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Association of serum neurofilament light with microglial activation in multiple sclerosis

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ABSTRACT

Background Translocator protein (TSPO)-PET and neurofilament light (NfL) both report on brain pathology, but their potential association has not yet been studied in multiple sclerosis (MS) in vivo. We aimed to evaluate the association between serum NfL (sNfL) and TSPO-PET-measurable microglial activation in the brain of patients with MS.

Methods Microglial activation was detected using PET and the TSPO-binding radioligand [¹¹C]PK11195. Distribution volume ratio (DVR) was used to evaluate specific [¹¹C]PK11195-binding. sNfL levels were measured using single molecule array (Simoa). The associations between [¹¹C]PK11195 DVR and sNfL were evaluated using correlation analyses and false discovery rate (FDR) corrected linear regression modelling.

Results 44 patients with MS (40 relapsing-remitting and 4 secondary progressive) and 24 age-matched and sex-matched healthy controls were included. In the patient group with elevated brain [¹¹C]PK11195 DVR (n=19), increased sNfL associated with higher DVR in the lesion rim (estimate (95% CI) 0.49 (0.15 to 0.83), p(FDR)=0.04) and perilesional normal appearing white matter (0.48 (0.14 to 0.83), p(FDR)=0.04), and with a higher number and larger volume of TSPO-PET-detectable rim-active lesions defined by microglial activation at the plaque edge (0.46 (0.10 to 0.81), p(FDR)=0.04 and 0.50 (0.17 to 0.84), p(FDR)=0.04, respectively). Based on the multivariate stepwise linear regression model, the volume of rim-active lesions was the most relevant factor affecting sNfL.

Conclusions Our demonstration of an association between microglial activation as measured by increased TSPO-PET signal, and elevated sNfL emphasises the significance of smouldering inflammation for progression-promoting pathology in MS and highlights the role of rim-active lesions in promoting neuroaxonal damage.

INTRODUCTION

In multiple sclerosis (MS), neuroaxonal damage is considered the substrate of permanent neurological disability.¹ Neurofilaments are neuronal intermediate filaments that are involved in the growth and stability of axons and also in the maintenance of mitochondrial stability and microtubule content.² After neuronal damage or neurodegeneration, neurofilaments can be released into interstitial tissue and eventually enter blood circulation.² With sensitive detection methods, the serum concentration of released neurofilaments can be reliably

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Postmortem studies have shown that microglial activation associates with progressive disease and axonal damage in multiple sclerosis (MS). Based on previous in vivo studies, microglial activation is a significant predictor of MS disease progression.

WHAT THIS STUDY ADDS

⇒ Our results provide novel in vivo evidence that in MS, activated microglia, especially within the perilesional normal appearing white matter and at chronic lesion rim, contribute to neuroaxonal damage resulting in release of neurofilament light (NfL).

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our results indicate that among patients with MS with no evidence of recent relapse activity or gadolinium enhancing lesions, increased NfL levels are an indication of an ongoing smouldering disease process.

measured. Neurofilament light chain (NfL) has been used as a biomarker of neuroaxonal damage in a variety of chronic neurodegenerative disorders, including MS.

In MS, neuroaxonal damage occurs in focal, acute inflammatory lesions but also more diffusively in the normal-appearing white matter (NAWM). Activated microglia are present throughout the volume of acute inflammatory lesions, in the rim of slowly expanding lesions and also more widely in the NAWM. Activated microglial cells can contribute to axonal damage by secreting proinflammatory cytokines, chemokines and free radicals, and by increasing the release of glutamate.³ The 18 kDa translocator protein (TSPO) is expressed on the mitochondrial membranes of activated innate immune cells. In MS, positron emission tomography (PET) imaging using the first-generation TSPO-binding radioligand [¹¹C]PK11195 has been widely used to detect the activation of brain innate immune cells in vivo.⁴ Specific [¹¹C]PK11195 binding is increased in the NAWM of patients with MS compared with the white matter of healthy individuals.^{5,6} Increased binding in the NAWM is associated with current higher disability scores and progressive MS,^{7–9} and is also predictive for future disability progression.⁶

The primary aim of this study was to investigate the association between serum NfL (sNfL) levels and TSPO-PET detectable microglial activation in the white matter of patients with MS. As a secondary aim, we evaluated the association of sNfL with different types of chronic T1 lesions, that is, inactive, overall-active and rim-active, determined based on TSPO-PET measurable innate immune cell activation within and surrounding lesions.¹⁰

MATERIALS AND METHODS

Study subjects

Study patients have been recruited between 2016 and 2019 from the outpatient clinic of the Division of Clinical Neurosciences at the University Hospital Turku, Turku, Finland based on MS diagnosis according to current diagnostic criteria and willingness to participate in a PET study. The patients have undergone [¹¹C]PK11195 PET imaging to detect brain innate immune cell activation, MRI to provide anatomical reference and to evaluate pathology related to MS, and clinical assessment by an experienced clinician to evaluate Expanded Disability Status Scale (EDSS) score using a standardised examination form (neurostatus.net).

For this study, the inclusion criteria for patients with MS were a blood sample obtained within 180 days of PET imaging. The exclusion criteria was a clinical relapse within 120 days before blood sampling and/or gadolinium-enhancing lesions. Healthy controls (HCs) with no known neurological symptoms or diseases were selected to match the age and sex of included patients.

MRI acquisition and creation of region of interest masks

Brain MRI scans of patients with MS were performed at Turku PET Centre with a 3 T Ingenuity TF PET/MR System scanner (Philips Healthcare, Cleveland, Ohio, USA). Axial T2, three-dimensional (3D) fluid-attenuated inversion recovery (FLAIR), 3D T1 and gadolinium-enhanced 3DT1 sequences with a spatial resolution of 1×1×1 mm were acquired. An eight-channel SENSE head coil was used. The details of the MRI protocol have been previously described.⁸ Brain MRI scans of HCs were performed in Turku, Finland either with a Gyroscan Intera 1.5 T Nova Dual scanner (n=8), 3 T Ingenuity TF PET/MR System scanner (n=10) or 3 T Ingenia (n=6) scanner (Philips Healthcare).

The detailed description of the semiautomated method that was used to create a combined T2 lesion region of interest (ROI) and a combined T1 lesion ROI mask images has been described previously.¹⁰ In brief, the Lesion Segmentation Tool (LST, www.statistical-modelling.de/lst.html, a toolbox running in SPM8) was used for the initial identification of T2 lesions from FLAIR images. T1 hypointense lesions were recognised visually on 3D T1 images with help of LST-identified T2 lesions. T1 lesion ROI mask images were manually shaped slice by slice using Carimas (<https://turkupetcentre.fi/carimas/>). Freesurfer V5.3 software (<http://surfer.nmr.mgh.harvard.edu/>) was used for creating binary brain ROI and for segmenting grey matter and white matter. The brain ROI includes the cerebrum, cerebellum and brain stem, but excludes ventricles. The 0–2 mm lesion rim ROI was created by dilating the T1 lesion ROI mask image by 2 voxels, after which the T1 lesion ROI was removed. Similarly, the 2–6 mm perilesional NAWM ROI was created by dilating the T1 lesion ROI by 6 voxels, after which both T1 lesion and 0–2 mm lesion rim ROIs were removed. The NAWM ROI was created by removing the combined T1 lesion mask, 0–2 mm

lesion rim mask, brain stem and cerebellar white matter from the white matter ROI. Examples of different ROI masks are illustrated in online supplemental eFigure 1. NAWM, cortical grey matter and total T1 lesion volumes (cm³) were acquired with Freesurfer software as previously described.⁷

PET acquisition and analysis

PET scans of patients with MS and HCs were performed with a brain-dedicated ECAT HRRT scanner (CTI/Siemens) as previously described.¹⁰ The intrinsic spatial resolution of the scanner is 2.5 mm. The [¹¹C]PK11195-radioligand was synthesised according to our previously described methodology.¹¹ The mean (SD) injected dose of [¹¹C]PK11195 was 488 (14.8) MBq for patients with MS and 489 (16.5) MBq for HCs (p=0.9).

PET images were reconstructed, smoothed, and coregistered as previously described.^{6,11} To evaluate specific binding of [¹¹C]PK11195 indicating innate immune cell activation, distribution volume ratio (DVR) was calculated in prespecified region of interests (ROIs) (NAWM, T1 lesion, 0–2 mm lesion rim, perilesional NAWM and brain). The [¹¹C]PK11195 DVR was determined using a supervised cluster algorithm (SuperPK software, SVCA4 classification).^{12,13}

Categorisation of individual lesions

T1 hypointense lesions were categorised into three subtypes (rim-active, overall-active and inactive) based on the proportion of active voxels in the lesion and at the rim as previously described.¹⁰ Briefly, in rim-active lesions the proportion of active voxels was considerably higher at the lesion edge. Overall-active lesions have considerable binding both in the lesion and at the edge. Only T1 lesions larger than 27 mm³ within cerebral white matter were included in the categorisation.

Measurement of sNfL chain

Blood was collected in 10 mL Vacuette serum clot-activator tubes (Greiner Bio-one, product number 455092) before 12 AM. Blood was allowed to clot for 30 min at room temperature and serum was stored in aliquots at –80°C in the Auria Biobank (Turku, Finland) within 2 hours of sampling. Frozen samples were shipped on dry ice to Basel, Switzerland, where sNfL concentrations were measured by a single molecule array assay (Simoa Technology).^{14,15}

Statistical analysis

The statistical analyses were performed using R (V4.1.1). Continuous variables are presented as median with IQR unless stated otherwise. The normality of variables was checked with Shapiro-Wilk's test. The 80th percentile of the HC brain DVR was used as a cut-off value to define patients with normal or increased microglial activation indicated as brain DVR(low) and brain DVR(high) subgroups. Wilcoxon rank-sum (Mann-Whitney U) test was used to compare patients with MS with HCs and brain DVR(low) subgroup with brain DVR(high) subgroup. Pearson correlation analysis was used to evaluate correlations of brain DVR with DVRs within other studied ROIs.

To test our main hypothesis, we calculated Pearson correlation coefficients (r) between the logarithm of sNfL levels and DVR values (normal distribution) in four prespecified ROIs. To evaluate the secondary aim of the study, Spearman correlation coefficients (ρ) between sNfL and lesion-type-related parameters were calculated. Spearman correlation was used due to the non-normal distributions of both sNfL and lesion-type parameters.

Multiple sclerosis

Linear regression modelling was used to further analyse associations between sNfL and demographical, clinical, volumetric and TSPO-PET-related parameters. First, univariate linear regression models with continuous and categorical variables were performed. The p values of univariate models were corrected using false discovery rate (FDR) method for the number of investigated variables (n=21). Then, to identify the most relevant factors, multivariate stepwise regression modelling was performed. The building of the model started with no predictors. All variables used in univariate models were considered when the multivariate model was built. New predictors were added one by one, if the Bayesian information criterion (BIC) value was lower than in the simpler model. The variable leading to the lowest BIC value was always chosen. The logarithm of sNfL was used as the response because non-transformed values led to non-normality of residuals. Similarly, in model-building, logarithm of lesion numbers and lesion volumes were used as predictors. To each value representing the number of

lesions, +1 was added and to each value representing the lesion volume +0.01 was added before logarithmic transformation to avoid a logarithm of 0, which cannot be calculated. Square root transformation was used for time since last relapse.

All tests were two tailed, and a p<0.05 was considered statistically significant for all analyses.

RESULTS

Characteristics of the study cohort

A total of 44 patients with MS and 24 age-matched and sex-matched HCs were included in the study. Most patients had relapsing-remitting disease and had either been on same disease-modifying treatment (DMT) or untreated on average 1337 days before study onset. The clinical and radiological characteristics of the study patients are presented in [table 1](#). The median time difference between imaging and sampling was 12 days (IQR 0–37, range 0–155). For 12 patients (27 %) the PET imaging

Table 1 Demographic, clinical and brain MRI volumetric characteristics of study subjects

	HC	MS*	Brain DVR(low)	Brain DVR(high)	P value†
n	24	44	25	19	
Sex, n (%)					0.16
Female	17 (70)	33 (75)	21 (84)	12 (63)	
Male	7 (30)	11 (25)	4 (16)	7 (37)	
Age	43.6 (35.4–46.9)	46.5 (42.4–50.9)	44.9 (40.7–50.7)	48.0 (43.7–51.3)	0.2
BMI‡	24.4 (22.4–26.5)	25.7 (22.7–30.0)	25.7 (22.9–33.2)	25.6 (21.7–28.9)	0.2
Disease type, n (%)					0.3
RRMS		40 (91)	24 (96)	16 (84)	
SPMS		4 (9)	1 (4)	3 (16)	
Duration, years§		13.3 (9.1–15.3)	12.6 (8.7–16.4)	13.4 (9.8–14.6)	0.9
EDSS		2.5 (2.0–3.0)	2.5 (1.5–3.0)	3.0 (2.5–3.75)	0.047
MSSS		3.0 (1.5–4.3)	2.9 (1.4–3.9)	3.05 (2.0–4.7)	0.097
Relapses¶		3.5 (2–6)	3 (2–5)	6 (2.5–6)	0.18
ARR		0.30 (0.21–0.43)	0.25 (0.20–0.32)	0.37 (0.30–0.44)	0.004
Time since last relapse, years**		6.0 (1.9–9.5)	6.7 (2.3–10.8)	3.7 (1.9–7.4)	0.3
Treatment, n (%)††					0.042‡‡
Untreated		12 (27)	10 (40)	2 (11)	
Teriflunomide§§		13 (30)	8 (32)	5 (26)	
Interferon-β		6 (14)	3 (12)	3 (16)	
Fingolimod		4 (9)	2 (8)	2 (11)	
Glatiramer acetate		4 (9)	1 (4)	3 (16)	
Dimethylfumarate		3 (7)	0 (0)	3 (16)	
Natalizumab		2 (5)	1 (4)	1 (5)	
Volumes, cm ³					
NAWM	489 (459–524)	459 (416–493)	456 (435–492)	462 (405–502)	0.8
GM cortex	466 (421–485)	431 (417–467)	431 (413–465)	429 (419–461)	0.9
T1 hypo lesion load		2.3 (1.2–4.8)	1.9 (1.1–2.8)	3.1 (1.7–11.7)	0.019

Values are expressed as median (IQR) unless specified otherwise. Patients with MS were divided into brain DVR(low) and brain DVR(high) subgroups based on the 80th percentile of healthy controls brain DVR.

*From comparison of HC and MS p=0.8 for sex, p=0.07 for age, p=0.2 for BMI, p=0.04 for NAWM volume and p=0.1 for GM cortex volume.

†From comparison of brain DVR(high) and brain DVR(low) subgroups with Fisher's test (categorical variables) or Wilcoxon (continuous variables), significant p-values are bolded.

‡One missing value in HC group and in MS group (BMI).

§From onset of symptoms.

¶Total number of relapses before study onset.

**One missing value in brain DVR(low) group.

††Ongoing at least 120 days before sampling unless specified otherwise.

‡‡Treated versus untreated.

§§Two patients had started teriflunomide within 120 days before blood sampling (previous treatment was IFN-β-1a).

ARR, annualised relapse rate; BMI, body mass index; DVR, distribution volume ratio; EDSS, Expanded Disease Status Scale; GM, grey matter; HC, healthy control; MS, multiple sclerosis; MSSS, Multiple Sclerosis Status Scale; NAWM, normal appearing white matter; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis.

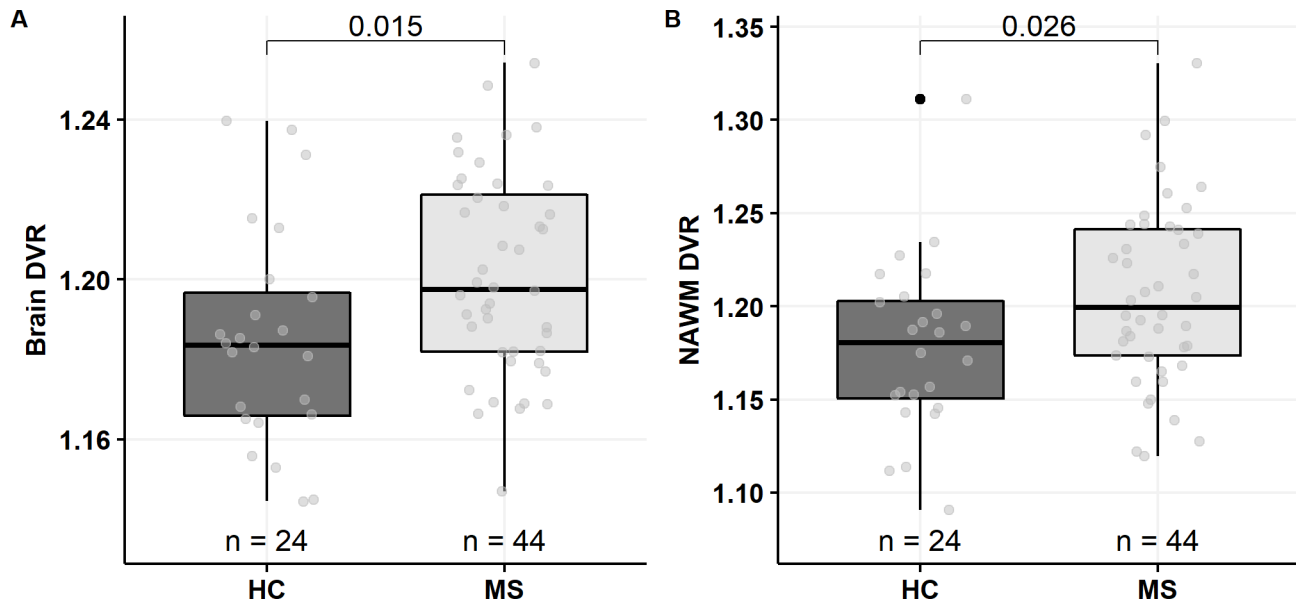


Figure 1 [^{11}C]PK11195 DVR values of patients with MS and HCs in the whole brain and normal appearing white matter. The [^{11}C]PK11195 DVR values reflecting innate immune cell activation were increased in the whole brain (A) and in the NAWM (B) of patients with MS compared with HCs. Wilcoxon rank-sum test was used for statistical analyses. In boxplots, the thick horizontal lines represent the medians, the boxes represent the IQRs and the end of the whiskers represent the minimum and maximum values, if outliers represented as black dots do not exist. DVR, distribution volume ratio; HC, healthy control; MS, multiple sclerosis; NAWM, normal appearing white matter.

and blood sampling were obtained on the same day, and for 36 patients (81 %) within 60 days. The median (IQR) sNfL level of patients with MS was 19.0 (9.1–24.3) pg/mL.

[^{11}C]PK11195-binding in the whole brain was increased in patients with MS compared with HC (1.20 vs 1.18, $p=0.015$, [figure 1](#)). Radioligand binding was also higher in the NAWM of patients with MS compared with the white matter of HCs (1.20 vs 1.18, $p=0.026$). Brain DVR values correlated with DVR values in the NAWM ($r=0.75$, $p<0.001$), lesion rim and perilesional NAWM (online supplemental eFigure 2).

Based on the brain DVR cut-off value of 1.205, nearly half of the patients (43 %, $n=19$, brain DVR(high) subgroup) had increased microglial activation in the brain compared with HC ($p<0.001$). There was no difference in the brain DVR between the brain DVR(low) subgroup and HC ($p=0.8$). When brain DVR(high) and brain DVR(low) groups were compared, it was noticed that, despite similar age and disease duration, brain DVR(high) patients had higher EDSS and annualised relapse rate ($p=0.047$ and $p=0.004$, respectively), and larger T1 lesion volume ($p=0.019$) ([table 1](#)). In addition, the number, volume and proportion of rim-active lesions were higher in the brain (DVR)high patients, whereas the proportion of inactive lesions was smaller ([table 2](#)). Similarly, the number of overall-active lesions was higher in the brain (DVR)high group. There was no statistically significant difference in the median sNfL level between the brain (DVR)low and brain (DVR)high groups (20.2 (12.1–24.4) vs 19 (15.1–23.5) pg/mL, $p=0.7$, respectively).

Values are expressed as median (IQR). Shown are p values from Wilcoxon test. T1 hypo intense lesions were categorised into three subtypes (rim-active lesions, overall-active lesions and inactive lesions) based on the proportion of active voxels in the lesion and at the rim as previously described.¹⁰ Only T1 lesions larger than 27 mm³ within cerebral white matter were included in the categorisation.

Association between sNfL and brain innate immune cell activation

Among the brain (DVR)high patients, higher sNfL levels correlated with increased [^{11}C]PK11195-DVR in NAWM ($r=0.48$, $p=0.036$) and perilesional NAWM ($r=0.58$, $p=0.009$), at the lesion rim ($r=0.59$, $p=0.008$) and in T1 hypointensive lesions ($r=0.49$, $p=0.034$) ([figure 2](#)). All correlations, except T1 DVR, were sustained in partial age-corrected correlation analyses (data not shown). Among the brain (DVR)low patients or all studied patients with MS, no correlations were observed (data not shown).

Association between sNfL and different lesion subtypes

Among the brain (DVR)high patients, increased sNfL levels correlated with a higher total number of T1 lesions ($p=0.5$, $p=0.031$, data not shown). When various subtypes of chronic

Table 2 Results of T1 lesion characterisation in brain (DVR)low and brain (DVR)high subgroups of patients with MS

	Brain DVR(low)	Brain DVR(high)	P value*
No of all T1 lesions	8 (2–18)	18 (10–30)	0.014
No of rim-active lesions	0 (0–2)	3 (1–5)	<0.001
Volume of rim-active lesions	0.0 (0.0–0.1)	0.2 (0.08–0.51)	<0.001
% of rim-active lesions	0.0 (0–9.5)	19.4 (12.7–23.2)	<0.001
No of inactive lesions	6 (0–7)	6 (3.5–8)	0.66
Volume of inactive lesions	0.4 (0–0.7)	0.4 (0.2–0.8)	0.71
% of inactive lesions	51.5 (0–71.4)	28.6 (19–43)	0.005
No of overall-active lesions	4 (1–7)	8 (3.5–20.5)	0.023
Volume of overall-active lesions	0.7 (0.2–1.4)	1.6 (0.3–7.4)	0.061
% of overall-active lesions	41.2 (25–52.4)	52 (33.9–61.3)	0.16

*Significant p -values are bolded.
DVR, distribution volume ratio; MS, multiple sclerosis.

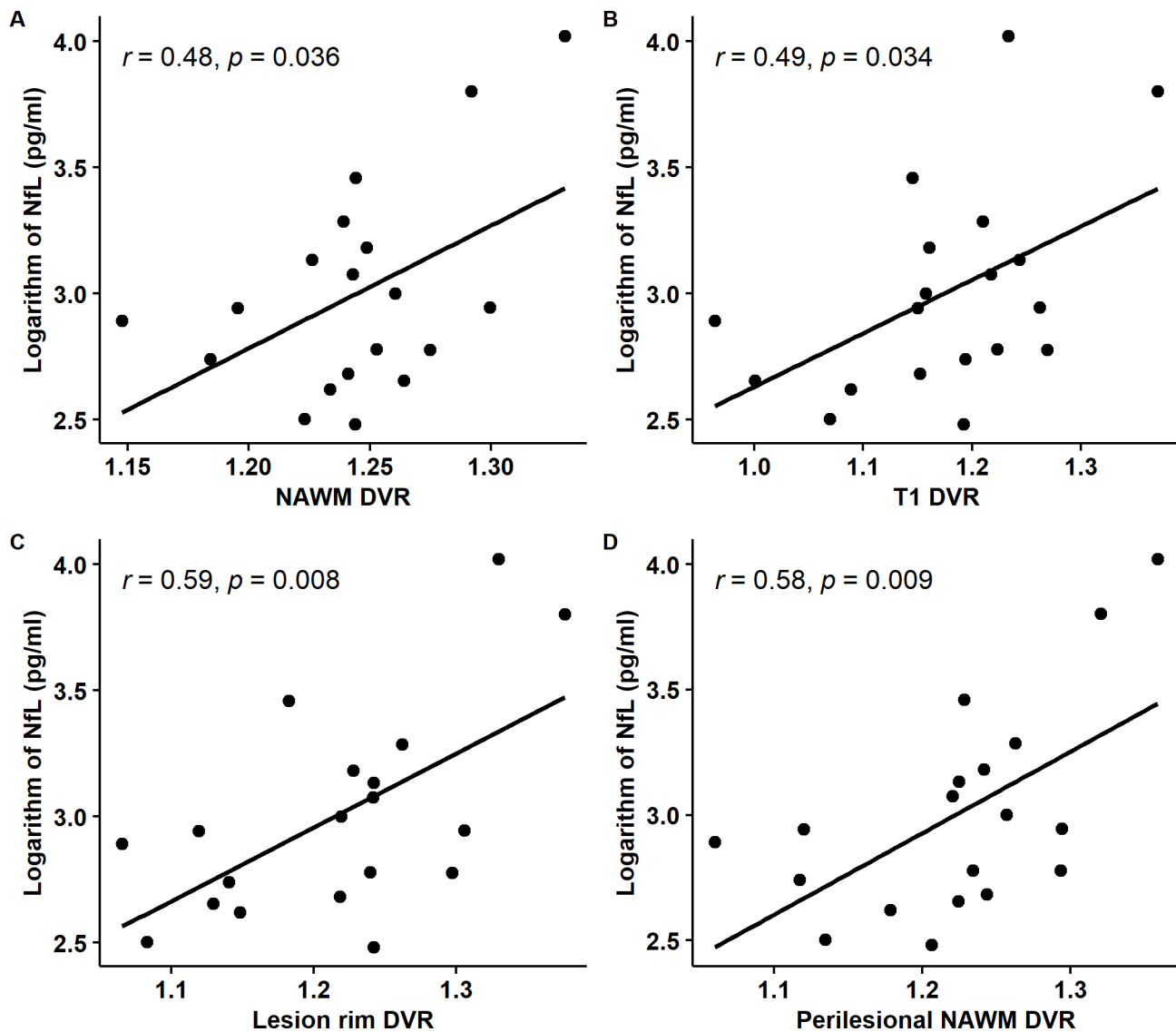


Figure 2 Pearson correlations between serum NfL and [¹¹C]PK11195 DVR values of the cerebral white matter regions of interest. Among patients with MS having increased innate immune cell activation within the brain (n=19) increased sNfL levels correlated with higher [¹¹C]PK11195 DVR values in the whole NAWM (A), in T1 hypo lesions (B), at the lesion rim (C) and in the perilesional NAWM (D). Logarithm of sNfL was used in order to achieve normally distributed data. DVR, distribution vol ratio (represents specific binding of [¹¹C]PK11195); MS, multiple sclerosis; NAWM, normal appearing white matter; NfL, neurofilament light; r, Pearson correlation coefficient; sNfL, serum NfL.

lesions were evaluated, it was noticed that increased sNfL correlated with a larger proportion of lesions containing activated microglia and a smaller proportion of inactive lesions ($\rho=0.64$, $p=0.003$ and $\rho=-0.64$, $p=0.003$, respectively, online supplemental eFigure 3). In addition, sNfL correlated with both the number and volume of lesions containing activated microglia ($\rho=0.6$, $p=0.006$ and $\rho=0.57$, $p=0.012$, respectively, online supplemental eFigure 4). In contrast, there were no correlations between sNfL and the number or volume of inactive lesions ($\rho=0.12$, $p=0.63$ and $\rho=-0.028$, $p=0.91$, data not shown).

Lesions containing activated microglia were further divided into rim-active and overall-active lesions, according to Nylund *et al.*¹⁰ Higher sNfL levels correlated with the number and volume of rim-active lesions ($\rho=0.46$, $p=0.045$ and $\rho=0.62$, $p=0.005$, respectively, figure 3). Similar correlations were observed between sNfL and the number and volume of overall-active lesions ($\rho=0.6$, $p=0.006$ and $\rho=0.58$, $p=0.01$, respectively,

figure 3). The significance of these correlations was sustained in age-corrected partial correlation analysis (data not shown).

Among all studied patients with MS increased sNfL correlated with larger volume of overall-active lesions and active lesions, whereas among the brain (DVR)low patients no correlations between sNfL and lesion subtype parameters were observed (data not shown).

Exploration of factors having an impact on sNfL using linear regression modelling

In an FDR corrected univariate linear regression model, increased sNfL associated with higher DVR in the lesion rim (estimate (95% CI) 0.49 (0.15 to 0.83), $p(\text{FDR})=0.04$) and perilesional NAWM (0.48 (0.14 to 0.83), $p(\text{FDR})=0.04$), with a higher number of rim-active and overall-active lesions (0.46 (0.10 to 0.81), $p(\text{FDR})=0.04$) and 0.47 (0.12 to 0.82), $p(\text{FDR})=0.04$), and with larger volume of rim-active lesions (0.50 (0.17 to

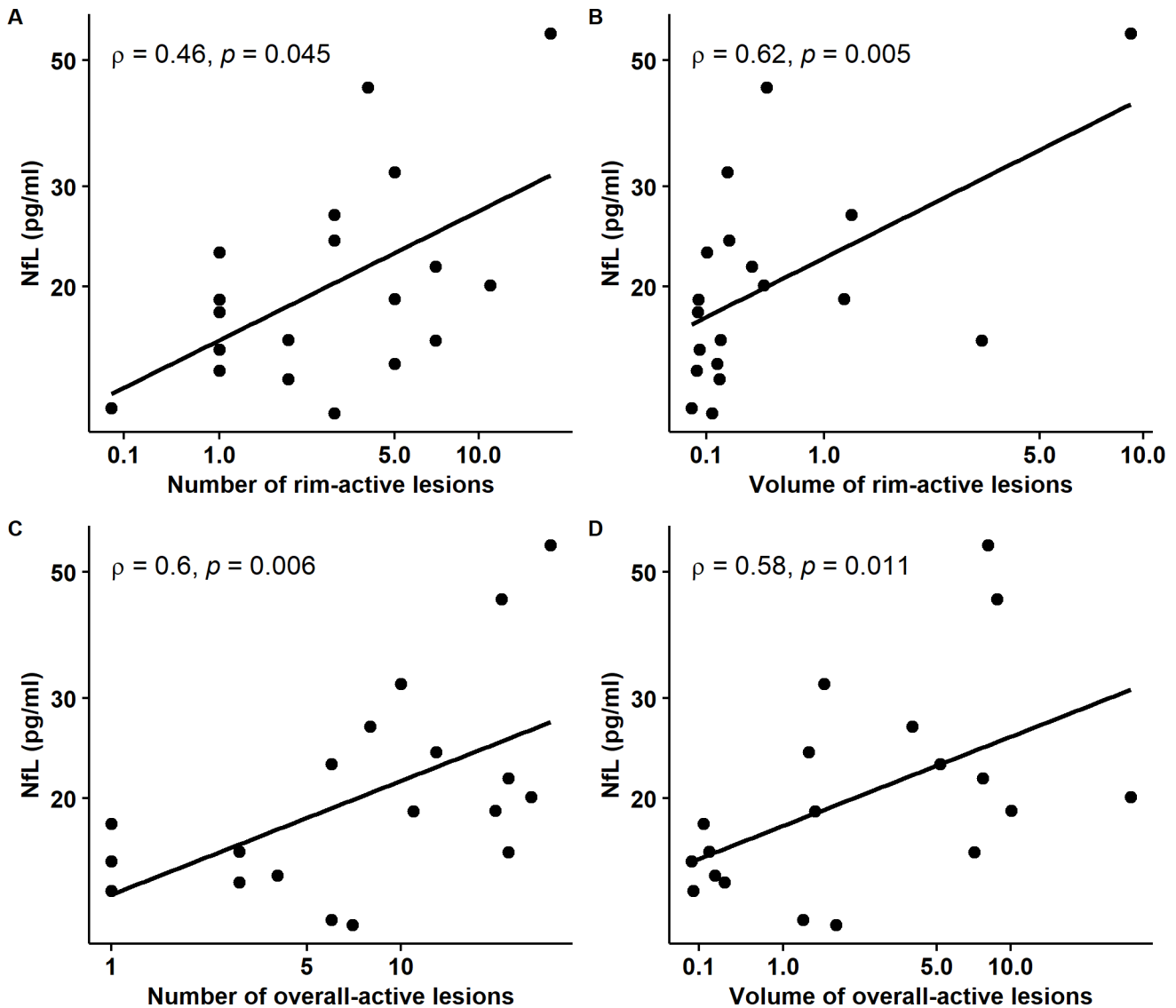


Figure 3 Spearman correlations between serum NfL and number and volume of active lesions. Among brain DVR(high) patients increased sNfL levels correlated with higher number and larger volume of rim-active lesions (A, B). Similarly, increased NfL correlated with higher number and larger volume of overall-active lesions (C, D). DVR, distribution vol ratio (represents specific binding of [^{11}C]PK11195); NAWM, normal appearing white matter; NfL, neurofilament light; ρ , Spearman correlation coefficient; sNfL, serum NfL.

0.84), $p(\text{FDR})=0.04$ (figure 4A, online supplemental eTable 1). The volume of rim-active lesions accounted for 37 %, and [^{11}C]PK11195-DVR at the lesion rim for 35% of the variance in sNfL. In addition, increased sNfL associated with larger T1 lesion load.

To identify the most relevant factor(s) affecting sNfL, all studied parameters were considered in building the multivariate stepwise model. The final model included the volume of rim-active lesions and DMT status at the time of study onset (figure 4C, online supplemental eTable 1). The final model explained 53% of the variance in sNfL.

DISCUSSION

This TSPO-PET study indicates an association between increased brain innate immune cell activation in patients with MS and sNfL, a biomarker of neuroaxonal damage. The strongest associations were observed between microglial activation at the rim of chronic T1 hypointense lesions and in the perilesional NAWM and sNfL. NfL is now considered the most promising biomarker candidate

for nervous system damage-related pathology in MS.¹⁶ Most pronounced changes in NfL levels are seen in association with focal inflammatory lesion-induced axonal damage.^{17 18} More modest, but significant alterations are seen in the context of smouldering disease and MS disease progression.^{14 19 20} Our study cohort had no concurrent gadolinium-enhancing lesions or recent relapses. Hence, the increases in sNfL concentration observed in this cohort are most likely associated with the smouldering disease process. However, we cannot entirely rule out the effect of possible new or enlarging T2-lesions on sNfL, as relevant longitudinal MRI had been performed only for a small subcohort of patients. TSPO-PET, on the other hand, can be used to quantitate innate immune cell activation in vivo, both in connection with chronic lesions and in the NAWM,¹¹ and TSPO-PET measurable microglial activation promotes progression independent of relapses.⁶ Thereby, the association of elevated sNfL concentrations with increased [^{11}C]PK11195-DVR both in the perilesional NAWM and in association with chronic lesions supports the detrimental role of the TSPO-PET-measurable innate immune cell activation.

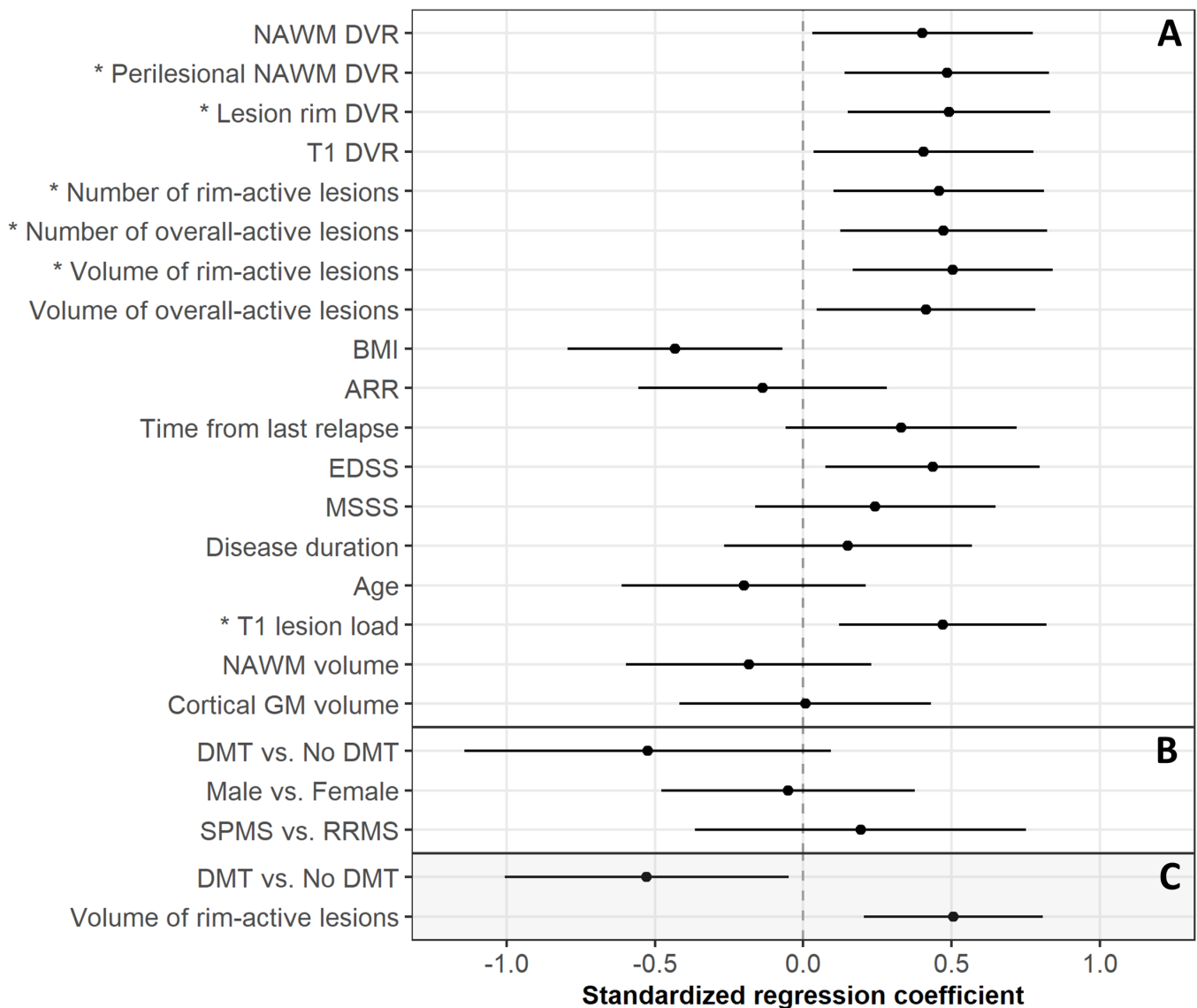


Figure 4 Forest plot illustrating the results of the linear regression modelling of sNFL with TSPO-PET related, demographical and clinical parameters. (A) Univariate linear regression models with continuous variables. The p values of univariate models were corrected using false discovery rate (FDR) method for the number of investigated variables ($n=21$). After FDR correction serum NFL associated with most of the studied TSPO-PET-related parameters, that is, perilesional NAWM and lesion rim DVRs, number of rim-active and overall-active lesions, and volume of rim-active lesions. Of the nine studied demographical or clinical parameters, NFL associated with T1 lesion load. *Denotes associations that remained significant in FDR correction. (B) Univariate linear regression models with categorical variables. NFL did not associate with DMT status at the time of study onset, sex or MS disease type. (C) Multivariate stepwise linear regression model. The model building started with a model without any predictors, and in each step the most suitable variable according to Bayesian information criterion was added to the model. All variables that were used in univariate models were considered, when multivariate model was build. The final model included the volume of rim-active lesions and DMT status. Results among brain DVR(high) patients ($n=19$) are shown. Dots represent standardised regression coefficients and lines represent the confidence intervals of these estimates. Logarithm of NFL was used as a response in all models as non-transformed values led to non-normality of residuals. Similarly, in model-building, logarithm of lesion numbers and lesion volumes were used as predictors. To each value representing the number of lesions, +1 was added and to each value representing the lesion volume +0.01 was added before logarithmic transformation to avoid a logarithm of 0, which cannot be calculated. ARR, annualised relapse rate between disease and study onset; BMI, body mass index; DMT, disease-modifying treatment; DVR, distribution volume ratio (represents specific binding of [^{11}C]PK11195); EDSS, expanded disability status scale; GM, grey matter; MSSS, Multiple Sclerosis Status Scale; NAWM, normal appearing white matter; PET, positron emission tomography; RRMS, relapsing remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; TPSO, translocator protein.

Based on postmortem MS studies, microglia in the NAWM have an activated morphology,⁴ and the number of microglia expressing the homeostatic marker P2RY12 is reduced compared with healthy individuals.²¹ In several neurodegenerative conditions, microglia express proinflammatory markers such as iNOS and p22phox, whereby they are capable of promoting neuro-axonal damage.^{21 22} Accordingly, our previous work showed

an association between [^{11}C]PK11195-DVR and widespread DTI-measurable structural abnormalities in the NAWM.⁸ This study demonstrated an association between sNFL and [^{11}C]PK11195-DVR in the perilesional NAWM areas, supporting the concept of stronger microglial activation in proximity of lesions²³ and perilesional NAWM with reduced myelin and axonal content, as compared with NAWM distant to lesions.²⁴

Chronic active or smouldering lesions are associated with MS progression. They can be detected in susceptibility MRI based on paramagnetic rims of iron-laden, activated microglia and macrophages with a proinflammatory phenotype.²⁵ TSPO-PET has similarly been used to phenotype chronic MS lesions to rim-active or inactive lesions.^{10 26} It is particularly the smouldering lesions, which are frequently associated with signs of axonal damage and demyelination,²⁷ and higher sNfL levels or age-adjusted percentiles in patients with MS are associated with the presence of iron rim lesions.^{28–30} NfL seems to be increased especially in patients having two or more paramagnetic rim lesions,^{29 30} and a recent autopsy-based study demonstrated an association between higher CSF NfL levels and larger proportions of active and mixed lesions containing foamy microglia/macrophages associating with acute axonal damage.³¹

In this study, we used an automated TSPO-PET-based method to quantitate the number of rim-active lesions.¹⁰ Our results demonstrated that increased sNfL is associated with both a higher number and larger volume of rim-active lesions. In addition, of all studied PET-related variables, the volume of rim-active lesions was the strongest factor affecting sNfL levels. It explained over 50% of the variance in logNfL, even when the DMT status was taken into account. These results corroborate the tissue-destructive nature of the rim-active lesions identified using TSPO-PET, consistent with the previously reported correlation of rim-active lesion load to brain atrophy measures in MRI.¹⁰ However, the association of sNfL with volume of overall-active lesions suggests that microglial activation both inside the lesions and/or in the adjacent area contribute to neuroaxonal damage. Not surprisingly, it has also been demonstrated that axonal injury within chronic focal lesions correlates with perilesional axonal injury.²⁴

Few studies have evaluated the association between NfL and soluble markers of innate immune cell activation in patients with MS.^{30 32 33} The results from these studies have been somewhat inconsistent, which could reflect the heterogeneity of innate immune cell phenotypes between different disease stages. Compared with studies using soluble CSF biomarkers to evaluate brain innate immune cell activation, the advantage of PET is the ability to not only quantify potential markers associating with innate immune cell activation but also localise the inflammatory process in the brain *in situ*. Our results show that higher sNfL levels associates with increased [¹¹C]PK11195 DVR, especially in the perilesional NAWM and at the lesion rim, where about 95% of TSPO-expressing cells are microglia or macrophages.³⁴ In patients with traumatic brain injury, increased plasma NfL correlated with higher [¹¹C]PBR28 (a second-generation TSPO-ligand) uptake.³⁵ On the other hand, in patients with early and prodromal Alzheimer's disease, elevated plasma NfL levels correlated with decreased cortical [¹¹C]PK11195 binding potential.³⁶

The median sNfL levels in our study cohort were comparable to levels obtained using Simoa NfL assay developed by Disanto *et al.*¹⁴ However, to our surprise, there was no difference in the median sNfL between brain DVR(low) and brain DVR(high) subgroups. This could be related to non-MS-related factors affecting sNfL, such as vascular risk factors and renal function,¹⁶ or MS-related activity not reflected by brain PET and MRI, such as spinal lesions. The lack of correlation between [¹¹C]PK11195 DVR and NfL among patients with unaltered microglial activation compared with controls could be related to relatively poor signal-to-noise ratio of the used ligand.

This study has some limitations that need to be taken into consideration when interpreting the results. The binding affinity

of [¹¹C]PK11195 is not optimal. However, based on our long-standing experience, [¹¹C]PK11195 can be reliably used with well-validated post-processing and image analysis pipeline.¹² The allowed time window of 180 days between blood sampling and PET was not ideal, and could have affected our results, although others have used a similar time window.²⁹ We used absolute NfL values instead of age-normalised and BMI normalised Z-scores of NfL, which can be considered a weakness. As TSPO is expressed also on a small proportion of astrocytes,³⁷ we intend next to evaluate the association between TSPO-PET and an astrocytic biomarker glial fibrillar acidic protein, that was recently shown to associate with progression independent of relapses.³⁸

In conclusion, our results provide *in vivo* evidence that in MS, activated microglia, especially within perilesional NAWM and chronic lesion rim contribute to neuroaxonal damage resulting in release of NfL. This emphasises the link between neuronal damage and microglial activation in MS. Our results also highlight the role of rim-active lesions in promoting neuronal damage and support the concept of early use of highly efficacious therapy to achieve complete suppression of focal lesional disease activity.³⁹

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