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Serum glial fibrillary acidic protein correlates with multiple sclerosis disease severity

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Abstract

Background: Cerebrospinal fluid (CSF) levels of two soluble biomarkers, glial fibrillary acidic protein (GFAP) and neurofilament light chain (NfL) have been shown to associate with multiple sclerosis (MS) disease progression. Now, both biomarkers can be detected reliably in serum and importantly, their serum levels correlate well with their CSF levels.

Objective: To evaluate the usability of serum GFAP measurement as a biomarker of progressive disease and disease severity in MS.

Methods: Clinical course, EDSS, disease duration, patient age and MRI parameters were reviewed in 79 MS patients in this cross-sectional hospital-based study. Serum samples were collected for measurement of GFAP and NfL concentrations using single molecule array (Simoa) assay. A cohort of healthy controls was evaluated for comparison.

Results: Higher serum concentrations of both GFAP and NfL were associated with higher EDSS, older age, longer disease duration, progressive disease course and MRI pathology.

Conclusion: Earlier studies have demonstrated that GFAP, unlike NFL, is not increased in association with acute focal inflammation-related nervous system damage. Our work suggests that GFAP serum level associates with disease progression in MS and could potentially serve as an easily measurable biomarker of CNS pathology related to disease

progression in MS.

Glossary

CNS = central nervous system; **CSF** = cerebrospinal fluid; **EDSS** = The Expanded Disability Status Scale; **GFAP** = glial fibrillary acidic protein; **MS** = multiple sclerosis; **MSSS** = multiple sclerosis severity score; **NfL** = neurofilament light chain

Introduction

Both inflammation and neurodegenerative mechanisms have important roles in the pathogenesis of multiple sclerosis (MS). Two thirds of the patients who initially present with a relapsing-remitting disease (RRMS) will develop a progressive disease course before age 75.[1] Time of transformation into progressive disease seems to be most of all age-dependent, and independent of disease duration or amount of inflammatory activity preceding progression.[1, 2] Identification of clinical disease progression or signs of pathology leading to clinical progression is insensitive by clinical or MRI evaluation, and is mostly done retrospectively.[3] It would be of great value if the risk of progression, or the level of progression-related CNS-pathology could be evaluated using easily measurable biomarkers.

The search for usable biomarkers for MS progression has been intensive for decades.[4] Most of the potential biomarkers discovered so far have had to be determined from cerebrospinal fluid (CSF) as their concentration in the peripheral blood is extremely low. Repeated CSF-sampling for follow-up of disease pathology is unpractical both in a clinical setting and in therapeutic trials of MS, and there is hence a great need for serum-measurable markers associated with MS disease pathology. Luckily, the methodology for measuring minute concentrations of brain-derived proteins in serum has evolved and is now widely available.[5, 6]

The two most promising biomarker candidates for nervous system damage-related pathology in progressive MS are neurofilament light (NfL) and the glial fibrillary acidic protein (GFAP). NfL is one of three neurofilament proteins (neurofilament heavy chain, NfH; neurofilament medium chain, NfM and neurofilament light chain, NfL). It is a major cytoskeletal intermediate filament protein in myelinated axons, and one of the most promising biomarker candidates to demonstrate neural damage in a variety of neuropathological conditions, including MS.[7, 8] GFAP is the major intermediate cytoskeletal protein of astrocytes. It is another biomarker which has been widely studied for detecting damage of the CNS particularly in traumatic brain injury (TBI).[9, 10] It is released into the intercellular space and CSF during astrocyte activation.[11, 12] Most of the previous studies on both NfL and GFAP have been conducted on CSF samples, but

both can now be detected also in serum.[5, 13] Importantly, for both NfL and GFAP the serum concentrations have been shown to reflect the levels in the CSF.[14-17] Higher concentrations of NfL in the CSF have been shown to associate with MS relapse and inflammatory activity,[13-15, 18, 19] and both NfL and GFAP in the CSF have been described to be associated with accumulating neurological disability.[14, 15, 18, 20, 21]

The usability of CSF GFAP and NFL concentrations as biomarkers for MS has been studied already since 1990s,[22, 23] but the results have been somewhat contradictory. This is probably mostly because of the use of different measurement techniques and the heterogeneity of the MS patient cohorts. In this study we measured these two potential biomarkers simultaneously from the serum samples of MS patients using ultrasensitive measurement technique, single molecule array (Simoa) assay. Our aim was to report the serum GFAP levels in different cohorts of MS patients and in healthy controls, and to evaluate the usability of serum GFAP measurement as a biomarker for disease progression-related CNS pathology in MS.

Materials and methods

79 MS patients were recruited to this hospital-based cross-sectional study from the Neurology outpatient clinic of the Division of Clinical Neurosciences at the Turku University Hospital, Turku, Finland. The Ethical Committee of the Hospital District of

Southwest Finland approved the study. A written consent was obtained from all patients. 46 patients had relapsing remitting disease (RRMS), and 33 had secondary progressive MS (SPMS). In addition, 13 healthy age-matched controls (HC) were included. 40 RRMS (85.11 %) and 11 SPMS (33.33 %) patients were on disease modifying therapy (DMT) (natalizumab n=10, interferon beta-1a n=8, glatiramer acetate n=6, rituximab n=5, dimethyl fumarate n=5, fingolimod n=14, teriflunomide n=3). Blood samples were collected from all patients and controls, and serum was stored at -40˚C within four hours of sampling. The patients were free of clinical relapses (new neurological symptoms lasting at least 24 hours) during the time of sampling. Clinical disease course, disease duration and patient age were reviewed, and EDSS score was assessed by the investigating neurologist. The demographics and clinical parameters of the cohorts are summarized in table 1. Concentrations of GFAP (commercially available kit) and NfL were measured from paired serum samples using single molecule array (Simoa) assay technology.[5, 14] The statistical analysis was performed using R statistical tool (3.5.0). For the correlation analysis Spearman's rank correlation coefficient was determined. The differences between groups were assessed using the Kruskal-Wallis test. Pairwise differences were assessed using the Wilcoxon rank-sum test. GFAP and NfL were used to predict the disease status using logistic regression and classification tables were constructed using probability 0.5 as the

threshold. Additionally, ROC curves and AUC values were used to assess the goodness of the predictive power.

MRI data was available from 6 HC, 46 RRMS and 19 SPMS patients at the time of serum sampling and clinical evaluation. The MRI sequences included 3DT1 and FLAIR sequences, wherefrom volumes of grey matter cortex (GMctx), normal appearing white matter (NAWM) and total T1 and T2 lesion volumes (cm^3) were defined using Freesurfer software and Lesion Segmentation Tool according to our previously reported methodology.[24] The raw data used in preparation of the article will be shared in anonymized format by request of a qualified investigator.

Results

Patients

The MS patients and HC did not differ in age or sex, but the SPMS patients were significantly older than the RRMS patients (table 1). The demographic details of the study subjects are listed in table 1.

[Table 1]

Serum GFAP and NfL

The levels of GFAP and NfL were strongly associated with each other (figure 1A). The level of serum GFAP was higher in MS patients compared to healthy controls, but there was no statistically significant difference in the NfL level compared to healthy controls

(table 1, figure 1B). Majority of the MS patients (64.56 %) were using disease modifying treatment (DMT), which allowed us to evaluate the effect of DMT on serum GFAP and NfL levels. The level of both GFAP and NfL was significantly higher in patients with no DMT compared to patients with treatment (figure 1C).

When evaluated within the MS subgroups, both GFAP and NfL were elevated among SPMS patients when compared to RRMS or controls (table 1, figure 1D). The level of neither biomarker within the RRMS group differed significantly from HC (table 1, figure 1D). As the levels of GFAP and NfL are known to increase with age, we corrected the results including age as a covariant to the statistical analysis. Age-correction did not change the statistical significances (table 1).

Association of GFAP and NfL with MRI parameters

The NfL level in RRMS was relatively low and comparable to HC in our cohort. As earlier studies have shown that NfL levels associate with MRI activity,[8, 13-16] we evaluated the association of focal inflammatory damage, as measured by total T1 and T2 lesion loads (cm^3) in brain MRI, with the serum levels of the two biomarkers. The lesion loads were significantly higher in SPMS compared to RRMS (table 1, figure 2A), and both T1 and T2 loads associated with higher GFAP levels (figure 2B). An increased T1 lesion load was associated with elevated NfL (figure 2B). GMctx volumes were

lower in SPMS compared to RRMS (table 1, figure 2A), but there was no statistically significant difference in the NAWM volumes. Both biomarkers were negatively associated with cortical grey matter (GMctx) but not with normal appearing white matter (NAWM) volume (figure 2B).

The correlation of serum GFAP and NfL with age, disease duration and disability

Serum levels of GFAP and NfL were associated with age (figure 3A) and elevated GFAP and NfL associated strongly with increased disability (higher EDSS score) and disease duration (figure 3B and C). The serum level of NfL also associated with MSSS (Multiple Sclerosis Severity Score) (figure 3D).

[Table 2]

Predictive power of serum GFAP and NfL levels

Finally, we conducted a statistical analysis to test the categorizing power of the two biomarkers. Area under curve (AUC) for serum levels of GFAP and NfL in relation to differentiating between RRMS and SPMS patients were 0.77 for GFAP, 0.76 for NfL, and 0.81 for GFAP and NfL together (figure 4). The specificity, sensitivity, and positive and negative predictive values for both GFAP and NfL are presented in table 2. The GFAP prediction matched for 19 SPMS patients but 14 SPMS patients were incorrectly categorized as RRMS. The values were almost identical for NfL (18 and 15). For RRMS however, GFAP level predicted 41 patients as RRMS and only 5 patients

incorrectly as SPMS. For NfL these numbers were 39 and 7. The sensitivity of differentiating between RRMS and SPMS patients was 57.58 % for GFAP and 54.55 % for NfL, with specificity 89.13 % and 84.78 %, respectively (table 2). The values were the same as the values for GFAP alone when GFAP and NfL were considered together (data not shown).

Discussion

This is the first study to report serum levels of GFAP in MS using the ultrasensitive Simoa assay. Our results indicate that both GFAP and NfL are significantly increased in SPMS compared to healthy controls and RRMS. Higher GFAP and NfL levels associate with advanced age, EDSS and disease duration, and with each other. The NfL levels associate also with MSSS. The statistical significance in the association with the disease severity remained when age was considered as covariant (data not shown). Our results are in line with previous results, where GFAP level in the CSF was shown to be a potential marker of progression in MS.[21] Association of EDSS with CSF levels of both GFAP[18, 21] and NfL[14, 15] has also been suggested in previous reports. Importantly, GFAP, unlike NfL is not known to increase during acute inflammation in the CSF[19, 22] or in the serum, nor is it known to be affected by DMT.[25, 26]

In our study, NfL levels in the RRMS group were low and after age-correction comparable to levels in the healthy control group. Considering the nature of our RRMS cohort this finding is not surprising. The RRMS patients were sampled during a relapse-free stage of the disease, and they mostly had clinically stable disease with no current MRI evidence of acute inflammation, i.e. there were no gadolinium-enhancing lesions. The RRMS patients had an overall low disease burden, which was also indicated by the low lesion volume in MRI. Most of the RRMS patients (85.11 %) were using a disease modifying therapy (DMT) at the time of sampling. The use of the DMT might also have contributed to the low NfL levels, as initiation of DMT has previously been shown to lead to reduction in the serum NfL level.[15] In our cohort, patients using DMT had significantly lower values of both NfL and GFAP compared to those without treatment.

We evaluated in this study how precisely serum biomarkers GFAP and NfL alone and together could classify MS patients with different disease courses within the study cohort. We found that GFAP was slightly more sensitive (57.58 % vs 54.55 %) and specific (89.13 % vs 84.78 %) in predicting the current disease course (RRMS vs SPMS) of the patients than NfL. The ROC curves indicated that GFAP alone is slightly better than NfL alone. Additionally, these two together give slightly greater AUC value than GFAP alone (0.77 vs 0.81). Although the two biomarkers might not be entirely optimal in describing our patient cohort, there may be other important indications for

their use in evaluation of MS patients. It appears that measuring of GFAP and NfL simultaneously may be useful for example for differentiating true relapses and acute inflammatory events associated with MS disease from other neurological symptoms. To give an example, an RRMS patient might have neurological symptoms which could be attributed to inflammatory disease activation. This could be confirmed by measurement of NfL and GFAP. A high NfL and low GFAP in this situation would support the suspicion of a relapse. Another scenario could be a situation when an individual is presenting with neurological symptoms compatible with MS. A high GFAP at an early stage of diagnostic work-up could be indicative of already prevalent neural pathology (which may or may not be visible in MRI). In this situation also NfL would probably be elevated, but if only NfL was measured, one could not differentiate whether the elevation was due to acute inflammation or to more chronic process of neurodegenerative pathology. Further studies are however needed before these serum biomarkers can be reliably used for evaluation of MS patients in the clinic. It will be important to evaluate serum GFAP levels also during relapses and in association with acute gadolinium-enhancing lesions. It will also be useful to measure serum GFAP longitudinally within different subtypes of MS, and before and after various treatments.

In conclusion, we demonstrate in this work that serum levels of both GFAP and NfL are markedly increased in SPMS patients compared to healthy controls and RRMS patients.

Simultaneous NfL and GFAP measurements in serum will be potentially useful for evaluation of the nature of the ongoing pathological process in the CNS with GFAP likely associating with progression-related pathology, and NfL being released also due to inflammation-associated neural damage. For validation as markers of disease activity and of disease progression, and possibly as predictors of disease progression, longitudinal studies measuring both biomarkers in a rich patient cohort of different disease types are still needed. The ease of blood sampling for measurement of MS pathology-related biomarkers (as opposed to CSF sampling, or MRI or PET imaging) enables frequent and sensitive monitoring of the disease pathology, and prompt treatment adjustments.

Author contributions

Heidi Högel, analysis and interpretation the data, drafting the manuscript

Eero Rissanen, acquisition of data, critical revision of manuscript

Christian Barro, acquisition of data, revision of manuscript

Markus Matilainen, statistical analysis, revision of manuscript

Marjo Nylund, acquisition of data, revision of manuscript

Jens Kuhle, study supervision, critical revision of manuscript for intellectual content

Laura Airas, study concept and design, study supervision, critical revision of manuscript for intellectual content

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Table 1: Demographic characteristics, biomarker concentrations and MRI results of the study subjects.

	Healthy controls (HC)	Patients (MS)	p value[1] (HCvsMS)	Disease type		p-value[1,4]		
				RRMS	SPMS	HCvsRRMS	HCvsSPMS	RRMSvsSPMS
n	13	79		46	33			
Age, median (range)	49.24 (25.43-69.47)	48.26 (28.43-73.60)	0.622	46.32 (28.43-56.13)	56.12 (31.61-73.60)	0.211	0.213	<0.001
Gender, no of females (%)	9 (69.23)	56 (70.89)	0.903[3]	36 (78.26)	20 (60.60)	0.998[3]	0.998[3]	0.265[3]
Disease duration, y	-	15.48 (9.25)	-	11.30 (5.20)	21.18 (10.52)	-	-	<0.001
Age at MS onset, median (range)	-	33.78 (19.31-50.64)	-	33.08 (19.31-48.19)	33.89 (20.45-50.64)	-	-	0.735
EDSS, median (range)	-	3.0 (0.0-8.0)	-	2.5 (0.0-5.0)	6.0 (2.0-8.0)	-	-	<0.001
MSSS, median (range)	-	3.79 (0.35-8.92)	-	3.05 (0.35-6.81)	5.33 (1.00-8.92)	-	-	<0.001
Immunomodulatory treatment, n (%)	-	51 (64.56)	-	40 (85.11)	13 (39.39)	-	-	-
Serum biomarkers, pg/ml								
GFAP, mean (SD)	79.31 (33.59)	120.00 (82.41)	0.031	89.24 (34.97)	162.88 (107.61)	0.293	0.002	<0.001
GFAP, median (range)	69.08 (42.23-136.98)	98.68 (28.55-508.50)		86.50 (28.55-211.36)	142.01 (60.84-508.50)			
log(GFAP)			0.008[2]			0.215[2,5]	0.001[2,5]	0.019[2,5]
NFL, mean (SD)	24.89 (7.37)	26.74 (16.90)	0.606	20.62 (10.60)	35.26 (20.23)	0.073	0.097	<0.001
NFL, median (range)	22.92 (11.46-36.57)	22.36 (6.59-97.59)		17.88 (6.59-55.80)	31.60 (9.49-97.59)			
log(NFL)			0.839[2]			0.552[2,5]	0.658[2,5]	0.044[2,5]
MRI volumes, cm^3								
n	6	65		46	19			
T1 hypo lesion load, mean (SD)	-	9.25 (11.75)	-	5.92 (7.35)	17.30 (16.11)	-	-	<0.001
T2 lesion load, mean (SD)	-	12.13 (13.29)	-	9.45 (10.76)	18.62 (16.60)	-	-	0.005
NAWM volume, mean (SD)	492.17 (29.83)	450.91 (62.67)	0.081	459.72 (60.09)	429.58 (65.27)	0.235	0.099	0.235
GMctx volume, mean (SD)	498.93 (63.35)	428.69 (47.57)	0.009	439.10 (39.86)	403.49 (55.92)	0.018	0.017	0.017

[1] Wilcoxon rank-sum test, [2] ANCOVA with age as a covariate, [3] Chi squared test, [4] Holm method for multiple comparisons, [5] Tukey method for multiple comparisons

Abbr. EDSS - expanded disability status scale, GFAP – glial fibrillary acidic protein, MSSS – multiple sclerosis severity score, NfL – neuronal filament light, NAWM – normal appearing white matter, GMctx – cortical grey matter

Table 2: The specificity, sensitivity and the predictive values calculated for GFAP and NfL. The first column shows the number of SPMS patients categorized as SPMS or RRMS and the second column the number of RRMS patients categorized as SPMS or RRMS by the prediction using the level of GFAP or NfL. The last column shows the total number of patients categorized as SPMS or RRMS according to level of the biomarker.

	SPMS	RRMS	Total
GFAP			
level predicting SPMS	19	5	24
level predicting RRMS	14	41	55
total	33	46	
Sensitivity	57.58 %		
Specificity	89.13 %		
Positive predictive value	79.17 %		
Negative predictive value	74.55 %		
NfL			
level predicting SPMS	18	7	25
level predicting RRMS	15	39	54
total	33	46	
Sensitivity	54.55 %		
Specificity	84.78 %		
Positive predictive value	72.00 %		
Negative predictive value	72.22 %		

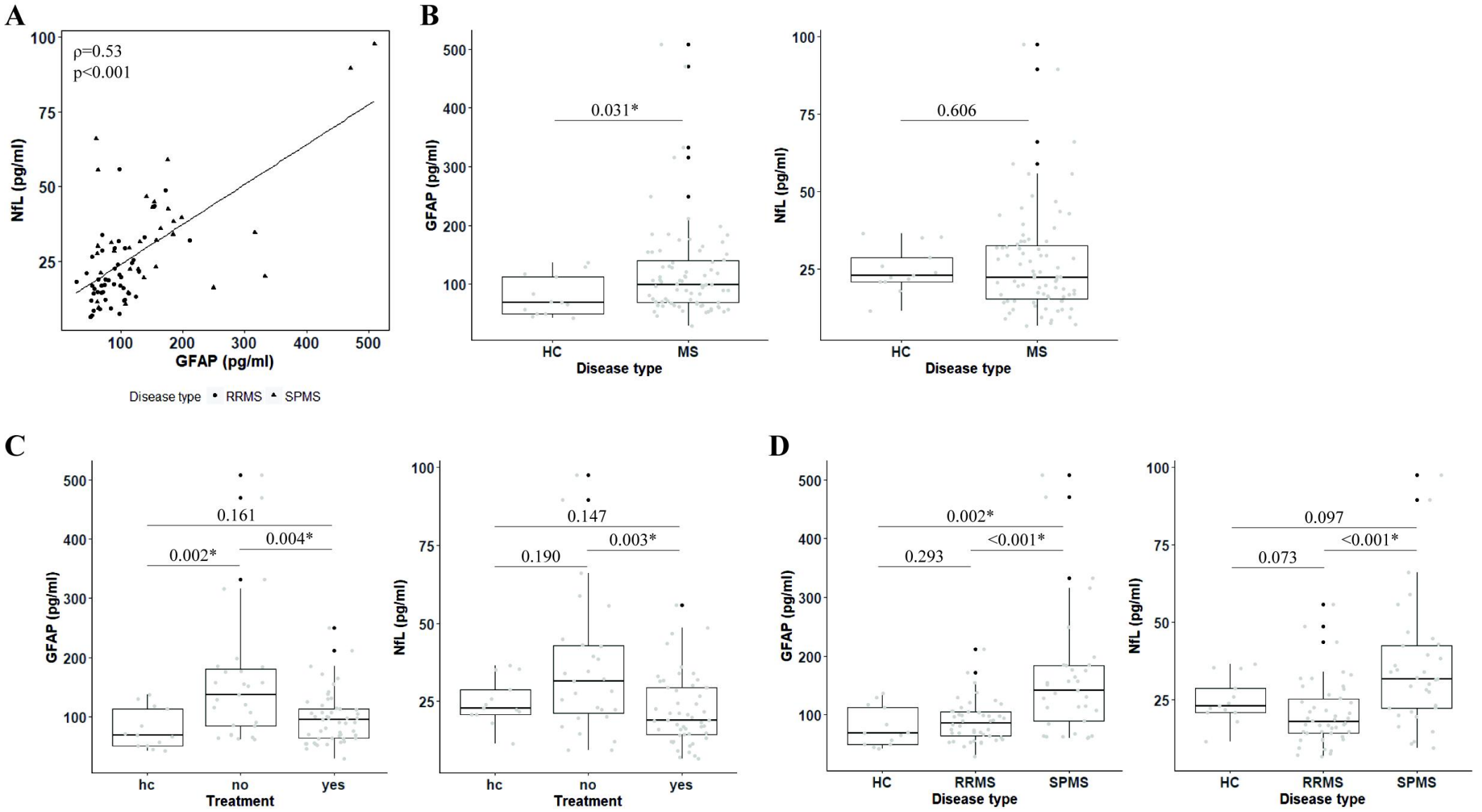
Figure legends

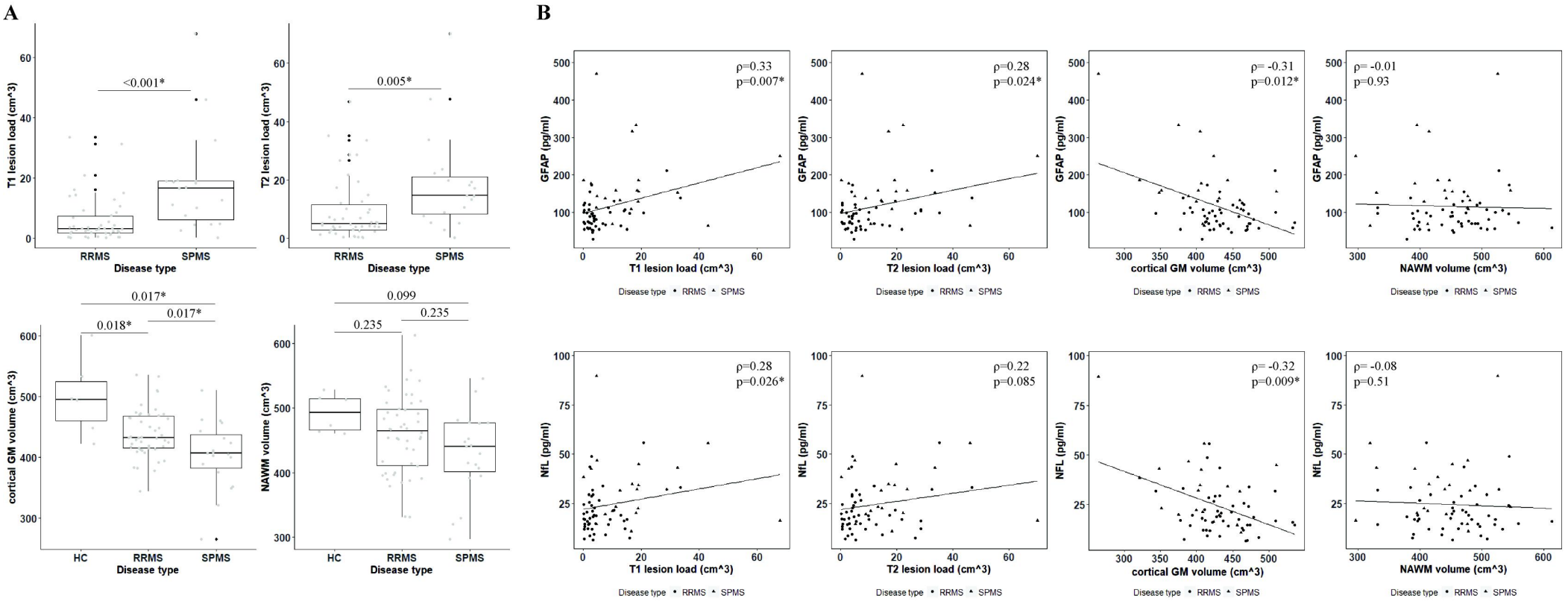
Figure 1: The serum biomarker levels of the study subjects. *(A)* The serum level of GFAP is strongly associated with NfL level (r=0.53, p<0.001). *(B)* Comparison of serum GFAP and NfL levels between healthy controls (HC) and MS patients. GFAP but not NfL levels are increased in MS patients (p=0.031). *(C)* The effect of disease modifying treatment (DMT) on the serum GFAP and NfL levels. The MS patients with treatment (Yes) have lower serum levels of both GFAP and NfL compared to the patients with no DMT (No) (GFAP no vs yes p=0.004, NfL no vs yes p=0.003). *(D)* Comparison of serum GFAP and NfL levels between the patient subgroups. Both GFAP and NfL levels are elevated only in SPMS patients (GFAP HC vs RRMS p=0.293, HC vs SPMS p=0.002*, RRMS vs SPMS p<0.001*; NfL HC vs RRMS p=0.073, HC vs SPMS p=0.097, RRMS vs SPMS p<0.001*). The asterisk (*) indicates statistical significance (p<0.05).

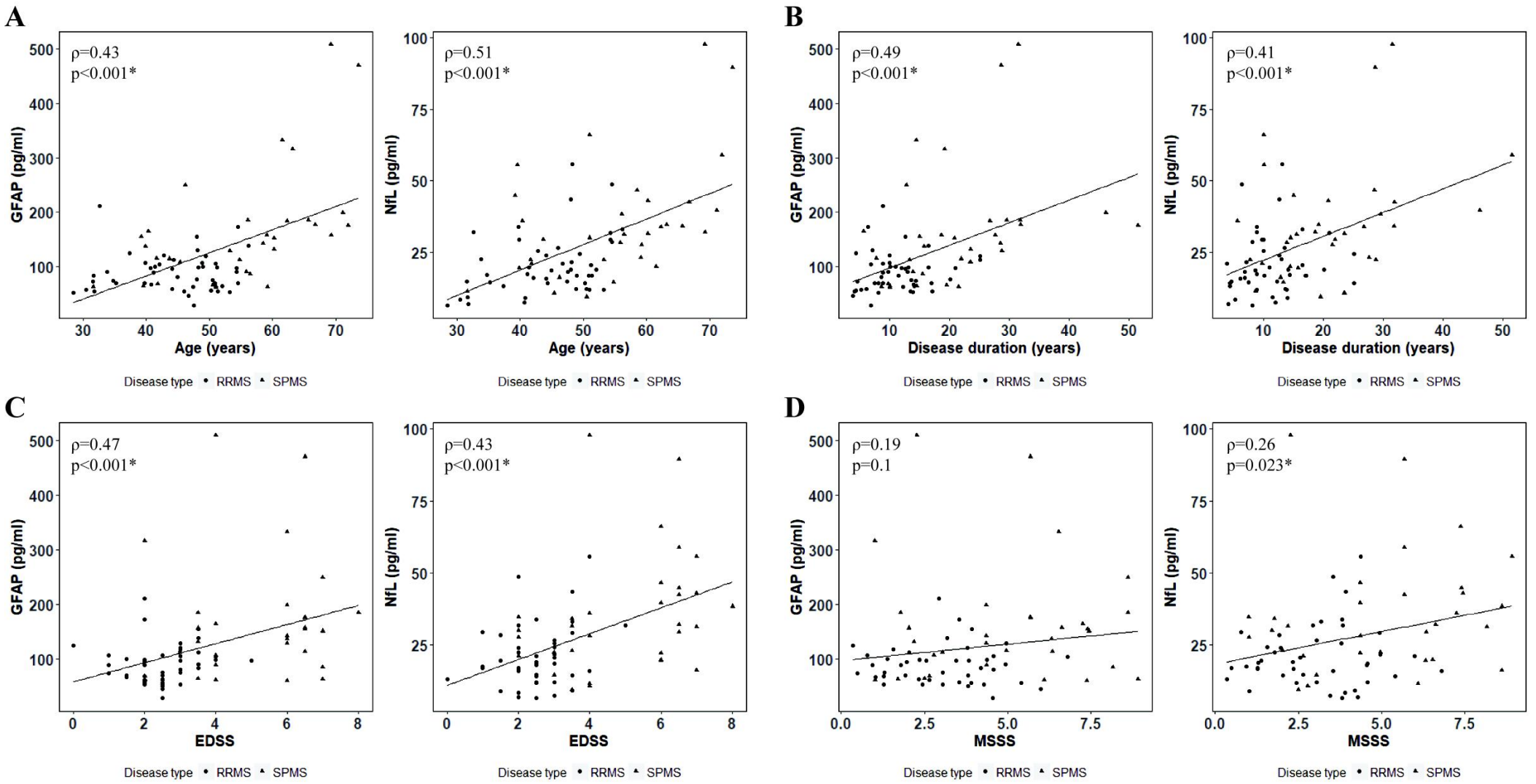
Figure 2: *(A)* Comparison of MRI measures between RRMS and SPMS and healthy controls (HC). Both T1 and T2 lesion loads (cm^3) are significantly higher in SPMS compared to RRMS (p<0.001* and p=0.005*). Also, the cortical grey matter (GMctx) volume (cm^3) is significantly lower in SPMS compared to HC (p=0.017*). The GMctx volume (cm^3) in SPMS is also lower compared to RRMS (p=0.017) and in RRMS compared to HC (p=0.018). The differences in normal appearing white matter (NAWM) volumes are not statistically significant. *(B)* GFAP levels associate positively with T1 (r=0.33 p=0.007*) and T2 (r=0.28 p=0.024*) lesion loads (cm^3), and NfL with T1 lesion load (cm^3) (r=0.28 p=0.026*). Both are also negatively associated with GMctx volume (cm^3) (GFAP r=-0.31 p=0.012*, NfL r=-0.32 p=0.009*) but not with NAWM volume (cm^3). The asterisk (*) indicates statistical significance (p<0.05).

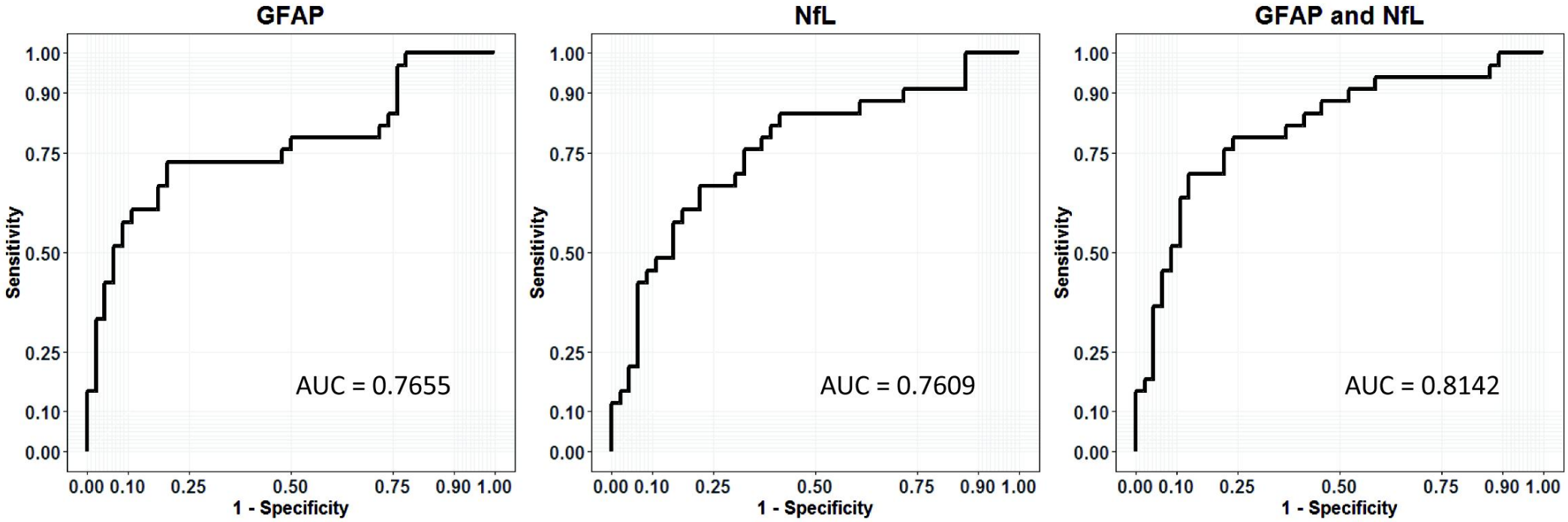
Figure 3: GFAP and NFL levels associate with *(A)* age (r=0.43 p<0.001* and r=0.51 p<0.001*), *(B)* disease duration (r=0.49 p<0.001* and r=0.41 p<0.001*) and *(C)* EDSS (r=0.47 p<0.001* and r=0.43 p<0.001*). *(D)* NfL level associates also with MSSS (r=0.26 p=0.023*) in MS patients. The asterisk (*) indicates statistical significance (p<0.05).

Figure 4: Receiver operating characteristics (ROC) curves with area under curve (AUC) for GFAP and NfL separately, and together indicating specificity and sensitivity to discriminate between RRMS and SPMS patients. AUC for serum GFAP was 0.766 (89.1 % specificity and 57.6 % sensitivity), for NfL 0.761 (84.8 % specificity and 54.6 % sensitivity), and together 0.814 (89.1 % specificity and 57.6 % sensitivity).


A
ρ=0.53
p<0.001
NfL (pg/ml)
GFAP (pg/ml)
Disease type RRMS SPMS
B
0.031*
GFAP (pg/ml)
HC
MS
Disease type
0.606
NfL (pg/ml)
HC
MS
Disease type
C
0.161
0.004*
0.002*
GFAP (pg/ml)
hc
no
yes
Treatment
0.147
0.003*
0.190
NfL (pg/ml)
hc
no
yes
Treatment
D
0.002*
<0.001*
0.293
GFAP (pg/ml)
HC
RRMS
SPMS
Disease type
0.097
<0.001*
0.073
NfL (pg/ml)
HC
RRMS
SPMS
Disease type


A
T1 lesion load (cm^3)
<0.001*
RRMS
SPMS
Disease type
T2 lesion load (cm^3)
0.005*
RRMS
SPMS
Disease type
cortical GM volume (cm^3)
0.017*
0.017*
0.018*
HC
RRMS
SPMS
Disease type
NAWM volume (cm^3)
0.099
0.235
0.235
HC
RRMS
SPMS
Disease type
B
GFAP (pg/ml)
ρ=0.33
p=0.007*
T1 lesion load (cm^3)
Disease type • RRMS ▲ SPMS
GFAP (pg/ml)
ρ=0.28
p=0.024*
T2 lesion load (cm^3)
Disease type • RRMS ▲ SPMS
GFAP (pg/ml)
ρ= -0.31
p=0.012*
cortical GM volume (cm^3)
Disease type • RRMS ▲ SPMS
GFAP (pg/ml)
ρ= -0.01
p=0.93
NAWM volume (cm^3)
Disease type • RRMS ▲ SPMS
NfL (pg/ml)
ρ=0.28
p=0.026*
T1 lesion load (cm^3)
Disease type • RRMS ▲ SPMS
NfL (pg/ml)
ρ=0.22
p=0.085
T2 lesion load (cm^3)
Disease type • RRMS ▲ SPMS
NFL (pg/ml)
ρ= -0.32
p=0.009*
cortical GM volume (cm^3)
Disease type • RRMS ▲ SPMS
NfL (pg/ml)
ρ= -0.08
p=0.51
NAWM volume (cm^3)
Disease type • RRMS ▲ SPMS


A
ρ=0.43
p<0.001*
GFAP (pg/ml)
Age (years)
Disease type RRMS SPMS
ρ=0.51
p<0.001*
NfL (pg/ml)
Age (years)
Disease type RRMS SPMS
B
ρ=0.49
p<0.001*
GFAP (pg/ml)
Disease duration (years)
Disease type RRMS SPMS
ρ=0.41
p<0.001*
NfL (pg/ml)
Disease duration (years)
Disease type RRMS SPMS
C
ρ=0.47
p<0.001*
GFAP (pg/ml)
EDSS
Disease type RRMS SPMS
ρ=0.43
p<0.001*
NfL (pg/ml)
EDSS
Disease type RRMS SPMS
D
ρ=0.19
p=0.1
GFAP (pg/ml)
MSSS
Disease type RRMS SPMS
ρ=0.26
p=0.023*
NfL (pg/ml)
MSSS
Disease type RRMS SPMS


GFAP
NfL
GFAP and NfL
Sensitivity
1 - Specificity
0.00
0.10
0.25
0.50
0.75
0.90
1.00
AUC = 0.7655
AUC = 0.7609
AUC = 0.8142