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Serum Glial Fibrillary Acidic Protein Compared With Neurofilament Light Chain as a Biomarker for Disease Progression in Multiple Sclerosis

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IMPORTANCE There is a lack of validated biomarkers for disability progression independent of relapse activity (PIRA) in multiple sclerosis (MS).

OBJECTIVE To determine how serum glial fibrillary acidic protein (sGFAP) and serum neurofilament light chain (sNfL) correlate with features of disease progression vs acute focal inflammation in MS and how they can prognosticate disease progression.

DESIGN, SETTING, AND PARTICIPANTS Data were acquired in the longitudinal Swiss MS cohort (SMSC; a consortium of tertiary referral hospitals) from January 1, 2012, to October 20, 2022. The SMSC is a prospective, multicenter study performed in 8 centers in Switzerland. For this nested study, participants had to meet the following inclusion criteria: cohort 1, patients with MS and either stable or worsening disability and similar baseline Expanded Disability Status Scale scores with no relapses during the entire follow-up; and cohort 2, all SMSC study patients who had initiated and continued B-cell–depleting treatment (ie, ocrelizumab or rituximab).

EXPOSURES Patients received standard immunotherapies or were untreated.

MAIN OUTCOMES AND MEASURES In cohort 1, sGFAP and sNfL levels were measured longitudinally using Simoa assays. Healthy control samples served as the reference. In cohort 2, sGFAP and sNfL levels were determined cross-sectionally.

RESULTS This study included a total of 355 patients (103 [29.0%] in cohort 1: median [IQR] age, 42.1 [33.2-47.6] years; 73 female patients [70.9%]; and 252 [71.0%] in cohort 2: median [IQR] age, 44.3 [33.3-54.7] years; 156 female patients [61.9%]) and 259 healthy controls with a median [IQR] age of 44.3 [36.3-52.3] years and 177 female individuals (68.3%). sGFAP levels in controls increased as a function of age (1.5% per year; $P < .001$), were inversely correlated with BMI (−1.1% per BMI unit; $P = .01$), and were 14.9% higher in women than in men ($P = .004$). In cohort 1, patients with worsening progressive MS showed 50.9% higher sGFAP levels compared with those with stable MS after additional sNfL adjustment, whereas the 25% increase of sNfL disappeared after additional sGFAP adjustment. Higher sGFAP at baseline was associated with accelerated gray matter brain volume loss (per doubling: 0.24% per year; $P < .001$) but not white matter loss. sGFAP levels remained unchanged during disease exacerbations vs remission phases. In cohort 2, median (IQR) sGFAP *z* scores were higher in patients developing future confirmed disability worsening compared with those with stable disability (1.94 [0.36-2.23] vs 0.71 [−0.13 to 1.73]; $P = .002$); this was not significant for sNfL. However, the combined elevation of *z* scores of both biomarkers resulted in a 4- to 5-fold increased risk of confirmed disability worsening (hazard ratio [HR], 4.09; 95% CI, 2.04-8.18; $P < .001$) and PIRA (HR, 4.71; 95% CI, 2.05-9.77; $P < .001$).

CONCLUSIONS AND RELEVANCE Results of this cohort study suggest that sGFAP is a prognostic biomarker for future PIRA and revealed its complementary potential next to sNfL. sGFAP may serve as a useful biomarker for disease progression in MS in individual patient management and drug development.

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Supplemental content

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The pathogenesis of multiple sclerosis (MS) involves both adaptive and innate immune disease mechanisms. The former is associated with recurring episodes of acute neurologic symptoms, relapses, and formation of localized lesions in the brain and spinal cord caused by invasion of blood-derived immune cells. In contrast, the latter has been suggested to drive more diffuse inflammation and neurodegeneration, also called smoldering MS,[1] that clinically presents as disease progression. Although high-efficacy therapies, such as B-cell-depleting treatment (BCDT), result in almost complete suppression of focal lesion formation, their effectiveness for preventing development of long-term disability is modest.[2,3] This therapeutic gap is mirrored by a diagnostic unmet need to assess progression. Serum neurofilament light chain (sNfL) is now well established as therapy response marker in active disease[4-6]; however, its capacity to reflect concurrent, or to predict progression, especially when acute inflammatory disease activity is suppressed by high efficacy therapies, is still under debate.[4,7-12]

Glial fibrillary acidic protein (GFAP) is an intermediate filament of astrocytes, equivalent to NfL in neurons, and has been proposed as a biomarker to identify present disease progression and to prognosticate future progression in MS.[13-18] Early studies measuring GFAP levels in the cerebrospinal fluid (CSF) of patients with MS found a correlation with neurologic disability in subsequent years; however, this was not the case for NfL levels.[14] Furthermore, high CSF GFAP levels were associated with faster progression to an Expanded Disability Status Scale (EDSS) score of 3 and 6,[19] and levels were higher in primary progressive MS than in relapsing-remitting MS (RRMS).[14,20,21] Moreover, there is also evidence of increased GFAP levels in the CSF of patients with progressive MS who had no recent relapses, showing the potential of GFAP levels for measuring pure progression.[13] In contrast, although NfL was a sensitive indicator of neuroaxonal injury during acute disease activity, ie, lesion formation and relapses, CSF levels of GFAP remained unaffected in this state.[20,22]

Based on different methodological approaches in 2 independent patient cohorts followed in the Swiss MS Cohort (SMSC), this study attempted a direct comparison of sGFAP and sNfL levels: how they reflect acute disease activity vs the identification and prognostication of future disease progression and whether their combination provides added value. In cohort 1, we (1) measured their levels in patients who either remained clinically stable or continued to accumulate more disability over time and (2) compared how they are impacted by acute inflammation in a cohort of patients with relapsing forms of MS (RMS). Cohort 2 comprised patients with MS receiving BCDT as a model of optimal suppression of acute disease activity to evaluate how sNfL and sGFAP levels, alone and in combination, are prognostic for future disability worsening and progression independent of relapse activity (PIRA).

Key Points

Question Are serum glial fibrillary acidic protein (sGFAP) and/or neurofilament light chain (sNfL) concentrations associated with and prognostic for disease progression in patients with multiple sclerosis?

Findings In this cohort study of 355 patients and 259 healthy controls (contributing 737 and 485 serum samples, respectively), elevated sGFAP *z* scores (corrected for confounding factors age, sex, and body mass index) identified current disease progression and were associated with future disease progression but not with acute inflammation. In addition, the association of sNfL levels with progression was less pronounced, whereas sNfL levels were strongly increased during relapse activity.

Meaning Results suggest that sGFAP is more strongly associated than sNfL with disease progression in MS, a finding that has clinical implications for patient management and development of novel drugs.

Methods

Study Design and Patients With MS

This cohort study, conducted from January 1, 2012, to October 22, 2022, was approved by the ethics committees of all participating centers. Patients in both cohorts provided written informed consent. A description of the SMSC and standard definitions are available in the eMethods in Supplement 1. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Cohort 1

Three groups of patients with MS with extreme phenotypes were compared: patients with either stable MS (stMS) or worsening disability[23] had similar baseline EDSS scores and no relapses during the entire follow-up; the focal inflammation group consisted of patients with relapsing MS from whom serum samples were acquired both during active disease phase (relapse and/or contrast-enhancing brain lesions) and remission. Patients with worsening progressive MS or stMS were matched for age, disease duration, EDSS scores, and T2-weighted lesion volume at baseline. Patients with worsening progressive MS presented with at least 1 PIRA event during follow-up. Further details are available in the eMethods in Supplement 1. Cohort 1 included patients of only White race and ethnicity. Other race and ethnic subgroups were too small for meaningful analysis.

Cohort 2

We included all SMSC patients who had initiated and continued BCDT (ocrelizumab or rituximab). sNfL and sGFAP levels were measured in the first sample available 8 months or more after treatment start (median [IQR], 12.2 [10.7-16.8] months). We included patients with RRMS and progressive MS. PIRA was defined by the occurrence of confirmed disability worsening (CDW) events in the absence of relapses between the visit defining baseline of the EDSS worsening event until its confirmation visit at least 6 months later. All other CDW events were defined to be relapse-associated worsening (RAW) events. Cohort 2 included patients of only White race and Hispanic ethnicity. Other race and ethnic subgroups were too small for meaningful analysis.

Healthy Controls

Blood samples from healthy controls (HCs) in the Genome-Wide Association Study of Multiple Sclerosis (GeneMSA[24,25]) were collected at the University Hospital Basel between July 7, 2004, and May 29, 2007. A family history or current diagnosis of MS, as well as other reported ongoing relevant illnesses (eg, diabetes, arterial hypertension), were considered exclusionary for this group.

sGFAP and sNfL Measurements

Blood samples were collected within 8 days from the clinical visit and stored at −80 °C following standardized procedures.[26] sGFAP and sNfL concentrations were measured in duplicate with the ultrasensitive single molecule array (Simoa) technology (Quanterix). In cohort 1, samples were measured using the singleplex Simoa GFAP Discovery Kit on the HD-X analyzer according to the manufacturer's instructions. sNfL levels had been measured in a previous study[4] using the Simoa Nf-Light kit. In cohort 2, samples were measured using the Neurology 2-plex B assay according to manufacturer's instructions (eFigure 1 in Supplement 1). Further details, including information on magnetic resonance imaging (MRI) assessment methods, are in the eMethods in Supplement 1.

Statistical Analysis

In HCs, the association between log-transformed biomarker concentrations as a dependent variable and age, sex, and body mass index (BMI; calculated as weight in kilograms divided by height in meters squared) as independent variables were analyzed using mixed models with a random intercept for person. In analogy with age- and BMI-adjusted sNfL reference values,[4] we calculated sGFAP *z* scores additionally adjusted for sex. A more detailed description of the statistical analysis is available in the eMethods in Supplement 1.

Cohort 1

Comparison of sGFAP and sNfL levels in stMS/worsening progressive MS and RMS cohorts vs HCs was performed using a linear mixed model with log-transformed sGFAP or sNfL levels as the dependent variable and age, BMI, sex, and phenotype group (stMS, worsening progressive MS; RMS in either remission or active disease state) as independent variables as well as a random intercept for the person to account for the repeated nature of the data. To assess the association of disease progression with sGFAP or sNfL levels (individual biomarkers as dependent variables), univariable and multivariable models with stMS vs worsening progressive MS status as well as age, sex, BMI, follow-up time, disease duration, disease-modifying treatment, and EDSS scores as independent variables were used. To evaluate the independent association between disease progression or active disease status and sGFAP or sNfL levels that is not explained by the other biomarker, the respective $\log_2$-transformed marker was additionally added to these models. The within-person variation of sGFAP or sNfL levels was assessed by the intraclass correlation coefficient (ICC) with 95% CI obtained by bootstrapping. Atrophy rates per year in the combined stMS and worsening progressive MS cohort were assessed with a linear mixed model. The associations between biomarker levels and gray matter volume and white matter volume loss were modeled using interaction terms between $\log_2$-transformed baseline sGFAP and sNfL levels, and follow-up time and estimates express the change in annualized atrophy rates per doubling in biomarker concentration. To compare the prognostic power of baseline sGFAP and sNfL levels for PIRA, univariable and multivariable Cox regression models were performed in the combined stMS and worsening progressive MS cohort.

Cohort 2

Biomarker levels in patients with and without later CDW were visualized using box plots and were considered increased compared with HC when being significantly above $z = 0$ in the univariate Wilcoxon signed rank tests (a *z* score of 0, corresponding to the 50th percentile, indicates the physiologic mean level of HC[4]). A cross-sectional analysis was performed using linear models with individual biomarker *z* score as the dependent variable and demographic and clinical variables as predictors. The association between biomarker levels and time to CDW was investigated using Kaplan-Meier curves and Cox regression models. Receiver operating characteristics (ROC) analyses were performed to identify optimal cut points for sGFAP and sNfL *z* score values to dichotomize the respective biomarker levels in high and low groups to prognosticate CDW. The performance of a composite of both biomarkers in prognosticating CDW was investigated by categorizing patients into 4 groups according to high and low levels for each biomarker, using the constellation of low sGFAP/low sNfL as a reference.

Sensitivity analyses were performed using only CDW due to PIRA (ie, excluding CDW due to RAW). A 2-sided *P* value ≤ .05 was considered statistically significant. Analyses were performed in R, version 4.2.0 (R Project for Statistical Computing).

Results

Serum GFAP and sNfL Concentrations in HCs

This study included a total of 355 patients (103 [29.0%] in cohort 1: median [IQR] age, 42.1 [33.2-47.6] years; 30 male individuals [29.1%]; 73 female individuals [70.9%] and 252 [71.0%] in cohort 2: median [IQR] age, 44.3 [33.3-54.7] years; 96 male individuals [38.1%]; 156 female individuals [61.9%]). The cohort of 259 HCs (485 samples) included 177 female individuals (68.3%) and had a median (IQR) age at baseline of 44.3 (36.3-52.3) years. sGFAP levels increased with age (1.5% per year; $P < .001$) (eFigures 2 and 3 in Supplement 1) and were inversely correlated with BMI (1.1% decrease per BMI unit, estimate 0.989; 95% CI, 0.979-0.998; $P = .01$). Across all ages, levels were 14.9% higher in women than in men ($P = .004$). sNfL levels increased by 2.5% per year of age and decreased by 2.2% per unit BMI (estimate 0.978; 95% CI, 0.969-0.986; $P < .001$) in both sexes. sGFAP and sNfL levels were moderately correlated at baseline (Spearman $\rho = 0.47$; $P < .001$).

Cohort 1

At baseline, patients with stMS and worsening progressive MS showed little difference in demographic, clinical, or MRI data,

Table 1. Patient Characteristics of Stable, Worsening Progressive Multiple Sclerosis (MS) and Relapsing MS Sampled During Remission and Active Disease

Variable	MS, No. (%)		*P* value	No. (%)		*P* value
	Stable	Worsening progressive		Remission	Active	
No. of patients	19	18	NA	66		NA
Samples, No.	169	184		66	66	
No. of samples per patient	9 (8-10)	10 (9-12.5)	.10	NA	NA	NA
Follow-up time, median (IQR) [range], y	7.1 (5.7-8.0) [4.1-9.0]	6.5 (5.2-7.7) [2.7-8.5]	.40	NA	NA	NA
Sex						
Female	12 (63.2)	11 (61.1)	<.99	50 (75.8)		NA
Male	7 (36.8)	7 (38.9)		16 (24.2)		
Age, median (IQR), y	44.2 (39.5-49.2)	43.8 (40.9-53.8)	.78	40.6 (30.2-46.4)	39.9 (29.2-45.4)	.62
Disease category at study entry						
RRMS	18 (94.7)	10 (55.6)	.02	62 (93.9)	62 (93.9)	.80
Progressive MS	1 (5.3)	8 (44.4)		4 (6.1)	4 (6.1)	
EDSS score, median (IQR)	3.0 (2.5-3.8)	4.0 (3.1-4.4)	.07	2.0 (1.5-3.0)	2.0 (2.0-3.0)	.25
Disease duration, median (IQR), y	9.4 (6.3-20.1)	13.70 (7.8-18.7)	.43	7.8 (3.8-14.7)	7.5 (3.4-14.1)	.50
DMT			.09			.001
Untreated	3 (15.8)	7 (38.9)		8 (12.1)	23 (34.8)	
Platform	5 (26.3)	0 (0)		4 (7.6)	9 (13.6)	
Oral	6 (31.6)	6 (33.3)		40 (60.6)	31 (47.0)	
Monoclonal antibody therapies	5 (26.3)	5 (27.8)		13 (19.7)	3 (4.5)	
Relapse[a]	NA	NA	NA	0 (0)	36 (54.5)	NA
Time since last relapse, median (IQR), d	NA	NA	NA	NA	16.0 (4.8-22.5)	NA
T2w lesion volume, median (IQR), mL	10.9 (2.7-19.7)	16.3 (12.8-44.7)	.21	5.2 (2.0-14.6)	5.9 (2.6-17.9)	0.48
EDSS score at last sampling, median (IQR)	2.5 (2.0-3.8)	6.0 (5.6-6.9)	<.001	NA	NA	NA
No. of PIRA events						
0	19 (100)	0 (0)	<.001	NA	NA	NA
1	0 (0)	6 (33.3)				
2	0 (0)	8 (44.4)				
3	0 (0)	4 (22.2)				
DMT at last visit						
Untreated	1 (5.3)	4 (22.2)	<.001	NA	NA	NA
Platform	4 (21.1)	0 (0)				
Orals	11 (57.9)	0 (0)				
mAB	3 (15.8)	14 (77.8)				
CEL at sample	1 (0.8)	2 (1.9)	.83	NA	NA	NA
New/enlarging T2w lesion at sample	13 (7.7)	20 (10.9)	.41			
Presence of CEL	NA	NA	NA	0 (0)	30 (45.5)	NA
Relapse and CEL				0 (0)	9 (13.6)	NA
T2w lesion volume, median (IQR), mL				5.2 (2.0-14.6)	5.9 (2.6-17.9)	.48

Abbreviations: CEL, contrast-enhancing lesion; DMT, disease-modifying treatment; EDSS, Expanded Disability Status Scale; mAB, monoclonal antibody therapies; MS, multiple sclerosis; NA, not applicable; PIRA, progression independent of relapse activity; RRMS, relapsing-remitting MS; w, weighted.

[a] Within 30 days.

except that treatment with monoclonal antibodies at last follow-up was more frequent in worsening progressive MS; the EDSS score remained stable in stMS (decreased from 3.0 to 2.5 at 7.1 years median follow-up), whereas in worsening progressive MS, it increased from a score of 4.0 to 6.0 with a median follow-up of 6.5 years (**Table 1**; eFigure 4 in Supplement 1). Worsening progressive MS showed more total brain volume loss (0.28% per year) vs stMS (estimate 0.997; 95% CI, 0.996-0.998; $P < .001$) (eFigure 5 in Supplement 1). Patients with RMS were more frequently untreated in active vs remission state (Table 1).

Comparison of sGFAP and sNfL Concentrations Between Patients and HCs

sGFAP levels were highest in worsening progressive MS (103.0 pg/mL with a 77% increase vs 51.8 pg/mL in HCs; $P < .001$), followed by RMS in active disease (59.1 pg/mL; $P < .001$), RMS during remission (52.9 pg/mL; $P = .01$), and

patients with stMS (63.2 pg/mL; $P = .12$) (eTable 1, eFigure 6 in Supplement 1). Conversely, sNfL levels were highest in active RMS (10.2 pg/mL, namely 98.6% as per adjusted estimate higher than in HCs, 6.3 pg/mL; $P < .001$), followed by worsening progressive MS (10.9 pg/mL; $P < .001$), stMS (7.2 pg/mL; $P = .03$), and RMS in remission (6.7 pg/mL; $P < .001$).

Serum GFAP and sNfL Levels in Worsening Progressive MS vs stMS

sGFAP and sNfL concentrations were increased by 64.2% and 42.2%, respectively, in worsening progressive MS vs stMS (**Table 2**, model 1, **Figure 1**A). After multivariable adjustment, these differences were 57.5% and 24.8%, respectively (Table 2, model 2, Figure 1B), also after additional correction for sNfL (50.9% increase in worsening progressive MS vs stMS), whereas the 25% increase of sNfL levels disappeared after additional sGFAP-level adjustment (Table 2, model 3, Figure 1C). Additional sensitivity analyses adjusting for T2-weighted lesion volume, and number of new and enlarged and contrast-enhancing brain lesions confirmed these results and showed comparably increased sGFAP levels in worsening progressive MS vs stMS (eTable 2 in Supplement 1). sGFAP levels in the worsening progressive MS cohort showed less within-person variability over time (ICC: estimate, 0.91; 95% CI, 0.83-0.94, ie, 91% of the variation in sGFAP levels is explained by variation between patients), whereas for sNfL ICC was 0.80 (95% CI, 0.72-0.85; difference, 11%; 95% CI, 2%-19%; $P = .02$).

sGFAP and sNfL Levels in RMS During Active Disease and Remission

sNfL concentrations were 58.4% increased in active disease vs remission, whereas this difference was 7.3% for sGFAP levels (eTable 3 in Supplement 1, model 1). After adjustment for potential confounders, these differences were 53.2% and 4.8%, respectively (eTable 3 in Supplement 1, model 2). Additional correction for sGFAP levels did not influence the association of focal inflammation status with sNfL levels (50.6% increase in active vs remission state), whereas association with sGFAP levels remained insignificant (eTable 3 in Supplement 1, model 3).

Association of Baseline sGFAP and sNfL Levels With Brain Volume Loss and PIRA

Each doubling of baseline sGFAP levels was associated with an additional loss of gray matter volume (−0.24% per year; 95% CI, −0.35% to −0.12%; $P < .001$) but not white matter volume (0.05%; 95% CI, −0.09% to 0.18%; $P = .48$), whereas doubling of baseline sNfL levels was associated with an additional loss of white matter volume (−0.26%; 95% CI, −0.38% to −0.15%; $P < .001$) but not gray matter volume (−0.01%; 95% CI, −0.11 to 0.09; $P = .78$) (eTable 4, eFigure 7 in Supplement 1). Baseline values of sGFAP levels had a better prognostic capacity for future PIRA (HR per doubling, 3.88; 95% CI, 1.69-8.86; $P = .001$; ie, an almost 4-fold risk of PIRA by doubling of baseline sGFAP concentration) than sNfL levels (HR, 1.77; 95% CI, 1.11-2.83; $P = .02$). In a combined model, with additional adjustment for age, sex, BMI, and disease duration, these findings were confirmed: sGFAP levels (HR, 3.63; 95% CI, 1.46-9.04; $P = .006$) and sNfL levels (HR, 1.90; 95% CI, 0.86-4.19; $P = .11$).

Cohort 2

Cohort Characteristics

We included 252 patients receiving BCDT who were relapse-free in the 6 months prior to sampling (ie, baseline). The majority of patients presented with RRMS (181 of 252 [71.8%]), whereas the remaining had progressive MS (34 [13.5%] secondary progressive MS; 37 [14.7%] primary progressive MS). A total of 43 of 252 (17.1%) experienced CDW during follow-up, of which 39 (90.7%) were due to PIRA and 4 (9.3%) due to RAW (eTable 5 in Supplement 1).

sGFAP and sNfL Levels and Development of Future CDW

In patients with MS overall, sGFAP levels were strongly increased compared with those of HCs (z score = 0) with a median (IQR) of 0.82 (−0.05 to 1.95) z score units above normal ($P < .001$), whereas the increase of sNfL levels was less pronounced (0.50; IQR, −0.25 to 1.32; $P < .001$). Development of CDW was associated with a higher sGFAP z score 12.2 (IQR, 10.7-16.8) months after BCDT start than in patients without future CDW (1.94; IQR, 0.36-2.23 vs 0.71; IQR, −0.13 to 1.73) (**Figure 2**A). Although sNfL z score were less but still significantly increased vs that in HCs, the difference between patients with vs those without CDW development was not significant (Figure 2B). This pattern was similar when RAW events were excluded (sGFAP levels: PIRA, 1.98; IQR, 0.33-2.27 vs no PIRA, 0.71; IQR, −0.11 to 1.73; $P = .003$; sNfL levels: PIRA, 1.09; IQR, 0.14-1.49 vs no PIRA, 0.44; IQR, −0.25 to 1.23; $P = .04$).

Next, we explored which demographic and disease-related variables were associated with increased biomarker levels in patients receiving BCDT compared with HCs using multivariable models (ie, using biomarker z scores as dependent variable (eTable 6 in Supplement 1). Models on the absolute sGFAP and sNfL concentrations are included in eTable 7 in Supplement 1. The model for sGFAP z score explained 13.3% of the variance and was driven by female sex, younger age, higher EDSS, and whether the patient developed CDW while receiving BCDT (CDW status in eTable 6 in Supplement 1). The same model with sNfL z score as the outcome explained 1.8% of its variance. Specifically, only sGFAP z scores, but not those of sNfL, were linked to the EDSS score and future CDW. Again, findings were similar in the PIRA only set (not shown).

Prognostic Value of sGFAP and sNfL Levels for Future CDW

Time-to-event analyses showed that 1 sGFAP z-score unit increase led to a 1.36-fold (HR, 1.36; 95% CI, 1.09-1.69; $P = .006$) increased risk of CDW (after correction for covariates: HR, 1.32; 95% CI, 1.06-1.66; $P = .01$). For sNfL z score, a numerically higher risk was found (HR, 1.25; 95% CI, 0.95-1.65; $P = .11$; after correction: HR, 1.27; 95% CI, 0.95-1.71; $P = .11$). When combining both sGFAP and sNfL z scores in 1 model, sGFAP was associated with disease worsening: HR, 1.34 (95% CI, 1.03-1.73; $P = .03$), but not sNfL (HR, 1.04; 95% CI, 0.75-1.43; $P = .82$).

Next, we used different z score cut points to see whether their increase was associated quantitatively to the risk of CDW. sGFAP z score cut points of 1, 1.5, and 2 led to gradually increasing CDW hazard ratios ranging from 2.1 to 3.4

Table 2. Multivariable Mixed Linear Models Investigating the Association of Worsening Status (Stable Multiple Sclerosis [MS] vs Worsening Progressive MS) With Log-Transformed Serum Glial Fibrillary Acidic Protein (sGFAP) and Serum Neurofilament Light Chain (sNfL) Levels

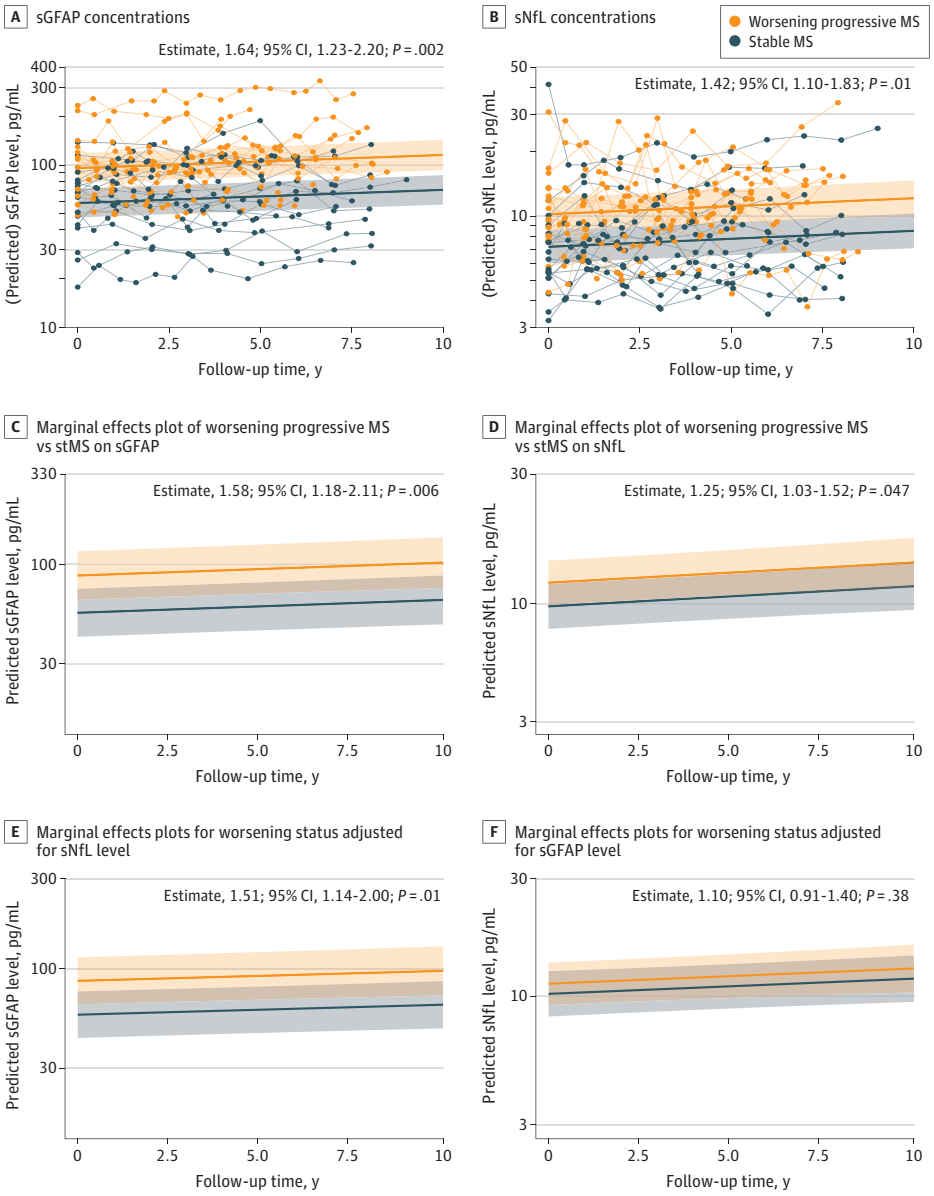
Model	Sample, No.	sGFAP, median (IQR), pg/mL	Estimate (95% CI)[a]	*P* value	sNfL, median (IQR), pg/mL	Estimate (95% CI)	*P* value
Model 1: simple							
Follow-up time	NA	NA	1.019 (1.011-1.026)	<.001	NA	1.017 (1.008-1.027)	<.001
Progression							
Stable MS	169	63.2 (43.4-90.7)	NA	NA	7.1 (5.4-9.4)	NA	NA
Worsening progressive MS	184	103.0 (81.3-132.5)	1.642 (1.226-2.199)	.002	10.9 (8.2-13.9)	1.422 (1.104-1.831)	.01
Model 2: multivariable							
Age at baseline	NA	NA	1.008 (0.993-1.023)	.35	NA	1.019 (1.009-1.029)	.002
Follow-up time	NA	NA	1.016 (1.007-1.025)	<.001	NA	1.019 (1.008-1.030)	.001
Sex							
Female	224	87.7 (57.3-109.7)	1.026 (0.764-1.378)	.87	8.4 (6.3-10.9)	0.875 (0.725-1.058)	.21
Male	129	84.3 (57.7-121.1)	NA		11.8 (5.8-16.7)	NA	
BMI[b]	NA	NA	0.991 (0.973-1.008)	.32	NA	0.969 (0.953-0.985)	<.001
Disease duration at baseline	NA	NA	1.002 (0.985-1.018)	.86	NA	1.005 (0.994-1.016)	.40
DMT							
Untreated	48	97.4 (63.8-112.9)	NA	NA	11.7 (8.7-16.4)	NA	NA
Platform	40	68.6 (57.7-90.4)	1.191 (1.048-1.356)	.009	9.7 (6.3-17.4)	0.956 (0.821-1.142)	.59
Orals	118	74.7 (39.4-97.4)	1.032 (0.933-1.139)	.54	7.7 (5.3-9.5)	0.921 (0.811-1.039)	.20
mAB	147	103.6 (68.4-136.5)	1.080 (0.997-1.171)	.06	9.4 (6.8-12.8)	0.938 (0.842-1.035)	.22
EDSS score	NA	NA	1.011 (0.982-1.041)	.46	NA	1.002 (0.969-1.039)	.92
Progression							
Stable MS	169	63.2 (43.4-90.7)	NA	NA	7.1 (5.4-9.4)	NA	NA
Worsening progressive MS	184	103.0 (81.3-132.5)	1.575 (1.178-2.106	.006	10.9 (8.2-13.9)	1.248 (1.024-1.521)	.05
Model 3: plus sNfL/sGFAP							
Age at baseline	NA	NA	1.004 (0.990-1.0.19)	.59	NA	1.016 (1.007-1.026)	.004
Follow-up time	NA	NA	1.012 (1.004-1.021)	.005	NA	1.014 (1.003-1.025)	.01
Sex							
Female	224	87.7 (57.3-109.7)	1.053 (0.792-1.400)	.74	8.4 (6.3-10.9)	0.868 (0.725-1.040)	.17
Male	129	84.3 (57.7-121.1)	NA	NA	11.8 (5.8-16.7)	NA	NA
BMI[b]	NA	NA	0.996 (0.979-1.013)	.66	NA	0.973 (0.958-0.989)	.002
Disease duration at baseline	NA	NA	1.001 (0.985-1.017)	.94	NA	1.005 (0.994-1.1015)	.42
DMT							
Untreated	48	97.4 (63.8-112.9)	NA	NA	11.7 (8.7-16.4)	NA	NA
Platform	40	68.6 (57.7-90.4)	1.214 (1.072-1.377)	.003	9.7 (6.3-17.4)	0.907 (0.782-1.082)	.24
Oral	118	74.7 (39.4-97.4)	1.045 (0.948-1.151)	.37	7.7 (5.3-9.5)	0.917 (0.812-1.032)	.17
mAB	147	103.6 (68.4-136.5)	1.090 (1.008-1.179)	.03	9.4 (6.8-12.8)	0.918 (0.827-1.010)	.10
EDSS score	NA	NA	1.012 (0.984-1.041)	.41	NA	0.999 (0.968-1.036)	.98
sNfL per doubling, pg/mL	NA	NA	1.141 (1.079-1.207)	<.001	NA	NA	NA
sGFAP per doubling, pg/mL	NA	NA	NA	NA	NA	1.217 (1.120-1.315)	<.001
Progression							
Stable MS	169	63.2 (43.4-90.7)	NA	NA	7.1 (5.4-9.4)	NA	NA
Worsening progressive MS	184	103.0 (81.3-132.5)	1.509 (1.139-1.998)	.01	10.9 (8.2-13.9)	1.099 (0.905-1.339)	.38

Abbreviations: BMI, body mass index; DMT, disease modifying treatment; EDSS, Expanded Disability Status Scale; mAB, monoclonal antibody therapies; NA, not applicable; sGFAP, serum glial fibrillary acidic protein; sNfL, serum neurofilament light chain.

[a] Estimates are back transformed and represent multiplicative effects.

[b] Calculated as weight in kilograms divided by height in meters squared.

Figure 1. Serum Glial Fibrillary Acidic Protein (GFAP) and Serum Neurofilament Light Chain (sNfL) in Worsening Progressive Multiple Sclerosis (MS) and Stable MS



Concentrations of sGFAP (A) and sNfL (B) in worsening progressive MS vs stable MS (stMS) over follow-up time. sGFAP and sNfL concentrations were increased by 64.2% and 42.2%, respectively, in worsening progressive MS vs stMS. Thin lines connect longitudinal data points of individual patients; thick lines show the group regression lines from Table 2, model 1. Only the regression lines are predicted. Estimates including 95% CI and *P* value of differences between worsening progressive MS vs stMS are added to the plots (B). Marginal effects plots of multivariable mixed models showing the association of worsening progressive MS vs stMS with sGFAP (C) and sNfL levels (D) over follow-up time. After adjustment for potential confounders, sGFAP and sNfL concentrations were increased by 57.5% and 24.8%, respectively, in worsening progressive MS vs stMS (Table 2, model 2). Marginal effects plots for worsening status with additional adjustment for sNfL (E) and sGFAP (F). Additional correction for sNfL levels had a minor association with the difference of sGFAP levels between stMS vs worsening progressive MS status (50.9% increase); however, additional correction for sGFAP eliminated the association of progression status with sNfL levels (Table 2, model 3).

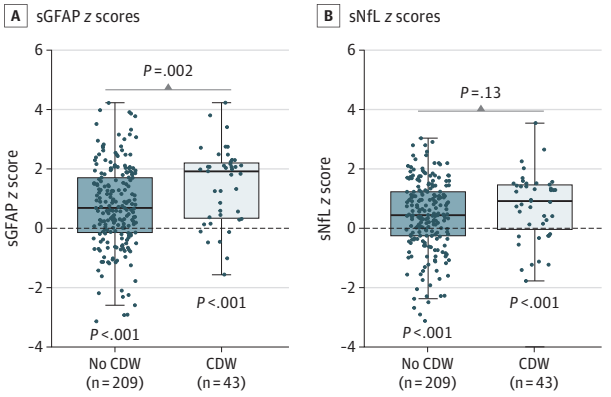
(eFigure 8A in Supplement 1). The associations were all significant for sGFAP, whereas for sNfL (eFigure 8B in Supplement 1), findings were less strong.

sGFAP and Prognostication of Worsening in a Combined Analysis of sNfL and sGFAP Levels

The risk of CDW in patients with high sGFAP levels (ie, *z* score >1.8, cutoff optimized in ROC analysis) compared with low sGFAP levels was 3-fold increased (HR, 3.25; 95% CI, 1.78-5.93; *P* < .001) in a time-to-event analysis. Patients with high sNfL levels (ie, *z* score >1.3) showed a 2-fold increased risk of future CDW (HR, 2.26; 95% CI, 1.24-4.14; *P* = .008) vs patients with low sNfL levels.

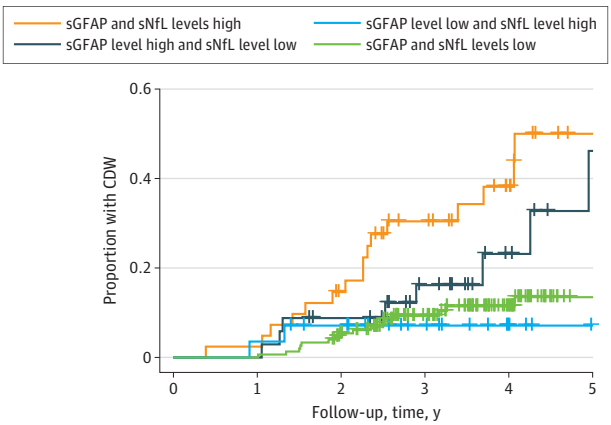
The combination of high sGFAP/high sNfL levels was associated with a 4-fold increased risk of worsening compared with low sGFAP/low sNfL levels (HR, 4.09; 95% CI, 2.04-8.18; *P* < .001 and PIRA only: HR, 4.71; 95% CI, 2.05-9.77; *P* < .001), that of high sGFAP/low sNfL levels showed slightly reduced association (**Figure 3**) (PIRA only: HR, 2.28; 95% CI, 0.92-5.64; *P* = .08). In contrast, the combination of low sGFAP/high sNfL levels did not show an increased risk for future CDW (PIRA only: HR, 1.17; 95% CI, 0.34-4.10; *P* = .80). The Kaplan-Meier analysis indicated that 4 years after initiation of treatment, 38% (95% CI, 20%-53%) of patients in the high sGFAP/high sNfL group will have CDW, compared with 23% (95% CI, 3%-39%) in the high sGFAP/low sNfL group, whereas this will be the case only

Figure 2. Serum Glial Fibrillary Acidic Protein (sGFAP) and Serum Neurofilament Light Chain (sNfL) *z* Scores in Patients With and Without Confirmed Disease Worsening During Follow-up While Receiving B-Cell–Depleting Therapy in Comparison to Healthy Controls



Box plot representation of sGFAP *z* scores (A) and sNfL *z* scores (B). Dashed lines indicate mean values in healthy controls (ie, *z* score = 0) and *P* values below indicate whether observed values differ from *z* scores 0 (Wilcoxon signed rank test). In patients with MS (without and with future confirmed disease worsening [CDW] development), sGFAP levels were increased compared with healthy controls (*z* scores healthy controls = 0; $P < .001$ for both), whereas the increase of sNfL was less pronounced ($P < .001$ for both). Development of CDW was associated with higher sGFAP *z* scores, which was not the case for sNfL.

Figure 3. Kaplan-Meier Curves Using Combined Biomarker Data to Predict Time to Confirmed Disease Worsening (CDW)



No. at risk						
sGFAP and sNfL levels high	41	40	33	22	13	4
sGFAP level high and sNfL level low	34	34	29	20	9	4
sGFAP level low and sNfL level high	28	27	23	16	9	5
sGFAP and sNfL levels low	149	149	132	94	51	19

Optimized cutoffs of serum glial fibrillary acidic protein (sGFAP) and serum neurofilament light chain (sNfL) *z* scores from receiver operating characteristic curve analysis, based on the Youden index, were used to dichotomize patient groups. High sGFAP/high sNfL levels were associated with a 4-fold (hazard ratio [HR], 4.09; 95% CI, 2.04-8.18; $P < .001$) increased risk of CDW compared with low sGFAP/low sNfL levels. The combination of high sGFAP/low sNfL levels showed a slightly reduced risk (HR, 2.32; 95% CI, 0.99-5.42; $P = .05$). The combination of low sGFAP/high sNfL levels, however, did not show an increased risk on CDW (HR, 1.03; 95% CI, 0.30-3.53; $P = .97$).

in 11% (95% CI, 6%-16%) if they fall into the low sGFAP/low sNfL group.

Discussion

The long-term course of disability in MS is driven by 2 partly independent pathomechanisms: focal lesional activity and brain-diffuse neurodegeneration.[23] sNfL has been established in recent years as a biomarker of ongoing neuronal damage in the course of the former process, whereas its association with progression as the clinical manifestation of the latter is relatively weaker.[12] The need for a biomarker that specifically reflects current and prognosticates future disability due to pure progression/PIRA has become urgent on the background that disability worsening often continues despite almost complete suppression of acute disease activity under high-efficacy therapies.[2,3] Increased CSF levels of GFAP have been proposed first by Axelsson et al[14] as a specific biomarker for progression. However, this finding was based on repetitive CSF analysis, which has precluded its entry into routine practice to close this diagnostic gap. Second, the relative contribution of lesional activity and RAW vs PIRA to the overall progression could not be determined in the mixed RRMS and progressive MS population studied. In this cohort study, we attempted to resolve the question about the mechanistic source of GFAP increase in MS by 2 orthogonal methodological approaches where relapse activity was absent in worsening progressive MS and stMS (cohort 1) or lesional activity and relapses were suppressed by BCDT in a mixed MS population (cohort 2). Current results suggest that increased levels of sGFAP were associated with pure progression/PIRA, although this biomarker is largely inert to acute disease activity. Higher baseline sNfL levels were prognostic for white matter volume loss, and baseline sGFAP specifically prognosticated gray matter loss, a previously proposed proxy for disease progression.[27,28] These findings from serum analysis are fully congruent with those of Axelsson et al in CSF.[14]

The increase of GFAP levels in the course of MS progression appears to result from astrocyte proliferation/activation and possibly injury.[29] This seems to be a brain-diffuse process, affecting mainly the normal-appearing white matter resulting in decreased diffusion tensor imaging derived measures.[16,30] In return, the minor increase of sNfL seen, eg, in patients with worsening progressive MS may result from continuous neuronal loss outside of acute lesion formation as part of the pathogenesis of progression due to subclinical neuroinflammation in chronic active lesions and in normal-appearing white matter.[31]

The association of sGFAP levels with future CDW and imaging features of progression is further supported by studies using serum samples.[16,17,32,33] However, these were incompletely controlled for confounding factors such as sex, age, and BMI, which resulted in significant overlap in GFAP levels across different MS groups and also controls, thus limiting clinical usefulness. Moreover, the comparison of raw biomarker concentrations vs *z* scores as an outcome highlights the advantage of

the latter in terms of pathogenetic relevance and ease of interpretability; covariates explained 29% of variation in raw sNfL concentration but only 1.8% of the variation in sNfL *z* score. For sGFAP levels, covariates similarly explained 25% of the variation in sGFAP concentrations but additionally also 13% in the variation of sGFAP *z* scores. Using corrected *z* scores, instead of absolute concentrations, to assess change of these biomarkers compared with normal values, thus substantially increase the sensitivity for detecting pathologic values, a prerequisite for its use in individual patients.

Important from a clinical perspective is that prognostication of future disability can be made based on a single GFAP measurement and from a biofluid (serum) that is easily accessible in clinical practice. A further aspect in our data set for the clinical use of sGFAP levels is the establishment of normative values of sGFAP that allow to define aberrations from physiological values corrected for confounding factors. Although age and BMI were known confounders, also based on the experience from the establishment of normative values for sNfL,[4] the 15% increase of sGFAP values in women vs men was an unexpected finding. Third, the combined evaluation of sNfL and sGFAP levels provides the highest predictive power for disability worsening, specifically in years 2 to 4, as a reflection of a comprehensive coverage of biological processes leading to disability worsening.

Limitations

This study has some limitations. One limitation is that we studied almost exclusively the effect of anti-CD20 antibodies as high-efficacy therapy but less so other types of disease-modifying treatments of this efficacy level (eg, natalizumab). Such evaluations will be necessary to expand on the limited data available whether disease-modifying treatment can lead to decrease of sGFAP levels[34] as a potential sign of attenuation of astrogliosis or pathological astrocyte activation. Second, the current normative database is derived from a relatively small cohort of HCs, where the impact of subclinical comorbidities could not be explored. A much larger cohort of persons, including those with other neurologic disease, may be needed to establish robust normal values for sGFAP levels.

Conclusions

In summary, the findings of this cohort study suggest that sGFAP levels may serve as a biomarker that reflects specifically chronic disease processes conveyed by astrocytes that manifest as pure progression/PIRA in MS. With this property, sGFAP levels are complementary to sNfL, whose levels are strongly associated with neuronal damage due to lesional disease activity.

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