



Published in final edited form as:

*Ann Neurol.* 2015 November ; 78(5): 710–721. doi:10.1002/ana.24497.

## Clinical and Pathological Insights into the Dynamic Nature of the White Matter Multiple Sclerosis Plaque

Josa M. Frischer, PhD<sup>1</sup>, Stephen D. Weigand, MS<sup>2</sup>, Yong Guo, PhD<sup>3</sup>, Nilufer Kale, MD<sup>3</sup>, Joseph E. Parisi, MD<sup>3,4</sup>, Istvan Pirko, MD<sup>3</sup>, Jay Mandrekar, PhD<sup>2</sup>, Stephan Bramow, MD<sup>5,6</sup>, Imke Metz, MD<sup>7</sup>, Wolfgang Brück, MD<sup>7</sup>, Hans Lassmann, MD<sup>8</sup>, and Claudia F. Lucchinetti, MD<sup>3</sup>

<sup>1</sup>Department of Neurosurgery Medical University Vienna, Vienna, Austria

<sup>2</sup>Department of Health Sciences Research, College of Medicine, Mayo Clinic, Rochester, Minnesota

<sup>3</sup>Department of Neurology, College of Medicine, Mayo Clinic, Rochester, Minnesota

<sup>4</sup>Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota

<sup>5</sup>Department of Neurology, Copenhagen University Hospital, Bispebjerg, Denmark

<sup>6</sup>Department of Pathology, Copenhagen University Hospital Rigshospitalet, Denmark

<sup>7</sup>Department of Neuropathology, University of Goettingen, Goettingen, Germany

<sup>8</sup>Department of Neuroimmunology, Center for Brain Research, Medical University of Vienna, Vienna, Austria

### Abstract

**Background**—An extensive analysis of white matter plaques in a large sample of MS autopsies provides insights into the dynamic nature of MS pathology.

**Methods**—120 MS cases (1220 tissue blocks) were included. Plaque types were classified according to demyelinating activity based on stringent criteria. *Early-active*, *late-active*, *smoldering*, *inactive*, and *shadow plaques* were distinguished. 2476 MS white matter plaques were identified. Plaque type distribution was analyzed in relation to clinical data.

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All Correspondence to: Claudia F. Lucchinetti, MD, Professor of Neurology, Chair, Department of Neurology, Mayo Clinic, College of Medicine, 200 First Street SW, Rochester, MN 55905, clucchinetti@mayo.edu.

#### Author's Contributions

*Study design:* CFL, HL, WB. *Data collection:* JMF, NK, SB, YG, IM, JEP, HL, CFL. *Data analysis:* SW, JM, JMF. *Data interpretation:* JMF, HL, WB, CFL. *Figures:* JMF, YG, SW, JM. *Drafting of the manuscript:* JMF, CFL, HL, SW, JEP, IP. *Critical revision of the manuscript:* All authors.

#### Potential Competing Interests Statement

In addition to the reported funding JMF, YG, SW, JM, SB, NK, and JEP have nothing to disclose. IP reports grants from Novartis, outside the submitted work. HL reports personal fees from TEVA, Novartis, Baxter, and AMGEN outside the submitted work. WB reports grants and personal fees from Teva Pharma, Biogen Idec, Novartis and personal fees from Merck-Serono, Bayer Vital, Genzyme outside the submitted work. IM reports personal fees from Biogen Idec, Bayer Healthcare, TEVA, Serono, Novartis, outside the submitted work. CFL shares in royalties from marketing of kits for detecting AQP4 autoantibody and reports research grants from Biogen Idec, Novartis and Alexion Pharmaceuticals outside the submitted work.

**Findings**—*Active plaques* were most often found in early disease, whereas at later stages, *smoldering*, *inactive* and *shadow plaques* predominated. The presence of *early-active* plaques rapidly declined with disease duration. Plaque type distribution differed significantly by clinical course. The majority of plaques in acute-monophasic and RRMS were active. Among SPMS cases with attacks, all plaque types could be distinguished including active plaques, in contrast to SPMS without attacks in whom inactive plaques predominated. *Smoldering* plaques were frequently and almost exclusively found in progressive MS. At 47-years of age, an equilibrium was observed between *active* and *inactive* plaques, whereas *smoldering* plaques began to peak. Men displayed a higher proportion of *smoldering* plaques.

**Interpretation**—Disease duration, clinical course, age and gender contribute to the dynamic nature of white matter MS pathology. *Active* MS plaques predominate in acute and early RRMS and are the likely substrate of clinical attacks. Progressive MS transitions to an accumulation of *smoldering* plaques characterized by microglial activation and slow expansion of pre-existing plaques. Whether current MS therapeutics impact this pathological driver of disease progression remains uncertain.

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## Introduction

Multiple Sclerosis (MS) is the most common cause of non-traumatic disability in young adults.<sup>1</sup> MS classically has been described as a chronic, inflammatory, demyelinating disease of the CNS with the focal white matter lesion, the “plaque”, as its hallmark pathology.<sup>2-4</sup> There is considerable heterogeneity in the clinical, radiographic and pathological features of MS. Relapses and progression are the two basic clinical phenomena. Relapses are considered to be the clinical expression of focal acute inflammatory demyelinating plaques disseminated in the CNS.<sup>2-4</sup> More recently, gray matter pathology and diffuse white matter injury have been linked to neurological disability and disease progression, and distinct interindividual patterns of demyelination and tissue injury have been described.<sup>2,3,5</sup> Still, the clinical correlate of active demyelination in the pathogenesis of progressive MS remains controversial.<sup>4,6</sup> Moreover, mechanisms that drive disease progression from the relapsing-remitting to the progressive stage and mechanisms that further drive disease progression in that stage are not fully elucidated.<sup>3</sup> Although the fundamental features of the MS plaque have been characterized, criteria for the classification of subtypes are inconsistent and different schemes have been applied, particularly with respect to clinical correlation and underlying pathogenic mechanisms of disease.<sup>2,4</sup> Consequently, there remains a significant ongoing need to systematically and rigorously analyze white matter MS plaques from different stages and phases of the disease.

In this study, we present an extensive analysis of white matter MS plaques in a large sample of human archival autopsied tissues. We evaluate plaque type distribution in relation to age, gender, clinical course and disease duration and thus gain new insights into the dynamic nature of the MS plaque.

## Patients and methods

### Sample retrieval and inclusion criteria

Study approval was granted by the ethics commission of the Medical University Vienna and by the Institutional Review Board of the Mayo Clinic Rochester. Studies were performed on paraffin-embedded archival autopsy tissues of the Centre for Brain Research, Medical University Vienna, Austria, and the Department of Neurology, Mayo Clinic, Rochester, USA. MS cases with sufficient material for histopathological work-up were identified. For each case included in the study, the total available archived material was analysed. Pathological evidence consistent with MS was confirmed by a certified neuropathologist. Clinical diagnosis was determined according to McDonald or Poser criteria.<sup>7,8</sup> Clinical course was defined by a certified neurologist and categorized as acute-monophasic MS, relapsing-remitting (RR), secondary progressive with attacks (SP+) or without attacks (SP-), primary progressive (PP), asymptomatic or uncertain.<sup>9</sup> Patients with clinical and/or pathologic features of acute disseminated encephalomyelitis or neuromyelitis optica and patients with confounding CNS pathologies such as whole brain radiation or large infarcts were excluded from this study.<sup>10-12</sup>

### Neuropathological techniques and immunohistochemistry

For classification of white matter plaques, sections were stained with hematoxylin-eosin (HE), luxol fast blue (LFB), Bielschowsky silver and by immunohistochemistry for CD68/KiM1P, proteolipid protein (PLP) and glial fibrillary acidic protein (GFAP). Active plaques were further classified by immunohistochemistry for myelin oligodendrocyte glycoprotein (MOG), cyclic nucleotide phosphodiesterase (CNPase) and myelin-associated glycoprotein (MAG). Immunohistochemistry was performed as described previously (Table 1).<sup>13</sup>

### Plaque type classification

White matter plaques were classified as described previously.<sup>13,14</sup> White matter *active* plaques were characterized by dense infiltrates of macrophages containing LFB-positive and PLP-reactive myelin degradation products (Figure 1A and left insert, Figure 1B). Among *active* plaques, *early-active* and *late-active* plaques were distinguished. *Early-active* plaques were further defined by macrophages containing both minor myelin proteins (CNPase, MOG, MAG; Figure 1A and right insert) as well as major myelin proteins (MBP, PLP). Macrophages in *late-active* plaques contained only major myelin proteins.<sup>14</sup> White matter *smoldering* (slowly expanding) plaques typically showed an inactive center with no or few macrophages, surrounded by a rim of activated microglia (Figure 1C, 1D).<sup>13,15</sup> White matter *inactive* plaques revealed a sharp plaque border without or only few macrophages or activated microglia (Figure 1E, 1F).<sup>13,14</sup> Completely remyelinated white matter plaques were classified as *shadow* plaques (Figure 1G, 1H).<sup>16,17</sup>

### Statistical analysis

Statistical analyses were performed by R version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). Plaque type distributions were grouped with patient characteristic using bar plots. Multinomial regression was performed to model plaque type

proportions as a function of one of the following predictors: disease duration, clinical course or age at death.<sup>18</sup> Plaque type was treated as a five-level or four-level categorical outcome or response variable and modeled with multinomial regression, an extension of binary logistic regression to the case where the outcome or response has more than two categories.<sup>18</sup> These regression models were used to provide estimates of the probability of each plaque type at each level of a covariate. These probabilities can be multiplied by 100 and interpreted as estimated percentages or relative frequencies. Multiple plaques from the same patient cannot be assumed to be statistically independent. A cluster bootstrap procedure was used to calculate 95% confidence intervals that account for intra-patient correlation among multiple plaques from the same individual. By taking this into account no undue weight is given to patients with more versus fewer plaques. This approach does not make assumptions about causes and effects but rather allows us to estimate and contrast the distribution of plaque type at different ages, among different clinical courses, or at different disease durations. In terms of the composition of the sample, a flexible restricted cubic spline modeling approach was used so that plaque type distribution estimates at longer disease durations were largely unaffected by how many subjects with shorter disease duration were included. Thus, fulminant MS cases have little to no influence on our estimates for disease durations beyond about 2 years.

To complement this plaque-level analysis, a similar but distinct patient-level analysis was performed. For each plaque type, patients were classified as having or not having at least one plaque of that type. We then modeled this binary outcome using logistic regression. Thus, the plaque-level and patient-level analyses provided complimentary information.

## Results

### Patient characterization

A total of 120 subjects (76 (63%) women; 44 (37 %) men) were studied (Table 2). Median age at death was 56 years (range, 17 – 86). Median disease duration in the total cohort was 12 years (range, 0 – 48) but differed ( $p < 0.0001$ ) among disease courses. The longest disease duration occurred among SPMS patients without attacks, followed by SPMS patients with attacks. Consequently, age at death also differed significantly ( $p < 0.0001$ ) by disease course. Patients with acute-monophasic disease died at an earlier age whereas especially SPMS patients without attacks were significantly older (Table 2).

### Plaque type distribution

Overall, 1220 blocks (median: 9 blocks per patient, range: 1–46) were analyzed including single hemispheric ( $n=19$ , 2%), double hemispheric ( $n=71$ , 6%), large ( $n=60$ , 5%) and routine blocks ( $n=1070$ , 87%). The majority of routine blocks ( $n=705$ , 58%) were taken from supratentorial locations, while additional blocks were from brainstem ( $n=179$ , 15%), cerebellum ( $n=95$ , 8%), spinal cord ( $n=112$ , 9%) or optic nerve ( $n=39$ , 3%). Double/single hemispheric blocks were cut either as coronal or horizontal sections at the thalamic level ( $n=37$ , 3%) or at a frontal or occipital level ( $n=24$ , 2%). Additional double hemispheric sections included cerebellum and brainstem ( $n=29$ , 2%).

In total, 2476 plaques were identified, including 869 (35%) *active* plaques, 374 (15%) *smoldering* plaques, 875 (35%) *inactive* plaques and 358 (14%) *shadow* plaques. Among *active* plaques, 432/869 (50%) plaques were *early-active*. Median plaque count per patient was 11 (range: 1–123).

In general, plaque type distribution was rather similar across block locations between supratentorial and infratentorial white matter (Figure 2). Especially active plaques were equally distributed across all block locations. Differences were observed only among smoldering and inactive lesions: Lesions in the spinal cord were more likely to be inactive. In contrast, no/ few smoldering plaques were found in the spinal cord or optic nerve. However, smoldering and inactive plaques were also both equally distributed between the supratentorial and the infratentorial white matter (Figure 2).

### Plaque type in relation to disease duration

Overall, the percentage of an individual patient's plaques that were active declined with disease duration (Spearman rank correlation  $r_s = -0.8$ ,  $p < 0.0001$ ). Most strikingly, the percentage of *early-active* plaques declined with disease duration (Spearman rank correlation  $r_s = -0.7$ ,  $p < 0.0001$ ). In patients with disease duration up to 1 year, the majority of plaques (318/425, 75%) were *early-active*, while in patients with disease duration of over 30 years, the percentage of *early-active* plaques (1/440, 0%) was nearly zero (Figure 3A). After 30 years of disease duration, *inactive* (221/440, 50%), *smoldering* (102/440, 23%) and *shadow* plaques (90/440, 20%) predominated.

A multinomial regression model was fit at the plaque-level (Figure 3B). The probability that an observed plaque was *early-active* rapidly declined with disease duration. Conversely, the corresponding curve for *inactive* and *shadow* plaques increased with *inactive* plaques clearly predominating from about 15 years onward. *Smoldering* plaques were seen mainly in patients with disease duration longer than 10 years (Figure 3B). Next, a logistic regression model was fit at the patient-level estimating the probability a patient had at least one of each type of plaque as a function of disease duration (Figure 3C). The probability of *early-* or *late-* active plaques in a patient again declined with disease duration, whereas the probability of inactive plaques inversely rose (Figure 3C). At disease onset, most patients had an *early-active* plaque. At 25 years of disease duration, the probability a patient harbouring at least one *early-active* plaque fell as low as 3%, while nearly all patients (94%) had an *inactive* plaque (Figure 3C). At the same stage, the probability of at least one *smoldering* plaque or one *shadow* plaque was 64% or 49%, respectively. *Smoldering* plaques peaked at approximately 20 years of disease duration (Figure 3C).

### Plaque type in relation to clinical course

Plaque type frequencies differed significantly by clinical course ( $p < 0.0001$ , Figure 4A), even after adjusting for age or disease duration. *Early-active* plaques (309/416, 74%) predominated in acute-monophasic MS. A striking two thirds of all plaques were *active* in RRMS compared to just one third of all plaques in SPMS with attacks. Moreover, an even distribution of *early-active* and *late-active* plaques was found in RRMS. In SPMS patients with attacks, *early-active* plaques still contributed a fair, albeit smaller, proportion of all

*active* plaques (Figure 4A). Generally, a rather even distribution of *active* plaques (236/719, 33%), *smoldering* plaques (122/719, 17%), *inactive* (217/719, 30%) and *shadow* plaques (144/719, 20%) was found in SPMS patients with attacks. Among SPMS patients without attacks, *inactive* (362/595, 61%) and *smoldering* plaques (128/595, 22%) predominated, and *active* plaques in general (45/595, 8%) were rarely seen. Among PPMS patients, a high number of *smoldering* plaques (106/377, 28%) but also *inactive* (118/377, 31%) and *shadow* plaques (97/377, 26%) were evident. *Active* plaques (56/377, 15%) were less frequent in PPMS. Interestingly, *smoldering* plaques occurred almost exclusively in patients with progressive MS. The dominant plaque type among incidental/asymptomatic MS patients was *inactive*.

### Plaque type in relation to age and gender

At age 47-years, percentages of *active* and *inactive* plaques were equal (see crossing point of two curves in Figure 4B). This age also marked the beginning of the peak of *smoldering* and *shadow* plaques. When estimating the probability that a patient had at least one of each plaque type as a function of age, similar findings as described above were observed (Figure 4C). Again, the probability curves for *active* and *inactive* plaques were inversed, intersecting at 49-years of age. Both *smoldering* and *shadow* plaques peaked around 50-years of age (Figure 4C). The probability of a specific plaque type as a function of age-at-death was analyzed separately for male and female patients (Figure 5). *Active* plaque frequencies declined similarly in men and women (Figure 5A). However, a clear difference between the sexes was seen with *smoldering* plaques. Between ages 45- and 55-years, men harboured a significant higher percentage of *smoldering* plaques than women ( $p=0.046$ , Figure 5B). At age 60 years and older, the probability of *inactive* plaques was lower for men than women (Figure 5C). In contrast, *shadow* plaques were rather equally distributed between the genders (Figure 5D).

### Discussion

Classification criteria for the white matter MS plaque are controversial and data on different courses and time points are lacking. Our analysis has focused on clinical and pathological features of an extensive collection of archival MS autopsy cases, representing a comprehensive cross section of different disease courses and time points of age and disease duration.<sup>6</sup> Blocks from the supra- and infratentorial white matter as well as the spinal cord and the optic nerve are included. In addition, double/single hemispheric sections provide an even more expansive sampling. Plaques types are rather equally distributed among block locations, showing that especially active plaques may occur anywhere in the CNS white matter. Clinical data are similar to previously published studies.<sup>1,6</sup> In comparison to their epidemiological occurrence, there is an overrepresentation of fulminant MS cases in this study.<sup>19</sup> However, this focus on the acute MS stage has been conducted to be able to fully evaluate and appreciate the differences between early and chronic MS. In addition, the statistical analysis directly modelled disease duration and age effects in a flexible way that fulminant MS cases have little to no influence on our estimates for disease durations beyond about 2 years. Moreover, our sample also represents the clinical RRMS-SPMS continuum.



Further, SPMS patients with attacks compared to SPMS patients without attacks represent earlier and later stages in disease progression.

Our data indicate that features of white matter MS pathology vary with time. *Active* plaques predominate in early MS, while *inactive* plaques are most frequently found in chronic MS with long standing disease duration. The probability of a patient harbouring an *active* plaque declines with time. More importantly, the probability of *early-active* plaques declines even more strikingly with disease duration. Thus, while *early-active* plaques are frequently found in early MS stages, they rarely occur among patients with long standing disease. *Early-active* plaques are a prerequisite for immunopathological pattern classification.<sup>20</sup> Therefore, our published data describing interindividual immunopathological heterogeneity in early active MS, in contrast to reported findings of immunopathological homogeneity among chronic MS patients may simply reflect the rarity of classifiable early active plaques in later stages of the disease.<sup>20,21</sup>

*Active* plaques typically are associated with gadolinium enhancement on MRI and most likely represent the pathological substrate of attacks.<sup>22,23</sup> In support of these data, *active* and *early-active* plaques represent the vast majority of plaques found in acute-monophasic and about two-thirds of those in relapsing-remitting MS. *Active* plaques are also common in SPMS patients with attacks, although *late-active* plaques predominate over *early-active* plaques. However, among SPMS patients without attacks, *active* plaques are rarely found whereas *inactive* plaques predominate. Interestingly, *smoldering* (slowly expanding) plaques are almost exclusively seen among progressive MS patients as well as in SPMS patients with and without attacks. Especially among PPMS patients, *smoldering* plaques are frequently found while *active* plaques contribute a smaller percentage of plaques.

*Smoldering* plaques have been associated with microglial activation, ongoing axonal injury and neurodegeneration.<sup>13,15</sup> Consequently, this chronic plaque type confirms slow but active demyelination among progressive MS patients and may also explain why worsening of pre-existing symptoms is a typical feature of progressive MS.<sup>24,25</sup> However, there are also lesions, which have a clear rim of activated microglia, but do not show evidence for ongoing demyelination and neurodegeneration, defined by the presence of early myelin degradation products in macrophages. It has been proposed that the adaptive and innate immune systems are involved in the immunopathogenesis of MS to different extents during the course of the disease.<sup>26</sup> The early, relapsing-remitting stage is associated with the antigen-specific T and B cell-mediated adaptive immune responses whereas the progressive phase is associated with innate immune responses characterized by chronic inflammation and microglial activation.<sup>26</sup> Thus, *smoldering* plaques provide further pathological evidence that the active demyelinating process seems to decelerate as the disease transforms into the progressive stage and the immune response may shift from adaptive to innate.<sup>26</sup>

In light of these pathological data, it is important to determine whether *smoldering* MS plaques can be distinguished on MRI. The high prevalence of *smoldering, slowly expanding* plaques with ongoing neurodegeneration in progressive MS may imply that a subset of pre-existing plaques increases in size. The presence of enlarging lesions has been demonstrated in several MRI studies.<sup>27,28</sup> Poor inter-rater correlation is a major problem that limits the

use of enlarging lesions in clinical practice.<sup>29</sup> This may be overcome by the use of 3D acquisition techniques and subtraction MRI, where registered volumes representing an earlier time point are subtracted from a later time point, enabling more objective visualization of enlarging lesions.<sup>30</sup> Gadolinium enhancement on MRI is considered absent among enlarging *smoldering* plaques.<sup>22</sup> USPIO, a negative contrast material may be able to visualize macrophages at the edges of smoldering plaques, although conclusive correlative pathological studies are lacking.<sup>31</sup> In 2010, an International CMSC Consensus Conference suggested that in addition to gadolinium enhancing lesions or new T2 lesions, enlarging T2 lesions should also be considered a marker that distinguishes relapsing from progressive forms of MS.<sup>32</sup> However, generalized brain atrophy is also considered an important correlate of MS disease progression.<sup>5,33</sup> The presence of tissue atrophy secondary to ongoing axonal degeneration and loss within the inactive plaque center may, with time, counterbalance plaque expansion at the *smoldering* plaque edge.<sup>34</sup> Therefore, in advanced stages of MS, plaque size by MRI might not, per se, increase, and the overall volumetric lesion load may paradoxically decrease.

In contrast to other plaque types, *shadow* plaque frequencies are similar across different disease courses, with the exception of the acute-monophasic stage, in which they are rarely found. Completely remyelinated *shadow* plaques as described in our study represent the late stage of remyelination.<sup>17,23</sup> In contrast, early remyelination has been described in early MS and active plaques.<sup>17</sup> However, remyelinated areas seem to be more susceptible to a second-hit inflammatory demyelinating attack than the normal-appearing white matter.<sup>35</sup> Since chronic MS has been associated with compartmentalized inflammation and a decrease in inflammation over time, it seems surprising that shadow plaques are not more common among progressive MS patients with longstanding disease.<sup>3,13</sup> As previously described, this failure to remyelinate axons might be caused by an age-dependent loss of trophic support from microglia or the local environment in demyelinated plaques.<sup>3,36</sup> However, we cannot know when the remyelination took place and how long such shadow plaques with reduced myelin density persist. Thus, the results could also mean that those shadow plaques, are lesions which have been formed in early disease stages and show persistent remyelination through the subsequent course of the disease.<sup>3,13,17,36</sup>

Observations from different studies have led to the hypothesis that the accumulation of disability in MS is mainly an age-related process, seemingly independent of the clinical subtype of MS.<sup>24</sup> In our cohort, the occurrence of *active* plaques declines with age, whereas *inactive* plaques increase. Around 47 years of age, an apparent equilibrium between active and inactive plaques is reached. In contrast, *smoldering* plaques begin to peak at that age. These observations are consistent with a recent study of over 900 MS cases which reported that the onset of progression is an age dependent milestone in MS, occurring at around 46 years of age in both SPMS and PPMS cases.<sup>37</sup> A conclusion from these data is that MS pathology transforms from new and active white matter plaques to slow expansion of pre-existing plaques in progressive MS. Consequently, a rather even distribution of the different white matter plaque types is seen at the beginning of the progressive stage. Previously published data show that there is a poor correlation between focal white matter lesions with cortical lesions and diffuse white matter injury.<sup>41</sup> Taking already existing data on diffuse



white matter injury and cortical demyelination into account, this transition of white matter pathology seen in our study also appears to be associated with extensive cortical demyelination and diffuse white matter injury in progressive MS.<sup>3,5</sup> Thus, the dynamic nature of MS pathology is reflected in the gray as well as in the white matter.<sup>3,5</sup>

In our pathological cohort, male patients show a higher fraction of *smoldering* plaques than female patients, especially at an age of 45 years and above. Several gender related differences in the natural history of MS have been described. Although MS is mainly reported in women, epidemiological studies show that women reach disability milestones later than men.<sup>1,38,39</sup> Men demonstrate a more progressive disease course, associated with a shorter time to and a younger age of conversion to secondary progressive MS.<sup>38,39</sup> Interestingly, a recent study reported that the expression of sex steroidogenic enzymes and hormone receptors in MS plaques and white matter demonstrated gender specific differences and further suggested a higher capacity for females to increase progesterone synthesis and signaling.<sup>40</sup> Progesterone and its metabolite DHP have been shown to ameliorate the clinical signs of EAE and decrease inflammatory activity of microglia.<sup>40</sup> These data may help explain the differences we observed in plaque types between genders.

## Conclusion

Our findings show that features of white matter MS pathology vary over time. *Active* plaques are the pathological substrate of attacks and predominate in acute or relapsing MS. *Smoldering*, *slowly expanding* plaques are almost exclusively found in progressive MS and contribute to disease progression. Gender and age specific differences in plaque development are evident. In chronic MS, the underlying pathology transforms from new and *active* white matter plaques to slow expansion of pre-existing plaques and *inactive* plaques. Our pathological data support the concept that as MS transitions into the progressive stage, immune responses may shift from adaptive to innate. Whether current MS therapeutics impact this pathological driver of disease progression remains uncertain.

## Acknowledgement and Funding

We would like to thank Marianne Leisser, Ulrike Koeck, Angela Kury and Patricia Ziemer for expert technical assistance. This work was supported by grants from NIH R01 NS 49577-6 (CFL), Novartis CFTY720DUS37T (CFL) and partly from the Austrian Science Fund, FWF Project P24245-B19 (HL) and FWF Project J3508-B24 (JMF). WB and IM were supported by grants from the German Ministry for Education and Research (BMBF, "German Competence Network Multiple Sclerosis" (KKNMS), Pattern MS/NMO) during the conduct of the study.

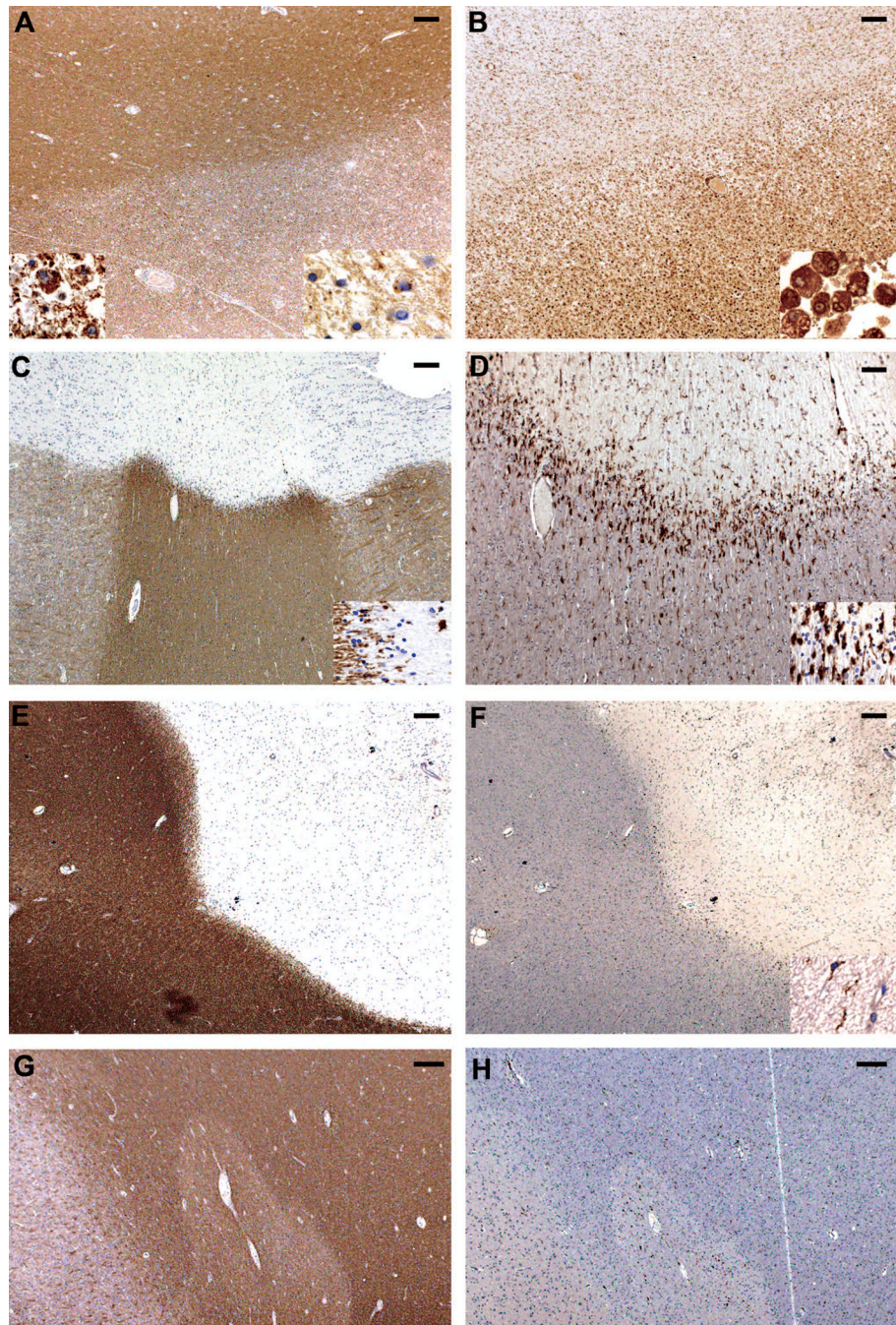
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### Figure 1. Characterization of plaque types

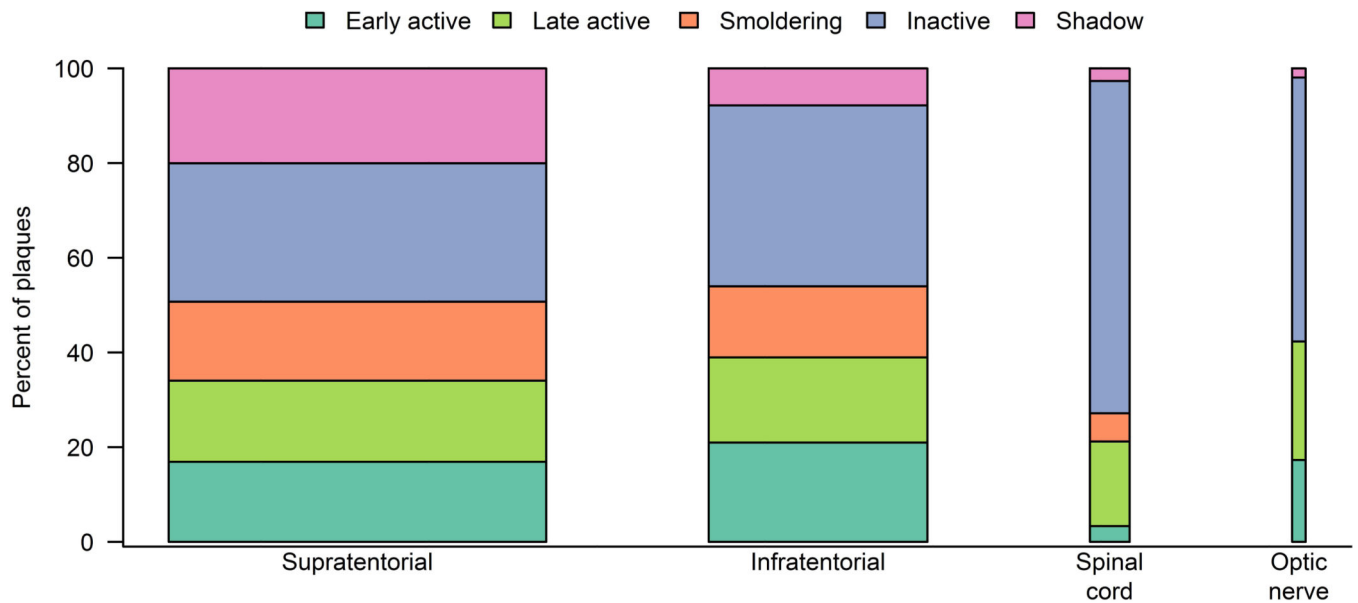
Figure 1 gives an overview of plaque types. Left pannel (A, C, E, G) depicts PLP staining. Right pannel (B, D, F, H) shows staining for KiM1P positive microglia / macrophages. Characterization of plaque types reflects the demyelinating activity of each plaque. Bar in A-C, E-H= 200µm, Bar in D=100 µm.

(A, B) Early active plaques (EAL) were defined by macrophages immunoreactive for minor myelin proteins (MOG positive macrophages right insert in A) as well as major myelin proteins (PLP positive macrophages left insert in A).

(C, D) Smoldering plaques (also called slowly expanding plaques) typically showed a rather inactive centre with no or few macrophages, surrounded by a rim of activated microglia. Only few of these macrophages or microglia cells contained early myelin degradation products. Inserts depict plaque edge.

(E, F) Inactive plaques revealed a sharp plaque border without or only few macrophages or activated microglia (insert).

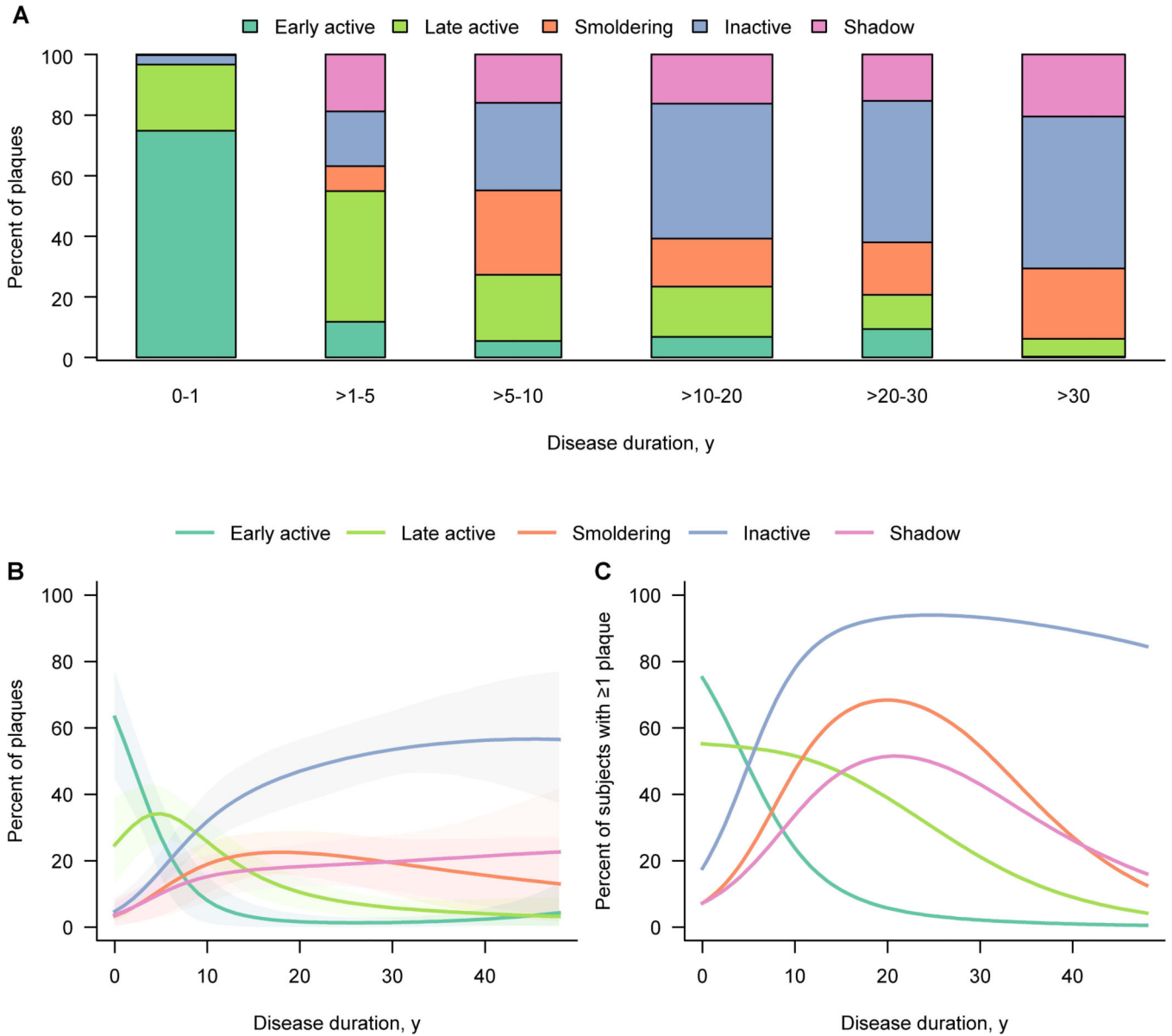
(G, H) Completely remyelinated plaques typically containing few macrophages without early myelin degradation products were classified as shadow plaques. Shadow plaques presented with a sharp plaque edge and were associated with fibrillary gliosis.



**Figure 2. Distribution of plaque location**

Figure 2 shows the distribution of plaque location. Bar widths indicate relative sample size. The majority of plaques (n=1439, 58%) were located supratentorial. A fair amount of plaques were found infratentorial (n=834, 34%). Additional plaques were found in the spinal cord (n=151, 6%) and in the optic nerve (n=52, 2%). Plaque type distribution was rather similar across block locations between supratentorial and infratentorial white matter. When using separate logistic regression models adjusted for age and disease duration, no regional differences were found for active plaques including specifically testing for early active or late active plaques. Differences were only observed among smoldering and inactive lesions: Lesions in the spinal cord were more likely to be inactive ( $p < 0.001$ ,  $p=0.002$ ) compared to supratentorial and infratentorial lesions. Lesions in the spinal cord were less likely to be smoldering ( $p=0.02$ ) compared to supratentorial lesions. However, smoldering and inactive plaques were also both equally distributed between the supratentorial and the infratentorial white matter.



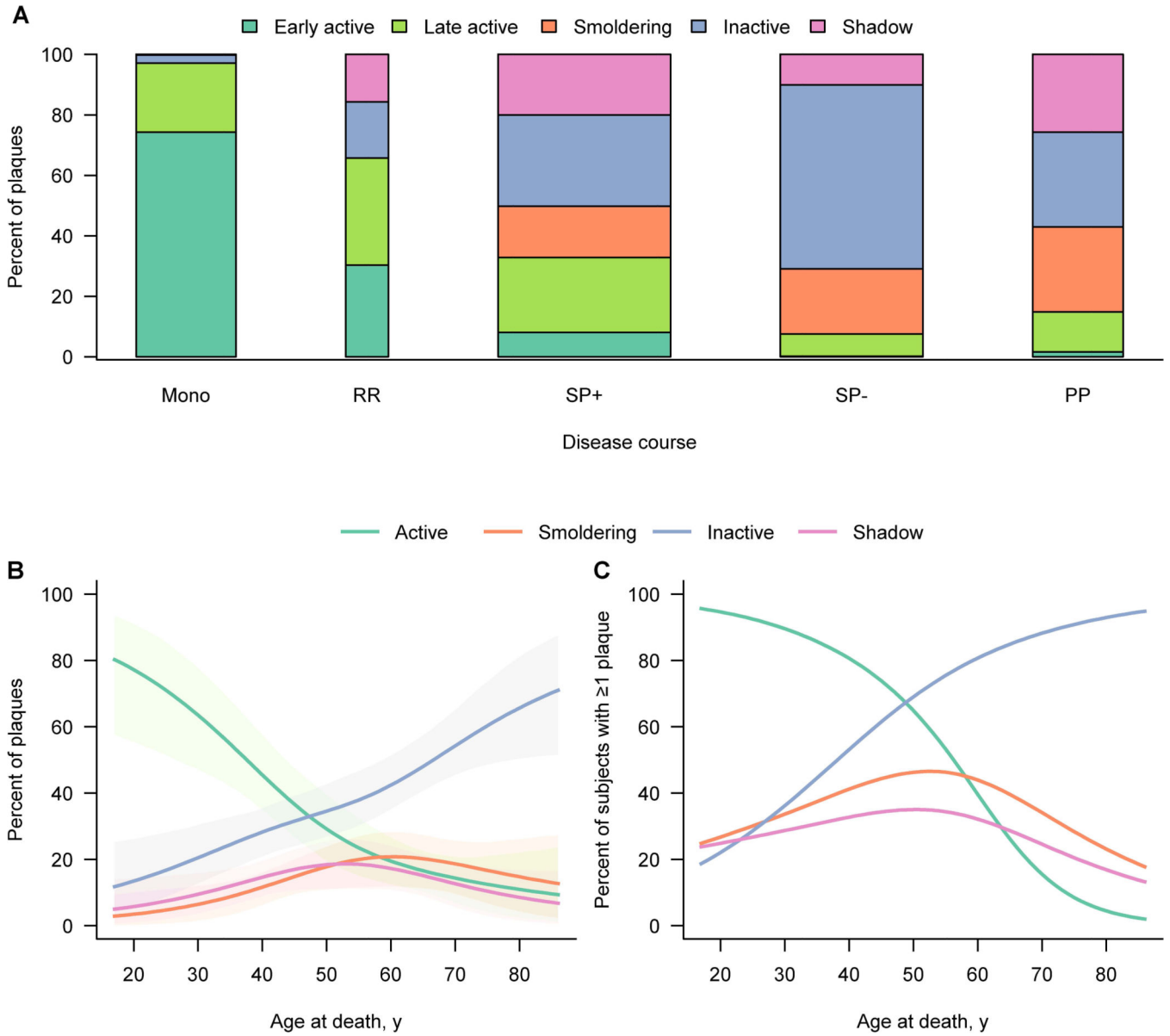


**Figure 3. Distribution of plaque types by disease duration**

(A) The bar plot shows the actual plaque distribution in relation to disease duration. The percentages shown for each subgroup or bar are calculated among all plaques for those subjects within the subgroup. Bar width is related to subgroup size. On multinomial modeling plaque type distribution differs significantly ( $p < 0.0001$ ) among disease duration subgroups.

(B) Graph B provides estimates of the percentage of plaques by type expressed as a function of the patient's disease duration using multinomial modeling accounting for clustering of plaques within patient. Percentages across the curves add to 100 percent at a given duration. Shaded regions represent 95% cluster bootstrap confidence intervals. Significant differences are observed ( $p < 0.0001$ ) based on multinomial modeling.

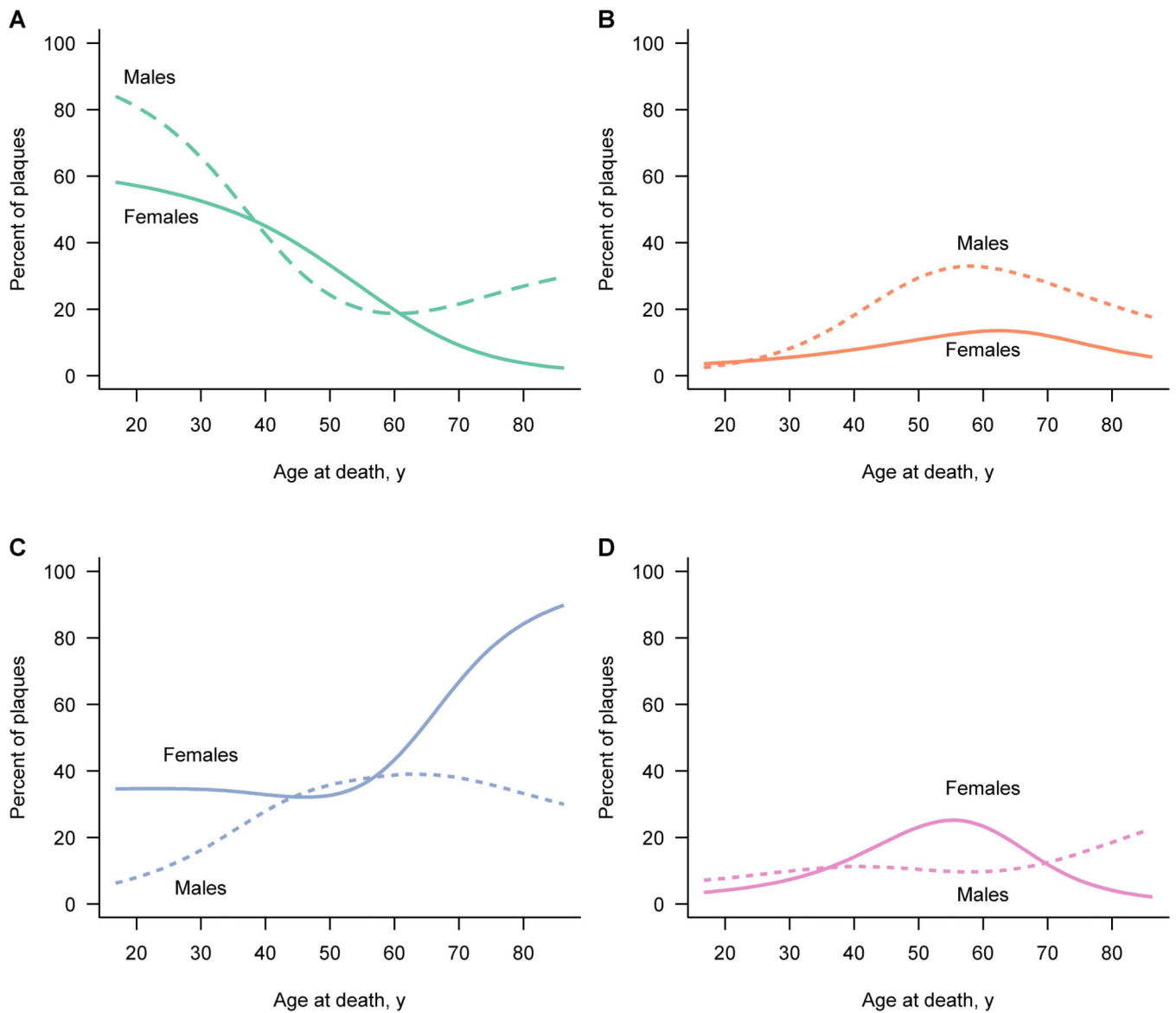
(C) In graph C the estimated proportion of patients with at least one of each plaque type as a function of disease duration is depicted. Estimates are based on separate logistic regression models at the patient level. Percentages across the curves add to more than 100 percent since subjects could have at least one of several types of plaques.



**Figure 4. Distribution of plaque types by disease course and age**  
 (A) The bar plot shows the actual plaque distribution in relation to clinical course. The percentages shown for each subgroup or bar are calculated among all plaques for those subjects within the subgroup. Bar width is related to subgroup size. On multinomial modeling plaque type distribution differs significantly ( $p < 0.0001$ ) among disease courses.  
 (B) Graph B provides estimates of the percentage of plaques by type expressed as a function of the patient's age at death using multinomial modeling accounting for clustering of plaques within patient. Percentages across the curves add to 100 percent at a given age. Shaded regions represent 95% cluster bootstrap confidence intervals. Early active and late active plaques are pooled as active plaques. Significant differences are observed ( $p < 0.0001$ ) based on multinomial modeling.

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(C) In graph C the estimated proportion of patients with at least one of each plaque type as a function of age is depicted. Estimates are based on separate logistic regression models at the patient-level. Percentages across the curves add to more than 100 percent since subjects could have at least one of several types of plaques. Early active and late active plaques are pooled as active plaques.



**Figure 5. Gender differences in plaque type distribution**

Figure 5 depicts the estimated percent of each plaque type by age at death for males and females separately based on a multinomial regression model accounting for clustering of plaques within patient. Estimates for women are denoted by a solid line while estimates for men are denoted by a dotted line.

**Table 1**

Primary antibodies used in this study

<i>Primary Antibodies</i>							
#	Antigen	Pre-treatment	Dilution	Antibody type	Target	Source	
1	PLP	ST, E	1:1000	mAb; mouse	proteolipid protein myelin	Serotec, Oxford, UK	
2	CD68	ST, E	1:100	mAb; mouse	sialomucin microglia, macrophages	Dako, Glostrup, Denmark	
3	KiMIP	ST, C	1: 5000	mAb; mouse	pan-microglia-macrophages	W.Brück Göttingen, Germany	
4	MAG	ST, E	1: 1000	mAb; mouse	myelin associated glycoprotein	Abcam, Cambridge, UK	
5	MOG	ST, C	1:1000	mAb; mouse	myelin oligodendrocyte glycoprotein	Abcam, Cambridge, UK	
6	CNPase	ST, E	1: 2000	mAb; mouse	2', 3'-cyclic nucleotide 3'-phosphodiesterase	Sternberger Monoclonals Inc., Lutherville, USA	
7	NF 150kD	ST, E	1:2000	Ab; rabbit	phosphorylated neurofilament chain M	Chemicon, Temecula, CA, USA	
8	GFAP	ST, E	1: 3000	Ab; rabbit	glial fibrillary acidic protein	Dako, Glostrup, Denmark	

Ab = polyclonal antibody; C = Citrate buffer (10mM, pH 6.0); E = EDTA (EDTA 1mM, TRIS 10mM), pH 9.0; mAb = monoclonal antibody; ST = Steamer



**Table 2**

Sample characterization

	Acute-Mono n=21	RR n=10	SP+ n=25	SP- n=29	PP n=20
<b>Age at death, y</b>					
Median (IQR)	45 (28, 48)	54 (42, 58)	50 (45, 60)	65 (58, 72)	58 (51, 75)
Range	17 to 78	19 to 69	28 to 76	31 to 84	34 to 86
<b>Gender</b>					
Female	11 (52%)	6 (60%)	17 (68%)	18 (62%)	13 (65%)
Male	10 (48%)	4 (40%)	8 (32%)	11 (38%)	7 (35%)
<b>Disease duration, y</b>					
Median (IQR)	0.2 (0, 0.3)	10 (3, 20)	16 (9, 28)	22 (16, 34)	14 (6, 27)
Range	0 to 1	0 to 43	1 to 48	10 to 45	2 to 47
<b>Cause of death</b>					
Directly MS related	21 (100%)	2 (20%)	2 (8%)	0 (0%)	0 (0%)
Cardiovascular failure	0 (0%)	4 (40%)	8 (32%)	14 (49%)	9 (45%)
Respiratory failure	0 (0%)	1 (10%)	6 (24%)	7 (24%)	5 (25%)
Other	0 (0%)	0 (0%)	5 (20%)	5 (17%)	1 (5%)
Not known	0 (0%)	3 (30%)	4 (16%)	3 (10%)	5 (25%)

Clinical course is categorized as acute-monophasic (n=21, 18%), relapsing-remitting (RR n=10, 8%), secondary progressive with attacks (SP+ n=25, 21%), secondary progressive without attacks (SP- n=29, 24%) and primary progressive (PP n=20, 17%). An acute-monophasic disease course leads to death under 1 year. A small group of 7 (6%) patients are classified as asymptomatic MS (not shown). In 8 (6%) patients the disease course could not be sufficiently evaluated by retrospective chart review (not shown). Cause of death distinguishes between directly related to MS, cardiovascular failure (due to peripheral septic conditions, myocardial infarction or congestive heart failure), respiratory failure (due to pulmonary embolism or pneumonia) and other causes (trauma, renal failure or malignancies other than CNS malignancies). In 15 (12%) patients cause of death is unavailable.