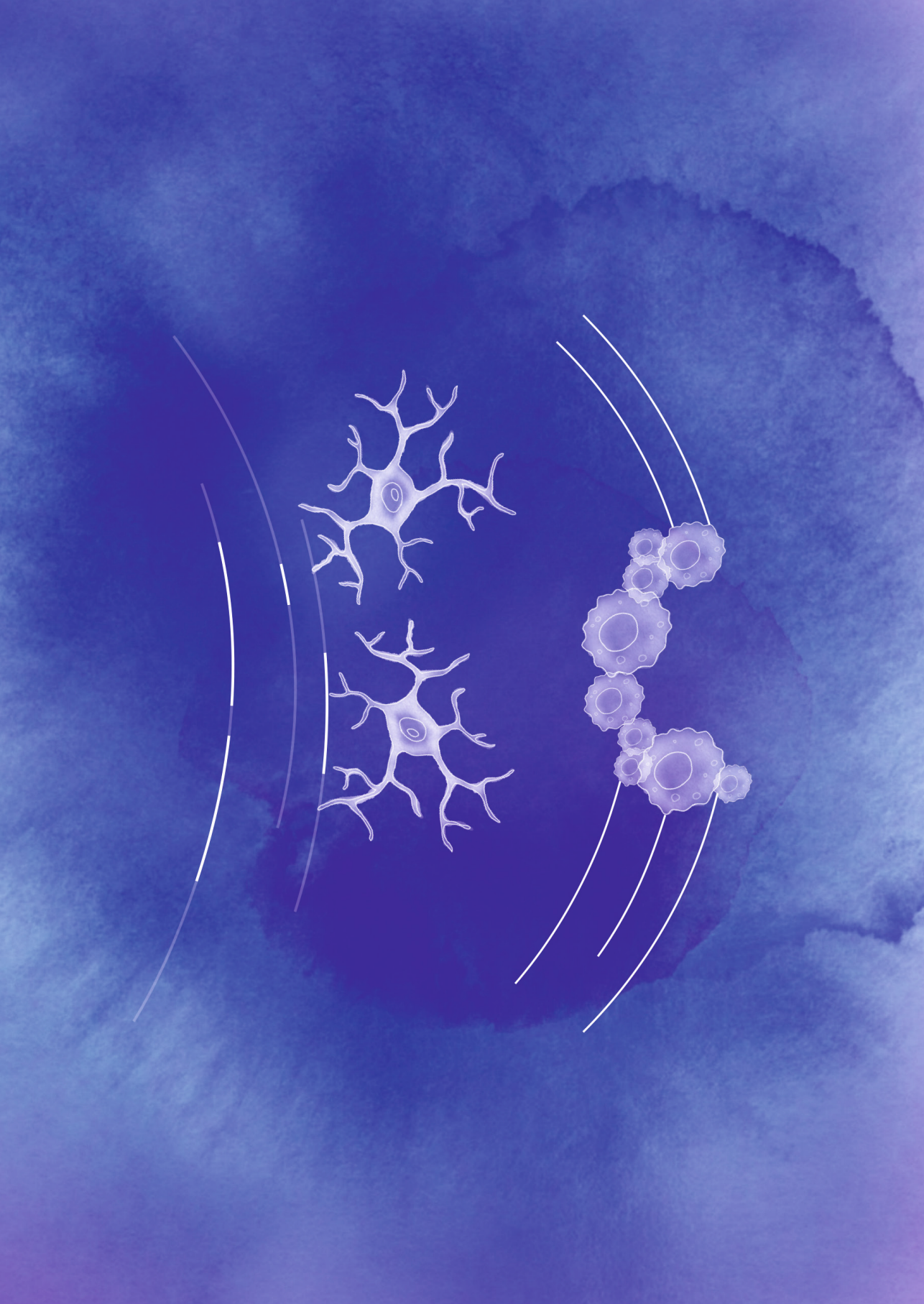
Progression of multiple sclerosis

The role of microglia and neurons

van den Bosch, A.M.R.

Publication date
2024



CHAPTER 6

Neurofilament light chain levels in multiple sclerosis correlate with lesions containing foamy macrophages and with acute axonal damage

Aletta M.R. van den Bosch[1]†, Nina L. Fransen[1]†, Matthew R.J. Mason[1], Annemieke Rozemuller[2], Charlotte E. Teunissen[3], Joost Smolders[1,4] and Inge Huitinga[1,5]

[1]Neuroimmunology Research Group, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands; [2]Dept. Pathology, Amsterdam UMC, Amsterdam, The Netherlands; [3] Neurochemistry lab, Dept. Clinical Chemistry, Amsterdam Neuroscience, Amsterdam UMC, Vrije Universiteit, Amsterdam, The Netherlands; [4] Dept. of Neurology and Immunology, MS center ErasMS, ErasmusMC, Rotterdam, The Netherlands; [5]Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

† These authors contributed equally to this work.

Neurology Neuroimmunology & Neuroinflammation 2022 Mar 3;9(3):e1154

ABSTRACT

To investigate whether white matter lesion activity, acute axonal damage, and axonal density in MS associate with CSF neurofilament light chain (NfL) levels. Of 101 brain donors with MS (n = 92 progressive MS, n = 9 relapsing-remitting MS), ventricular CSF was collected, and NfL levels were measured. White matter lesions were classified as active, mixed, inactive, or remyelinated, and microglia/macrophage morphology in active and mixed lesions was classified as ramified, ameboid, or foamy. In addition, axonal density and acute axonal damage were assessed using Bielschowsky and amyloid precursor protein (APP) (immune) histochemistry. CSF NfL measurements of donors with recent (<1 year) or clinically silent stroke were excluded. CSF NfL levels correlated negatively with disease duration (p = 6.9e-3, r = 0.31). In donors without atrophy, CSF NfL levels correlated positively with the proportion of active and mixed lesions containing foamy microglia/macrophages (p = 9.85e-10 and p = 1.75e-3, respectively), but not with those containing ramified microglia. CSF NfL correlated negatively with proportions of inactive (p = 5.66e-3) and remyelinated lesions (p = 0.03). In the normal appearing pyramid tract, axonal density negatively correlated with CSF NfL levels (Bielschowsky, p = 0.02, r = -0.31), and the presence of acute axonal damage in lesions was related to higher NfL levels (APP, p = 1.17e-6). The amount of acute axonal damage was higher in active lesions with foamy microglia/macrophages and in the rim of mixed lesions with foamy microglia/macrophages when compared with active lesions containing ramified microglia/macrophages (p = 4.6e-3 and p = 0.02, respectively), the center and border of mixed lesions containing ramified microglia/macrophages (center: p = 4.6e-3, border, p = 4.6e-3, and n.s., p = 4.6e-3, respectively), the center of mixed lesions containing foamy microglia/macrophages (p = 4.6e-3 and p = 0.02, respectively), inactive lesions (p = 4.6e-3 and p = 4.6e-3, respectively), and remyelinated lesions (p = 0.03 and p = 0.04, respectively). Our results demonstrated that active and mixed white matter MS lesions with foamy microglia show high acute axonal damage and correlate with elevated CSF NfL levels. Our data support the use of this biomarker to monitor inflammatory demyelinating lesion activity with axonal damage in MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) with focal demyelinating lesions throughout the CNS.[1,2] Pathologically, MS is characterized by different types of lesions that can be staged by the presence and morphology of microglia/macrophages in relation to demyelination.[3,4] Furthermore, some patients show diffuse atrophy with axonal loss throughout the CNS.[3,5] We and others found that at time of death, there is substantial inflammatory lesion activity,[6] and 57% of all lesions in the MS autopsy cohort of the Netherlands Brain Bank (NBB) are either active or mixed.[3] The level of inflammatory lesion activity is correlated to a more severe disease course,[3] and in this study we assess if axonal damage coincides with the inflammatory lesion activity in the same MS autopsy cohort.

Neurofilaments are neuron-specific structural scaffolding protein components of the cytoskeleton that are essential for axonal growth and maintenance.[7] During axonal damage, neurofilaments are released into the cerebrospinal fluid (CSF). Therefore neurofilaments are a potential molecular fluid biomarker for the extent of axonal damage.[8] Indeed, levels of neurofilament light (NfL) in the CSF or blood plasma/serum have been used as general indicators or predictors of neuronal damage in various neurological disorders and events, among which MS, Alzheimer's Disease and traumatic brain injury.[8–16] In MS, NfL can serve as a prognostic biomarker in both relapsing remitting and progressive MS, and for monitoring clinical relapses and treatment response.[13] Previous studies have focused on understanding the value of NfL measurements in MS by combining conventional MRI measurements and clinical data with NfL in the blood and/or CSF.[17–21] NfL levels correlate with clinical relapses and radiological biomarkers of inflammatory disease activity, as it reflects the amount of gadolinium-enhancing lesions, [18,20] the amount of paramagnetic rim MRI lesions in the absence of gadolinium-enhancing lesions, [22] as well as disease progression in terms of the T2 lesion load,[12,20] T2 lesion volume[18,20] and presence of atrophy of the brain and spinal cord.[18,21,23]

These observations raise the question whether neuropathological hallmarks of inflammatory lesion activity, neuroaxonal damage or neurodegeneration correlate with axonal damage as reflected by CSF NfL levels in MS. Here, we assessed the relationship between CSF NfL levels and disease severity, axonal loss in the normal appearing white matter (NAWM), and white matter lesion characteristics including microglia/macrophage activation score (MMAS) and acute axonal damage as measured with amyloid precursor protein (APP) in lesions in a well characterized MS autopsy cohort of 101 cases of the NBB.

MATERIALS AND METHODS

Donors

For inclusion in this study, we screened N=182 MS brain donors that came to autopsy at the Netherlands Brain Bank (NBB) between 1991 and 2015.[3] MS pathology was confirmed by a certified neuropathologist and donors with clinical or pathological features of encephalomyelitis were excluded. Donors were also excluded if there were clinical signs of dementia or if a neuropathologist diagnosed pathological dementia based on senile pathology of α-synuclein presence, Tau+ tangles and β-amyloid plaques (Braak > 2, Thal fase >2). Cases of whom no CSF was available for analysis were excluded. The included study cohort consisted of 101 MS donors (*n* = 92 progressive MS, *n* = 9 relapsing-remitting MS). Clinical disability status was scored following the Kurtze's Expanded Disability Status Scale (EDSS), and the time from first symptoms to EDSS-6 and EDSS-8 was determined, together with the age at onset and the total duration of disease from onset of first symptoms. Disease severity score was calculated as 5 – log (years to EDSS6 + 1). Donors were scored for minor senile pathology (Braak ≤ 2, Thal fase ≤ 2), previous history of stroke in their clinical and post-mortem neuropathological files and for the presence of atrophy as based on the post-mortem macroscopic examination. Donor demographics are summarized in **Table 1**. The exclusion criteria and studied characteristics that were related to NfL levels of the various subgroups is visualized in the flowchart in **Fig. 1**.

Tissue dissection and sample collection

Donors provided informed consent for brain autopsy and for the use of material and clinical data for research purposes in compliance with national ethical guidelines. The NBB autopsy procedures were approved by the Ethical Committee of the VU University Medical Center in Amsterdam, the Netherlands. CSF of all donors was collected from the ventricles and stored at -80°C. Of *n* = 43 MS donors blood was collected from the heart, centrifuged at 3.000 rpm for 15 minutes, and the plasma was aliquoted and stored at -80°C. Regarding the higher number of donors with available CSF samples compared to plasma samples, we focused our current analysis on CSF samples. Blocks were dissected as previously described[3] from standardized locations from the brain stem and the spinal cord, as well as any MS plaques visible macroscopically or on MRI guidance from 1-cm-tick coronal brain slices cut throughout the brain. For the NAWM cohort (Fig. 1), of all donors the brain stem sample containing the pyramid tract was analyzed, as this area is standardly dissected and therefore comparable between donors, and because the axons are oriented in the same direction when cut longitudinal facilitating comparable quantifications. Donors

were excluded if there was a lesion present in the pyramid tract or if the pyramid tract was missing, resulting in a NAWM cohort of *n* = 57.

Table 1: MS donor characteristics of the various subgroups studied.

	All donors		**Donors without recent (<1 year) or clinically silent stroke**			
	All	**NAWM pyramid tract present**	**All**	**Without atrophy**	**With atrophy**	**NAWM pyramid tract present**
	N=101	*N=57*	*N=75*	*N=57*	*N=18*	*N=44*
Age (years, SD)	64,5 (12,90)	69,10 (11,28)	63,39 (12,57)	62,33 (12,30)	66,72 (13,20)	66,76 (11,53)
Sex (F%)	64,36	59,09	64,00	59,65	77,78	60,61
PMD (hours, SD)	8,01 (2,70)	7,81 (3,18)	7,80 (2,72)	7,91 (2,83)	7,46 (2,41)	7,83 (3,43)
Brain weight (grams, SD)	1182,4 (135,86)	1198,53 (136,48)	1168,89 (134,53)	1202,09 (122,88) *	1063,78 (116,84) *	1190,93 (127,37)
pH of CSF (SD)	6,47 (0,25)	6,48 (0,27)	6,46 (0,25)	6,49 (0,25)	6,37 (0,24)	6,49 (0,30)
Storage time (years, SD)	13,64 (6,23)	14,51 (6,05)	13,65 (6,41)	13,21 (6,11)	15,06 (7,31)	14,60 (5,80)
Age at onset (years)	33,56 (10,52)	35,39 (11,57)	33,54 (11,07)	32,89 (10,79)	35,5 (11,98)	35,03 (11,85)
Years to EDSS6	17,52 (12,12)	18,39 (11,57)	16,85 (11,92)	17,72 (12,90)	13,94 (7,36)	16,72 (11,13)
Microglia/ macrophage score (SD)	0,31 (0,27)	0,26 (0,28)	0,30 (0,27)	0,33 (0,28)	0,23 (0,22)	0,25 (0,27)
Lesion load BRS (SD)	6,66 (7,75)	4,12 (4,89)	6,86 (8,05)	6,31 (7,70)	8,56 (9,08)	4,19 (4,92)

**Brain weight is significantly lower in donors with atrophy compared to donors without atrophy (p=3.34e-5, Generalized Linear Model). Abbreviations: NAWM = normal appearing white matter, SD = standard deviation, F = female, PMD = post mortem delay, CSF = cerebrospinal fluid, EDSS6: (Kurtze's) Expanded Disability Status Scale*

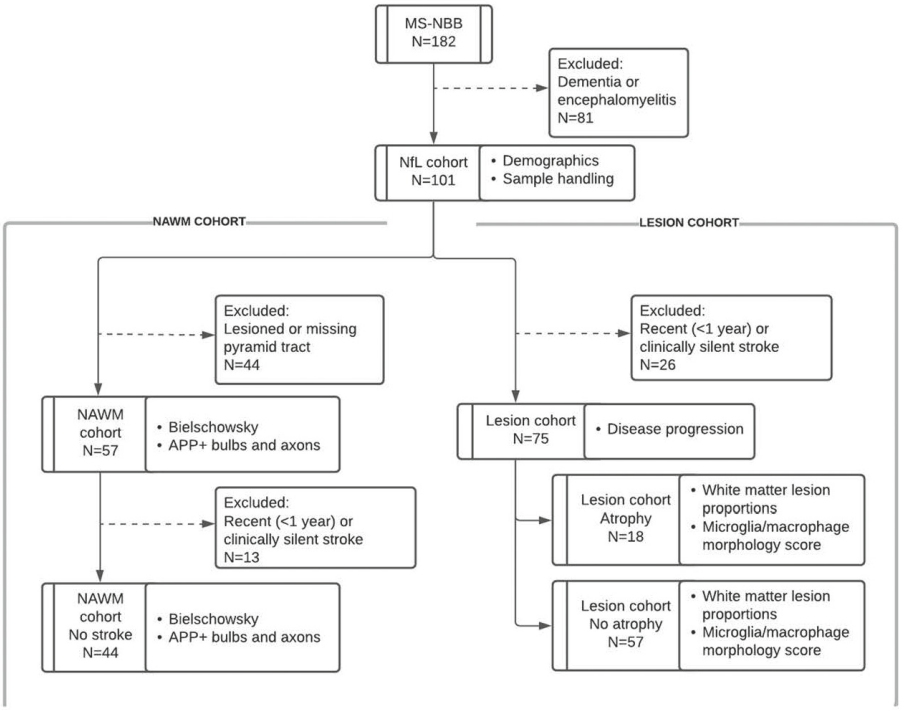


Figure 1: Flowchart of inclusion criteria and tests performed on the subgroups in the NAWM cohort and the lesion cohort. NAWM: normal appearing white matter.

Neurofilament measurements

CSF (n = 101) and paired plasma samples (n = 43) were run simultaneously using a single molecule array (Simoa) assay to measure the NfL levels on a HD-X instrument (Quanterix, Billerica, MA, USA) using the manufacturer's instructions[21,22]. The Simoa assay is a highly sensitive sandwich based enzyme-linked immune sorbent assay (ELISA). The immunocomplex beads fir into one femtomolar-sized chamber, resulting in highly concentrated reaction volume and signal per well. This technology allows an up to 1000-fold increase in sensitivity, with multiple studies reporting strong correlations of CNS-proteins between CSF and blood samples[24]. Post-mortem CSF and plasma samples were standardly diluted 1:100 and 1:4 respectively, and further diluted conform the assay's dynamic range of 0,686 – 500 pg/mL, with a dilution linearity (Mean %L between LLOQ and ULOQ) within the acceptable range of 85-115%.

Lesion characterization

Characterization of MS lesions had been carried out previously by Luchetti *et al*. for each donor on all available archived material (3819 lesions in total) following the system of Van der Valk *et al*. (2000) and Kuhlmann *et al*. (2017). Double immunohistochemistry for proteolipid protein (PLP) (MCA839G, AD Serotec, Oxford, UK, with DAB) and human leukocyte antigen (HLA-DR-DQ) (M0775, CR3/43, DAKO, Denmark, with DAB + nickel) [3 w]ere [performed]. Reactive sites and white matter lesion types were discriminated based on demyelination and HLA-DR+ microglia/macrophages. In reactive sites there is no demyelination and there is accumulation of microglia/macrophages. In active lesions there is partial demyelination and accumulation of microglia/macrophages throughout. In mixed lesions there is a fully demyelinated, gliotic center with a border of accumulated microglia/macrophages. The microglia/macrophages in active and mixed lesions are scored 0 if the majority is ramified, 0.5 if the majority is ameboid or 1 if the majority is foamy. In inactive lesions there is complete demyelination of the lesion and there is no presence of microglia/macrophages. In remyelinated lesions there is partial myelination and there are sparse microglia/macrophages throughout[3,26,27].

Calculation of lesion load and proportions of lesions

All white matter lesion parameters were calculated per donor previously by Luchetti *et al*. (2018). In the brainstem, a standardly dissected area, of each donor the lesion load has been calculated as the sum of all white matter lesions present, and the reactive load has been calculated as the numbers of reactive sites identified.[3] The MMAS of each donor has been calculated by dividing the sum of de score of the microglia/macrophages values (0, 0.5, 1) in active and mixed lesions by the amount of active and mixed lesions. Proportions of active, mixed and inactive lesions throughout the CNS were calculated per donor as the sum of number of the specific type of lesions divided by the number of all lesions (active, mixed, inactive and remyelinated lesions), discriminating between ramified, ameboid or foamy microglia/macrophages in active and mixed lesions. The proportion of remyelinated lesions was calculated per donor as the number of remyelinated lesions divided by the sum of inactive and remyelinated lesions.[3]

Quantification of axonal density and APP presence

To quantify axon density, Bielschowsky silver staining was performed on 8 μm paraffin-embedded pyramid tract of the NAWM cohort (n = 57). Deparaffinization and rehydration was performed in a series of xylene and alcohol. Incubation with pre-heated 20% silver nitrate solution in H2O at 40°C was performed for 20 minutes. Tissue was placed in H2O, and 32% ammonium hydroxide solution droplets were added before incubation with silver nitrate solution and incubated at 40°C for 20 minutes. Incubation with 1% ammonium

solution was performed for 1 minute. Developer solution (50% nitric acid (65%) + 0.8% formaldehyde (40%) + 0.2% citric acid in depH2O) was added to the ammonium silver nitrate solution and incubated for 10 minutes. 5% sodium thiosulfate solution was incubated for 5 minutes and dehydration was performed in alcohol series and xylene. Images were taken on an Axioskop (Zeiss) at 10x magnification an automated random selection of ROI's comprising >40% of the area was made, and the percentage of area covered by axons at 40x magnification was analyzed with the Image Pro Cell Count Grid software (Media Cubernetics).

For APP [28,29] 8μm paraffin-embedded NAWM sub-cortical white matter lesion (n = 50) sections were deparaffinized in a series of xylene and alcohol. Antigen retrieval was performed by microwaving citrate buffer pH6.0 for 10 minutes at 800W before blocking with SUMI (0.25% gelatin + 0.5% Triton-X + 10% NHS in TBS). Primary antibodies (APP: MAB348 Millipore, 1:1000) were diluted in SUMI and incubated overnight. After blocking for endogenous peroxidase (0.03% H2O2 in SUMI), biotinylated secondary antibody 1:400 in SUMI was incubated for 1 hour and avidin-biotin-complex (1:800 in TBS) was incubated for 45 minutes, then visualized with DAB (50% diaminobenzidine + 0.03% H2O2 in TBS) and counterstained with hematoxylin. In the NAWM, donors were scored for presence of APP+ bulbs or axons. In the different white matter lesion types the number of APP+ bulbs and axon fragments relative to the area were counted, with the center and border of mixed lesions separately, and in the perilesional white matter of the same tissue block. The counts were normalized to the APP+ events in the perilesional white matter and to the area of the lesion.

Statistical analysis

NfL measurements of the CSF and plasma, as well as lesion and reactive site load of the brainstem were natural log-transformed to normalize the data for further analysis. Correlations between continuous variables were tested with Pearson Correlation Coefficient. Differences between dichotomous variables and NfL concentrations were tested with Wilcoxon Rank sum test. Differences between dichotomous variables and proportion measures were tested with Binomial Generalized Linear Models (GLMs). Correlations between proportion measures and continuous variables were tested with Quasibinomial GLMs. Associations between dichotomous variables were tested with Fisher's Test. If multiple groups were tested at once, a Kruskal-Wallis rank sum test was used in combination with pairwise Wilcoxon Rank Sum Tests with corrections for multiple testing. All statistics were performed in RStudio Desktop (version 1.2.5033, Rstudio, Inc., Boston, MA, USA), using key packages ggplot2, devtools, car and lsmeans.

RESULTS

Donor demographics and sample handling

Correlations of CSF NfL levels with donor demographics and sample handling are summarized in **Table 2**. CSF NfL levels were not correlated with age, sex, pH of the CSF, weight of the brain, post-mortem delay or storage time. Compared to donors without stroke, NfL levels were significantly higher in donors with recent stroke (<1 year before death) (Pairwise Wilcoxon Rank Sum Tests with corrections for multiple testing, P = 2.4e-3) and with clinically silent stroke (P = 3.5e-4), and there were no differences found in donors with stroke 1-5 years or >5 years before death. As recent and clinically silent stroke elevate CSF NfL levels, this may confound the data. Therefore, for subsequent MS pathology-specific analysis, MS donors with recent (<1 year) and clinically silent stroke were excluded from further analyses (Fig. 1, n = 26 excluded, n = 75 remained). Minor senile pathology (Braak score 1-2 and/or Thal phase 1-2) did not correlate with NfL levels. Lastly, atrophy was validated by the lower brain weight of donors with atrophy than without atrophy (Wilcoxon Rank sum test P = 7.4e-5).

Correlations of CSF NfL with disease progression measures are summarized in **Table 2**. CSF NfL levels of MS donors at time of death did not correlate with age at onset of MS or severity score as calculated with time to EDSS 6, but did correlate negatively with the duration of disease, being higher in patients who had a shorter disease duration (Pearson's Correlation Test, P = 6.9e-3, r = -0.29), also after correction for age (P = 0.04, r = -0.24).

Axonal damage in the normal appearing white matter

Of 44 MS donors, in the dissected medulla oblongate the pyramid tract was missing or a lesion was present in the pyramid tract. In the remaining 57 donors, axonal density, quantified as the percentage of Bielschowsky positive area (high axonal density: **Fig. 2A**, low axonal density: **Fig. 2B**) in the NAWM, negatively correlated with NfL (Fig. 2C, Pearson's Correlation Test, P = 0.02, r = -0.31). In donors without recent or clinically silent stroke (n = 44), axonal density in the NAWM was not correlated to CSF NfL levels (data not shown).

In the NAWM (n = 57), presence of APP+ axons and bulbs was scored (no APP present: Fig 2D, APP+ axons and bulbs present: Fig. 2E). CSF NfL levels were significantly higher in donors with presence of APP+ axons or bulbs compared to those without (P = *1.17e-6)*. In donors without recent or clinically silent stroke (n = 44), donors with presence of APP+ axons or bulbs also had significantly higher CSF NfL levels compared to donors with without APP+ axons or bulbs present (data not shown, P = 2.3e-4).

Table 2: Correlations of clinical and pathological donor characteristics with CSF NFL levels of all MS donors.

			CSF NfL mean (SD) pg/mL (log)	**P**	**r**
Age			-	0.85	-0.02
Sex	M		7.57 (1.25)	0.35	-
	F		7.75 (1.23)	-	-
pH of CSF			-	0.25	-0.12
Brain weight			-	0.31	0.10
PMD			-	0.38	0.09
Storage time			-	0.36	0.09
Plasma NFL (pg/mL (log))			-	**1.9e-4**	0.54
Minor senile pathology	No		7.71 (1.37)	0.78	-
	Yes		7.66 (1.09)	-	-
Stroke (years from death)	No		7.25 (0.98)	-	-
	Yes	<1	9.42 (1.40)	**2.4e-3**	-
		1-5	7.47 (0.87)	0.46	-
		>5	7.68 (1.33)	0.72	-
		Clinically silent	8.50 (1.13)	**3.5e-4**	-
Atrophy	No		7.73 (1.27)	0.31	-
	Yes		7.49 (1.02)	-	-
Age at onset (years)*			33.53 (11.07)	0.33	0.12
Severity score*			3.85 (0.31)	0.16	0.16
Disease duration (years)*			30.31 (12.80)	**6.9e-3**	-0.31

*All associations were tested with either Pearson Correlation Coefficient. Wilcoxon Rank sum test was used if two groups were compared, and Pairwise Wilcoxon Rank Sum Test corrected for multiple testing for stroke at different time points. Abbreviations: SD = standard deviation, M = male, F = female, PMD = post mortem delay, CSF = cerebrospinal fluid. * Donors without recent (<1 year) or clinically silent stroke*

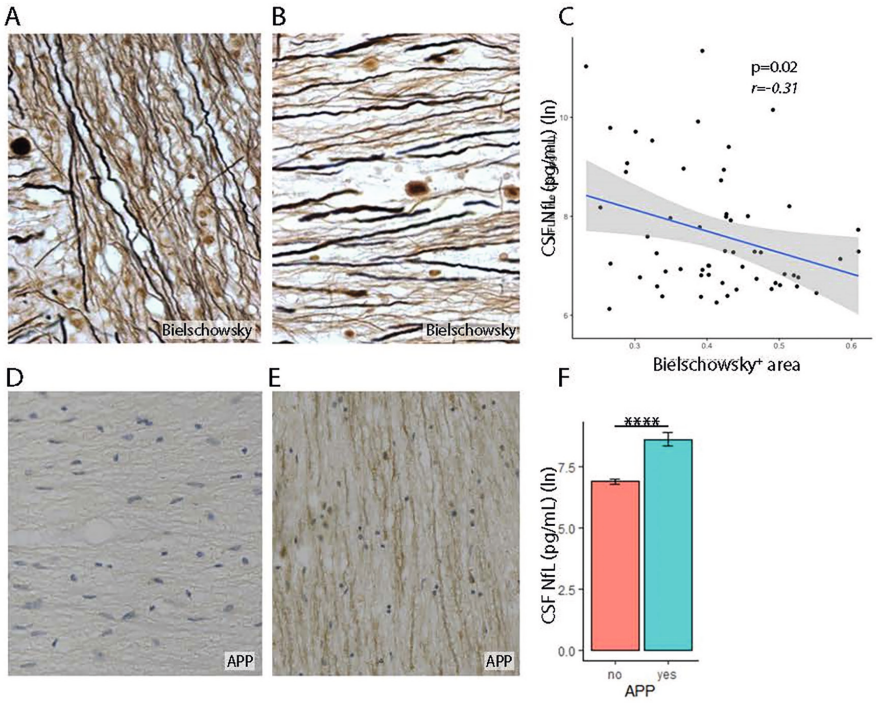


Figure 2: NfL levels are negatively correlated with axonal density and are increased with presence of acute axonal damage in the normal appearing white matter. For axonal density, a Pearson's Correlation Test was performed, and the regression line is visualized with the gray area indicating the 95% confidence interval. For APP+ a Wilcoxon Rank Sum test with correction of multiple testing was performed. Images are taken at 40x magnification. **(A)** Representative image of a high number of Bielschowsky+ axons and (**B)** low number of Bielschowsky+ axons. **(C)** Axonal density, as measured by Bielschowsky+ axons, was negatively correlated with CSF NfL (p=0.02, r=0.31). **(D)** Representative image of NAWM without APP+ axons or bulbs, **(E)** representative image of NAWM with APP+ axons and bulbs. **(F)** The CSF NfL levels of donors with APP+ axons or bulbs were significantly higher that of those without APP+ axons or bulbs (*P* = 1.17e-6).

White matter inflammation and lesion activity

As brain atrophy on MRI is related to NfL levels [9], we have analyzed correlations of CSF NfL in MS donors with macroscopic brain atrophy and without atrophy separately. Donor demographics were not different between donors with atrophy and without atrophy, as visualized in **Table 1**. CSF NfL levels of MS donors without atrophy were similar to donors with atrophy (Wilcoxon Rank sum test, **Table 2**). Donors with and without atrophy have comparable lesion measures (**Table 3**).

Fig. 3 shows ramified microglia/macrophages with MMAS score 0 (**Fig. 3A**) and foamy microglia/macrophages with MMAS score 1 (**Fig. 3B**), and shows microscopic images of an active lesion containing ramified (**Fig. 3C**) and foamy (**Fig. 3D**) microglia/macrophages, a mixed lesion containing ramified (**Fig. 3E**) and foamy (**Fig. 3F**) microglia/macrophages, an inactive lesion (**Fig. 3G**) and a remyelinated lesion (**Fig. 3H**). As shown in **Table 3**, correlations of CSF NfL with lesion measures found in all donors become stronger when donors with atrophy are removed. In donors without atrophy, CSF NfL levels correlated with several lesion type proportions.

In donors without atrophy, the MMAS positively correlated with CSF NfL (GLM, $P = 1.2e\text{-}6$, **Fig. 3I**). CSF NfL levels correlated with the proportion of all active lesions (GLM, $P = 6.35e\text{-}3$) and this correlation was considerably stronger for active lesions containing foamy microglia/macrophages (GLM, $P = 9.85e\text{-}10$, **Fig. 3K**). CSF NfL levels did not correlate with the proportion of all mixed lesions but was significantly correlated to the proportion of mixed lesions containing foamy microglia/macrophages (GLM, $P = 1.75e\text{-}3$, **Fig. 3M**). CSF NfL levels did not correlate significantly with the proportion of active or mixed lesions containing ramified microglia/macrophages (**Fig. 3J & 3L**). Reciprocally, CSF NfL levels negatively correlated with the proportion of inactive lesions (GLM, $P = 1.75e\text{-}3$, **Fig. 3N**) and remyelinated lesions (GLM, $P = 0.03$, **Fig. 3O**). Lastly, CSF NfL did not correlate with the lesion load or the proportion of reactive sites in the brain stem. In donors with atrophy, only a weak correlation was found of CSF NfL levels with the proportion of active lesions containing foamy microglia/macrophages (GLM, $P = 0.03$), and no correlation was found with other lesion proportions.

Table 3: Correlations of lesion proportions and NfL levels in the CSF in MS donors without recent or clinically silent stroke, differentiated between donors with and without atrophy

		Donors without recent or clinically silent stroke							
Lesion type	***Subtype***	**All (n=75)**			**With atrophy (n=18)**		**Without atrophy (n=57)**		**With vs without atrophy**
		Total #lesions	Average load / Proportion / score (SD)	P	Average load / Proportion / score (SD)	P	Average load / Proportion / score(SD)	P	P
Lesion load (BRS)		659	1.51 (1.14)	0.36	1.65 (1.27)	0.37	1.46 (1.10)	0.68	0.55
Reactive load (BRS)		149	0.55 (0.74)	0.85	0.73 (0.87)	0.20	0.49 (0.68)	0.47	0.24
Active		874	0.21 (0.23)	**0.01**	0.18 (0.22)	0.64	0.22 (0.23)	**6,35e-3**	0.51
	Ramified	*299*	0.08 (0.15)	0.33	0.09 (0.16)	0.73	0.08 (0.15)	0.31	0.70
	Foamy	*356*	0.07 (0.13)	**6.25e-10**	0.03 (0.06)	**0.03**	0.08 (0.14)	**9.85e-10**	0.14
Mixed		985	0.27 (0.27)	0.50	0.22 (0.29)	0.15	0.28 (0.27)	0.14	0.40
	Ramified	*416*	0.13 (0.17)	0.43	0.07 (0.08)	0.41	0.14 (0.19)	0.41	0.08
	Foamy	*194*	0.04 (0.06)	**0.02**	0.02 (0.05)	0.13	0.04 (0.07)	**1,75e-3**	0.14
Inactive		901	0.32 (0.27)	**0.04**	0.34 (0.30)	0.85	0.32 (0.26)	**5,66e-3**	0.78
Remyelinated		548	0.20 (0.22)	0.16	0.19 (0.21)	0.14	0.20 (0.22)	**0.03**	0.39
MMAS score		1859	0.31 (0.27)	**7.47e-7**	0.23 (0.22)	0.28	0.33 (0.28)	**1.2e-6**	0.07

Associations of lesion load and reactive load are tested with Pearson Correlation, and the rest were tested with quasibinomial Generalized Linear Models
Abbreviations: SD = standard deviation, BRS = brain stem, vs = versus.

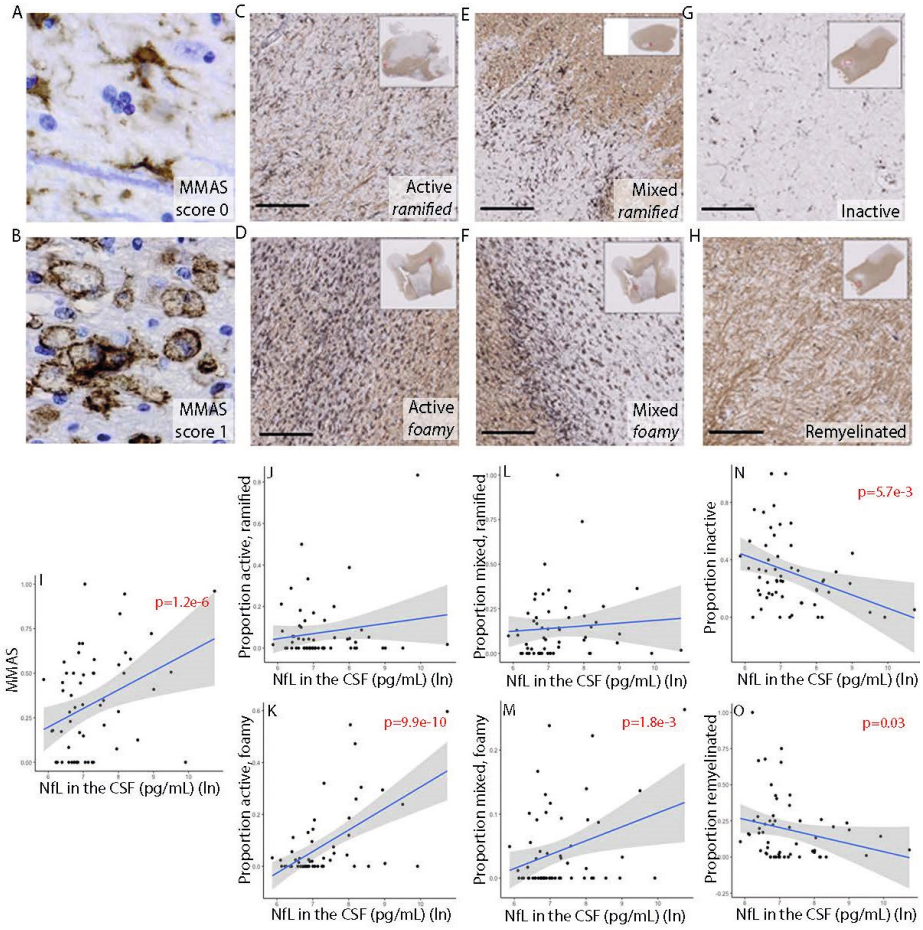


Figure 3: CSF NfL levels correlate with pathological hallmarks of inflammatory lesion activity. Correlations for different lesion measures were tested with a general linear model and correlation between NfL levels in the CSF and plasma was tested with a Pearson's Correlation Test , and the regression line is visualized with the gray area indicating 95% confidence interval. Representative immunohistochemical stainings show **(A)** ramified microglia/macrophages with microglia/macrophage activation score (MMAS) 0 and **(B)** foamy microglia/macrophages with MMAS 1. Representative immunohistochemical stainings stained for PLP in brown and HLA-DR+ microglia in black with a scalebar of 200µm of active lesions with **(C)** ramified microglia/macrophages, **(D)** foamy microglia/macrophages, mixed lesions with **(E)** ramified microglia/macrophages and **(F)** foamy microglia/macrophages, **(G)** inactive lesions and **(H)** remyelinated lesions. In donors without atrophy, CSF NfL levels positively correlated with **(I)** MMAS (p=1.2e-6), and proportions of **(K)** active lesions (p=9.9e-10) with foamy microglia/macrophages, **(M)** mixed lesions with foamy microglia/macrophages (p=1.8e-3), and CSF NfL levels negatively correlated with the proportions of **(N)** inactive lesions (p=5.7e-3) and **(O)** remyelinated lesions (p=0.03).

Plasma NfL levels and white matter inflammation and lesion activity

Regarding the more frequent use of plasma samples compared to CSF samples for NfL analyses in clinical monitoring of MS activity, we have explored the relation of plasma NfL with neuropathological substrates in the small MS cohort with available plasma samples (N=43). Unfortunately the sample size of donors without recent (<1 year) or clinically silent stroke with plasma samples was low (n=26). Plasma and CSF NfL levels correlated significantly (Fig. 3P, Pearson's Correlation Test, P = 1.9e-4, r =0.54). Correlations between plasma NfL levels and lesion load, reactive site load, lesion proportions and the MMAS score are summarized in Supplementary table 1. In donors without atrophy (n=22) the correlation between plasma NfL and the MMAS score and the proportion of active lesions with foamy microglia/macrophages showed generally similar trends as CSF NfL (Supplementary figure 1).

Acute axonal damage in subcortical white matter lesions

To confirm the association of foamy macrophages with increased axonal damage, as reflected by higher CSF NfL levels, we performed APP stainings in different lesion types. As visualized in **Fig. 4**, in active lesions containing foamy microglia/macrophages the amount of APP+ bulbs and axon fragments, normalized to the amount of APP+ bulbs and axon fragments in the perilesional white matter and the area of the lesion, is significantly higher compared to active lesions containing ramified microglia/macrophages (pairwise Wilcoxon Rank Sum Tests with corrections for multiple testing, P = 4.6e-3), active lesions containing ameboid microglia/macrophages, the border and the center of mixed lesions containing ramified microglia/macrophages (P = 4.6e-3 & P = 4.6e-3 resp), the center of mixed lesions containing foamy microglia/macrophages (P = 4.6e-3), inactive lesions (P = 4.6e-3) and remyelinated lesions (P = 0.03). The amount of APP+ bulbs and axon fragments is significantly higher in the border of mixed lesions containing foamy microglia/macrophages compared to active lesions containing ramified microglia/macrophages (P = 0.02), the center of mixed lesions with ramified microglia/macrophages (P = 4.6e-3), the center of mixed lesions with foamy microglia (P = 0.02), inactive lesions (P = 4.6e-3) and remyelinated lesions (P = 0.04).

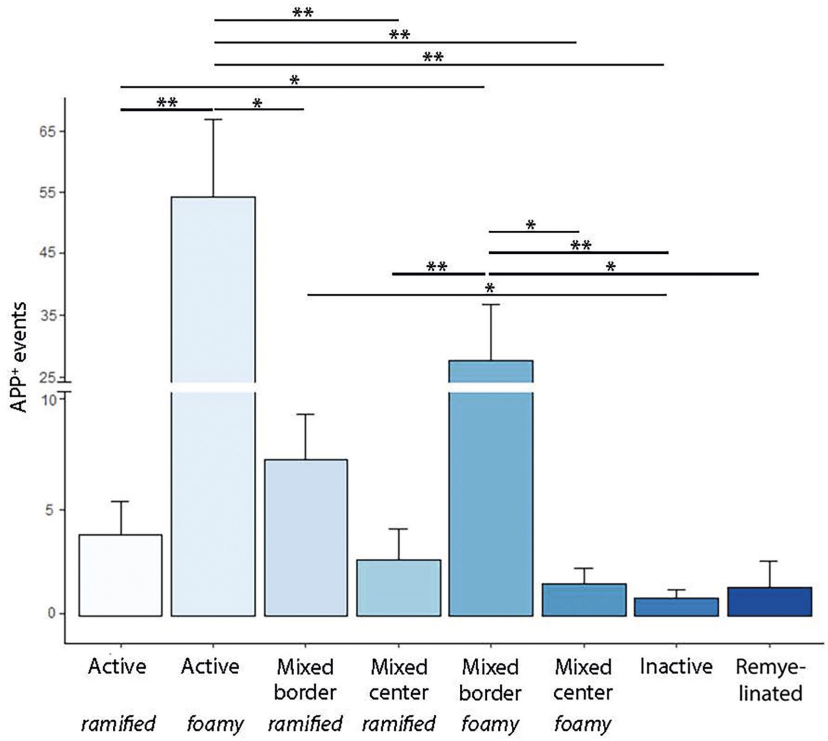


Figure 4: Acute axonal damage most prevalent in active lesions with foamy microglia/macrophages and the border of mixed lesions with foamy microglia/macrophages. A Kruskal-Wallis test followed by pairwise Wilcoxon Rank sum tests with correction for multiple testing was performed. In active lesions containing foamy microglia/macrophages, the amount of APP+ bulbs and axon fragments is significantly higher compared to active lesions containing ramified microglia/macrophages (p=4.6e-3), the border and the center of mixed lesions containing ramified microglia/macrophages (p=4.6e-3 & p=4.6e-3 resp.), the center of mixed lesions containing foamy microglia/macrophages (p=4.6e-3), inactive lesions (p=4.6e-3) and remyelinated lesions (p=0.03). The amount of APP+ bulbs and axon fragments is significantly higher in the border of mixed lesions containing foamy microglia/macrophages compared to active lesions containing ramified microglia/macrophages (p=0.02), the center of mixed lesions with ramified and foamy microglia/macrophages (p=4.6e-3, p=0.02, resp.), inactive lesions (p=4.6e-3) and remyelinated lesions (p=0.04). The amount of APP+ bulbs and axon fragments in the border of mixed lesions containing ramified microglia/macrophages is significantly higher compared to inactive lesions (p=0.04)

DISCUSSION

Here we present a quantitative neuropathological analysis of an extensive MS autopsy cohort of 101 brain donors and 3819 lesions showing that acute axonal damage relating to active inflammatory lesion activity and reduced axonal density associate with increased CSF NfL levels. Our key findings are that 1) CSF NfL levels were negatively correlated with disease duration. 2) CSF NfL levels were positively correlated with increased axonal loss and with acute axonal damage in the normal appearing white matter. 3) CSF NfL levels were positively correlated with proportions of active and mixed lesions containing foamy microglia/macrophages, and negatively correlated with inactive and remyelinating lesion proportions. 4) Active lesions with foamy microglia/macrophages and the border of mixed lesions with foamy microglia/macrophages have a higher amount of APP+ bulbs and axonal fragments compared active and mixed lesions containing ramified microglia, inactive lesions and remyelinated lesions. These findings show that specifically MS lesions containing lipid laden foamy microglia/macrophages are associated with acute axonal damage, and that proportion of such lesions positively correlate with CSF NfL levels. Furthermore, CSF Nfl levels correlate with fast progression of MS. Together, this validates CSF NfL as a quantifiable biomarker for inflammatory white matter lesion activity driven axonal damage and disease progression in MS.

In our cohort, CSF NfL levels were not confounded by donor demographics nor by post-mortem delay or storage time. In line with clinical studies, we found post mortem CSF NfL levels positively correlated with post mortem plasma NfL levels, with the same order of magnitude.[19–21] In contrast to some previous studies, but in line with the clinical cohorts of Kuhle *et al.* (2019), Uher *et al.* (2020) and Bridel *et al.* (2019), we did not observe an effect of age on CSF NfL levels in MS[12,14,18,19,21] Most likely, increases in CSF NfL due to acute axonal damage and demyelinating activity are superimposed on the increases due to ageing in our autopsy cohort.[12] Similar to previous clinical studies, there were no sex-related differences in post-mortem CSF NfL levels.[14,15,19,21] The pH of the CSF, the brain weight and the storage time did not correlate with CSF NfL levels further validating our post mortem sampling method.

We confirmed a history of a recent stroke and a clinically silent stroke as confounding factors when studying MS-specific pathologies in relation to CSF NfL, and these donors were therefore excluded from MS-specific analyses.[30–33] Brain atrophy in MS can also be a source of increases in NfL,[23] but in our study it did not have an effect on CSF NfL levels. Most likely, this is due to the spatial and temporal variations in atrophy that are not taken into account with the yes/

no score used in this study. However, CSF NfL changes due to atrophy, the irreversible neurodegenerative component of MS[34], superimposed on the effect of lesion characteristics on CSF NfL. Therefore, the relation between MS lesion characteristics and CSF NfL levels were analyzed separately in donors with and without atrophy in our study.

CSF and plasma NfL levels have previously been correlated with progression of MS-related disability during life.[11,19,21] Here we show that CSF NfL levels were negatively correlated with the disease duration reflecting a more severe course, but not the age at onset or the disease severity score as calculated with time to EDSS6. This suggests that donors with a shorter disease duration had experienced more neurodegeneration in the year before death compared to donors with a longer disease duration. CSF NfL levels reflect recent acute MS-related axonal damage[15,30], likely reflecting lesion activity causing axonal damage within a period of approximately the previous year and are therefore not likely retrospectively reflecting the speed of disease progression before reaching EDSS6.

In the NAWM, CSF NfL negatively correlated with Bielschowsky+ axons and therefore positively correlated with increased axonal loss. CSF NfL levels were significantly higher in donors with APP+ axons or bulbs in the NAWM compared to donors without APP+ axons or bulbs (P = 1.17e-6). This is most likely due to both Wallerian degeneration and neuro-axonal damage.[35,36]

Previously was shown that APP+ axons and bulbs are more frequently present in the border of mixed lesions than in the center[22,37]. Here, we corroborate these findings, and additionally show in active lesions and the border of mixed lesions with foamy macrophages significantly more APP+ acute axonal damage compared to active lesions and the border of mixed lesions with ramified microglia/macrophages. Therefore, the strong positive correlation between CSF NfL levels and the proportion of active and mixed lesions with foamy microglia/macrophages is most likely due to the increased acute axonal damage in these lesions. The correlation of CSF NfL levels and proportions of (mixed) active MS lesions is in line with radiological studies showing correlations between NfL with MRI biomarkers of inflammatory disease activity in terms of the amount of gadolinium-enhancing lesions and paramagnetic rim MRI lesions. Interestingly, our data suggest that foamy and ramified microglia may have different implications in terms of neuro-axonal damage, likely relating to functional differences between lipid laden foamy versus ramified microglia/macrophages[38].

Our study suggests several important issues about the use of CSF NfL as a biomarker for acute axonal damage in MS relating to lesion activity. Firstly, we show that recent or clinically silent stroke and atrophy influence the relation between NfL CSF levels and MS lesion activity and acute axonal damage. Thus, these are comorbid conditions to use CSF NfL to monitor lesion activity and related axonal damage in MS patients. Sequential NfL measurements within individual patients should solve this problem. Secondly, CSF samples were available of more brain donors than plasma samples and therefore we studied NfL levels in CSF. However, we show that CSF NfL levels and plasma NfL correlate and that plasma NfL levels show a similar trend as CSF NfL levels towards a positive correlation with the MMAS score and the proportion of active lesions with foamy microglia/macrophages. Future studies should attempt to establish the relation of plasma NfL with neuropathological substrates in MS, as measurements of plasma NfL would be easier to apply clinically than of CSF NfL. Lastly, due to a smaller group of donors with atrophy compared to donors without atrophy, it is possible that differences between these two groups are due to a loss of statistical power in the group with atrophy. However, as the relations between CSF NfL and lesion measures of all donors together are less significant than the relations between CSF NfL and lesion measures of only donors without atrophy, this suggests that these groups are fundamentally different.

In summary, in an MS autopsy cohort of 101 cases we show that CSF NfL levels negatively correlate with disease duration and positively correlate neuropathologically with proportions of active and mixed lesions containing foamy microglia/macrophages and acute axonal damage, validating NfL as marker of disease activity.

Data availability

The data presented in this study are available on request from the corresponding author.

Acknowledgements

The authors are grateful to the brain donors with multiple sclerosis who donated their brain for research and to the team of the Netherlands Brain Bank for their excellent service.

Funding

MS Research grant 17-975 and MoveS inspiration Grant 19-1079.

Disclosure

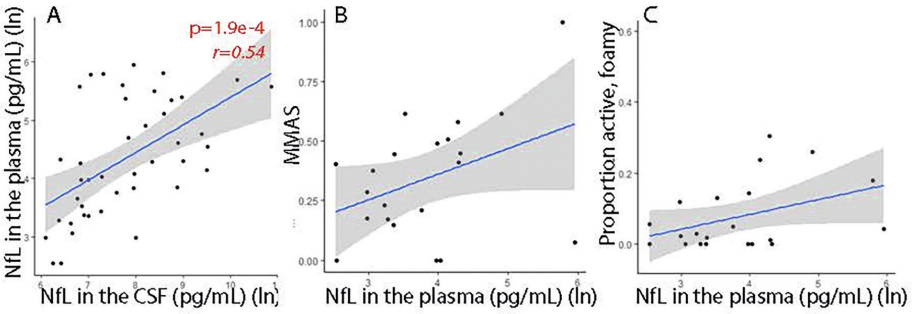
The authors report no disclosures relevant to the manuscript.

REFERENCES

1. Lassmann, H. Multiple sclerosis pathology. *Cold Spring Harb. Perspect. Med.* 8, 1–15 (2018).
2. Lassmann, H., Raine, C. S., Antel, J. & Prineas, J. W. Immunopathology of multiple sclerosis: Report on an international meeting held at the Institute of Neurology of the University of Vienna. *J. Neuroimmunol.* 86, 213–217 (1998).
3. Luchetti, S. *et al.* Progressive multiple sclerosis patients show substantial lesion activity that correlates with clinical disease severity and sex: a retrospective autopsy cohort analysis. *Acta Neuropathol.* 135, 511–528 (2018).
4. Stadelmann, C., Wegner, C. & Bruek, W. Inflammation, demyelination, and degeneration - Recent insights from MS pathology. *Mol. Basis Dis.* 1812, 275–282 (2011).
5. Frischer, J. M. *et al.* Clinical and Pathological Insights into the Dynamic Nature of the White Matter Multiple Sclerosis Plaque. *Ann Neurol* 78, 710–721 (2015).
6. Lucchinetti, C. *et al.* Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann. Neurol.* 47, 707–717 (2000).
7. Yuan, A., Rao, M. V., Veeranna & Nixon, R. A. Neurofilaments at a glance. *J. Cell Sci.* 125, 3257–3263 (2012).
8. Ferreira-atuesta, C., Reyes, S., Giovanonni, G. & Gnanapavan, S. The Evolution of Neurofilament Light Chain in Multiple Sclerosis. *Front. Neurosci.* 15, 1–13 (2021).
9. Khalil, M. *et al.* Neurofilaments as biomarkers in neurological disorders. *Nat. Rev. Neurol.* 14, 577–589 (2018).
10. Ziemssen, T., Akgün, K. & Brück, W. Biomarkers in multiple sclerosis. *J. Neuroinflammation* 9, 1–11 (2019).
11. Varhaug, K. N., Torkildsen, Ø., Myhr, K. M. & Vedeler, C. A. Neurofilament light chain as a biomarker in multiple sclerosis. *Front. Neurol.* 10, 1–6 (2019).
12. Uher, T. *et al.* Neurofilament levels are associated with blood – brain barrier integrity , lymphocyte extravasation , and risk factors following the first demyelinating event in multiple sclerosis. 1–12 doi:10.1177/1352458520912379.
13. Kapoor, R. *et al.* Serum neurofilament light as a biomarker in progressive multiple sclerosis. *Neurology* 95, 436–444 (2020).
14. Bridel, C. *et al.* Diagnostic Value of Cerebrospinal Fluid Neurofilament Light Protein in Neurology: A Systematic Review and Meta-analysis. *JAMA Neurol.* 76, 1035–1048 (2019).
15. Shahim, P. *et al.* Serum neurofilament light protein predicts clinical outcome in traumatic brain injury. *Sci. Rep.* 6, 1–9 (2016).
16. Zetterberg, H. *et al.* Association of Cerebrospinal Fluid Neurofilament Light Concentration With Alzheimer Disease Progression. *JAMA Neurol.* 73, 60–67 (2016).
17. Gafson, A. R. *et al.* Neurofilaments: neurobiological foundations for biomarker applications. *Brain* 143, 1975–1998 (2020).
18. Kuhle, J. *et al.* Blood neurofilament light chain as a biomarker of MS disease activity and treatment response. *Neurology* 92, E1007–E1015 (2019).
19. Piehl, F. *et al.* Plasma neurofilament light chain levels in patients with MS switching from injectable therapies to fingolimod. *Mult. Scler. J.* 1–9 (2017) doi:10.1177/https.
20. Barro, C. *et al.* Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis. *Brain* 141, 2382–2391 (2018).

21. Disanto, G. *et al.* Serum Neurofilament light: A biomarker of neuronal damage in multiple sclerosis. *Ann. Neurol.* 81, 857–870 (2017).
22. Maggi, P. *et al.* Chronic White Matter Inflammation and Serum Neurofilament Levels in Multiple Sclerosis. *Neurology* 0, 10.1212/WNL.0000000000012326 (2021).
23. Kuhle, J. *et al.* Serum neurofilament is associated with progression of brain atrophy and disability in early MS. *Neurology* 88, 826–831 (2017).
24. Kuhle, J. *et al.* Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. *Clin. Chem. Lab. Med.* 54, 1655–1661 (2016).
25. Bridel, C., Verberk, I. M. W., Heijst, J. J. A., Killestein, J. & Teunissen, C. E. Variations in consecutive serum neurofilament light levels in healthy controls and multiple sclerosis patients. *Mult. Scler. Relat. Disord.* 47, 102666 (2020).
26. Kuhlmann, T. *et al.* An updated histological classification system for multiple sclerosis lesions. *Acta Neuropathol.* 133, 13–24 (2017).
27. Van Der Valk, P. & De Groot, C. J. A. Staging of multiple sclerosis (MS) lesions: Pathology of the time frame of MS. *Neuropathol. Appl. Neurobiol.* 26, 2–10 (2000).
28. Lindner, M., Fokuhl, J., Linsmeier, F., Trebst, C. & Stangel, M. Chronic toxic demyelination in the central nervous system leads to axonal damage despite remyelination. *Neurosci. Lett.* 453, 120–125 (2009).
29. Nystad, A. E., Torkildsen, Ø. & Wergeland, S. Effects of vitamin D on axonal damage during de- and remyelination in the cuprizone model. *J. Neuroimmunol.* 321, 61–65 (2018).
30. Gattringer, T. *et al.* Serum neurofilament light is sensitive to active cerebral small vessel disease. *Neurology* 89, 2108–2114 (2017).
31. Lewis, S. B., Wolper, R. A., Miralia, L., Yang, C. & Shaw, G. Detection of phosphorylated NF-H in the cerebrospinal fluid and blood of aneurysmal subarachnoid hemorrhage patients. *J. Cereb. Blood Flow Metab.* 28, 1261–1271 (2008).
32. Zanier, E. R. *et al.* Neurofilament light chain levels in ventricular cerebrospinal fluid following acute aneurysmal sybarachnoid hemorrhage. 8, 157–159 (2011).
33. Uphaus, T. *et al.* NfL (Neurofilament Light Chain) Levels as a Predictive Marker for Long-Term Outcome After Ischemic Stroke. *Stroke* 50, 3077–3084 (2019).
34. Andravizou, A. *et al.* Brain atrophy in multiple sclerosis: mechanisms, clinical relevance and treatment options. *Autoimmun. Highlights* 10, (2019).
35. Allen, I. V., McQuaid, S., Mirakhur, M. & Nevin, G. Pathological abnormalities in the normal-appearing white matter in multiple sclerosis. *Neurol. Sci.* 22, 141–144 (2001).
36. Singh, S. *et al.* Relationship of acute axonal damage, Wallerian degeneration, and clinical disability in multiple sclerosis. *J. Neuroinflammation* 14, 1–15 (2017).
37. Ferguson, B., Matyszak, M. K., Esiri, M. M. & Perry, V. H. Axonal damage in acute multiple sclerosis lesions. *Brain* 120, 393–399 (1997).
38. Zia, S. *et al.* Microglia Diversity in Health and Multiple Sclerosis. *Front. Immunol.* 11, 1–14 (2020).

SUPPLEMENTARY FILES



Supplementary figure 1: Plasma NfL levels correlate with CSF NfL levels. (A) Plasma NfL levels and CSF NfL levels are positively correlated (p=1.9e-4). Plasma NfL levels are not significantly correlated to **(B)** the MMAS score or **(C)** the proportion of active lesions with foamy microglia/macrophages (p=0.07), but show generally similar trends as CSF NfL levels.

Supplementary table 1: Correlations of lesion proportions and NfL levels in the plasma in MS donors without recent or clinically silent stroke, differentiated between donors with and without atrophy

		Donors without recent or clinically silent stroke							
Lesion type	***Subtype***	**All (n=27)**			**With atrophy (n=5)**		**Without atrophy (n=22)**		**With vs without atrophy**
		Total #lesions	Average load / Proportion / score (SD)	P	Average load / Proportion / score (SD)	P	Average load / Proportion / score(SD)	P	P
Lesion load (BRS)		358	0.94 (0.96)	0.38	1.01 (0.90)	0.60	1.00 (1.00)	0.79	**0.04**
Reactive load (BRS)		86	0.34 (0.44)		0.41 (0.47)		0.39 (0.50)		0.22
Active		427	0.22 (0.18)	0.19	0.22 (0.18)	0.58	0.22 (0.17)	0.24	0.75
	Ramified	*180*	0.09 (0.12)	**5.5e-3**	0.10 (0.14)	0.30	0.09 (0.12)	**2.5e-3**	0.89
	Foamy	*127*	0.06 (0.08)	0.13	0.07 (0.08)	0.98	0.08 (0.09)	0.07	0.65
Mixed		537	0.29 (0.23)	0.41	0.32 (0.21)	0.41	0.31 (0.23)	0.94	0.38
	Ramified	*267*	0.15 (0.14)	0.37	0.19 (0.12)	0.99	0.18 (0.16)	0.72	0.09
	Foamy	*98*	0.04 (0.06)	0.24	0.04 (0.05)	0.24	0.04 (0.05)	0.66	0.22
Inactive		460	0.37 (0.25)	0.14	0.34 (0.22)	0.87	0.36 (0.23)	0.18	0.44
Remyelinated		297	0.19 (0.18)	0.87	0.17 (0.13)	0.26	0.19 (0.15)	0.32	0.96
MMAS score		964	0.33 (0.24)	0.18	0.31 (0.23)	0.56	0.33 (0.25)	0.22	0.85