Gö-VIP-7: Dr. med. Sabine Pfeifenbring

Institut für Neuropathologie

Extensive Acute Axonal Damage in Pediatric Multiple Sclerosis Lesions.
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Autoren: Sabine Pfeifenbring 1†#, Reem F. Bunyan 2#, Imke Metz 1, Christian Röver 3, Peter Huppke 4, Jutta Gärtner 4, Claudia F. Lucchinetti 5+, Wolfgang Brück 1+

(1) Institut für Neuropathologie, Universitätsmedizin Göttingen
(2) Department of Neurology, Neurosciences Center, King Fahad Specialist Hospital Dammam, Dammam, Saudi Arabia
(3) Institut für Medizinische Statistik, Universitätsmedizin Göttingen
(4) Klinik für Kinder- und Jugendmedizin, Universitätsmedizin Göttingen
(5) Department of Neurology, Mayo Clinic College of Medicine, Rochester, MN

†Korrespondierender Autor

#geteilte Erstautorenschaft

+geteilte Letztautorenschaft

Zusammenfassung des wissenschaftlichen Inhalts

(Dr. med. Sabine Pfeifenbring)

Die Multiple Sklerose (MS) ist eine autoimmune, chronisch verlaufende Erkrankung des Zentralnervensystems (ZNS). Histologisch finden sich entzündlich entmarkende Läsionen mit einer unterschiedlich ausgeprägten axonalen Schädigung. Die Erkrankung beginnt meist im jungen Erwachsenenalter. In 3-10% der Fälle tritt die MS bereits im Kindes- und Jugendalter auf. Über 90% der kindlichen MS-Patienten haben einen schubförmig remittierenden Verlauf, wobei insbesondere Kinder mit einem präpubertärem Krankheitsbeginn häufig schwere klinische Schübe und große, multifokale MRT-Läsionen zeigen. Histopathologische Studien zur kindlichen MS, die helfen könnten, die klinischen und bildgebenden Unterschiede zu verstehen, fehlten bislang. In der Arbeit von Pfeifenbring et al. konnten wir nun zeigen, dass das Ausmaß des akuten axonalen Schadens in MS-Läsionen bei Kindern mit präpubertärem Krankheitsbeginn signifikant stärker ist als bei Kindern und Jugendlichen mit (post)pubertärem Krankheitsbeginn und Erwachsenen. Das Ausmaß des akuten axonalen Schadens korrelierte positiv mit dem Schweregrad der Behinderung zum Zeitpunkt des Schubes (Expanded Disability Status Scale). Zudem wiesen die Hirnläsionen von Kindern mit präpubertärem Krankheitsbeginn eine deutliche stärke Infiltration durch schaumzellige Makrophagen auf, was auf eine stärkere Abräumungs- und Entzündungsreaktion hinweist. In unserer Arbeit konnten wir somit zum ersten Mal zeigen, dass den klinischen und bildgebenden Besonderheiten der kindlichen MS Unterschiede in der Pathologie zugrunde liegen.

Weitere Informationen:

Universitätsmedizin Göttingen
Institut für Neuropathologie
Dr. med. Sabine Pfeifenbring
Telefon: 0551/39-22700
Robert-Koch-Straße 40
37099 Göttingen
Sabine.pfeifenbring@med.uni-goettingen.de

Extensive Acute Axonal Damage in Pediatric Multiple Sclerosis Lesions

Sabine Pfeifenbring, MD,[1] Reem F. Bunyan, MD,[2] Imke Metz, MD,[1] Christian Röver, PhD,[3] Peter Huppke, MD,[4] Jutta Gärtner, MD,[4] Claudia F. Lucchinetti, MD,[5] and Wolfgang Brück, MD[1]

Objective: Axonal damage occurs early in multiple sclerosis (MS) and contributes to the degree of clinical disability. Children with MS more often show disabling and polyfocal neurological symptoms at disease onset than adults with MS. Thus, axonal damage may differ between pediatric and adult MS patients.
Methods: We analyzed axonal pathology in archival brain biopsy and autopsy samples from 19 children with early MS. Lesions were classified according to demyelinating activity and presence of remyelination. Axonal density and extent of acute axonal damage were assessed using Bielschowsky silver impregnation and immunohistochemistry for amyloid precursor protein (APP), respectively. Axonal injury was correlated with the inflammatory infiltrate as well as clinical characteristics. Results were compared with data from adult MS patients.
Results: Acute axonal damage was most extensive in early active demyelinating (EA) lesions of pediatric patients and correlated positively with the Expanded Disability Status Scale at attack leading to biopsy/autopsy. Comparison with 12 adult patients showed a 50% increase in the extent of acute axonal damage in EA lesions from children compared to adults, with the highest number of APP-positive spheroids found prior to puberty. The extent of acute axonal damage correlated positively with the number of lesional macrophages. Axonal density was reduced in pediatric lesions irrespective of the demyelinating activity or the presence of remyelination. Axonal reduction was similar between children and adults.
Interpretation: Our results provide evidence for more pronounced acute axonal damage in inflammatory demyelinating lesions from children compared to adults.

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Multiple sclerosis (MS) is an autoimmune, inflammatory demyelinating disease of the central nervous system (CNS) and the most common disabling neurological disease in young adults.[1,2] Pediatric MS with a clinical onset before the age of 18 years occurs in about 3 to 10% of MS patients.[3] More than 90% of pediatric MS patients have a relapsing–remitting disease course.[3,4]

The clinical course differs between pediatric and adult MS patients. First, children more often present with an acute disease onset associated with disabling clinical symptoms.[3–8] A polyfocal presentation at disease onset is more common in children (48.9%) than in adults (12%).[5] Second, children show a significantly higher relapse rate early in the disease.[4,9–12] Third, the remission after a severe relapse is better in children than in adults,[4,5,7,8] with 62 to 66% of pediatric patients[13,14] recovering completely from initial relapses compared to 46% of adult patients.[14] Furthermore, the mean time of recovery after a relapse is shorter in pediatric MS (4.3 weeks) than in adult MS (6–8 weeks).[11] Finally, children show a slower rate of disease progression[7,15] and take approximately 10 years longer to reach the secondary progressive disease phase compared to adults. However,

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Address correspondence to Dr Pfeifenbring, Department of Neuropathology, University Medical Center Göttingen, Robert-Koch-Straße 40, 37099 Göttingen, Germany. E-mail: sabine.pfeifenbring@med.uni-goettingen.de

From the [1]Department of Neuropathology, University Medical Center, Georg August University, Göttingen, Germany; [2]Department of Neurology, Neurosciences Center, King Fahad Specialist Hospital Dammam, Dammam, Saudi Arabia; [3]Department of Medical Statistics, University Medical Center, Georg August University, Göttingen, Germany; [4]Department of Pediatrics and Adolescent Medicine, University Medical Center, Georg August University, Göttingen, Germany; and [5]Department of Neurology, Mayo Clinic College of Medicine, Rochester, MN

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given their younger age at disease onset, they are approximately 10 years younger when they enter this phase of the disease.[16] Overall, children develop irreversible physical disability more slowly than adults.[9,13]

Defining the factors that are associated with the clinical differences between pediatric and adult MS is of special interest. Pathological studies in particular represent a promising way of gaining more insight. To date, there has been no comprehensive histological study of pediatric MS available in the literature. This study investigates axonal pathology in pediatric inflammatory demyelinating lesions consistent with MS. Axonal loss, one of the hallmarks of MS lesions, is an important pathological correlate of nonremitting clinical disability and disease progression.[17–19] Hence, there may be differences in axonal damage between pediatric and adult MS patients.

Various histological and immunohistochemical staining methods are available to assess axonal damage. The classical methods for visualizing the axonal network are silver impregnation[20] and immunohistochemistry for neurofilaments.[21] Irreversible loss of axons may be determined by analyzing the reduction in axonal density, whereas the presence of axonal spheroids is a reflection of impaired axonal transport associated with acute and possibly reversible axonal injury. Immunostaining with the precursor of the beta-amyloid protein (amyloid precursor protein [APP]) or other anterogradely transported proteins, such as synuclein, are good markers to evaluate the extent of acute axonal damage, because anterograde transport is interrupted and APP and/or synuclein accumulate focally as spheroids.[22–24] These APP-positive spheroids may persist for up to 30 days.[22] The accumulation of APP may be reversible[24] and in part account for the improvement of neurological symptoms observed after a relapse.

In the present study, we analyzed axonal pathology in pediatric MS lesions and compared the findings with data from adult MS patients to determine whether differences contribute to different clinical outcomes.

Patients and Methods

Patients

This study was performed on archival biopsy and autopsy tissue, which was collected as part of the Multiple Sclerosis Lesion Project, an international collaborative effort to study the pathologic, clinical, and radiologic correlates of MS lesions. Biopsies were performed for diagnostic reasons, to exclude neoplastic, inflammatory, or infectious disorders. Samples were sent to the Department of Neuropathology in Göttingen, Germany or to the Department of Neurology in Rochester, Minnesota for extramural pathological diagnostic consultation. No biopsies were performed for research purposes. The study was approved by the ethical review committees of the University Medical Center Göttingen (# 19/09/10) and the Mayo Clinic (IRB # 2067-99).

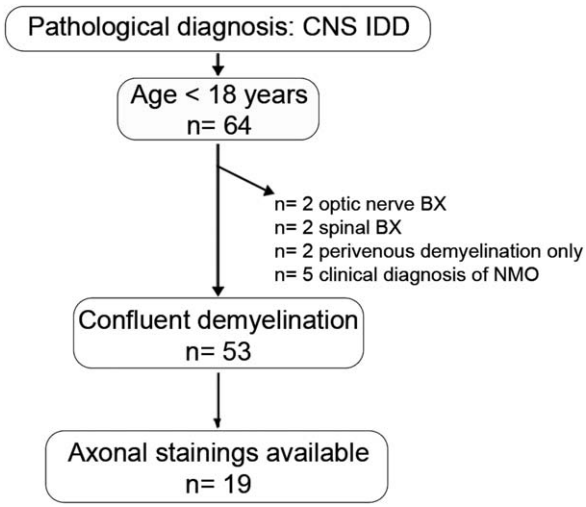

FIGURE 1: Case ascertainment. BX = biopsy; CNS = central nervous system; IDD = inflammatory demyelinating disease; NMO = neuromyelitis optica.

Inclusion criteria for the current study were: (1) brain biopsy or autopsy performed, availability of formalin-fixed, paraffin-embedded material; (2) pathological diagnosis of CNS inflammatory demyelinating disease; (3) age < 18 years; (4) pathological evidence of confluent demyelination consistent with MS; (5) no clinical, radiological, serological, or pathological evidence of neoplasm, infection, or vascular or nondemyelinating inflammatory etiology; (6) no structural or immunocytochemical evidence for an inflammatory demyelinating disease induced by a virus infection, such as progressive multifocal leukoencephalopathy or subacute sclerosing panencephalitis; and (7) sufficient tissue sample per paraffin block for detailed analysis of axonal damage.

Excluded were patients with clinical diagnosis of neuromyelitis optica or acute disseminated encephalomyelitis. Nineteen patients met inclusion criteria (Fig 1).

To compare the axonal pathology of pediatric and adult MS patients, we included archival biopsy samples from 12 adult patients diagnosed with inflammatory demyelinating disease consistent with MS (median age = 37 years, range = 24–54 years; Table 1). Adult patients were selected based on age at disease onset (range = 20-55 years), sex ratio (similar to the pediatric cohort), disease duration (<3 months), presence of an early active demyelinating lesion and periplaque white matter (PPWM), and presence of sufficient tissue for analysis of axonal damage.

Clinical data were obtained via face-to-face clinical evaluation, telephone follow-up, and medical record review. The following data were recorded: date of birth, date of biopsy/autopsy, age at biopsy/autopsy, sex, date of symptom onset, date of attack leading to biopsy/autopsy, clinical course, clinical

TABLE 1. Characteristics of Pediatric and Adult Patient Cohorts

Clinical Characteristics	Pediatric Patients, n = 19	Adult Patients, n = 12
Median age at BX/AX, yr (range)	13 (4–17)	37 (24–54)
Male:female	10:9	5:7
Clinical diagnosis in the further disease course by revised McDonald criteria for MS 2010,[25] No. [%]		
Clinically isolated syndrome suggestive of MS	8 [42.1]	6 [50]
MS	11 [57.9]	6 [50]
IgG oligoclonal bands in cerebrospinal fluid around the date of biopsy, No. [%]		
Present	2 [10.5], both patients diagnosed with MS	6 [50]
Not present	11 [57.9]	3 [25]
Unknown	6 [31.6]	3 [25]
Median days from first symptoms to BX/AX (range)	20 (3 days–4.5 years)	20 (7–49 days)
Median days from attack leading to BX/AX to date of BX/AX itself (range)	20 (3–68 days)	20 (7–49 days)
BX/AX site, No. [%]		
Frontal	12 [63.2]	9 [75]
Parietal	3 [15.8]	1 [8.3]
Temporal	1 [5.3]	0
Basal ganglia	1 [5.3]	1 [8.3]
Occipital	0	1 [8.3]
Unknown	2 [10.4]	0
EDSS at attack leading to BX/AX, median (range)	6 (1–10)	5 (0–9.5)
EDSS at last follow-up, living patients, median (range)	2 (0–4)	3 (1–9.5)
Median months from first symptoms to follow-up (range)	30.5 (1 month–17.3 years); CIS cohort 17 (1 month–17.3 years)	3.75 (1 month–15.75 years); CIS cohort 5.25 (2–40 months)
Anti-inflammatory treatment before BX/AX, No. [%]		
Prednisolone	5 [26.3]	3 [25]
Prednisolone and intravenous immunoglobulin	2 [10.5]	0
Prednisolone and plasmapheresis	0	1 [8.3]
None	11 [57.9]	6 [50]
Unknown	1 [5.3]	2 [16.7]

diagnosis in the further disease course (revised McDonald Criteria for multiple sclerosis 2010[25]), presence of oligoclonal bands (OCBs) of immunoglobulin G (IgG) in cerebrospinal fluid (CSF) around the date of biopsy, time from first symptoms to biopsy/autopsy, interval from attack leading to biopsy/autopsy to date of biopsy/autopsy itself, biopsy/autopsy site, estimated Expanded Disability Status Scale (EDSS) at attack leading to biopsy/autopsy, estimated EDSS at last follow-up, time from first symptoms to last follow-up, and anti-inflammatory treatment before biopsy/autopsy (prednisolone, prednisolone plus intravenous Ig, or prednisolone plus plasmapheresis). EDSS scores were estimated retrospectively from medical records. Age < 11 years was selected as an approximation of prepuberty. Patients' clinical characteristics are summarized in Table 1.

TABLE 1. Continued

Preoperative MRI Findings	Pediatric Patients; Index Lesions with Different Demyelinating Activities	Adult Patients; All Index Lesions Were Early Active Demyelinating
Index lesion size: T2–T2 Margins, No. [%]	n = 11	n = 10
0.3–2cm	2 [18.2]	5 [50]
2.1–5cm	7 [63.6]	4 [40]
>5cm	2 [18.2]	1 [10]
Gadolinium-enhancing index lesion, No. [%]	10 [83.3], n = 12	10 [100], n = 10
Index lesion edema present, No. [%]	8 [72.7], n = 11	9 [90], n = 10
Number of further T2 lesions, No. [%]	n = 14	n = 11
0	4 [28.6]	5 [45.5]
1	7 [50]	0
2–5	1 [7.1]	3 [27.3]
6–10	1 [7.1]	0
11–25	0	1 [9.1]
>26	1 [7.1]	1 [9.1]

AX = autopsy; BX = biopsy; CIS = clinically isolated syndrome; EDSS = Expanded Disability Status Scale; IgG = immunoglobulin G; MRI = magnetic resonance imaging; MS = multiple sclerosis.

The biopsied index lesion and other lesions were identified on preoperative magnetic resonance imaging (MRI). The index lesion was evaluated for size on T2-weighted imaging (margins of the discernible lesion without edema = 0.3–2cm, 2.1–5cm, >5cm) and presence of gadolinium enhancement and of edema. The number of other T2 lesions was counted (see Table 1).

Data analysis of the pathological, clinical, and radiographic material was performed by blinded researchers (R.F.B., I.M., P.H., J.G., C.F.L., W.B., and S.P.).

Histopathology

Specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Slices 4μm thick were stained with hematoxylin and eosin, Luxol fast blue/periodic acid–Schiff, and Bielschowsky silver impregnation. Immunohistochemical staining was performed using an avidin–biotin technique. The following primary antibodies were used for classification of lesions: anti–myelin basic protein (anti-MBP; Dako, Glostrup, Denmark), anti–proteolipid protein (anti-PLP; Biozol Diagnostica, Eching, Germany), anti–2′,3′-cyclic nucleotide 3′-phosphodiesterase (anti-CNPase; Covance, Princeton, NJ), anti–myelin oligodendrocyte glycoprotein (anti-MOG; Abcam, Cambridge, UK), anti–myelin-associated glycoprotein (anti-MAG, Abcam), anti-KiM1P (macrophages, Dr Radzun, University of Göttingen, Germany), anti-CD3 (T cells, Dako), and anti-CD8 (cytotoxic T cells, Dako). To analyze the acute axonal injury, we used an antibody against the beta-amyloid precursor protein (anti-APP; Chemicon, Millipore, MN).

Classification of Inflammatory Demyelinating Lesions

All MS lesions (pediatric and adult) were first classified according to demyelinating activity, as previously described.[2] Early active demyelinating lesions were infiltrated by numerous macrophages that contained cytoplasmic myelin degradation products immunoreactive for minor myelin proteins (CNPase, MAG, MOG; Fig 2) and major myelin proteins (PLP, MBP). In late active demyelinating lesions, the macrophages contained only major myelin proteins, but no minor myelin proteins, corresponding to more advanced myelin degradation. If macrophages had neither minor nor major myelin proteins within their cytoplasm, lesions were classified as inactive. Early remyelinating lesions were characterized by thin and irregularly formed myelin sheaths often accompanied by a dense infiltration of inflammatory cells such as macrophages and T lymphocytes. Periplaque white matter showed no signs of demyelination.

We analyzed 30 tissue blocks from 18 biopsies and 1 autopsy. There were up to 3 tissue blocks analyzed per patient, but only 1 for the majority of patients. Due to the limited sample number, the comparison of lesions with and without remyelination for the analysis of axonal damage was only done for early active lesions. The single pediatric MS autopsy case revealed 4 different lesions. The extent of acute axonal damage was assessed in each lesion according to demyelinating activity.

Morphometry

Axonal damage was analyzed using 2 different methods: Bielschowsky silver impregnation to determine the extent of axonal

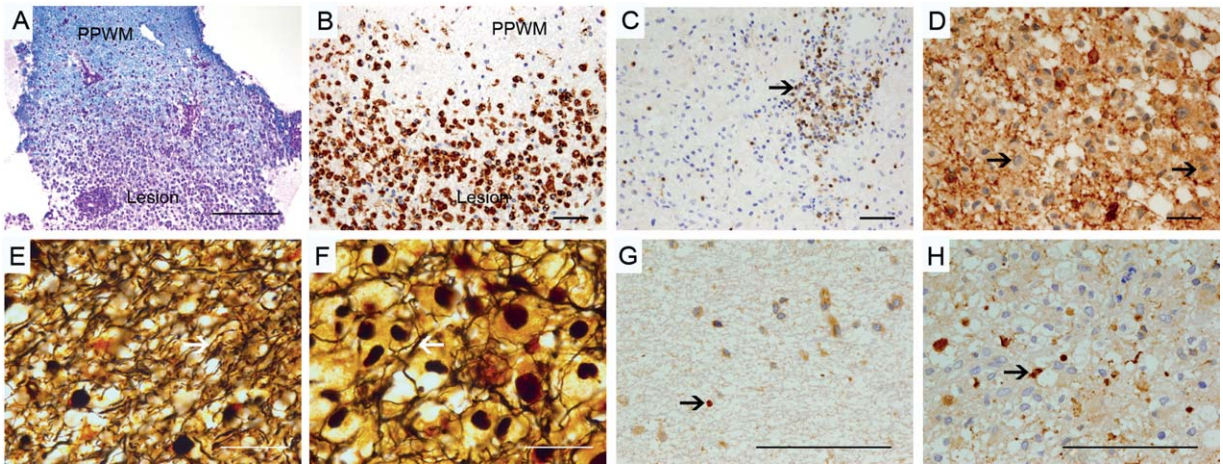

FIGURE 2: The pediatric multiple sclerosis (MS) lesions were confluently demyelinated (A; Luxol fast blue/periodic acid–Schiff). The inflammation within the lesions was mainly composed of foamy macrophages (B; KiM1P) and T lymphocytes (C; CD3, indicated by *arrow*). More than half of the lesions were classified as early active demyelinating as defined by the ongoing macrophage degradation of minor myelin proteins (D; 2′,3′-cyclic nucleotide 3′-phosphodiesterase, indicated by *arrows*). The relative axonal density was measured within the lesion and the periplaque white matter (PPWM) to compute the axonal reduction. Axonal density was reduced in all pediatric MS lesions compared to the PPWM (E = PPWM, F = lesion; Bielschowsky silver impregnation, axons are stained black, indicated by *arrows*). The number of amyloid precursor protein (APP)-positive axons and spheroids as a marker for acute axonal damage was measured in the lesions and the PPWM; acute axonal injury was higher in the lesions compared to the PPWM (G = PPWM, H = lesion; immunohistochemistry for APP, indicated by *arrows*). Scale bars: A = 200 μm, B/C = 50 μm, D–F = 20 μm, G/H = 100 μm.

reduction, and immunohistochemistry for APP to examine the extent of acute axonal damage.

Axonal density was determined in a subset of patients in whom corresponding PPWM was present in the tissue sample (see Fig 2). Counting was performed at ×1,000 magnification in at least 10 randomly selected microscopic fields using a 25-point Zeiss (Oberkochen, Germany) eyepiece. The number of grid points crossing axons was counted and expressed as a fraction of the total number of grid points. Median lesional axonal reduction was expressed as the median percentage reduction in axonal density in the lesion relative to the corresponding PPWM from the same patient.

The number of APP-positive axons was determined in at least 10 standardized microscopic fields of 0.01mm^2 each defined by an ocular morphometric grid under a ×100 objective in both the lesions and the PPWM (see Fig 2), and expressed as the median number of APP-positive axons/mm^2. Macrophages, T cells, and cytotoxic T cells were identified by immunohistochemistry using the corresponding antibodies and were counted at ×400 magnification in at least 10 standardized microscopic fields, each defined by an ocular grid.

Statistics

Mixed-effects models were used for analysis, where several measurements from each patient were available to account for correlations.[26] In addition, comparisons were performed using nonparametric methods (Mann–Whitney *U* test, Spearman rank correlation, Kruskal–Wallis test, chi-square test).[27] All tests were classified as significant if $p < 0.05$. IBM SPSS Statistics 19 software version 19 (Armonk, NY) and R version 3.1.0[28] were used. Prism software version 5 (GraphPad, San Diego, CA) was used for graphic presentation.

Results

Majority of Pediatric Lesions Were Early Active Demyelinating

Among the pediatric cohort, the stages of 24 lesional areas could be categorized according to demyelinating activity (13 lesions [54.2%] early active stage, 6 [25%] late active stage, and 5 [20.8%] inactive). Early remyelination was seen in 8 (61.5%) of 13 early active demyelinating lesions, 4 (66.7%) of 6 late active demyelinating lesions, and 3 (60%) of 5 inactive demyelinated lesions. Three early active lesions and 1 late active lesion could be differentiated in the single pediatric MS autopsy case. PPWM was present in 15 of the 19 patients evaluated.

Extensive Acute Axonal Damage in Pediatric MS Compared to Adult MS

Acute axonal damage is increased significantly, by 50%, in early active demyelinating lesions of pediatric patients (median = 1,665 APP-positive axons/mm^2) compared to adult patients (median = 1,100 APP-positive axons/mm^2, $p = 0.0455$). Furthermore, there was a significant negative correlation between the extent of acute axonal damage and the age at biopsy/autopsy ($r = -0.5519$, $p = 0.0035$, Fig 3).

Prepubertal Patients Revealed More Acute Axonal Damage Than (Post)Pubertal Patients

The acute axonal damage in early active demyelinating lesions was significantly higher in the prepubertal age

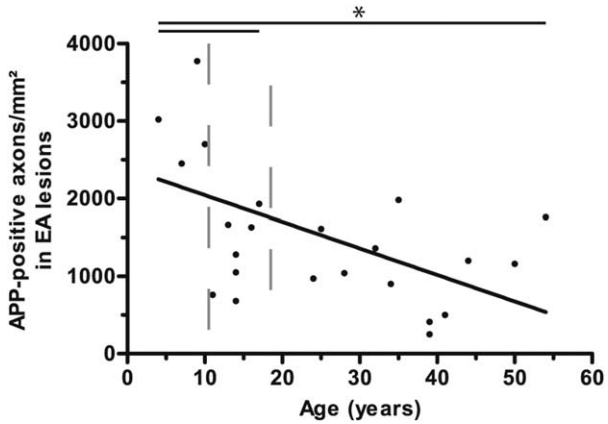

FIGURE 3: The extent of acute axonal damage in early active demyelinating lesions (EA) depends on age at biopsy/autopsy (Spearman rank correlation, $r = -0.5519$, $p = 0.0035$). The numbers of amyloid precursor protein (APP)-positive axons/mm^2 were significantly higher in the prepubertal age group (<11 years of age) compared to the pubertal age group (11–17 years of age, $p = 0.0061$) and adult patients (≥18 years of age, $p = 0.0044$). *p < 0.007.

group (<11 years of age, median = 2,860 APP-positive axons/mm^2) compared both to the pubertal age group (11–17 years of age, median = 1,280 APP-positive axons/mm^2, $p = 0.0061$) and to adult patients (median = 1,100 APP-positive axons/mm^2, $p = 0.0044$, see Fig 3). The numbers of APP-positive axons/mm^2 were similar in children with a pubertal disease onset (range = 683–1,932 APP-positive axons/mm^2) and adult patients (range = 250–1,980 APP-positive axons/mm^2).

Active Demyelinating Lesions of Pediatric MS Patients Showed the Most Extensive Acute Axonal Damage

The highest number of APP-positive axons/mm^2 was found in early active demyelinating lesions, with a median of 1,665 APP-positive axons/mm^2 (Fig 4A). A median of 1,190 APP-positive axons/mm^2 was measured in late active demyelinating lesions and a median of 870 APP-positive axons/mm^2 was determined in inactive lesions. Comparisons between plaques of different demyelinating activities showed no significant differences in the extent of acute axonal damage ($p > 0.05$), which may be due to the fact that we were only able to analyze a limited number of later stage demyelinating activity. The number of APP-positive axons/mm^2 was similar in early active demyelinating lesions with and without remyelination (median = 1,665 APP-positive axons/mm^2, 1,782 APP-positive axons/mm^2, respectively, $p > 0.05$, see Fig 4B). Within the PPWM, acutely damaged axons were seen in low numbers (median = 192 APP-positive axons/mm^2). The number of APP-positive axons was significantly higher in early active demyelinating lesions with or without remyelination, in late active demyelinating lesions, and in inactive demyelinated lesions compared to the PPWM ($p < 0.003$). The single pediatric MS autopsy case showed similar numbers of APP-positive axons/mm^2 in different early and late active lesions (Supplementary Table 1).

Extent of Acute Axonal Damage in Early Active Demyelinating Lesions Correlated with Degree of Macrophage Infiltration

To assess whether axonal injury is associated with the inflammatory infiltrate in pediatric and adult MS lesions, we performed correlation analyses between the numbers of inflammatory cells/mm^2 and axonal reduction as well as the extent of acute axonal damage within the same lesions on adjacent histological slides. The acute axonal damage in early active demyelinating lesions of pediatric

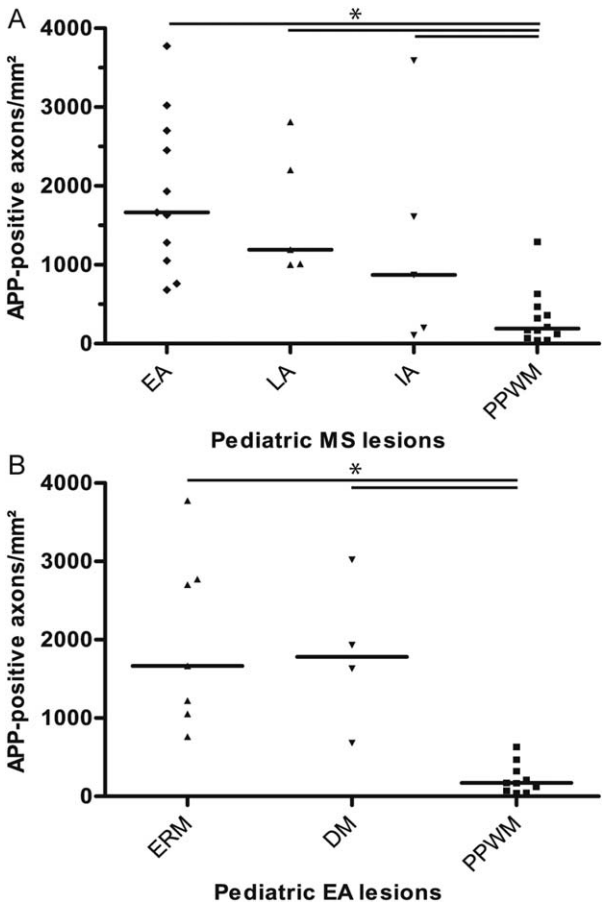

FIGURE 4: Acute axonal damage in pediatric patient lesions classified according to demyelinating activity (A) and presence of remyelination (B) as well as in the corresponding periplaque white matter. Acute axonal damage is significantly higher in early active demyelinating lesions (EA) with and without remyelination (B), late active demyelinating lesions (LA; A), and inactive demyelinated lesions (IA; A) than in the periplaque white matter (PPWM; $p < 0.003$). Comparisons between lesion types revealed no significant differences. APP = amyloid precursor protein; DM = early active lesions without remyelination; ERM = early active lesions with remyelination; MS = multiple sclerosis. (A) *$p < 0.05$. (B) *$p < 0.003$.

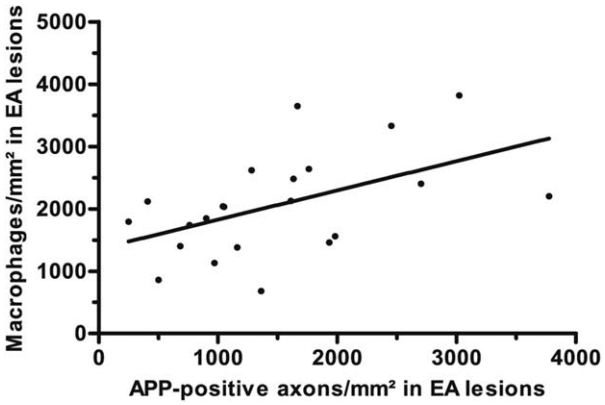

FIGURE 5: The degree of macrophage accumulation is associated with the extent of acute axonal damage in early active demyelinating lesions (EA) of pediatric and adult multiple sclerosis patients (Spearman rank correlation, $r = 0.5381$, $p = 0.0098$). APP = amyloid precursor protein.

and adult MS patients correlated significantly with the number of macrophages ($r = 0.5381$, $p = 0.0098$, Fig 5). The highest number of macrophages was measured in early active demyelinating lesions of prepubertal patients (mean = 2,937 macrophages/mm^2), whereas the macrophage infiltration was lower in MS patients with a pubertal (mean = 2,198 macrophages/mm^2, $p > 0.05$) or adult disease onset (mean = 1,652 macrophages/mm^2, $p = 0.0109$). No significant correlation was found between the numbers of CD3-positive T cells or CD8-positive cytotoxic T cells and APP-positive spheroids or the numbers of T cells, cytotoxic T cells, and macrophages and the extent of axonal reduction.

Higher T2 Lesion Load and Greater Index Lesion Size in Pediatric MS Compared to Adult MS

In the pediatric cohort, 71.4% showed multifocal ($\geq$2) MRI lesions in the preoperative MRI scan compared to 54.5% in adult patients ($p > 0.05$). Index lesion size was greater in pediatric patients (81.8% size > 2cm) compared to adult patients (50% size > 2cm, $p > 0.05$). Edema and gadolinium enhancement of the index lesion was more often present in adult patients than in pediatric patients, which can be explained by the different demyelinating activities of the index lesion present in both cohorts (see Table 1).

Disability at Attack Leading to Biopsy/Autopsy Correlates with Acute Axonal Damage in Early Active Demyelinating Lesions

To determine whether clinical characteristics of pediatric MS patients are associated with axonal injury, clinical parameters were correlated with the extent of axonal reduction and the number of APP-positive spheroids in lesions of different demyelinating activities.

A higher EDSS at attack leading to biopsy/autopsy positively correlated with a higher number of APP-positive spheroids in early active demyelinating lesions ($r = 0.6368$, $p = 0.0143$, Fig 6). The extent of acute axonal damage did not differ between male and female patients, and also did not correlate with biopsy/autopsy site, interval between symptom onset and biopsy/autopsy, clinical course, treatments administered, or EDSS at last follow-up.

No statistically significant correlations were found between axonal reduction and age at biopsy/autopsy, the interval between symptom onset and biopsy/autopsy, EDSS at attack leading to biopsy/autopsy, and EDSS at last follow-up. Gender-specific differences were also not observed. There were no significant correlations between the axonal reduction and the site of biopsy/autopsy, the clinical diagnosis of clinically isolated syndrome or MS in the further disease course, or a prior anti-inflammatory treatment.

Early Active Demyelinating Lesions of Pediatric Patients Showed a 49% Reduction in Axonal Density Compared to PPWM

Axonal density was reduced in all pediatric lesions compared to the PPWM (range = 14–74%). Early active demyelinating lesions showed a 49% reduction in axonal density compared to the PPWM (Fig 7A). Late active demyelinating lesions revealed a 42% axonal reduction and inactive demyelinated lesions a 44% axonal reduction. The axonal reduction was similar in early active demyelinating lesions with and without remyelination (51% and 44%, respectively, $p > 0.05$, see Fig 7B). No significant differences were found between plaques of different demyelinating activities ($p > 0.05$).

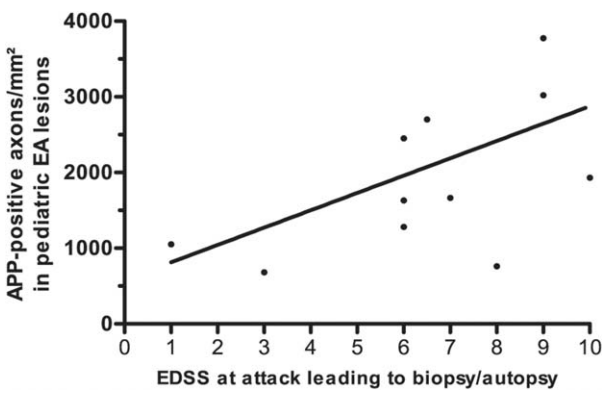

FIGURE 6: The number of amyloid precursor protein (APP)-positive spheroids in early active demyelinating lesions (EA) of pediatric patients correlated significantly with the Expanded Disability Status Scale (EDSS) at attack leading to biopsy/autopsy (Spearman rank correlation, $r = 0.6368$, $p = 0.0143$).

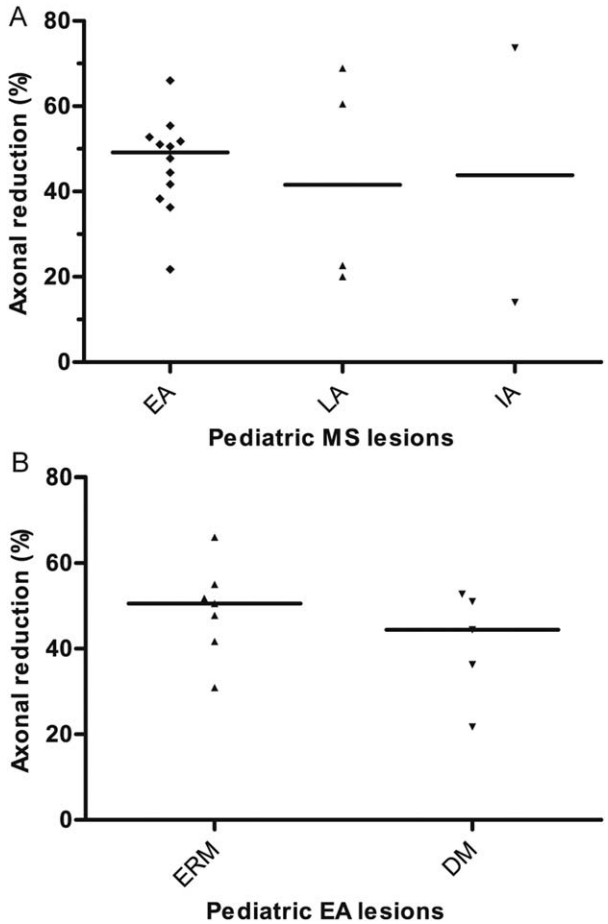

FIGURE 7: Axonal reduction in pediatric patient lesions compared to the periplaque white matter. Comparisons between lesion types showed no significant differences. DM = early active lesions without remyelination; EA = early active demyelinating lesions; ERM = early active lesions with remyelination; IA = inactive demyelinated lesions; LA = late active demyelinating lesions; MS = multiple sclerosis.

Discussion

Our study is the first to focus on the quantification of axonal damage in early MS lesions from pediatric patients. It is of particular relevance because the different clinical features between pediatric and adult MS patients may in part be related to a different extent of axonal pathology. In our study, we compared the axonal damage in MS lesions derived from pediatric patients with data from 12 adult MS patients as well as with published data (Tables 1–3).[23,29,30]

Our results demonstrated for the first time that the extent of acute axonal damage is increased by 50% in early active demyelinating lesions of pediatric patients compared to adults (see Table 3).[23,29] Furthermore, the extent of acute axonal damage correlated negatively with the age at biopsy/autopsy. In addition, we showed that the extent of acute axonal damage is associated with active demyelination in pediatric MS lesions, similar to what has been previously reported for adult patients.[19,22,23,29,31] The highest numbers of APP-positive axons/mm^2 were found in early active demyelinating lesions.

Interestingly, differences in extent of acute axonal damage and degree of macrophage infiltration between pediatric and adult MS patients were most obvious in MS patients with a prepubertal disease onset (<11 years of age; see Supplementary Table 2 for case descriptions of the prepubertal patients with high acute axonal damage). Lesions of prepubertal MS patients revealed the most extensive acute axonal damage and the densest infiltration of macrophages (see Table 3). Consistent with these pathological findings, differences in clinical presentation and laboratory and imaging findings of prepubertal and pubertal MS onset are illustrated by several clinical studies.[4] Children with disease onset before puberty are more likely to present with encephalopathy and polyfocal clinical features with the first relapse.[3,7–9,32,33] Hence, the first clinical event is often moderate to severe in prepubertal children.[8] Furthermore, fever and impaired cognitive functioning is more common in younger children.[3] Age also modifies the CSF profile at disease onset.[8] Patients <11 years of age have a higher percentage of neutrophils in CSF and are less likely to show OCBs or elevated IgG index than patients with onset between ages 11 and 17 years.[4,8,34] Prepubertal patients can present with widespread demyelination at disease onset as seen on MRI. The brain lesions are often larger and confluent, with less well-defined borders, which differs from findings in patients with pubertal MS onset. A significant number of these lesions can vanish on follow-up scans, unlike those seen in adolescents.[35] Hence, our study demonstrates for the first time that there is also a difference in pathology between MS patients with prepubertal and postpubertal disease onset. The reasons for these differences are still unknown. The often observed presence of neutrophils in the CSF of prepubertal patients suggests a prominent activation of the innate immune system, whereas the adaptive immune system usually becomes activated in postpubertal patients.[4] In a subset of prepubertal MS patients, we observed neutrophils and eosinophils in low numbers within the lesions. This different activation pattern and the still ongoing maturation of the immune system could account for the disease phenotype.[7,34] Furthermore, the incomplete myelination[35] and an age-dependent immunogenicity of specific CNS regions[14] may affect the severity of clinical symptoms and the lesion location. Encephalopathy and seizures, both often present in prepubertal patients, indicate widespread involvement of the CNS.[7,11] Hormonal changes related to puberty might also modulate the immune cells and levels of cytokines in the periphery and in the brain.[7,14] However, in contrast to

TABLE 2. Reference Groups of Published Pediatric and Adult Patients

Clinical Characteristics	Dziedzic 2010[29]	Bitsch 2000[30]	Ferguson 1997[23]
Patients, No.	63	42	18
Median age at biopsy, yr (range)	35 (10–72)	33 (11–64), unknown n = 5	Not mentioned
Sex	65.1% female, 34.9% male	66.7% female, 26.2% male, 7.1% unknown	Not mentioned
Clinical diagnosis	Clinically isolated syndrome n = 38, 60.3%; relapsing–remitting n = 19, 30.2%; secondary progressive n = 6, 9.5%	Laboratory-supported clinically definite n = 17, 40.5%; clinically definite n = 5, 11.9%; clinically probable n = 2, 4.8%; clinically possible n = 12, 28.6%; unknown n = 6, 14.3%	Not mentioned
Time from symptoms to biopsy, median (range)	1.9 months (3.6 days–19 years)	90 days (37 days–14 years)	Not mentioned
Treatment before biopsy	Not mentioned	None n = 18, 42.9%; prednisolone n = 13, 31%; intravenous immunoglobulin n = 1, 2.4%; interferon-β1b n = 1, 2.4%; unknown n = 9, 21.4%	Not mentioned
Material			
Tissue	Biopsy tissue	Biopsy tissue	Autopsy tissue
Lesions, No.	Early active demyelinating n = 33; inactive demyelinated n = 11	Early active demyelinating n = 24; late active demyelinating n = 13; inactive demyelinated n = 20	Acute lesions n = 6; active chronic lesions n = 6; chronic lesions n = 7
Periplaque white matter, No.	40	38	0

MS with a pubertal disease onset, this is unlikely to be the case in a prepubertal disease onset.

The inflammatory infiltrate is one of the factors influencing axonal injury in MS lesions.[18,31,36] We found a significant positive correlation between extent of acute axonal damage and degree of macrophage infiltration in pediatric and adult MS lesions, which is in line with previous studies of adult MS patients.[22,23,30,31] In general, the degree of macrophage accumulation was higher in pediatric MS lesions than in adult MS lesions (see Table 3).[2,23] Thus, pediatric MS lesions seem to be more inflammatory with regard to the microglia/macrophage response. Furthermore, in line with previous MRI studies,[9,10,12,37,38] we observed a higher T2 lesion load in the pediatric cohort than in the adult cohort. Pediatric patients had multifocal MRI lesions in the preoperative scan more often than adult biopsied patients, indicating a more active disease in pediatric MS. In addition, index lesion size was >2cm more often in pediatric MS than in adult MS. The increased inflammation might therefore be a factor contributing to the extensive acute axonal damage in pediatric MS. In contrast to reports on adult MS patients,[22,30,31] we did not detect a significant correlation between the extent of acute axonal damage and

TABLE 3. Extent of Acute Axonal Damage, Reduction in Axonal Density, and Degree of Macrophage Infiltration in Pediatric and Adult MS Patients

	Pediatric MS Patients				Adult MS Patients					
						Published Data				
	EA	LA	IA	PPWM	EA	EA	LA	IA	PPWM	Acute Lesions
Median APP-positive axons/mm^2	1,665; prepubertal patients = 2,860; pubertal patients = 1,280	1,190	870	192	1,100	730, Dziedzic 2010[29]		560, Dziedzic 2010[29]	~100, Dziedzic 2010[29]	~900, Ferguson 1997[23]
Median lesional axonal reduction, %	49; prepubertal patients = 52; pubertal patients = 44	42	44		39	~35, Bitsch 2000[30]; 42, Dziedzic 2010[29]	~60, Bitsch 2000[30]	50, Bitsch 2000,[30] Dziedzic 2010[29]		
Mean macrophages/mm^2	2,395; prepubertal patients = 2,937; pubertal patients = 2,198	2,163	2,012	366	1,652	1,528, Bruck 1995[2]	1,722, Bruck 1995[2]	1,046, Bruck 1995[2]	178, Bruck 1995[2]	~1,700, Ferguson 1997[23]

APP = amyloid precursor protein; EA = early active demyelinating; IA = inactive demyelinated; LA = late active demyelinating; MS = multiple sclerosis; PPWM = periplaque white matter.

the number of CD8-positive cytotoxic T cells. Consistent with previous studies on adult MS patients,[30] our study of pediatric MS lesions showed that the inflammatory infiltrate is not associated with axonal reduction. In addition, we found no evidence that prior anti-inflammatory treatment influenced the inflammatory infiltrate or extent of axonal damage, as treatments before biopsy/autopsy were similar in both pediatric and adult MS patients (see Tables 1 and 2).[30] Nearly half of the pediatric and adult MS patients[30] received no anti-inflammatory treatment before biopsy/autopsy, and one-third of the pediatric and adult MS patients[30] were treated with high-dose corticosteroids before biopsy/autopsy. The investigated lesions of both pediatric and adult patients represent acute lesion stages, and it is not known how the extent of acute axonal damage seen here correlates with the accumulation of long-term axonal loss over time. Early MS lesions are highly inflammatory, with a dense infiltration of foamy macrophages, and most have a profound edema, both of which extend the axonal network. Furthermore, tissue damage is still ongoing in early active demyelinating lesions. To analyze the impact of inflammation on irreversible neurodegeneration in MS lesions, the inflammation of early MS lesions should be related to the axonal density in chronic, longstanding plaques.

Our study also revealed that the higher extent of acute axonal damage observed in early active demyelinating lesions of pediatric patients was associated with a higher EDSS at attack leading to biopsy/autopsy. Therefore, it is possible that acute axonal damage is among the factors contributing to the severe disease onset often observed in pediatric MS patients. With regard to the favorable outcome, it remains unclear to what extent the acute axonal damage is reversible and whether this increased acute axonal damage leads to more axonal loss in the long term. We did not observe marked differences in extent of axonal reduction in early MS lesions between pediatric and adult MS patients (see Table 3).[29,30] To the best of our knowledge, there is only 1 imaging study available that investigated the amount of brain injury occurring in pediatric and adult MS.[37] Adults with pediatric onset MS showed increased tissue damage and reduced remyelination capacity, as measured by magnetization transfer ratio (MTR).[37] MTR is a

histopathologically validated imaging technique to evaluate tissue integrity, with reduced MTR values showing tissue destruction such as axonal loss or demyelination and increased MTR values corresponding to tissue repair by remyelination.[39,40] In adults with pediatric onset MS, the MTR values tended to be lower within T2 lesions, normal-appearing gray matter, and normal-appearing white matter as compared to adults with adult onset MS.[37]

The limitations of our study include the small sample size and potential selection bias given that these patients were biopsied in the course of their diagnostic evaluation. The need for a biopsy is generally limited to patients with an unusual disease course, atypical MRI, or treatment failure. In most cases, biopsy was done to exclude tumor. The majority of our investigated pediatric patients had a moderate to severe disease onset, as indicated by a median EDSS of 6 at attack leading to biopsy/autopsy. Hence, our cohort may represent a group of children with very active disease. However, this bias may be minimized because we compared our cohort with adult biopsied MS patients, who may also have an unusual disease presentation leading to brain biopsy (median EDSS of 5 at attack leading to biopsy). Most of our pediatric patients developed clinically definite MS[25] in the further disease course, and the EDSS at last follow-up is similar to published data.[41–44] With regard to the clinical characteristics and MRI findings, our investigated pediatric cohort is similar to clinical studies of pediatric MS.[4,9,16] To diminish the impact of lesional activity on extent of acute axonal damage, the correlation analyses could only be performed with early active demyelinating lesions (APP staining: prepubertal patients $n = 4$, pubertal patients $n = 7$), reducing the sample size for analysis. Tissue of pediatric MS lesions is quite rare, especially in prepubertal patients. However, we analyzed all available biopsy and autopsy samples of pediatric patients (prepubertal patients $n = 8$, pubertal patients $n = 11$) collected over several years (1997–2014) at 2 centers (Department of Neuropathology in Göttingen, Germany and Department of Neurology in Rochester, MN).

In conclusion, our findings demonstrate that the often observed severe disease onset in pediatric MS patients may in part be explained by the higher degree of acute axonal damage. Furthermore, acute axonal damage was greatest in prepubertal children. Given the favorable clinical outcome often observed following a severe first clinical event and the slower subsequent disease progression, acute axonal damage may potentially be reversible, and the childhood brain may show a greater plasticity compared to adults.[45,46] Further studies, especially in autopsy material, will be necessary to draw any conclusions regarding relative axonal density in classic chronic MS plaques of pediatric patients. As the first histological study focusing on early MS lesions of children, the present work contributes to our understanding of the potential mechanisms leading to the different clinical courses in pediatric versus adult MS patients.

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Authorship

I.M., C.F.L., W.B., and S.P. designed the study. Data collection was performed by R.F.B., I.M., C.F.L., W.B., and S.P. Data were analyzed by R.F.B., I.M., C.R., C.F.L., W.B., and S.P. and interpreted by all authors. Figures were created by I.M., C.R., C.F.L., W.B., and S.P. All authors were involved in the preparation and writing of the manuscript. S.P. and R.F.B. are co–first authors. C.F.L. and W.B. are co–last authors.

Potential Conflicts of Interest

I.M.: speaking fees, Biogen Idec, Bayer Healthcare, Teva, Serono, Novartis; grant, Biogen Idec. J.G.: research support, advisory board, speaking fee, travel support, Novartis; speaking fee, travel support, Merck-Serono; clinical study advisory board, Bayer Vital; personal fees, Sanofi-Aventis, Teva. C.F.L.: grants, NIH, National MS Society, Novartis, Department of Defense; expenses reimbursed for participation in seminar, Biogen Idec, CogState, QuestCor; honorarium for participation in online CME course, BNAC. W.B.: grants, Teva, Biogen Idec, Genzyme, Novartis; scientific advisory boards, Teva, Biogen Idec, Genzyme/Sanofi, Novartis; speaking fees, Bayer Vital, Biogen Idec, Merck Serono, Teva, Genzyme/

Sanofi, Novartis; editorial boards, *Acta Neuropathologica*, *Neuropathology and Applied Neurobiology*, *Multiple Sclerosis International*, *Therapeutic Advances in Neurological Disorders*.

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