD 3 hours and 29 minutes).

Based on immunohistochemical stainings for major histocompatibility complex II (microglia and macrophages) and proteolipid protein (PLP) (myelin/demyelination) (see Immunohistochemistry), a distinction was made between active, chronic active, or inactive WMLs and RAM-CLs or non–RAM-CLs (figures 1 and 2, respectively).

Standard protocol approvals, registrations, and patient consents. The study was approved by the local institutional ethics review board. Before death, all donors or their next of kin provided written informed consent for brain autopsy and use of material and clinical information for research purposes.
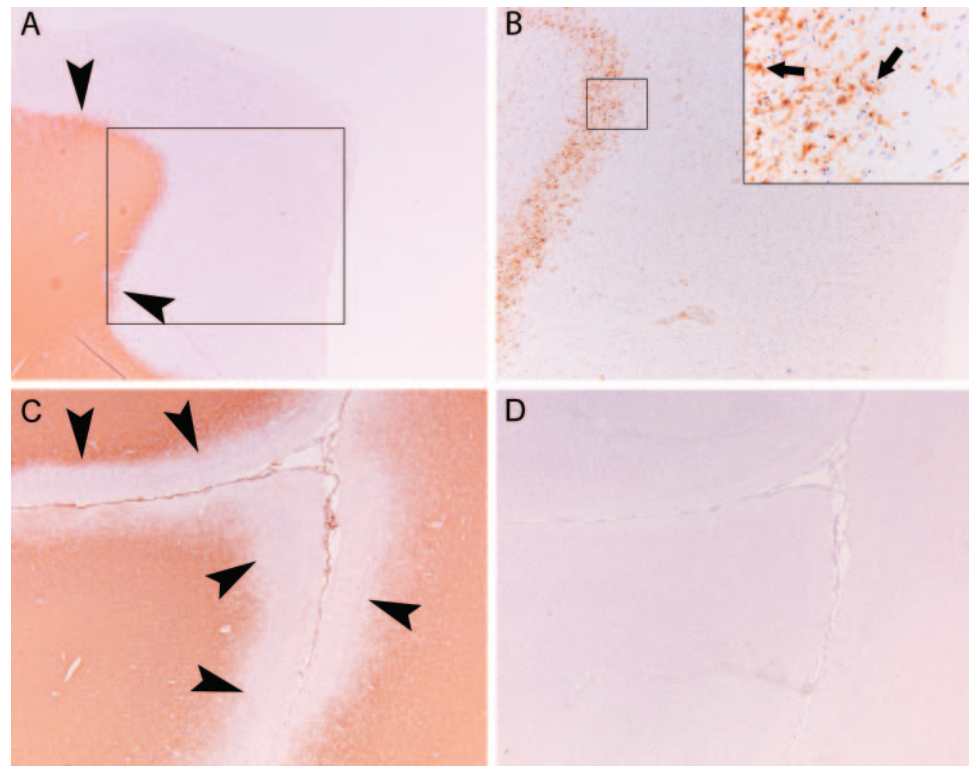
Figure 1   White matter lesions
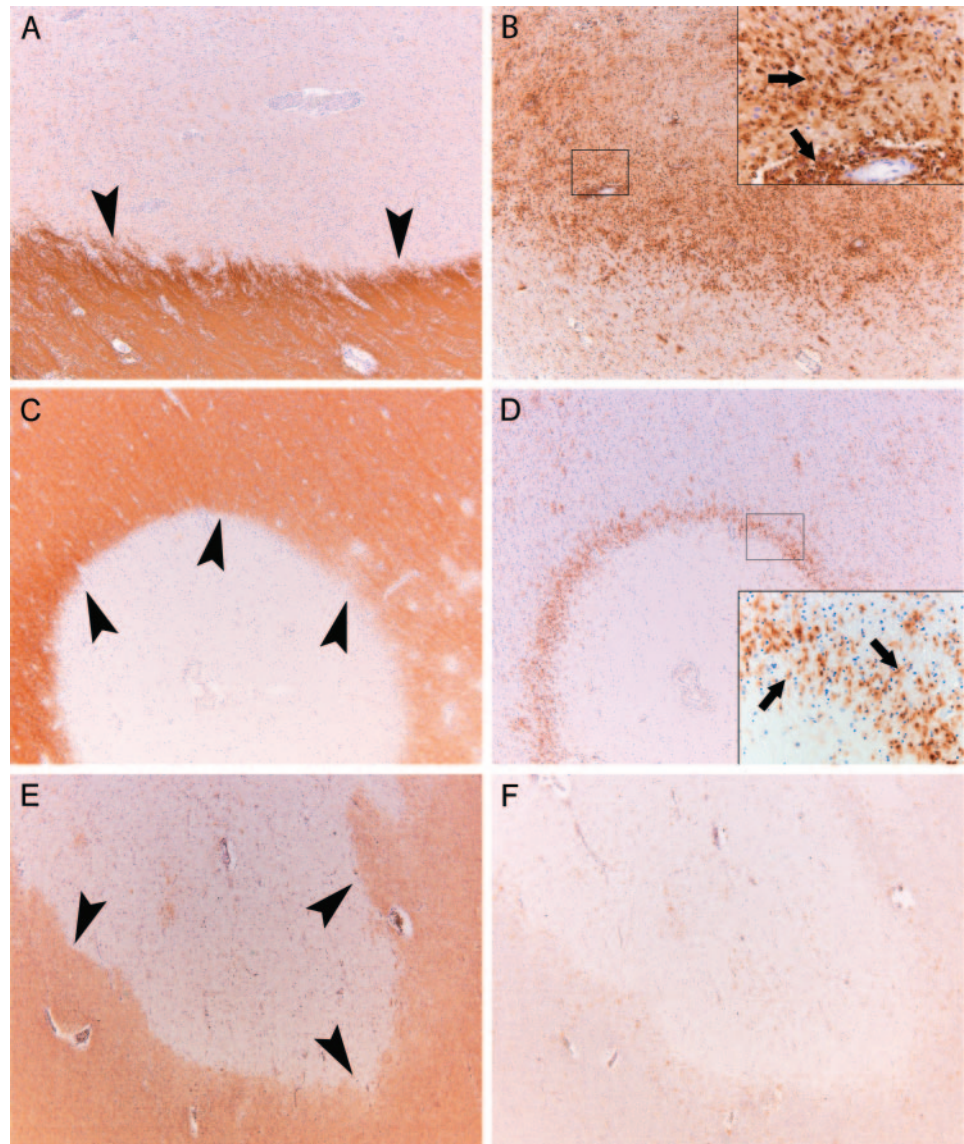
Based on immunohistochemical stainings with anti-proteolipid protein (A, C, and E) and anti-human leukocyte antigen DR (B, D, and F), white matter lesions were subdivided into active (A and B), chronic active (C and D), or inactive (E and F) white matter lesions.

Immunohistochemistry. Five-micrometer-thick paraffin sections were deparaffinized in a series of xylene (four 5-minute rinses), 100% ethanol, 96% ethanol, and 70% ethanol. Endogenous peroxidase activity was blocked by incubating the sections in methanol with 0.3% H_2O_2 for 30 minutes. For primary immunostaining with anti-PLP (clone Plpc1; mouse immunoglobulin G2a, 1:500; Serotec, Oxford, UK) sections were rinsed 3 times for 10 minutes each with phosphate-buffered saline (PBS) followed by incubation with primary antibodies diluted in PBS containing 1% bovine serum albumin (BSA) for 1 hour. For primary immunostaining with anti−human leukocyte antigen (HLA)-DR, sections were pretreated with microwave antigen retrieval (3 minutes at 900 W and 30 minutes at 180 W). After pretreatment, sections were cooled to room temperature, rinsed with PBS (three 10-minute rinses), and incubated with anti−HLA-DR (mouse immunoglobulin G2b, 1:50; gift from Dr. Hilgers, VU Medical Center, Amsterdam, the Netherlands)

diluted in PBS containing 1% BSA for 1 hour. Then sections were rinsed again with PBS (three 10-minute rinses) and incubated with EnVision horseradish peroxidase complex (DAKO, Glostrup, Denmark) and finally with 3,3′ diaminobenzidine-tetrahydrochloride dihydrate (DAKO) as a chromogen. After a short rinse with tap water, sections were counterstained with hematoxylin for 1 minute and intensely washed with tap water for 5 minutes.

Morphometric analysis and quantification. Based on anti-PLP and anti−HLA-DR immunostainings, all tissue sections were investigated for the presence of active, chronic active, or inactive WMLs (figure 1) and subsequently counted (table 2). Of note, leukocortical lesions were regarded as WMLs expanding into the cortical gray matter. Furthermore, a subset of the tissue blocks, containing cortical gray matter (see Postmortem tissue and lesion classification), was analyzed for the presence of

Based on immunohistochemical stainings with anti-proteolipid protein (A and C) and anti-human leukocyte antigen DR (B and D), cortical lesions were subdivided into cortical lesions with rims of activated microglia (RAM) (A and B) or non-RAM cortical lesions (C and D).

RAM-CLs or non–RAM-CLs. The tissue sections harboring CLs were photographed with a digital camera and a prepared digital image was printed. The cortical area demyelinated in the plane of the section was analyzed using light microscopy, and the same orientation was applied for the tissue on printed slides. Based on the serial section stained for HLA-DR, a distinction was made between RAM-CLs and non–RAM-CLs. The area of subpial demyelination was morphometrically measured on the digital images using ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/index.html).

Statistical analyses. GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) was used for the statistical analyses. For normal distribution of data, analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons was used to compare differences among the RAM-CL group, non–RAM-CL group, and non-CL group. When normality was not found, the nonparametric Kruskal-Wallis test was used with a Dunn correction for multiple comparisons. Possible correlations were investigated with the nonparametric Spearman rank correlation (when data were not normally distributed) or Pearson correlation test (when data were normally distributed). Results were considered significant when $p < 0.05$.

RESULTS **Clinical and pathologic descriptives of the MS autopsy cases.** Of the 41 patients with MS included in this study, 23 patients with MS had experienced a primary progressive (PP) disease course, 11 patients with MS had a secondary progressive (SP) disease course, 1 patient with MS had a progressive

relapsing (PR) course, and for 6 patients with MS the disease course was not specified or could not be retrospectively determined from the medical records. Mean age at death was 63.4 years (SD 13.0 years), and the mean disease duration was 29.6 years (SD 12.7 years). Of the 41 patients with MS included, 22 patients with MS harbored extensive subpial demyelination as described previously,[18] and these patients were designated as the CL group for further analyses. Nineteen patients with MS showed only very limited cortical demyelination, and these patients were regarded as the non-CL group. Overall, there was no difference in age at death or disease duration between these 2 groups.

On average, of the patients with MS in the CL group, 23.0% (SD 11.6%) of the cerebral cortex was demyelinated (vs 0.5% [SD 0.7%] in the non-CL group). A total of 639 tissue blocks were investigated within the CL group, and these blocks showed 208 inactive, 252 chronic active, and 59 active WMLs (figure 1, table 2). In 12 patients of this group, microglia activation at the borders of the CLs was regularly found (figure 2, A and B, table 2); this subgroup was therefore regarded as the RAM-CL group (mean percentage of cortical demyelination with RAM was 37.3%) (figure 2, A and B, table 2), whereas in the

Group and patient	Cortical demyelination, %	Cortical demyelination with RAM, %	Active WMLs	Chronic active WMLs	Inactive WMLs
Non–RAM-CL					
1	9.9	0	0	0	3
2	24.6	0	0	2	14
3	18.3	0	0	3	29
4	11.0	0	0	0	4
5	10.5	0	0	0	15
6	20.9	0	0	3	15
7	17.6	0	0	0	2
8	20.5	0	0	0	11
9	13.4	0	0	0	6
10	24.0	0	0	7	1
RAM-CL					
1	42.0	20	4	6	11
2	8.9	13	7	1	15
3	26.0	39	17	11	0
4	34.7	71	20	24	1
5	28.8	19	0	31	1
6	13.7	18	1	23	16
7	32.6	62	3	28	4
8	29.7	15	3	44	3
9	57.8	54	0	17	4
10	17.0	53	4	45	19
11	22.2	7	0	0	6
12	21.8	76	0	7	28
Non-CL					
1	0.5	0	0	2	8
2	2.3	0	0	0	21
3	0	0	0	0	0
4	1.1	0	2	16	13
5	0	0	0	0	22
6	1.4	0	0	0	6
7	0.8	0	1	2	4
8	0	0	0	0	0
9	0	0	0	0	0
10	0	0	0	2	0
11	1.1	0	1	0	10
12	0.5	0	0	0	5
13	1.0	0	0	0	17
14	0	0	0	0	2
15	1.0	0	1	33	9
16	0	0	0	6	2
17	0	0	1	0	1
18	0	0	3	3	0
19	0	0	0	0	3

Table 2 — Cortical demyelination and WMLs in MS

Abbreviations: CL = cortical lesion; MS = multiple sclerosis; RAM = rim of activated microglia; WML = white matter lesion.

remaining 10 patients of the CL group no microglia activation at the border of the CLs was found at all (regarded as the non–RAM-CL group) (figure 2, C and D, table 2). Interestingly, in the RAM-CL group, a significantly larger proportion of the cerebral cortex was demyelinated (mean 27.9%) compared with that in the non–RAM-CL group (mean 17.1%; $p = 0.03$). Neither RAM-CLs nor non–RAM-CLs showed any preference for a specific topographical distribution or localization within the cerebral cortex. Hence, either subpial lesion type (i.e., RAM-CLs and non–RAM-CLs) could be found at the surface of the cortex and also within deep sulci (data not shown). In the non-CL group, a total of 397 tissue blocks were investigated in which 123 inactive, 64 chronic active, and 9 active WMLs were subsequently found (figure 1, table 2).

Clinical profiles: comparisons between the RAM-CL group, non–RAM-CL group, and non-CL group. Comparing age at death among the 3 defined groups revealed overall differences between the RAM-CL, non–RAM-CL, and non-CL groups (ANOVA, $p = 0.004$). Post hoc testing showed that a lower mean age at death was found in the RAM-CL group than in the non–RAM-CL group or the non-CL group (mean age of the RAM-CL group was 53.5 years, mean age of the non–RAM-CL group was 68.7 years, and mean age of the non-CL group was 66.9 years; RAM-CL group vs non–RAM-CL group: $p < 0.05$ and RAM-CL group vs non-CL group: $p < 0.01$) (figure 3). Interestingly, no difference in age at death was found between the non–RAM-CL group and the non-CL group (figure 3A). Furthermore, differences in disease duration were also found among the 3 groups (ANOVA, $p = 0.024$). Post hoc, significantly shorter disease duration was found for the RAM-CL group (mean 20.9 years) than for the non-CL group (mean 34.5 years, $p < 0.05$) (figure 3B). After correction for multiple comparisons, no significant difference was found in disease duration between the RAM-CL group and the non–RAM-CL group (mean 32.8 years) (figure 3B). Finally, the age at disease onset did not differ among the 3 groups (mean RAM-CL group 31.5 years, mean non–RAM-CL group 35.9 years, and mean non-CL group 34.6 years; Kruskal-Wallis test, $p = 0.50$; data not shown).

Patients with PP MS were distinctly overrepresented in the non-CL group (16 of 19 patients). One patient had an SP disease subtype and one patient had a PR disease subtype. For one patient the disease subtype could not be retrospectively determined. In the RAM-CL and non–RAM-CL groups, no specific MS disease subtype was found to be clearly dominant.

Figure 3 Younger age at death and a shorter disease duration in patients with MS harboring cortical lesions with rims of activated microglia (RAM)
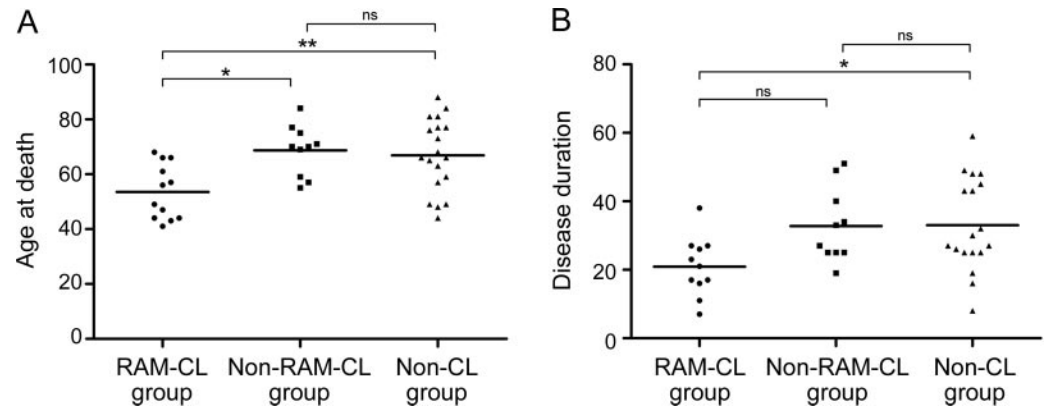
(A) Patients with MS harboring RAM cortical lesions (RAM-CLs) had a younger mean age at death (mean 53.5 years) than patients with only non-RAM-CLs (mean 68.7 years) or the group without CLs (non-CL) (mean 66.9 years) (RAM-CL group vs non-RAM-CL group $p < 0.05$ and RAM-CL group vs non-CL group $p < 0.01$). (B) Patients with MS harboring RAM CLs also experienced a shorter disease duration (mean 20.9 years) than the non-CL group (mean 34.5 years) and non-RAM-CL group (mean 32.8 years) (RAM-CL group vs non-CL group $p < 0.05$ and RAM-CL group vs non–RAM-CL group not significant). *$p < 0.05$; **$p < 0.01$.

Correlations of pathologic and clinical characteristics. Several pathologic and clinical parameters were analyzed for possible associations. As expected based on the data presented above, the presence of RAM-CLs was associated with a younger age at death in the CL group (Spearman $\rho = -0.70$, $p = 0.0003$). The extent of RAM-CLs was also associated with a shorter disease duration in the CL group (Spearman $\rho = -0.60$, $p = 0.004$). The age of disease onset was not associated with the extent of microglia activation at CL borders (Pearson $r = -0.13$, $p = 0.57$).

The number of chronic active WMLs also correlated significantly with a younger age at death (Spearman $\rho = -0.79$, $p < 0.0001$) and shorter disease duration (Spearman $\rho = -0.70$, $p = 0.0005$) in the CL group. However, the number of chronic active WMLs was not associated with either age at death or disease duration in the non-CL group (Spearman $\rho = -0.20$, $p = 0.20$ and Spearman $\rho = 0.12$, $p = 0.62$, respectively). In the CL group, the number of active WMLs was also associated with a younger age at death and a shorter disease duration, although this association was much weaker (Spearman $\rho = -0.42$, $p = 0.05$ and Spearman $\rho = -0.56$, $p = 0.008$, respectively). No association between age at death or disease duration and the number of inactive WMLs was found (Spearman $\rho = -0.01$, $p = 0.96$ and Spearman $\rho = -0.08$, $p = 0.73$, respectively). In the non-CL group, the presence of inactive WMLs correlated with a younger age at death (Spearman $\rho = -0.53$, $p = 0.02$), whereas disease duration was not associated with the number of inactive WMLs (Spearman $\rho = -0.25$, $p = 0.32$).

Interestingly, the presence of chronic active WMLs was associated with a higher load of RAM-CLs in the RAM-CL group (Spearman $\rho = 0.74$, $p < 0.0001$). Furthermore, leukocortical lesions were most prominently present in the RAM-CL group (128 leukocortical lesions) and to a lesser extent in the non–RAM-CL group (15 leukocortical lesions) and non-CL group (17 leukocortical lesions). Overall (i.e., in the CL group), no correlation was found between the number of inactive WMLs and non–RAM-CLs (Spearman $\rho = 0.07$; $p = 0.76$).

DISCUSSION This study investigated brain material from 41 well-characterized MS autopsy cases and showed that there is no difference regarding age at death or disease duration between patients with many CLs (termed the CL group) and patients with few CLs (termed the non-CL group). In a subset of patients within the CL group (i.e., 12 patients), a significant proportion of the CLs were characterized by a rim of activated microglia at their border (the RAM-CL group).[11,14] The CLs of the remaining 10 patients included in the CL group were completely devoid of any sign of microglia activation at their borders (the non–RAM-CL group). There was significantly more demyelination of the cerebral cortex in the RAM-CL group than in the non–RAM-CL group. Regarding age at death and age at disease onset, no differences were observed between the non-CL group and the non–RAM-CL group. However, a significant younger age at death was found in patients with MS with RAM-CLs compared with patients with MS in the non–RAM-CL group or in the

non-CL group. In addition, a shorter disease duration was found in patients with MS in the RAM-CL group compared with patients with MS in the non-CL group or in the non–RAM-CL group (although the latter comparison did not reach statistical significance, most likely due to the small sample size [n = 10]). Remarkably, patients with PP MS were distinctly overrepresented in the non-CL group. Although tissue selection was performed blinded to the patients' clinical statuses, it remains to be determined whether this finding is of clinical significance and could, for example, be explained by the fact that patients with PP MS tend to have a less inflammatory profile.[19] In line with our results (7 of 22 patients in the CL group experienced a PP disease course), previous studies have clearly indicated that extensive cortical demyelination can also occur in patients with PP MS.[9,10,14] In the general MS population, 10%–15% of patients with MS experience a PP disease course.[19] Accordingly, the same percentage of patients with PP MS should be found in our postmortem sample. However, the percentage of patients with PP MS in our tissue bank was higher than the expected 10%–15%, which might partly explain the overrepresentation of patients with PP MS in our non-CL group.

Analysis of several histopathologic characteristics among the 3 different groups indicated that the presence of RAM-CLs was significantly associated with a younger age at death as well as a shorter disease duration. For the CL group, the presence of chronic active WMLs was also associated with a younger age at death and shorter disease duration. Furthermore, we found that the presence of chronic active WMLs was associated with a higher RAM-CL load. Previous studies have suggested a significant role for (slowly expanding) chronic active WMLs in MS disease progression.[20] Based on our neuropathologic data, we here suggest that CLs with RAM may similarly contribute to MS disease progression and disease severity.

The underlying etiopathogenic mechanisms of cortical demyelination are not clear, although several studies have suggested that cortical demyelination may be the result of inflammation in the leptomeninges.[10–12] In our previous work, we were not able to confirm this association.[13] Recent work by Lucchinetti et al.[17] based on biopsy material from patients with early MS with tumefactive lesions showed that CLs not only occur early in the disease course but also are the result of an active inflammatory demyelinating process.[17] The researcher described the presence of myelin-laden macrophages and T-cell influx in CLs, something that is extremely rare in CLs at the end stage of disease.[11,14] In contrast, microglia activa-

tion at the border of subpial CLs is far more regularly found at autopsy.[11,14] Although the exact pathophysiologic role that activated microglia might play in these CLs is unclear, the strong positive correlation with chronic active WMLs found in this study may suggest an overall more pronounced activity of the innate immunity in disease progression in a subset of patients with MS. Previously, Magliozzi et al.[21,22] showed in a subset of patients with SP MS that microglia activation and a more severely damaged cerebral cortex is associated with a less favorable disease course and that these microglia may express harmful molecules such as inducible nitric oxide and tumor necrosis factor. Future researchers should detail whether the presence of (activated) microglia in MS CLs is mainly harmful[23,24,25] or whether these cells may also have neuroprotective effects in the MS cerebral cortex.[14,21,22,26,27]

In addition, as shown previously in animal models of MS (namely, experimental autoimmune encephalomyelitis),[28] the initiation of cortical demyelination was shown to be influenced by certain major histocompatibility complex haplotypes. As such, it would be of major interest to see whether genetic markers that have ensued from recent MS susceptibility studies[29–32] are associated with the differences in neuropathologic features as seen in the different MS subgroups described here. More specifically, it would be important to explore whether there are haplotype differences between patients with and without extensive cortical demyelination and between patients exhibiting RAM-CLs vs patients with non–RAM-CLs.

Our study provides new insight into the clinicopathologic associations of CLs with RAM and MS disease course, as well as into the relationship between gray and white matter pathologic lesions.

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