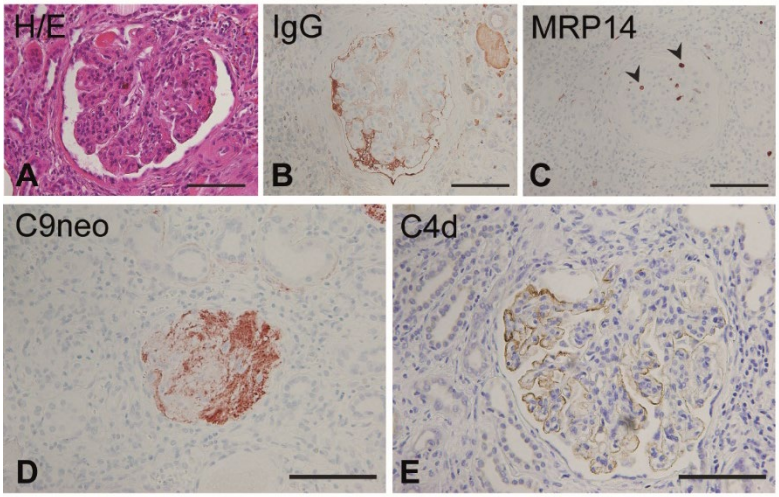
SUPPLEMENTARY MATERIAL

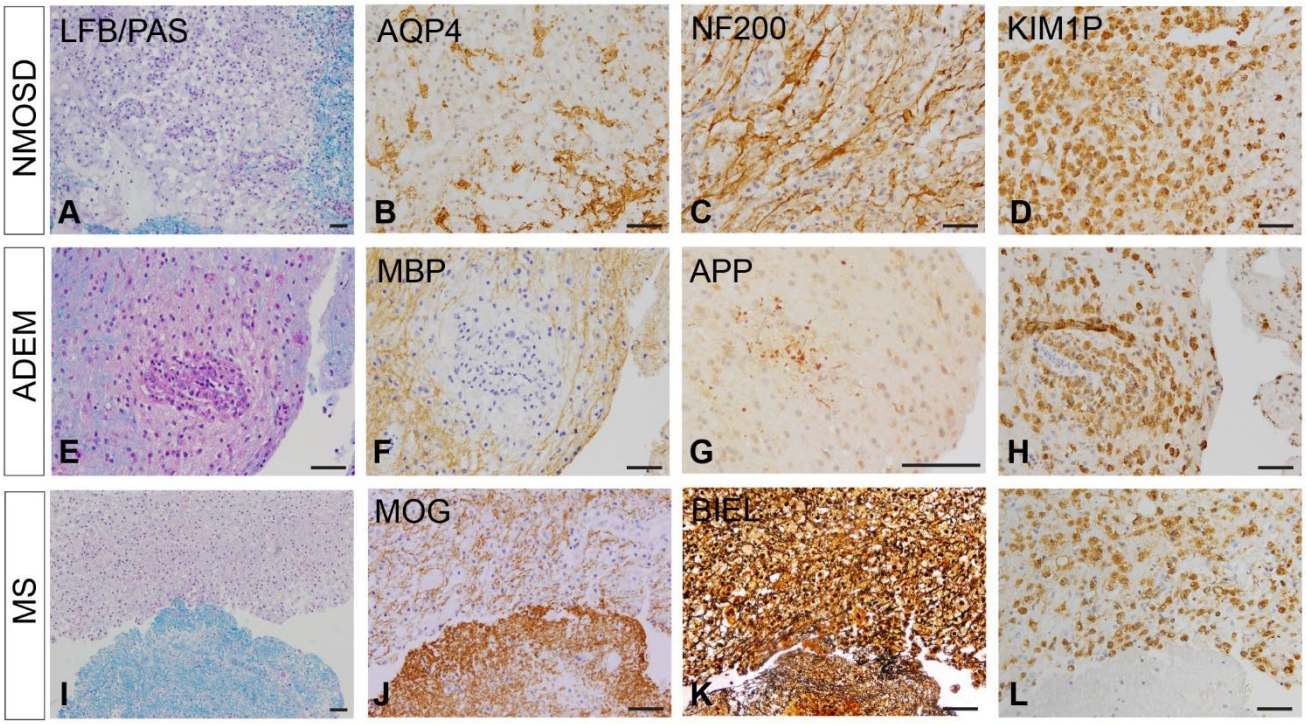
Landt *et al.*, Complement C4d informs the differential diagnosis of inflammatory demyelinating CNS diseases

eFigure 1



eFigure 1 C9neo and C4d deposition in antibody-mediated kidney graft rejection. Formalin-fixed paraffin-embedded sections of an allogenic kidney transplant demonstrating glomerulitis with inflammatory infiltrates (**a**). IgG deposition in glomerular capillaries (**b**). MRP14-positive monocytes and granulocytes (**c**, arrowheads); prominent C9neo (**d**) and C4d (**e**) deposition in glomerular capillaries. Scale bars: 50 µm.

eFigure 2



eFigure 2 Representative lesion characteristics of IIDD biopsies. NMOSD lesion (upper row) showing demyelination (**a**) with loss of astrocytes (**b**), axonal distension (**c**) and diffuse macrophage infiltration (**d**). ADEM lesion (middle row) with perivenous demyelination (**e** and **f**), perivenous acute axonal damage (**g**) and perivenous macrophages (**h**). MS lesion (bottom row) with confluent demyelination (**i** and **j**), relative axonal preservation (**k**) and diffuse macrophage infiltration (**l**). Scale bars: 100 µm.

APP: amyloid precursor protein; Biel: Bielschowsky silver impregnation; LFB/PAS: Luxol fast blue/periodic acid Schiff; Mϕ: macrophage; MBP: myelin basic protein; MOG: myelin oligodendrocyte glycoprotein; NF: neurofilament

eFigure 3

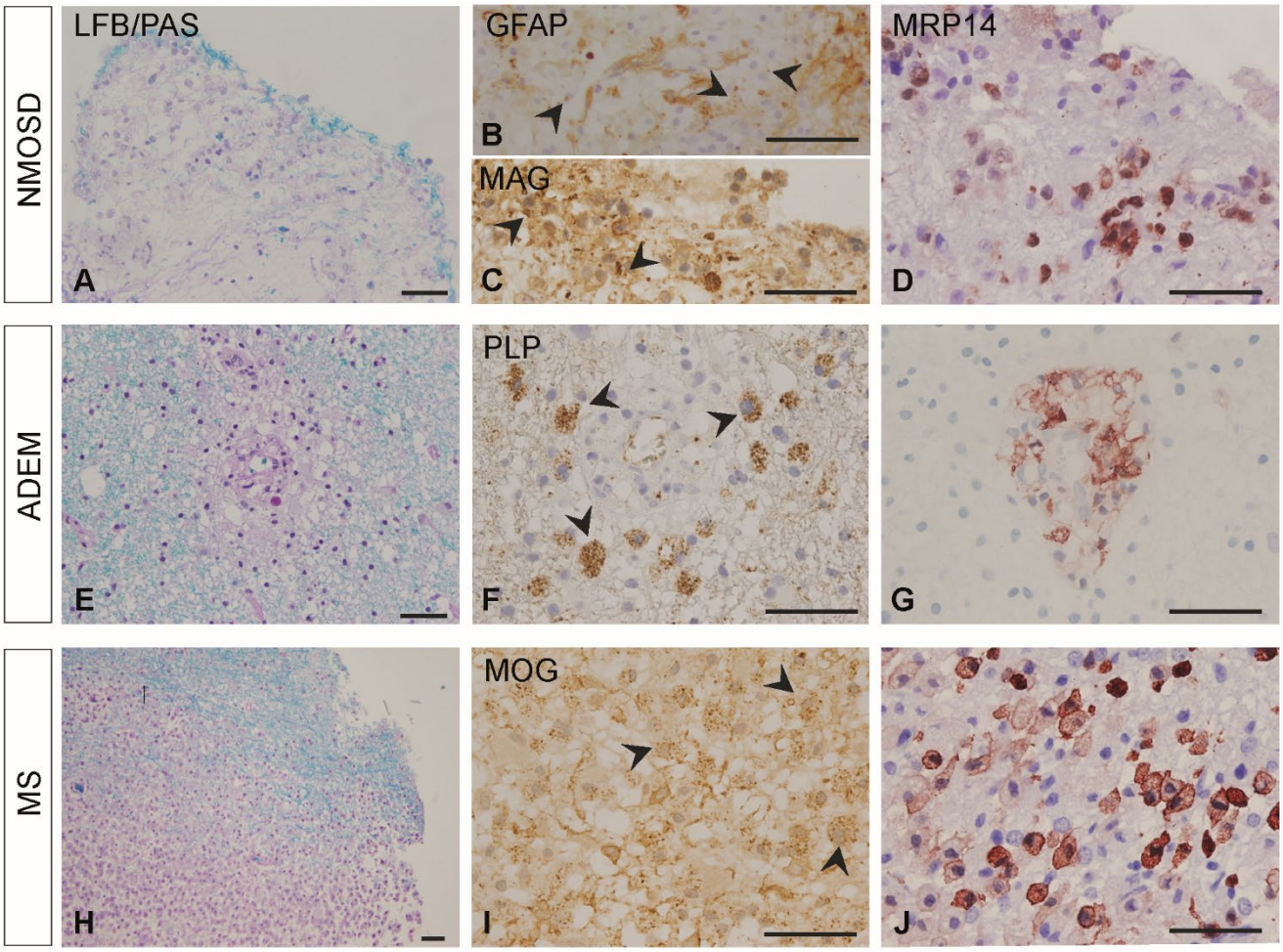
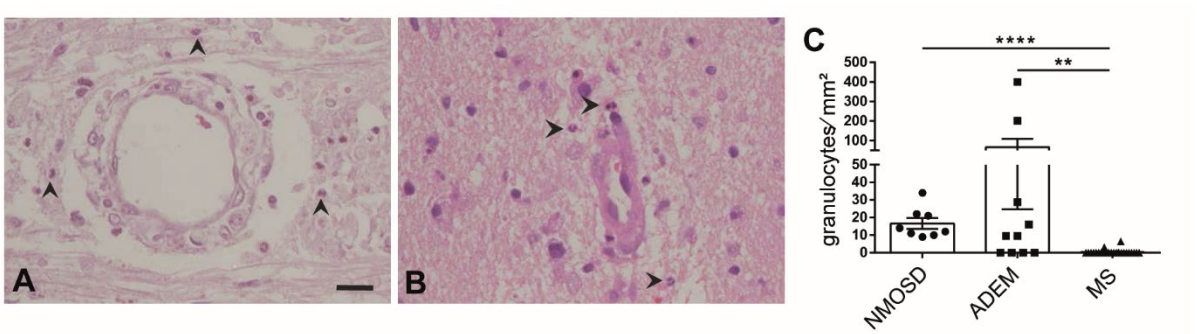


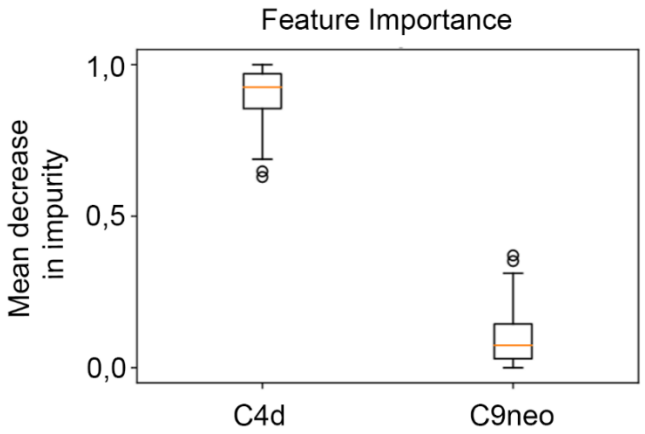
Figure 3 Lesion staging of NMOSD, ADEM and early MS biopsies. (**a-d**, upper row) early active NMOSD lesion (**a**, LFB/PAS) with phagocytosis of astrocyte remnants (**b**, GFAP) and myelin (**c**, MAG, arrows). Recently invaded monocytes and granulocytes depicted by MRP14 IHC (**d**). (**e-g**, middle row), active ADEM lesion with myelin phagocytosis (**f**, PLP) by macrophages (arrows). Perivascular MRP14 immunoreactivity depicts recently extravasated monocytes (**g**). (**h-j**, bottom row) shows early active demyelinating MS lesion with MOG-laden (**i**, arrows) and MRP14-positive phagocytes (**j**). GFAP, MAG, PLP, MOG: brown; MRP14: red. Scale bars = 100 µm.

eFigure 4



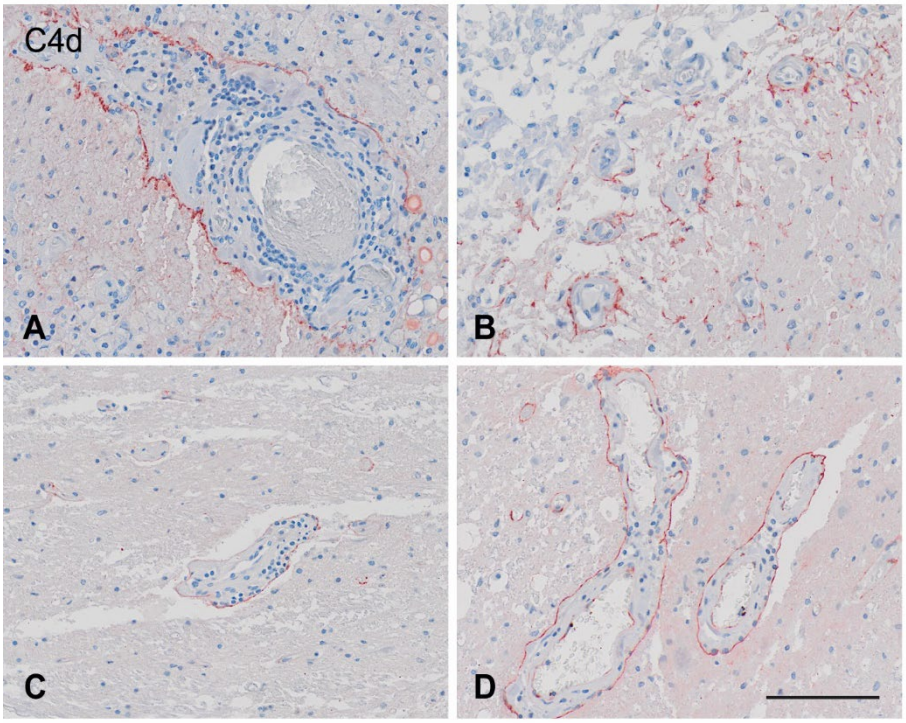
eFigure 4 Neutrophilic granulocytes in IIDD biopsies. Perivascular neutrophilic granulocytes (marked by arrowheads; H&E) within biopsy lesions of NMOSD (**a**) and ADEM (**b**). Quantification of PMN densities in demyelinated lesions of NMOSD, ADEM and early active MS (**c**). ** $P < 0.01$, **** $P < 0.0001$, Kruskal-Wallis test, followed by Dunn's multiple comparison test. Scale bar: 20 µm.

eFigure 5



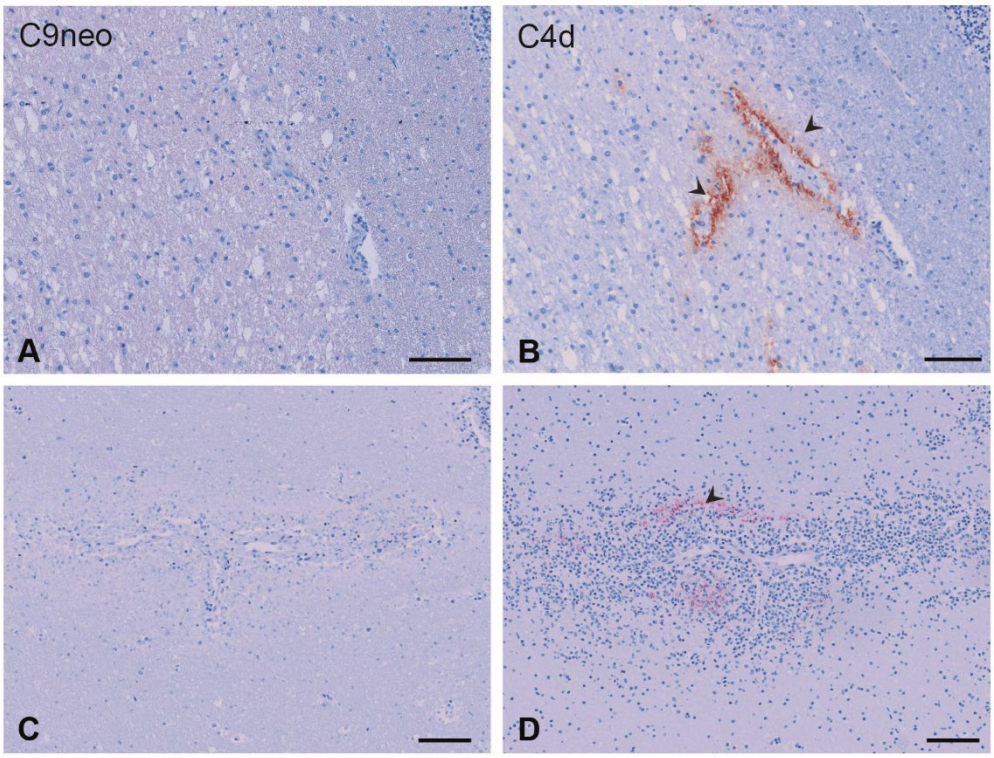
eFigure 5 Feature importance of C4d and C9neo IHC in random forest classification of IIDD. Feature importance of C4d compared to C9neo IHC for a IIDD decision tree identified by bootstrapped random forest classifiers (n = 120). C4d IHC shows a significantly higher mean decrease in impurity (P < 0.0001).

eFigure 6



eFigure 6 C4d deposition at the perivascular glia limitans in spinal tissue of NMOSD. C4d at the perivascular glia limitans in the spinal cord of four different NMOSD patients (**a-d**). Scale bar: 100 µm.

eFigure 7



eFigure 7 C4d deposition in MOG-Ab positive ADEM autopsies. Absence of C9neo deposition (**a** and **c**) in two patients with MOG-Ab positive ADEM and perivenous deposition of C4d (**b** and **d**, arrowheads). Scale bars: 100 µm.

eTable 1 Biopsy patients – clinical characteristics and morphometric evaluation.

NMOSD

NMO #	Disease duration [months]	Acute symptom onset [weeks]	Symptoms	MRI findings	Index lesion	Astro-cyte loss	MRP14	Phago-cytosis myelin	Phago-cytosis astroc.	C9neo	C4d	PMNs in ROI/ mm^2
1	120	1	Dysphagia, leg-accentuated tetraparesis	Multiple lesions: R frontal, corpus callosum, spinal cord with CE	Corpus callosum	Yes	+++	++	+	Neg.	Neg.	11
2	6	3.5	ON, cognitive impairment, aphasia	Multiple lesions: periventricular, basal ganglia, brainstem	Periventri-cular	Yes	N/A	+	+	Neg.	Neg.	10
3	108	3	ON, dysarthria, sensorimotor paraparesis	Confluent supra- and infratentorial lesions with marked bilateral occipital distribution, partly CE	N/A	Yes	+++	++/+	+	Neg.	+++	9
4	48	4	ON, aphasia, ata-xia, sensorimotor tetraparesis	Multiple lesions: ON, periventricular, L occipital with CE, spinal cord	N/A	Yes	+	+++	+++	Neg.	+	14
5	120	11	ON, cognitive impairment, neck pain, sensorimo-tor tetraparesis	L parietal lesion, myelitis	L parietal	Yes	++	+++	-	+	+++	12
6	N/A	N/A	Suspected NMO	Posterior corpus callosum	Corpus callosum	Yes	N/A	++	+	Neg.	+	34
7	>24	N/A	Paraparesis, dysaesthesia, neck pain	Multiple spinal cord lesions	Spinal cord T 6/7	Yes	+++	+	+	+++	+	22
8	N/A	N/A	Transverse myelitis with sensorimotor paraparesis	Transverse myelitis T5-6	Spinal cord T 5/6	Yes	+	++	+	++	+++	21

Abbreviations: ON = optic neuritis; CE = contrast enhancing; R/L = right/left, WM = white matter

ADEM

ADEM #	Disease duration [weeks]	Symptoms	MRI findings	Index lesion	Perive-nous DM	MRP14	Phago-cytosis myelin	C9neo	C4d	PMNs in ROI/ mm^2
1	N/A	Hemiparesis, personality change	R parietooccipital, partly CE, profound edema	N/A	Yes	++	+	Neg.	++	9.6
2	N/A	Suspected ADEM	N/A	N/A	Yes	+	++	Neg.	+	0
3	N/A	Suspected ADEM	N/A	N/A	Yes	Neg.	+	N/A	Neg.	9.6
4	1	Bilateral subacute amaurosis, confusion	Multiple lesions: posterior horn of R lateral ventricle with CE, infratentorial lesions, partly CE and progressive	L cere-bellum	Yes	+++	+/++	Neg.	+++	201.6
5	2	Headache, aphasia, apraxia, reduced consciousness	Bihemispheric edema-tous brain lesions with predominant parieto-occipital distribution	R frontal	Yes	+	+++	Neg.	+	0
6	N/A	Suspected ADEM	Multiple supra- and infratentorial lesions, incl. brainstem	R frontal	Yes	Neg.	+	Neg.	Neg.	0
7	62/30	Suspected recurrent ADEM	N/A	N/A	Yes	Neg.	+	Neg.	++	28.8
8	1	Headache, drowsi-ness, suspected ADEM	N/A	N/A	Yes	+	++	Neg.	Neg.	0
9	N/A	Headache, drowsiness	Mass-occupying, edematous WM lesion, with ventri-cular compression	Frontal	Yes	+++/++	+++	Neg.	Neg.	16
10	12	Fever, drowsiness, suspected ADEM	Multiple WM lesions incl. brainstem and spinal cord, CE	N/A	Yes	++	++/+++	+++	+++	400

MS

MS #	Disease duration [months]	Disease course	Symptoms	MRI findings	Index lesion	Myelin loss (MBP)	MRP14	Phagocytosis myelin MOG// CNP	C9neo	C4d	PMNs in ROI/ mm^2
1	2.5	RRMS	Transient left-sided sensorimotor hemiparesis	Multiple R fronto-parietal lesions, partly CE	R frontal	Yes	+	+// +	Neg.	Neg.	0
2	0.75	RRMS	L ON, R sensorimotor hemiparesis, hemidysesthe-sia, unsteady gait	Multiple lesions: L frontoparietal, brainstem, spinal cord	L frontal	Yes	+++	++// +	Neg.	Neg.	0
3	12	RRMS	Seizure, R numbness and paresthesia, aggressive behavior	CE parietal lesion	Parietal	Yes	+++	+++// +++	Neg.	Neg.	0
4	0.25	RRMS	ON, reduced attention span, R hemiparesis, aphasia, cerebellar ataxia, hypoacu-sis	Multiple lesions: frontoparietal, temporal, cerebellar and pontine, partly CE	N/A	Yes	+	+++// +	Neg.	Neg.	0
5	2	RRMS	ON, cognitive deficits	R occipital	R occipital	Yes	+	++// ++	Neg.	Neg.	0
6	7.5	RRMS	ON, epilepsy, dizziness, dysesthesia	Multiple lesions: R periventricular with CE, R hemispheric	R temporal	Yes	+++	+// ++	Neg.	Neg.	0
7	0.5	CIS	Headache, L sensorimotor hemiparesis, visual impairment	R parietal	R parietal	Yes	++	+++// +++	Neg.	Neg.	0
8	2	RRMS	R hemihypesthesia, dizziness	Multiple WM brain lesions and 5 spinal; partially CE	R occipital	Yes	+++	+++// +	Neg.	Neg.	6.4
9	1	CIS	R sensorimotor hemiparesis	L periventricular WM lesion with weak CE	L periven-tricular	Yes	+	+// +	Neg.	Neg.	0
10	1	RRMS	L hemiparesis, facial nerve palsy, dysarthria	R frontotemporal and juxtacortical lesions	N/A	Yes	++	++// ++	Neg.	Neg.	0
11	132	RRMS	L hemihypesthesia, abducens nerve palsy, cere-bellar ataxia, unsteady gait	Supra- and infratentorial lesions bilaterally, marked periventricular distribution	N/A	Yes	+++	+// +	Neg.	Neg.	0

MS #	Disease duration [months]	Disease course	Symptoms	MRI findings	Index lesion	Myelin loss (MBP)	MRP14	Phagocytosis myelin MOG// CNP	C9neo	C4d	PMNs in ROI/ mm^2
12	216	SPMS	Decreased alertness, cognitive impairment, aphasia, confinement in bed	Multiple cerebral and spinal cord lesions	N/A	Yes	+	++// +/++	Neg.	Neg.	0
13	120	SPMS	Spastic paraparesis, gait ataxia, fatigue	Multiple supra- and infratentorial WM lesions	N/A	Yes	+	++// ++	Neg.	Neg.	0
14	144	RRMS	Diplopia, L sensorimotor hemiparesis	Multiple lesions: R frontal, corpus callosum, brainstem, cervical spinal cord	R frontal	Yes	+	+/++// +	Neg.	Neg.	0
15	48	SPMS	Hyp- and paresthesia, bladder dysfunction	Multiple WM bihemispheric and spinal lesions with CE	N/A	Yes	+++	+++// ++	Neg.	Neg.	0
16	1	RRMS	Decreased alertness, aphasia, apraxia, **R** hemiparesis	Multiple supra-and infratentorial lesions, open-ring CE	R frontal	Yes	++	+// +	Neg.	Neg.	0
17	0.75	CIS	L hemiparesis	R parietal	R parietal	Yes	+	+// +	Neg.	Neg.	0
18	N/A	N/A	N/A	R central	R central	Yes	+	++// +	Neg.	Neg.	N/A
19	30	RRMS	R ON, L sensorimotor hemiparesis, headache, epileptic seizures	Multiple lesions: tume-factive R frontal with perifocal edema, peri-ventricular, corpus callosum, spinal cord	R frontal	Yes	+++	+++// +	Neg.	Neg.	0
20	3	RRMS	L ON	Multiple WM brain lesions: juxtacortical, brainstem, partly CE	R temporal	Yes	++	+++// +++	Neg.	Neg.	0
21	2	RRMS	L sensorimotor hemi-paresis	Multiple WM lesions: predominantly R, partly CE	R frontal	Yes	++	+// ++	Neg.	Neg.	0

MS #	Disease duration [months]	Disease course	Symptoms	MRI findings	Index lesion	Myelin loss (MBP)	MRP14	Phagocytosis myelin MOG// CNP	C9neo	C4d	PMNs in ROI/ mm^2
22	0.3	RRMS	Cognitive impairment, disorientation, incomplete INO	Multiple CE lesions: L frontoparietal, bilateral mesiotemporal, spinal cord	L fronto-parietal	Yes	+++	+++// ++	Neg.	Neg.	3.2
23	1	CIS	L brachiofacial hemiparesis	Singular tumefactive right parietal WM lesion with CE and perifocal edema	R parietal	Yes	++	++// +	Neg.	Neg.	0
24	0.3	CIS	R sensorimotor hemiparesis, memory deficits	L periventricular, frontoparietal lesion with CE and edema	L fronto-parietal	Yes	++	++// +	Neg.	Neg.	0
25	2	CIS	R leg paresis and R leg paresthesia	Tumefactive L frontal lesion without CE	L frontal	Yes	+	++// +++	Neg.	Neg.	0
26	144	RRMS	Nystagmus, ataxia, dysarthria, depression, spastic tetraparesis, bladder dysfunction	Multiple supra- and infratentorial WM lesions; large bifrontal CE WM lesion	R frontal	Yes	++	+++// +++	Neg.	Neg.	0

eTable 2 Autopsy patients in the study.

NMOSD

NMO #	Disease duration [months]	Onset of acute symptoms [weeks]	Symptoms/ cause of death	AQP4- ab	C4d at superficial glia limitans
1	96	2	Blindness; spastic tetraparesis; neurogenic bladder with incontinence; cardio-respiratory failure due to **septic shock**	N/A	Positive
2	18	10	Optic neuritis, tetraparesis, incontinence; **respiratory failure**	N/A	Positive
3	5	1	Tetraparesis; **loss of brainstem functions**, coma	N/A	Positive
4	9	N/A	Sensorimotor paraparesis; N/A	Yes	Positive
5	288	6	Blindness, sensorimotor paraparesis; **cardiac failure**	Yes	Positive
6	4	4	Blurred vision, vertigo, sensorimotor tetraparesis; bronchopneumomia, **respiratory failure**	N/A	Positive
7	48	4	Optic neuritis, dysarthria, difficulties in swallowing, paraparesis; **respiratory failure**	N/A	Positive
8	42	N/A	Reduced consciousness, sensorimotor paraparesis; **respiratory failure**	Yes	Positive
9	N/A	N/A	N/A	N/A	Positive

ADEM

ADEM #	Disease duration [days]	Symptoms/ cause of death	MOG-ab	C4d at superficial glia limitans
1	10	Encephalopathy, coma; **brain edema**	N/A	Negative
2	5	Encephalopathy, coma; **brain edema**	N/A	Negative
3	14	Personality change, progressive L hemiparesis; **brain edema**	N/A	Negative
4	N/A	N/A	N/A	Negative
5	~ 5	Headache, fever, somnolence; **brain edema, herniation**	Yes	Negative
6	60	Encephalopathy, dysarthria, hemiparesis; **sepsis, cardio-respiratory failure**	Yes	Negative

MS

MS #	Disease duration [months]	Disease course	Symptoms/ cause of death	C4d at superficial glia limitans
1	108	SPMS	Diplopia, cerebellar ataxia, sensorimotor tetraparesis, bladder incontinence, epilepsy; **status epilepticus**	Negative
2	12	RRMS	Dysarthria, sensorimotor tetraparesis, bladder incontinence; **pulmonary embolism**	Negative
3	N/A	RRMS	Optic neuritis, paraparesis; **pulmonary embolism**	Negative
4	36	PPMS	Sensory and motor deficits, walking problems, dysmetria, incontinence; **respiratory failure**	Negative
5	84	RRMS	Intracerebral hemorrhage with intraventricular extension, coma; **cardiopulmonary arrest**	Negative
6	132	PPMS	Cerebellar ataxia, gait instability, INO, dysphagia, cachexia; bronchopneumonia, **respiratory failure**	Negative

eTable 3 Antibodies used for immunohistochemistry.

Target protein (hu)	Host species, antibody type	Concentration	Pretreatment	Source
AQP4	Rabbit	1:200	Citrate + MW	Sigma Aldrich/Merck, Darmstadt, GER
C4d (clone A24-T)	Rabbit, monoclonal	1:50, 10 min	Dako Omnis buffer pH9, 30 min	Zytomed, Berlin, GER; stained in Dako Omnis machine, GV800 kit, Agilent/Pathology UMG
C4d	Rabbit, polyclonal	1:50	Tris-EDTA + MW	Cell Marque™ Tissue Diagnostics
C4d (SP91)	Rabbit, monoclonal	1:25	Citrate + Tris-EDTA	Sigma Aldrich/Merck, Darmstadt, GER
C9neo (clone B7)	Mouse, monoclonal	1:50	Protease	B.P. Morgan, Cardiff, UK
CNPase (clone SMI91)	Mouse, monoclonal	1:200	Citrate + MW	Covance
GFAP	Rabbit	1:1000	None	Dako, Glostrup, DK
IgG	Rabbit	1:4000	Citrate + MW	Dako, Glostrup, DK
KiM1P	Mouse	1:50	Citrate + MW	Institute of Pathology, University of Kiel, GER
Pan-laminin	Rabbit, polyclonal	1:100	Citrate + Tris-EDTA	Thermo Fisher, Waltham, USA
MBP	Rabbit	1:2000	None	Dako, Glostrup, DK
MOG	Rat	1:1000	Citrate + MW	Institute of Neuropathology, University Medical Center Göttingen, GER
MRP14/S100A9	Mouse	1:500	Protease	Acris/Origene, Herford, GER
PLP	Mouse	1:500	Citrate + MW	Biorad, CA, USA

Abbreviations: MW = microwave

eReferences

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