Original research

Serum biomarker levels predict disability progression in patients with primary progressive multiple sclerosis

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ABSTRACT

Background We aimed to investigate the potential of serum biomarker levels to predict disability progression in a multicentric real-world cohort of patients with primary progressive multiple sclerosis (PPMS).

Methods A total of 141 patients with PPMS from 18 European MS centres were included. Disability progression was investigated using change in Expanded Disability Status Scale (EDSS) score over three time intervals: baseline to 2 years, 6 years and to the last follow-up. Serum levels of neurofilament light chain (sNfL), glial fibrillar acidic protein (sGFAP) and chitinase 3-like 1 (sCHI3L1) were measured using single-molecule array assays at baseline. Correlations between biomarker levels, and between biomarkers and age were quantified using Spearman's r. Univariable and multivariable linear models were performed to assess associations between biomarker levels and EDSS change over the different time periods.

Results Median (IQR) age of patients was 52.9 (46.4–58.5) years, and 58 (41.1%) were men. Median follow-up time was 9.1 (7.0–12.6) years. Only 8 (5.7%) patients received treatment during follow-up. sNfL and sGFAP levels were moderately correlated (r=0.43) and both weakly correlated with sCHI3L1 levels (r=0.19 and r=0.17, respectively). In multivariable analyses, levels of the three biomarkers were associated with EDSS changes across all time periods. However, when analysis was restricted to non-inflammatory patients according to clinical and radiological parameters (n=64), only sCHI3L1 levels remained associated with future EDSS change.

Conclusions Levels of sNfL, sGFAP and sCHI3L1 are prognostic biomarkers associated with disability progression in patients with PPMS, being CHI3L1 findings less dependent on the inflammatory component associated with disease progression.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Neurofilament light chain (NfL), glial fibrillar acidic protein (GFAP) and chitinase 3-like 1 (CHI3L1) are known prognostic biomarkers in patients with relapsing forms of multiple sclerosis. However, their role in patients with progressive multiple sclerosis has been far less explored.

WHAT THIS STUDY ADDS

⇒ The present study provides for the first time important information on the potential for serum levels of NfL, GFAP and CHI3L1 to predict disability progression in a multicentric real-world cohort of patients with primary progressive multiple sclerosis (PPMS).

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The study paves a way for determining serum levels of NfL, GFAP and CHI3L1 in patients with PPMS in order to predict disability progression.

INTRODUCTION

Substantial progress has been made in recent years in the identification and characterisation of prognostic body fluid biomarkers in patients with multiple sclerosis (MS).[1] Despite numerous studies in the field, there is still a lack of sensitive and specific biomarkers for patients with progressive MS. In a recent study conducted at the Centre d'Esclerosi Múltiple de Catalunya (Cemcat; Barcelona, Spain),[2] we investigated the potential for blood neurofilament light chain (NfL) levels to predict disability progression in a well-characterised unicentric cohort of 51 patients with progressive MS who participated in a phase II randomised, double-blind, placebo-controlled trial of interferon-beta at the Cemcat.[3] In the study, baseline serum NfL levels predicted long-term disability progression after a mean follow-up time of 14 years.[2] However, performance of blood NfL levels as prognostic biomarker in a real-world setting of patients with progressive

MS is yet to be explored. Here, we aimed to investigate the potential for serum NfL levels to predict disability progression in a multicentric cohort of patients with primary progressive MS (PPMS) gathered from several European MS centres. For the present study, in addition to NfL, we also measured the serum levels of glial fibrillar acidic protein (GFAP) and chitinase 3-like 1 (CHI3L1), two biomarkers known to play prognostic roles in patients with MS.[4 5]

METHODS

Patients

A multicentric cohort of 141 patients with PPMS from 18 European MS centres was used in the study. Selection of patients was performed based on availability of serum samples at a baseline time point and follow-up longer than 5 years preferably without receiving disease modifying therapies. None of these patients participated in the previous single-centre cohort study conducted at the Cemcat.[2] The study was approved by the corresponding Hospital Ethics Committee, and participants gave written informed consent.

Clinical assessments and definition of disability progression

Expanded Disability Status Scale (EDSS) scores were recorded at baseline, 2 and 6 years and at the time of last visit. All EDSS scores were obtained by experienced neurologists during routine clinical visits. No need for EDSS confirmation in a follow-up visit was required, as even though transient EDSS increases or improvements could still occur, given the long-standing progressive phenotype of the patients included in the present study, this situation is far less probable than in patients with relapsing-remitting MS (RRMS) early in their disease course, particularly in the EDSS ranges considered in this cohort. The change in relevant EDSS units from baseline to the other three time points was calculated as follows: a relevant change for baseline EDSS <6.0 required an increase of ≥ 1 EDSS unit, whereas for baseline EDSS ≥ 6.0, increases of ≥ 0.5 EDSS units were considered relevant. Changes bridging EDSS=6.0 were calculated as a sum of respective partial changes, for example, a change from EDSS 4.5 to 6.5 corresponds to a change in $1.5+1=2.5$ relevant units (ie, from 4.5 to 6.0 and from 6.0 to 6.5). Changes in relevant EDSS units were then annualised by correcting for the time between baseline EDSS and follow-up EDSS.

MRI activity

In order to evaluate the potential for serum biomarkers to predict disability progression independent of clinical or MRI signs of disease activity, patients with PPMS were classified into inflammatory and non-inflammatory according to the presence or absence of the following criteria: (1) relapses in the 3 months before sample collection; (2) contrast-enhancing lesions in a baseline brain and/or spinal cord MRI performed in proximity to sample collection (within a time window of ≤ 3 months); (3) appearance of new T2 lesions or contrast-enhancing lesions in the follow-up brain and/or spinal cord MRIs performed according to the standard clinical practice at each participating centre. The presence of one MRI at baseline or during follow-up with signs of disease activity was sufficient to classify the patient as inflammatory. On the other hand, patients with PPMS without radiological disease activity at baseline and during follow-up were classified as non-inflammatory.

Biomarker determinations

Peripheral blood was collected by standard venipuncture and allowed to clot spontaneously for 30 min. Serum was then obtained by centrifugation and stored frozen at −80°C until used. None of the patients included in the study received treatment with corticosteroids or immunomodulatory and/or immunosuppressive therapies before baseline blood collection. Serum levels of NfL and GFAP were measured using commercially available immunoassay kits (cat#103 186 and #102 336 Quanterix, Billerica, Massachusetts, USA) run on the fully automated ultrasensitive Simoa HD-1 Analyzer (Quanterix). Samples were run in duplicate in accordance with manufacturers' instructions with appropriate standards and internal controls. The intra-assay and inter-assay coefficients of variation were 6% and 10% for NfL, and 5% and 12% for GFAP, respectively.

To measure serum CHI3L1 levels, a Simoa-based assay was developed based on the use of a SiMoA homebrew assay starter kit (Cat. #101351, Quanterix) following the manufacturer's guidelines, using the monoclonal mouse antihuman CHI3L1 (Cat. #4813, Quidel, San Diego, California, USA) and the polyclonal rabbit antihuman (Cat. #4815, Quidel) for capture and detection, respectively. Briefly, capture antibody (0.2 mg/mL) and beads were prepared by buffer exchange into the Quanterix-recommended bead conjugation buffer using Amicon Ultra-0.5

Table 1 Demographic, clinical and radiological characteristics of patients with PPMS

Characteristics	Whole cohort
N	141
Age (years)*	52.9 (46.4–58.5)
Male/female (n (% men))	58/83 (41.1)
Disease duration (years)†	4.6 (2.0–9.4)
Follow-up time (years)‡	9.1 (7.0–12.6)
EDSS at baseline	4.0 (3.0–5.5)
EDSS at 2 years§	4.5 (3.5–6.0)
EDSS at 6 years§	6.0 (4.6–7.0)
EDSS at last visit	6.5 (5.0–7.5)
Relapses in the previous 3 months (n (%))¶	2 (1.4)
Treatment during follow-up (n (%))**	8 (5.7)
MRI findings (n (%))	
Inflammatory	42 (29.8)
Non-inflammatory	64 (45.4)
Unknown	35 (24.8)
IgG oligoclonal bands (n (%))††	103 (79.2)
sNfL levels (pg/mL)	10.0 (7.4–13.3)
sGFAP levels (pg/mL)	96.9 (65.1–131.3)
sCHI3L1 levels (ng/mL)	44.1 (32.4–73.9)

Data are expressed as median (IQR).
*Refers to age at sample collection.
†Refers to the time between disease onset and sample collection.
‡Refers to the time between samples collection and the time of last visit.
§Information missing in 8 (5.7%) and 11 (7.8%) patients, respectively. Missing information at 2 years was due to the fact that EDSS was not recorded at this particular time point. Missing information at 6 years was due to a combination of patients with no EDSS recorded at that particular time point, and patients with follow-up below 6 years.
¶Refers to presence of relapses in the 3 months prior to sample collection.
**Two patients received intravenous pulses of cyclophosphamide for 1 year; two patients received intravenous pulses of methylprednisolone regularly; one patient received azathioprine for 4 years; one patient received two cycles of rituximab; one patient was treated with interferon-beta for 2 years; one patient was treated with laquinimod for 11 months.
††Information was not available for 11 (7.6%) patients.
EDSS, Expanded Disability Status Scale; sCHI3L1, serum levels of chitinase 3-like 1; sGFAP, serum levels of glial fibrillar acidic protein; sNfL, serum levels of neurofilament light chain.

centrifugal filters. Conjugation of the capture antibody to beads was based on 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide chemistry and performed according to Quanterix manual protocol. The detection antibody was generated by buffer exchanging into the biotinylation reaction buffer provided by Quanterix prior to conjugation to NHS-PEG4-biotin. Unreacted material was removed through additional buffer exchange using Amicon filter devices. The detection antibody was used at 0.3 μg/mL final concentration in the assay and streptavidin covalently coupled to β-D-galactosidase (SBG) concentration was 150 pM. The recombinant CHI3L1 protein (Cat. #A442, Quidel) was used for the standard curve preparation. A three-step configuration protocol was optimised for the assay.

Statistical methods

Correlations between biomarker levels, and between biomarker concentrations and age were visualised using scatter plots with concentrations displayed on the log10-scale due to their non-normal distribution. Accordingly, the correlation was quantified using Spearman's r. Linear regression models were used to identity factors influencing biomarker levels (dependent variable in individual analyses; log-transformed). Estimates were back-transformed and represent multiplicative effects. Then, linear regression models were used to assess the association between annualised EDSS change (dependent variable) and biomarker levels in individual models for each biomarker and each time interval (ie, baseline to year 2, baseline to year 6 and baseline to last follow-up). Biomarker levels were log2-transformed and estimates therefore represent additive effects on the annualised EDSS change per biomarker doubling. Estimates including 95% CIs both from univariable and multivariable analyses (adjusted for age, sex and baseline EDSS) were provided and visualised graphically. The adjusted R square is provided as a measure of how well a model fits the data thereby quantifying the proportion of the variance in the endpoint explained by the model. Adjusted R square values of models using a single versus all biomarkers are provided and models are compared using analysis of variance (ANOVA). Disease duration turned out to be not relevant and was not used as a covariate in any of the analyses. A subgroup analysis was performed using only patients with no signs of inflammation. All analyses were performed in R V.4.3.1.

RESULTS

Demographic and clinical characteristics of patients with PPMS at baseline

Table 1 summarises the demographic and clinical information of the whole cohort of patients with PPMS. Samples from patients with PPMS were collected in the period between 1995 and 2018. At baseline (ie, sampling), the median age of patients was 52.9 (IQR, 46.4–58.5) years, and 58 (41.1%) were men. Median disease duration was 4.6 (IQR, 2.0–9.4) years and median EDSS 4.0 (IQR, 3.0–5.5). Only 2 (1.4%) patients had a relapse in the 3 months prior to baseline sample collection, and 79.2% of patients with PPMS were positive for IgG oligoclonal bands.

Associations between serum biomarker levels and demographic and clinical variables at baseline

At baseline, median serum NfL level was 10.0 pg/mL (IQR, 7.4–13.3 pg/mL), median serum GFAP level was 96.9 pg/mL (IQR, 65.1–131.3 pg/mL) and median CHI3L1 level 44.1 ng/mL (IQR, 32.4–73.9 ng/mL) (table 1). As shown in figure 1, serum NfL and GFAP levels were moderately correlated (Spearman r=0.43; p<0.0001), whereas serum CHI3L1 levels were weakly correlated with serum NfL levels (Spearman r=0.19; p=0.022) and serum GFAP levels (Spearman r=0.17; p=0.042).

As shown in online supplemental figure 1, the three serum biomarker levels weakly correlated with age (Spearman r=0.28; p<0.001 for NfL; Spearman r=0.29; p<0.001 for GFAP; Spearman r=0.21; p=0.012 for CHI3L1). In multivariable analysis (table 2), serum GFAP levels were 24.4% lower in males (estimate: 0.754; 95% CI: 0.625 to 0.914; p=0.0042). Serum NfL levels increased by 7.2% per EDSS unit (estimate: 1.072; 95% CI: 1.008 to 1.141; p=0.0276) and serum GFAP levels increased by 6.4% per EDSS unit (estimate: 1.064; 95% CI: 1.001 to 1.130; p=0.0451). A trend towards a significant increase in serum CHI3L1 levels per EDSS unit was also observed (6.6% per EDSS unit; estimate: 1.066; 95% CI: 0.997 to 1.139); p=0.0601). Disease duration at baseline did not influence serum biomarker levels (table 2).

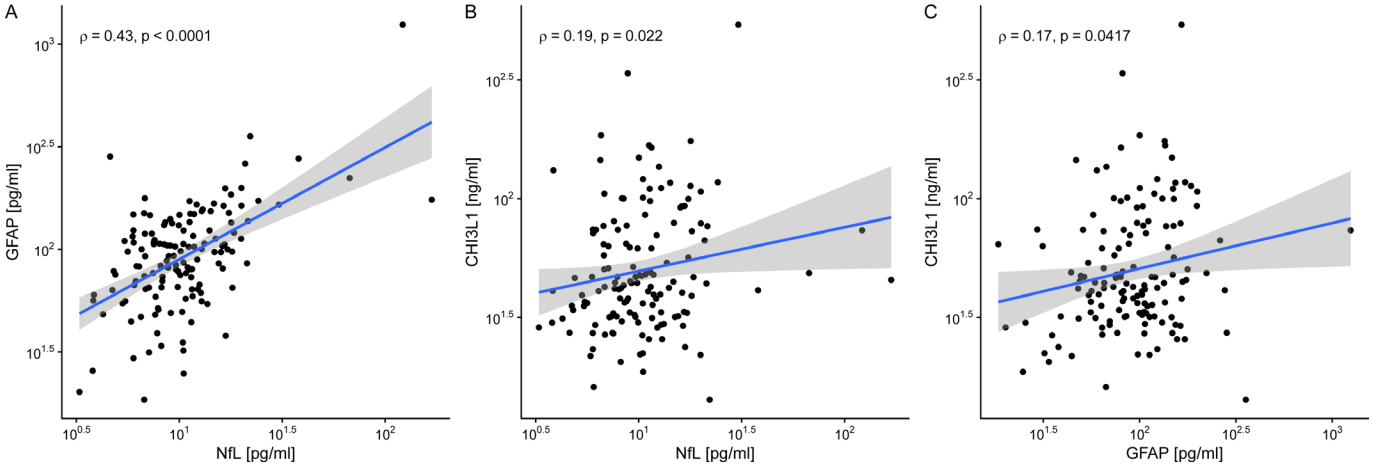

Figure 1 Correlations between serum NfL, GFAP and CHI3L1 levels. Scatter plots (with axis on log10-scale) showing correlations between NfL and GFAP serum levels (A), NfL and CHI3L1 serum levels (B) and GFAP and CHI3L1 serum levels (C). Serum levels of NfL and GFAP are expressed in pg/mL, and serum levels of CHI3L1 in ng/mL. CHI3L1, chitinase 3-like 1; GFAP, glial fibrillar acidic protein; NfL, neurofilament light chain; r, Spearman's rank correlation coefficient.

Table 2 Associations between serum biomarker levels and demographic and clinical variables at baseline

Variables	Estimate	sNfL (pg/mL) 95% CI	P value	Estimate	sGFAP (pg/mL) 95% CI	P value	Estimate	sCHI3L1 (ng/ml) 95% CI	P value
Multivariable									
Gender (male)	0.923	0.760 to 1.122	0.4192	0.756	0.625 to 0.914	0.0042	0.936	0.760 to 1.152	0.5280
Age	1.014	1.003 to 1.024	0.0134	1.009	0.998 to 1.019	0.1027	1.012	1.000 to 1.023	0.0450
Disease duration	0.989	0.973 to 1.005	0.1787	1.004	0.988 to 1.020	0.6078	0.993	0.976 to 1.010	0.4044
EDSS	1.072	1.008 to 1.141	0.0276	1.064	1.001 to 1.130	0.0451	1.066	0.997 to 1.139	0.0601

Estimates from individual linear models are back transformed and represent multiplicative effects. Reading example: NfL level on average increase by 7.2% per one unit increase in EDSS and GFAP are on average 24.4% lower in males.
Significant p values are shown in bold.
EDSS, Expanded Disability Status Scale; sCHI3L1, serum levels of chitinase 3-like 1; sGFAP, serum levels of glial fibrillar acidic protein; sNfL, serum levels of neurofilament light chain.

Associations between baseline serum biomarker levels and annualised change in relevant EDSS units

Patients were followed for a median time of 9.1 (IQR, 7.0–12.6) years, and median EDSS scores at 2 years, 6 years and at the time of last visit were 4.5 (IQR, 3.5–6.0), 6.0 (IQR, 4.6–7.0) and 6.5 (IQR, 5.0–7.5), respectively (table 1). Only 8 (5.7%) patients received treatment for a short period of time during follow-up.

Figure 2 and online supplemental table 1 show the results of the univariable and multivariable analyses after adjusting by age, sex and EDSS at baseline. In multivariable analysis, serum biomarker levels were all significantly associated with disability progression at 2 and 6 years. At 2 years (figure 2A), doubling of baseline serum biomarker levels was associated with an increase of 0.11 relevant EDSS units per year for NfL levels (estimate 0.11; 95% CI: 0.02 to 0.20; p=0.0226), 0.11 relevant EDSS units per year for GFAP (estimate 0.11; 95% CI: 0.01 to 0.20; p=0.0354) and 0.13 relevant EDSS units per year for CHI3L1 (estimate 0.13; 95% CI: 0.05 to 0.22; p=0.0032). At 6 years (figure 2B), doubling of baseline serum biomarker levels was associated with an increase of 0.09 units per year for NfL (estimate 0.09; 95% CI: 0.03 to 0.15; p=0.0021), 0.07 units per year for GFAP (estimate 0.07; 95% CI: 0.01 to 0.13; p=0.0328) and 0.06 units for CHI3L1 (estimate 0.06; 95% CI: 0 to 0.11; p=0.0451).

In the multivariable analysis for last follow-up, serum NfL and GFAP levels were strongly associated with EDSS change. As shown in figure 2C and online supplemental table 1, doubling of baseline serum NfL levels was associated with an annualised change of 0.08 relevant EDSS units (estimate 0.08; 95% CI: 0.04 to 0.12; p=0.0003), and a similar association was found for GFAP (estimate 0.08; 95% CI: 0.04 to 0.12; p=0.0005). A trend towards a significant association with disability progression was observed for CHI3L1, and doubling of baseline serum CHI3L1 levels was associated with an increase of 0.04 units per year (estimate 0.04; 95% CI: −0 to 0.08; p=0.0531).

Sensitivity analysis excluding the eight patients who received treatment during follow-up did not alter the results (data not shown). A model combining the three biomarkers in the whole cohort resulted in slightly better model fits compared with the single biomarker models, particularly at the time of the last follow-up (online supplemental table 2).

Associations between baseline serum biomarker levels and disability progression in patients with non-inflammatory PPMS

As shown in table 1, 64 (45.5%) of patients with PPMS were classified as non-inflammatory according to clinical disease activity at baseline, and radiological disease activity at baseline and/or during follow-up. Online supplemental table 3 shows demographic and clinical information of the non-inflammatory PPMS group. At baseline, the median age of patients was 54.0 (IQR, 49.0–59.1) years, and 27 (42.2%) were men. Median disease duration was 5.3 (IQR, 3.2–8.6) years and median EDSS 4.0 (IQR, 3.0–5.1). Median follow-up time in patients with non-inflammatory PPMS was 8.7 (IQR, 7.0–11.2) years, and median EDSS scores at 2 years, 6 years and at the time of last visit were 4.0 (IQR, 3.5–6.0), 6.0 (IQR, 4.0–6.6) and 6.2 (IQR, 4.5–7.0) respectively. Only 1 (1.6%) patient was treated during follow-up. Baseline median serum NfL levels were 10.2 pg/mL (IQR, 7.3–13.1 pg/mL), median serum GFAP levels 95.2 pg/mL (IQR, 65.4–131.0 pg/mL) and median CHI3L1 levels 43.3 ng/mL (IQR, 31.8–67.9 ng/mL) (online supplemental table 3).

In the subgroup of patients with non-inflammatory PPMS, only serum CHI3L1 levels were significantly associated with disability progression (figure 3; online supplemental table 3). In multivariable analysis at 2 years (figure 3A), doubling of baseline serum CHI3L1 levels was associated with an increase of 0.14 relevant EDSS units per year (estimate 0.14; 95% CI: 0.04 to 0.23; p=0.0076). Similarly, at the time of last follow-up (figure 3C), doubling of baseline serum CHI3L1 levels was associated with an increase of 0.07 relevant EDSS units per year (estimate 0.07; 95% CI: 0.01 to 0.13; p=0.0338). A trend towards a significant association with disability progression was observed at 6 years (figure 3B; p=0.0504). In contrast, serum NfL and GFAP levels were not significantly associated with disability progression at any of the time points in univariable or multivariable analyses in patients with non-inflammatory PPMS (figure 3; online supplemental table 4).

Sensitivity analysis excluding the only patient with non-inflammatory PPMS who received treatment during follow-up did not modify the results (data not shown). A model with the combination of the three biomarkers was not adding additional relevant information compared with the single biomarker model with CHI3L1 (online supplemental table 2).

DISCUSSION

The prognostic role of molecular biomarkers predicting disability worsening in patients with progressive MS has been far less explored compared with patients with relapsing forms of the disease.[1 6 7] In a recent single-centre study conducted at the Cemcat in a cohort of 51 patients with progressive MS who participated in a clinical trial of interferon-beta, high serum NfL levels determined at trial onset predicted long-term disability progression after 14 years defined by the presence of progression rates above the 75th percentile.[2] The question that remained from this study was whether these results obtained in a unicentric cohort and in the well-controlled setting of a clinical trial would also be observed in a real-world setting of patients with progressive MS. The potential for serum NfL levels to predict disability progression was also confirmed in a multicentric

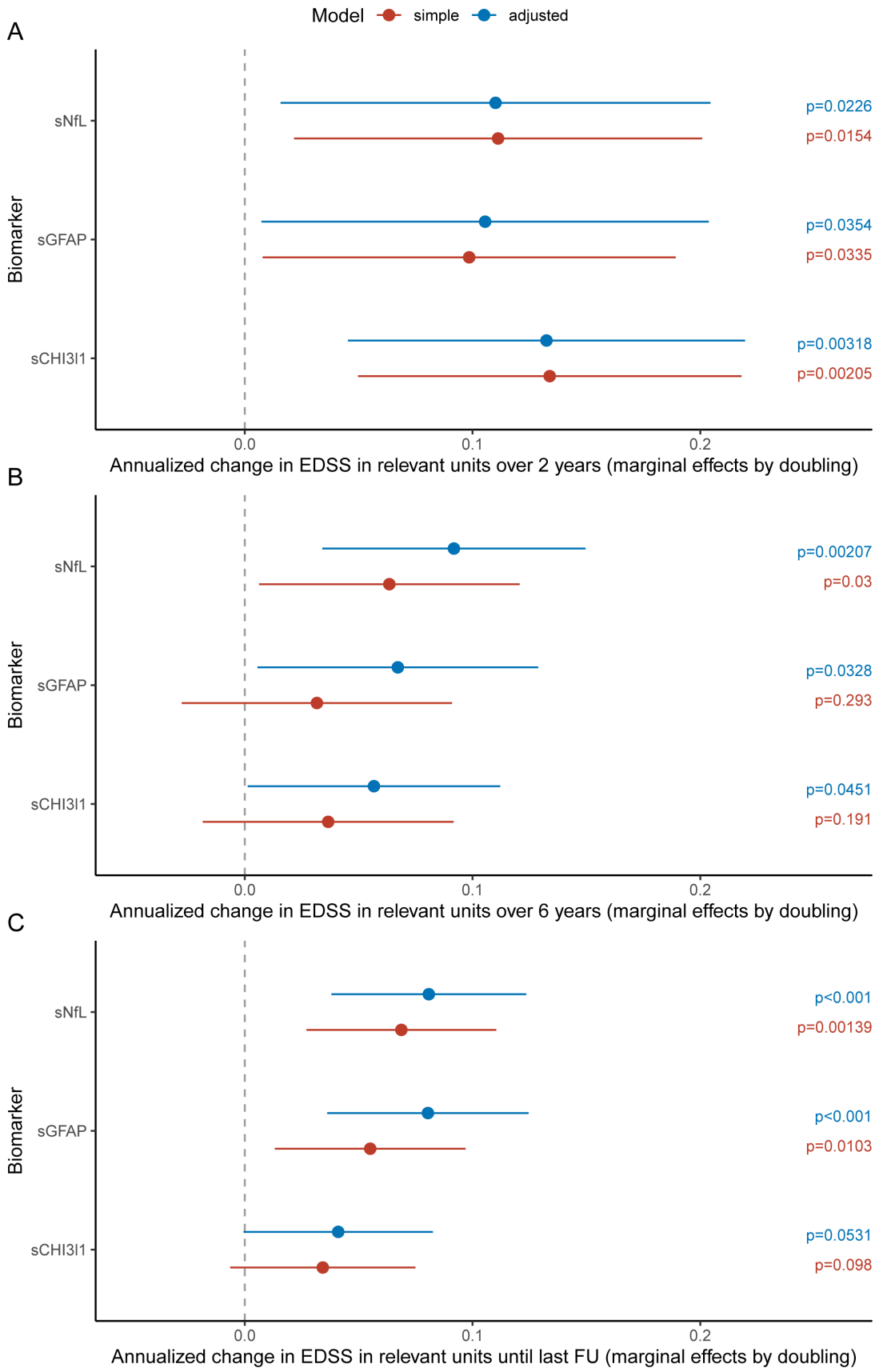

Figure 2 Association between serum biomarker levels and disability progression in the whole cohort of patients with PPMS. Forest plots showing the marginal effects per doubling in biomarker concentration on disability progression expressed as the annualised change in EDSS between baseline and the following three time points: at 2 years (A), 6 years (B) and at the time of last follow-up (C). Graphs show mean effects with the 95% CIs in univariable ('simple') and multivariable ('adjusted') models after adjusting for age, sex and baseline EDSS. EDSS, Expanded Disability Status Scale; FU, follow-up; sCHI3L1, serum levels of chitinase 3-like 1; sGFAP, serum levels of glial fibrillar acidic protein; sNfL, serum levels of neurofilament light chain.

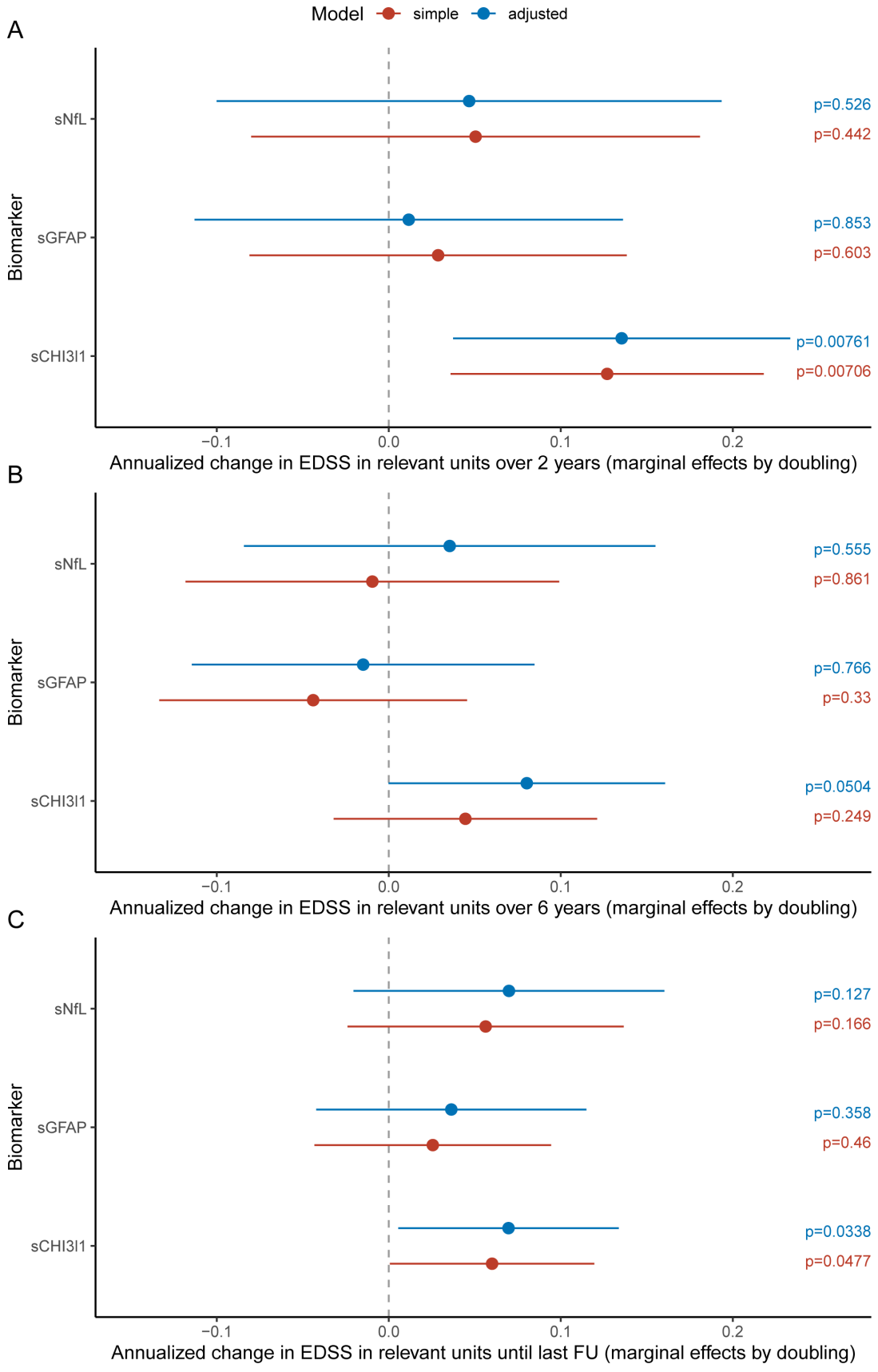

Figure 3 Association between serum biomarker levels and disability progression in patients with non-inflammatory PPMS. Forest plots showing the marginal effects per doubling in biomarker concentration on disability progression expressed as the annualised change in EDSS between baseline and the following three time points: at 2 years (A), 6 years (B) and at the time of last follow-up (C). Graphs show mean effects with the 95% CIs in univariable ('simple') and multivariable ('adjusted') models after adjusting for age, sex and baseline EDSS. EDSS, Expanded Disability Status Scale; FU, follow-up; sCHI3L1, serum levels of chitinase 3-like 1; sGFAP, serum levels of glial fibrillar acidic protein; sNfL, serum levels of neurofilament light chain.

cohort of 141 patients with PPMS with a median follow-up of 9 years. Interestingly, baseline serum NfL levels predicted disability progression defined by a change in relevant EDSS units not only in the long term after 9 years, but also in the short and medium term after 2 and 6 years respectively. It is important to remark that, in contrast to the previous study where 57% of the patients received interferon-beta during the 2-year trial duration,[2] a treatment with potential to modulate NfL levels,[2 8] in the present study only 6% of the patients with PPMS were treated for a short period of time during follow-up. Admittedly, the clinical relevance of present findings should be considered in light of the limited change of EDSS in patients with progressive disease, particularly in the high ranges of EDSS (at and above 6.5 points). It is probable that the use of other clinical parameters, including digital tools, with more granular assessments of disability, and focusing on the type of progressive impairment that this group of patients usually presents (upper limb mobility), would be more rewarding to capture significant increases in disability. In this regard, the fact that we have been able to demonstrate a predictive role of serum biomarkers even with the EDSS score indicates that clinical relevance is warranted.

Considering the high degree of heterogeneity inherent to MS and the complexity of disease progression from a pathophysiological point of view, it is very likely that biomarkers other than NfL are also associated with disability outcomes in patients with progressive MS. Bearing this in mind, for the present study we also measured the serum levels of GFAP and CHI3L1 to explore their association with disability outcomes. It should be mentioned that for the present study we developed a Simoa-based assay to measure the serum levels of CHI3L1, which permitted to quantify CHI3L1 levels in a more robust manner compared with the commercially available ELISAs. For CHI3L1, the Simoa technology has the advantage of a wider dynamic range and the ability to measure a higher number of samples in the same experiment, hence reducing the final variability of the experiment. Noteworthy, both baseline serum GFAP and CHI3L1 levels were also found to be associated with disability progression in patients with PPMS though with some peculiarities. Serum GFAP levels behaved similarly to serum NfL levels and were significantly associated with disability progression in the multivariable analysis at all time points. In contrast, the effect of serum CHI3L1 levels on disability outcomes was clearly more noticeable in the short-term after 2 years, and remained as a trend in the long term.

The above-mentioned findings related with serum levels of biomarkers and disability outcomes were observed in the whole cohort of 141 patients with PPMS. Of note, when analysis was restricted to the subgroup of patients with non-inflammatory disease according to clinical and radiological parameters, only serum CHI3L1 levels remained significantly associated with disability progression. The significant effect of serum NfL and GFAP levels on disability progression disappeared after removal of more patients with inflammatory PPMS. In our multicentric cohort, the percentage of patients with relapses and radiological disease activity is comparable to other published cohorts of patients with PPMS.[9] However, this part of the study has some limitations. The number of patients with non-inflammatory PPMS remaining for analysis is reduced to a little less than half of the whole cohort (45%), and there is a relatively high proportion of patients with no MRI information (25%) and therefore excluded from analysis. In addition, since evidence of radiological disease activity depends on the time when MRI is performed, possibility exists that a number of patients with non-inflammatory PPMS are wrongly classified. Our results are in line with the findings of a previous study conducted in a subset of patients with secondary progressive MS (SPMS) who participated in the ASCEND clinical trial evaluating the effect of natalizumab on disease progression, in which changes in serum NfL levels were not associated with disability progression in the absence of relapses and radiological inflammatory activity.[10]

GFAP, a type-III intermediate filament protein, is emerging as prognostic biomarker in a wide range of neurological conditions.[11] A number of studies carried out in patients with relapsing and progressive forms of MS have found associations between higher cerebrospinal fluid (CSF) and blood GFAP levels and disease progression and disability outcomes.[4 12–17] Interestingly, a combined elevation of serum GFAP and NfL levels was associated with a significant increase in disability worsening and progression independent of relapse activity in a large cohort of patients with relapsing MS.[4] However, similar to the findings with serum NfL levels in patients with SPMS from the ASCEND trial, serum GFAP concentration was not predictive of future disability progression in the absence of relapses and MRI inflammatory activity in this smaller cohort of patients.[18]

CHI3L1 is by far the most characterised chitinase-like protein, and it can be considered a biomarker of microglial and astrocyte activation.[5] The majority of CHI3L1 studies in MS have been conducted in the CSF, where higher CSF CHI3L1 levels in early phases of the disease were associated with an increased risk for disability progression[19] and were also an independent predictor of conversion to SPMS.[20] Also, in a small cohort of 25 patients with PPMS with 1-year follow-up, CSF CHI3L1 levels correlated with EDSS scores at baseline and 12 months, and tended to be associated with an increased risk for disability progression.[21] In contrast, the prognostic role of blood CHI3L1 levels predicting disability progression in patients with MS has been far less explored. In two cross-sectional studies, blood CHI3L1 levels were higher in patients with progressive MS compared with patients with RRMS.[22 23] Correlations between blood CHI3L1 levels with EDSS scores were found to be significant in patients with RRMS[22 24] and also in patients with progressive MS.[23] Interestingly, in the latter study[23] CHI3L1 levels and EDSS scores correlated only in serum but were not observed in the CSF.

The findings in the non-inflammatory group of patients with PPMS may indicate that serum CHI3L1 levels are less affected by the inflammatory component associated with disease progression compared with serum NfL and GFAP levels. This is supported by the stronger correlations observed between serum NfL and GFAP levels but weaker with respect to serum CHI3L1 levels, which may suggest that the latter is tagging more specifically the neurodegenerative component associated with disability progression. However, these findings with serum CHI3L1 levels in patients with non-inflammatory PPMS should be validated in future cohort studies.

In summary, serum levels of NfL, GFAP and CHI3L1 are prognostic biomarkers in patients with PPMS and can be used to predict short-term, medium-term and long-term disability progression in these patients. Among the three biomarkers, serum CHI3L1 levels seem to be less dependent on the inflammatory component observed in patients with progressive MS.

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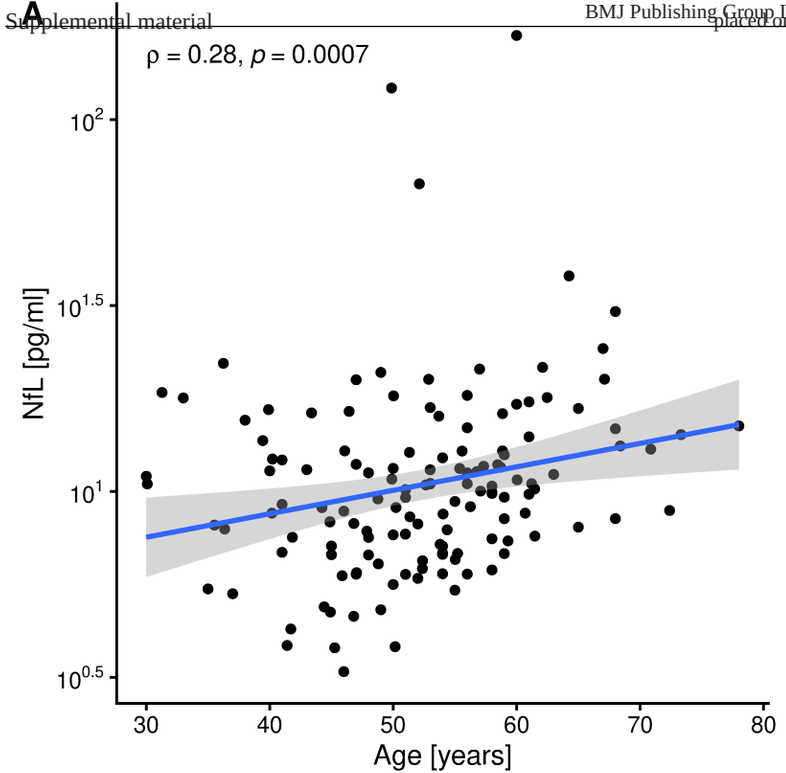

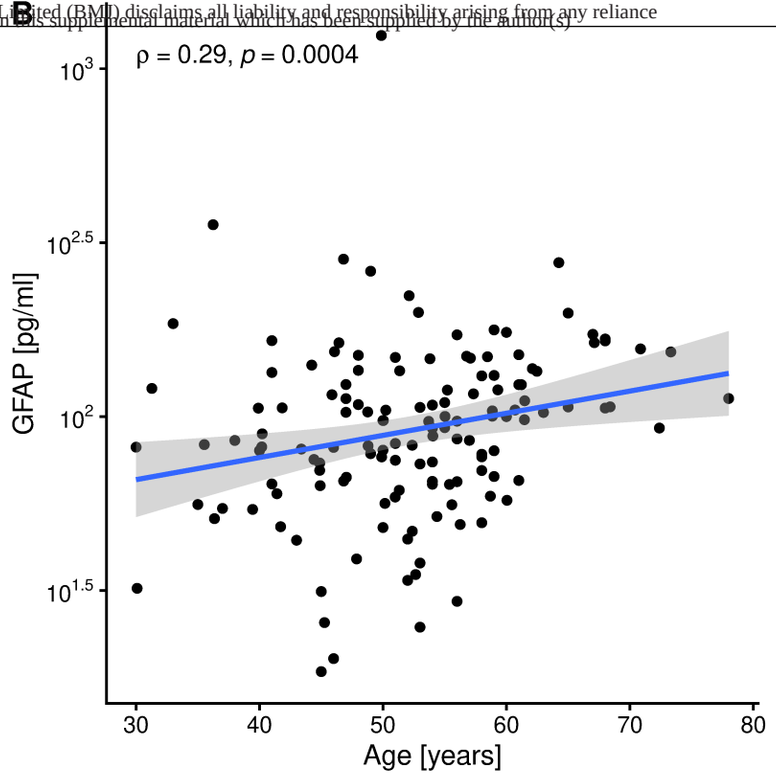

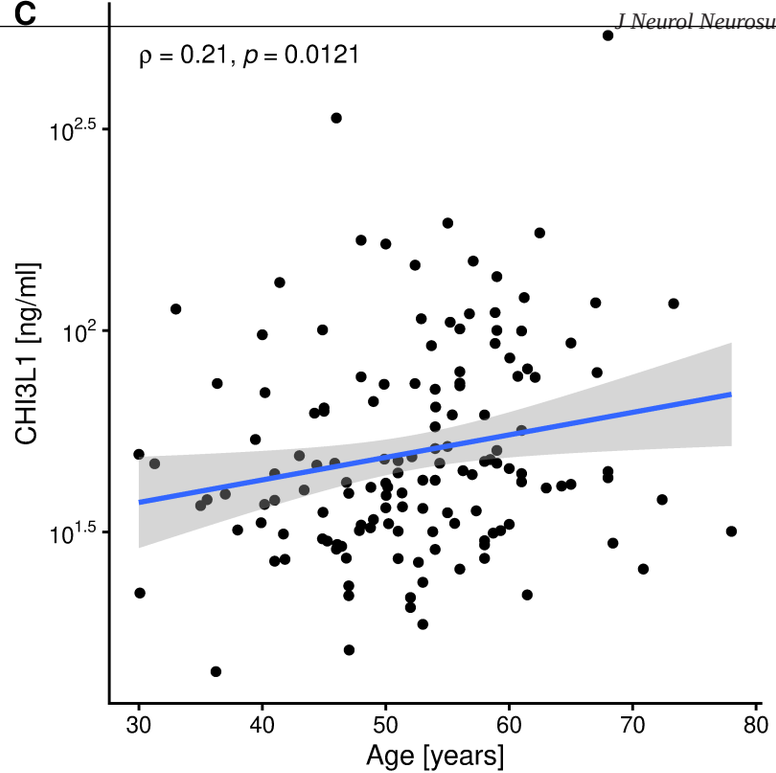

Online Supplemental Table 1. Associations between serum biomarker levels and disability progression in the whole PPMS cohort.

Variables	sNfL (pg/ml)			sGFAP (pg/ml)			sCHI3L1 (ng/ml)		
	estimate	95% CI	P value	estimate	95% CI	P value	estimate	95% CI	P value
2 years									
Univariable									
- Serum biomarker per doubling	0.11	0.02 - 0.20	**0.0154**	0.10	0.01 - 0.19	**0.0335**	0.13	0.05 - 0.22	**0.0020**
Multivariable									
- EDSS at baseline	-0.01	-0.06 - 0.04	0.6507	-0.01	-0.06 - 0.04	0.6341	-0.01	-0.06 - 0.04	0.6854
- Gender (male)	0.03	-0.13 - 0.19	0.6967	0.06	-0.10 - 0.22	0.4673	0.03	-0.12 - 0.19	0.6788
- Age	0.00	-0.01 - 0.01	0.4687	0.00	-0.00 - 0.01	0.3726	0.00	-0.01 - 0.01	0.4752
- Serum biomarker per doubling	0.11	0.02 - 0.20	**0.0226**	0.11	0.01 - 0.20	**0.0354**	0.13	0.05 - 0.22	**0.0032**
6 years									
Univariable									
- Serum biomarker per doubling	0.06	0.01 - 0.12	**0.0300**	0.03	-0.03 - 0.09	0.2932	0.04	-0.02 - 0.09	0.1907
Multivariable									
- EDSS at baseline	-0.02	-0.05 - 0.01	0.1610	-0.02	-0.05 - 0.01	0.2027	-0.02	-0.05 - 0.01	0.2271
- Gender (male)	0.09	-0.01 - 0.19	0.0690	0.10	0.00 - 0.20	**0.0495**	0.08	-0.01 - 0.18	0.0908
- Age	-0.01	-0.01 - 0.00	0.0681	-0.00	-0.01 - 0.00	0.1275	-0.00	-0.01 - 0.00	0.1358
- Serum biomarker per doubling	0.09	0.03 - 0.15	**0.0021**	0.07	0.01 - 0.13	**0.0328**	0.06	0.00 - 0.11	**0.0451**
Last follow-up									
Univariable									
- Serum biomarker per doubling	0.07	0.03 - 0.11	**0.0014**	0.06	0.01 - 0.10	**0.0103**	0.03	-0.01 - 0.07	0.0980
Multivariable									
- EDSS at baseline	-0.01	-0.03 - 0.01	0.4694	-0.01	-0.03 - 0.01	0.3899	-0.00	-0.03 - 0.02	0.6755
- Gender (male)	0.09	0.02 - 0.16	**0.0141**	0.11	0.04 - 0.19	**0.0029**	0.08	0.01 - 0.16	**0.0254**
- Age	-0.00	-0.00 - 0.00	0.7579	-0.00	-0.00 - 0.00	0.9071	0.00	-0.00 - 0.00	0.9302
- Serum biomarker per doubling	0.08	0.04 - 0.12	**0.0003**	0.08	0.04 - 0.12	**0.0005**	0.04	-0.00 - 0.08	0.0531

Estimates for the biomarker represent the additive increase in annualized relevant EDSS units when doubling biomarker concentrations. Reading example from the multivariable analysis: doubling of biomarker levels was associated with the following estimated increase in relevant EDSS units per year: 0.09 for NfL and GFAP (or 0.8 per 10 years) and 0.4 for CHI3L1. EDSS: Expanded Disability Status Scale. 95% CI: 95% confidence interval. Significant p values are shown in bold. sNfL: serum levels of NfL. sGFAP: serum levels of GFAP. sCHI3L1: serum levels of CHI3L1.

Online Supplemental Table 2. Combination of biomarkers to predict disability progression expressed as the annualized change in EDSS between baseline and 2 years, 6 years, and at the time of last follow-up.

Biomarkers	2 years	6 years	Last follow-up
Whole cohort			
sNfL	*0.02*	0.093	*0.098*
sGFAP	*0.014*	*0.057*	*0.091*
sCHI3L1	0.047	*0.053*	*0.032*
sNfL & sGFAP & sCHI3L1	**0.07**	**0.099**	**0.127**
Non-inflammatory cohort			
sNfL	*-0.044*	-0.007	-0.019
sGFAP	*-0.051*	-0.012	-0.046
sCHI3L1	**0.073**	**0.055**	**0.018**
sNfL & sGFAP & sCHI3L1	0.043	0.034	0.002

Data represent adjusted R-square values (i.e. the proportion of the variance in the endpoint explained by the model) of models with annualized change in EDSS as dependent variable and with the respective biomarker as a predictor (or all biomarkers, lines 4 and 8) adjusted for age, sex, and baseline EDSS (i.e. corresponding to adjusted models in Figure 2 and 3). Numbers in bold represent the largest adjusted R square values per endpoint. Reading example for the model predicting annualized change in EDSS between baseline and last follow-up in the whole cohort: While the adjusted model for sNfL, sGFAP and sCHI3L1

explain 9.8%, 9.1% and 3.2% of the variation in the endpoint, respectively, the combined model explains 12.7% while taking into accounting for the number of different variables (adjusted R square). Numbers in italic indicate that the respective adjusted single biomarker model explains significantly worse the data compared to the model with all 3 biomarkers.

Online supplemental Table 3. Demographic and clinical characteristics of non-inflammatory PPMS patients.

Characteristics	Non-inflammatory cohort
N	64
Age (years)[a]	54.0 (49.0 - 59.1)
Male / female (n (% men))	27 / 37 (42.2)
Disease duration (years)[b]	5.3 (3.2 - 8.6)
Follow-up time (years)[c]	8.7 (7.0 - 11.2)
EDSS at baseline	4.0 (3.0 - 5.1)
EDSS at 2 years[d]	4.0 (3.5 - 6.0)
EDSS at 6 years[e]	6.0 (4.0 - 6.6)
EDSS at last visit	6.2 (4.5 - 7.0)
Treatment during follow-up (n (%))[f]	1 (1.6)
sNfL levels (pg/ml)	10.2 (7.3 - 13.1)
sGFAP levels (pg/ml)	95.2 (65.4 - 131.0)
sCHI3L1 levels (ng/ml)	43.3 (31.8 - 67.9)

Data are expressed as median (interquartile range). [a]Refers to age at sample collection (baseline). [b]Refers to the time between disease onset and sample collection (baseline). [c]Refers to the time between samples collection and the time of last visit. [d,e]Information missing in 1 (3.1%) and 2 (6.2%) patients, respectively. [f]One patient received intravenous pulses of cyclophosphamide for 1 year. EDSS: Expanded Disability Status Scale. sNfL: serum levels of NfL. sGFAP: serum levels of GFAP. sCHI3L1: serum levels of CHI3L1.

Online Supplemental Table 4. Associations between serum biomarker levels and disability progression in non-inflammatory PPMS patients.

Variables	sNfL (pg/mL)			sGFAP (pg/mL)			sCHI3L1 (ng/mL)		
	estimate	95% CI	P value	estimate	95% CI	P value	estimate	95% CI	P value
2 years									
Univariable									
- Serum biomarker per doubling	0.05	-0.08 - 0.18	0.4421	0.03	-0.08 - 0.14	0.6027	0.13	0.04 - 0.22	**0.0071**
Multivariable									
- EDSS at baseline	0.01	-0.05 - 0.06	0.8486	0.01	-0.05 - 0.07	0.7499	0.00	-0.05 - 0.06	0.9303
- Gender (male)	-0.09	-0.28 - 0.11	0.3817	-0.08	-0.29 - 0.12	0.4162	-0.08	-0.26 - 0.11	0.4019
- Age	-0.00	-0.01 - 0.01	0.6745	-0.00	-0.01 - 0.01	0.7941	-0.01	-0.02 - 0.01	0.3301
- Serum biomarker per doubling	0.05	-0.10 - 0.19	0.5264	0.01	-0.11 - 0.14	0.8526	0.14	0.04 - 0.23	**0.0076**
6 years									
Univariable									
- Serum biomarker per doubling	-0.01	-0.12 - 0.10	0.8610	-0.04	-0.13 - 0.05	0.3297	0.04	-0.03 - 0.12	0.2492
Multivariable									
- EDSS at baseline	-0.01	-0.06 - 0.03	0.6345	-0.01	-0.05 - 0.04	0.7571	-0.01	-0.05 - 0.03	0.6008
- Gender (male)	0.05	-0.10 - 0.20	0.5360	0.04	-0.12 - 0.19	0.6482	0.05	-0.10 - 0.20	0.4988
- Age	-0.01	-0.02 - 0.00	0.1657	-0.01	-0.01 - 0.00	0.2328	-0.01	-0.02 - 0.00	0.0644
- Serum biomarker per doubling	0.04	-0.08 - 0.16	0.5554	-0.01	-0.11 - 0.08	0.7658	0.08	-0.00 - 0.16	0.0504
Last follow-up									
Univariable									
- Serum biomarker per doubling	0.06	-0.02 - 0.14	0.1658	0.03	-0.04 - 0.09	0.4599	0.06	0.00 - 0.12	**0.0477**
Multivariable									
- EDSS at baseline	0.00	-0.03 - 0.04	0.8510	0.01	-0.03 - 0.04	0.6871	0.01	-0.03 - 0.04	0.7497
- Gender (male)	0.02	-0.10 - 0.14	0.7494	0.03	-0.10 - 0.16	0.6357	0.02	-0.10 - 0.14	0.7495
- Age	-0.00	-0.01 - 0.00	0.5017	-0.00	-0.01 - 0.01	0.7178	-0.00	-0.01 - 0.00	0.4382
- Serum biomarker per doubling	0.07	-0.02 - 0.16	0.1272	0.04	-0.04 - 0.11	0.3581	0.07	0.01 - 0.13	**0.0338**

Estimates for the biomarker represent the additive increase in annualized relevant EDSS units when doubling biomarker concentrations. EDSS: Expanded Disability Status Scale. 95% CI: 95% confidence interval. Significant p values are shown in bold. sNfL: serum levels of NfL. sGFAP: serum levels of GFAP. sCHI3L1: serum levels of CHI3L1.