Axonal damage accumulates in the progressive phase of multiple sclerosis: A 3–year follow–up study

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Abbreviations: 9HPT =nine–hole PEG test, AI = ambulation index, BSP = brain–specific proteins, CSF = cerebrospinal fluid, CTRL = control. EDSS = expanded disability status scale, ELISA = enzyme linked immunoab-sorbant assay, IEF = isoelectric focusing, MS = multiple sclerosis, Nf = neurofilament, NfH = neurofilament heavy chain, NfL = neurofilament light chain, OND = other neurological diseases, PP = primary progressive, RR = relapsing remitting, SP = secondary progressive, NfH^{SMI34} = NfH detected with SMI34 antibody, NfH^{SMI35} = NfH detected with SMI35 antibody,

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Abstract

Neurofilament phosphoforms (Nf) are principal components of the axoskeleton released during axonal injury. Cerebrospinal fluid (CSF) levels of Nf phosphoforms might be a useful surrogate marker for disability in multiple sclerosis (MS), aid in distinguishing clinical subtypes and provide valuable prognostic information.

Thirty–four MS patients were included in a 3–year follow–up study along with 318 non–inflammatory neurological controls. CSF levels of 2 Nf heavy chain (NfH) phosphoforms (NfH^{SMI35}, NfH^{SMI34}) were quantified at baseline and 3–year follow–up using new ELISA techniques. Levels of NfH phosphoforms, the degree of phosphorylation (NfH^{SMI34} : NfH^{SMI35} ratio) and changes of NfH levels between baseline and follow–up ($\triangle$NfH) were then related to the clinical phenotype (relapsing remitting 'RR' or progressive 'SP/PP' disease), to 3 clinical scales (Kurtzke's EDSS, the ambulation index 'AI' and the 9 hole peg test '9HPT') and to progression of disability.

A significantly higher proportion (59%) of patients with SP/PP disease experienced an increase in NfH^{SMI35} levels between baseline and follow–up compared to those with RR disease (14%, p<0.05). CSF NfH^{SMI34} levels were higher in SP/PP patients at baseline (11 pg/mL) compared to RR patients (9 pg/mL, p<0.05) and NfH^{SMI35} levels were higher at follow-up (129 pg/mL) compared to levels below assay sensitivity (p<0.05). NfH^{SMI35} correlated with the EDSS (R=0.54, p<0.01), the AI (R=0.42, p<0.05) and the 9HPT (R=0.59, p<0.01) at follow–up.

The increase of NfH during the progressive phase of the disease together with the correlation of NfH^{SMI35} with all clinical scales at follow–up suggests that cumulative axonal loss is responsible for sustained disability and that high NfH^{SMI35} levels are a poor prognostic sign.

Axonal pathology remains the "Achilles' heel" of neurology. The new insights form recent studies into the "axonal death cascade"[1] in multiple sclerosis (MS) are that a high number of transected axons are already present in acute lesions[2,3] (independent of demyelination[4]), in patients with a short clinical course,[2,5] and as a result of electrical activity in a hostile micro–environment.[6] Axonal loss results in atrophy of the spinal cord,[7] cerebellum[8] and cortex,[9] all of which correlate with disability.[7–9] *In vivo* quantification of axonal damage is a key tool required for monitoring and understanding axonal pathology in complex diseases such as MS.

Neurofilaments (Nf) constitute a major component of the axoskeleton and are promising candidates for quantification of axonal damage because axonal transection results in disintegration of the distal axon membrane and Nf breakdown.[?,10] Nf are released into the adjacent compartment, i.e. the cerebrospinal fluid (CSF) were they can be measured.[11,12]

This prospective study was stimulated by 3 questions:[13,14] (1) Can *clinical subtypes* of MS be distinguished on the basis of axonal damage (disease heterogeneity[15])? (2) Does *disability* correlate with markers of axonal pathology? And (3) Can we predict loss of function by using biomarkers for axonal injury?

1 Methods

This study was approved by the local Ethics Committees. Written informed consent was obtained from all patients.

Patients Thirty–four patients from a previously reported cohort[16,17] with clinically definite MS[18] were followed up clinically after three years. A second CSF sample was available in 29 patients at time of study. MS patients

were classified as having relapsing remitting (RR, n=11), or progressive (SP/PP, n=23) disease.[19] For the CSF analysis patients with primary (PP) and secondary (SP) progressive disease were pooled because of small numbers. However, a detailed subgroup analysis for the classification of RR, SP and PP MS patients at baseline as well as a subgroup analysis of RR , SP (including those who converted from RR at baseline to SP at follow–up) and PP at follow–up will also be presented. In nine of the MS patients treatment with interferon beta (IFNβ) had been started since the recruitment in 1996. The control group consisted of 318 patients with other, non–inflammatory neurological diseases (OND) from the National Hospital of Neurology and Neurosurgery. Restricted sample volume meant not all assays could be performed on each sample and the numbers available for each comparison are presented in Table 1.

Clinical assessment   An Expanded Disability Status Scale score (EDSS)[20] ranging from 0 (normal) to 10 (death due to multiple sclerosis), an ambulation index (AI) ranging from 0 (no impairment) to 9 (restricted to wheelchair without independent transfer) and a 9–hole PEG test (9HPT) measuring upper limb motor function[21] were performed on all patients within one week of each lumbar puncture. Patients were classified as clinically advancing if they worsened on the EDSS scale by at least 1 point for an EDSS $<$ 5.5 or at least 0.5 point for an EDSS $\geq$ 5.5.

Assays   Samples of CSF were obtained by routine lumbar puncture. Aliquots of CSF were stored at -70$°$C until assayed. Levels of NfH phosphoforms were quantified using an in–house ELISA technique based on commercially available antibodies.[22] This ELISA has been optimised for the cap-

ture antibody SMI35 which recognises a range of NfH phosphoforms (170 kDa, pI 6.2 to 210 kDa, pI 5.1). In contrast the capture antibody SMI34 only recognises extensively phosphorylated NfH.[23] Unfortunately non–phosphorylated Nf are susceptible to proteases,[23–25] of which the CSF is a rich source. For this reason NfH=SMI32 was not measured in the present study and a ratio of NfH^{SMI34} to NfH^{SMI35} was used to approximate the phosphorylation status (see below). Albumin in CSF and serum was determined by standard Laurell 'rocket' electro–immunoassay.

Data analysis   All statistical analyses and graphs were done using SAS software (version 8.2, SAS Institute, Inc., Cary, North Carolina, USA). Because of non–Gaussian distribution the median values and the 25–75 % interquartile range (IQR) were shown. Independent variables were compared using the non-parametric two-sample exact Wilcoxon rank-sum test. If significance was based on small numbers the results were checked by the one–tailed Fisher's exact test. The linear relationship between continuous variables was evaluated using the Spearman correlation coefficient. Multiple correlations were corrected using the Bonferroni method. Linear regression analysis was performed using the least–squares method.

The change of NfH levels between baseline and follow–up was expressed as the difference: $\Delta NfH = NfH_{follow-up} - NfH_{baseline}$ A positive number indicated an increase in the NfH follow–up levels. Because the interassay CV for NfH is 10.6%, only an increase of at least 11% was considered for further statistical analysis.[22] The phosphoform ratio is an estimate of the degree of phosphorylation and was expressed as a cross–sectional measure: $RATIO = \frac{NfH^{SMI34}}{NfH^{SMI35}} \times 10$. A decrease of the ratio indicates an overall reduction in the level of phosphorylation. Values with zero denomi-

nator (or NfH at baseline and follow–up below assay sensitivity) could not be calculated and were excluded from this analysis.

2 Results

The demographic data at baseline are shown in Table 1. As expected at baseline EDSS, AI and 9HPT were worse in patients with SP/PP disease than in those with RR disease. The CSF levels of $NfH_{baseline}^{SMI35}$ were higher in the OND than in the MS patients (p<0.01, Table 2 A). The CSF levels of $NfH_{baseline}^{SMI34}$ were similar in OND subjects compared to MS patients. No correlations were found between any Nf phosphoform or their ratio with either age, disease duration, time from last relapse, relapse frequency or the $\frac{\text{CSF albumin}}{\text{Serum albumin}}$ ratio (data not shown). The shortest relapse free time was 3 month at baseline in 2 patients. At follow up 1 patient with SP/PP MS suffered from a superimposed relapse 2 weeks prior to CSF sampling. There was no correlation with time from relapse in either SP/PP MS patients or RR MS patients for either sampling point.

Axonal damage accumulates in SP/PP disease   A significant increase in NfH^{SMI35} from baseline to follow–up was observed in a higher proportion of patients with SP/PP MS (13/22, 59%, Figure 1) when compared to RR patients (1/7, 14%, p<0.05). At follow–up median CSF NfH^{SMI35} levels were higher in patients with SP/PP disease compared to patients with RR disease (Table 2 B, p<0.05).

An increase of NfH^{SMI34} was observed in a similar proportion of patients with RR (5/7, 71%) and with SP disease (15/22, 68%). The CSF $NfH_{baseline}^{SMI34}$ was higher in patients with SP/PP than with RR disease (Table 2 A, p<0.05). The proportion of RR patients with an increase in

NfH^{SMI34} (71%) was higher than the proportion with an increase in NfH^{SMI35} (14%, p<0.05). The $\frac{NfH^{SMI34}}{NfH^{SMI35}}$ ratio decreased in 6/7 (86%) of patients with RR and 12/22 (55%) with SP disease. Neither of these comparisons reached statistical significance.

Axonal injury correlates with disability NfH^{SMI35} levels correlated with all clinical scales at follow–up (Figure 2 A,B,C). The correlation was strongest for for the 9HPT (R=0.59, p=0.001), followed by the EDSS (R=0.54, p<0.01) and the AI (R=0.42, p<0.05). The correlation with the AI was lost after Bonferroni correction. One outlier was observed for the 9HPT (Figure 2 C). Exclusion of this outlier did not change the significance of the analysis (R=0.55, p<0.01). The NfH phosphoform ratio correlated with the EDSS (R=0.52, p<0.05) at follow–up, but this significance was lost after the Bonferroni correction. No significant correlation was found for any NfH phosphoform with the change of EDSS, AI or 9HPT over the 3–year period (data not shown) At baseline no such correlations were demonstrated after Bonferroni correction for any of the NfH phosphoforms or their ratio.

Axonal injury and prognosis There was a tendency for higher median CSF NfH^{SMI35} levels in MS patients who progressed on the EDSS scale within 3–years (107 pg/mL) when compared to those who remained stable (38 pg/mL). However this difference did not reach statistical significance for either the total MS cohort or the clinical subtypes (Table 3). However, using an arbitrary cut–off level of 20 pg/mL (assay sensitivity) on the baseline cohort, the positive predictive value of high NfH^{SMI35} levels for predicting progression on the EDSS scale within 3–years was 100% for RR MS and 20% for SP/PP MS patients with a specificity of 100% and 20% and a

sensitivity of 87.5% and 75%, respectively.

Three RR patients converted to SP disease in the 3–year observation period. Patients with RR disease who converted to SP disease had higher median CSF NfH^{SMI35} levels (123 pg/mL) when compared to non–converting RR patients (49 pg/mL) at baseline. Again this difference did not reach statistical significance.

Patients treated with IFNβ had a lower median EDSS on baseline (2.0 *versus* 5.5; p<0.05), follow–up (4.0 *versus* 4.5; p<0.05) and were less progressive (improvement by a median of 1.5 points on the EDSS compared to no change; p<0.05) compared to untreated patients. IFNβ treated patients had lower CSF NfH$^{SMI34}_{baseline}$ levels (7 pg/mL) compared to non–treated patients (11 pg/mL, p<0.01). No such differences were found for NfH$^{SMI35}_{baseline}$ or the NfH phosphoform ratio.

Subgroup analysis Because there are differences in pathogenesis between SP and PP MS, particularly with reference to the degree of inflammation, CSF levels of NfH^{SMI35} , NfH^{SMI34} and the ratio were also examined for all individual subgroups.

At baseline there was a significant difference between these groups for NfH^{SMI34} (F(2,26)=5.00, p<0.05). The post–hoc analysis revealed that CSF NfH^{SMI34} levels were higher in patients with PP disease (mean=26.6 pg/mL) compared to patients with SP (mean=10.19 pg/mL, p<0.01) or RR disease (mean=9.30 pg/mL, p<0.01).

At follow–up no such difference was found, probably due to small numbers and therefore no post–hoc analysis was performed.

3 Discussion

This study (1) provided evidence that accumulation of axonal damage as estimated by serial CSF NfH^{SMI35} levels predominated in SP/PP MS, (2) reveals a correlation of CSF NfH^{SMI35} levels with the degree of disability on 3 clinical scales (EDSS, AI, 9HPT) and (3) failed to demonstrate that CSF NfH phosphoforms might predict the development of new disability in patients with MS. We interpret these findings on basis of the epidemiologically supported hypothesis that axonal damage is a gradual cumulative process during the disease course[26,27] and that loss of neurological function is a direct consequence of axonal injury.[2,13,14]

Firstly, NfH^{SMI35} levels increased from baseline to the 3–year follow up sampling in about half of the MS patients. A significantly higher proportion of these patients had SP/PP than RR disease. Additionally the mean NfH$^{SMI35}_{follow-up}$ levels were significantly higher in SP/PP rather than in RR disease. This marked increase of NfH$^{SMI34}_{baseline}$ suggests that NfH phosphorylation may increase with disease duration. This interpretation contrasts with the consistent immunocytochemical observation that injured and demyelinated axons stain for non–phosphorylated NfH (NfH=SMI32).[2,28–31] However, none of these studies presented quantitative data comparing the amount of axons staining for phosphorylated versus non–phosphorylated NfH. A further complicating factor is that proteolytic enzyme activity is a prominent feature of the MS plaque[32,33] and potentially affects the levels particular of non–phosphorylated NfH which is susceptible to proteolysis.[23–25] To address this question we are currently analysing the quantitative distribution of NfH phosphoforms (NfH^{SMI32}, NfH^{SMI34} and NfH^{SMI35}) in microdissected brain tissue homogenate from a previously published

cohort.[16,34,35] An increase of NfH phosphorylation supports our finding and can be explained by targeted phosphorylation of the KSP repeats of the NfH and NfM tail domains by ERK1/2.[36] Fibrin upregulates ERK1/2[37] and has shown to be deposited on injured axons.[34,35,38] Additionally the MAP kinases SAPKs and ERK1/2 are activated by glutamate[39–43] which in turn leads to Ca–influx, slowing of axonal Nf transport and increased Nf phosphorylation.[44,45] Glutamate toxicity is an important pathological feature in MS, metabotropic glutamate receptor group I alpha is upregulated on axons in multiple sclerosis[28] and experimental treatment with the AMPA/kainate antagonist NBQX reduces axonal damage in experimental autoimmune encephalomyelitis.[29] The present CSF results are also be consistent with the postmortem observation that axonal damage increases with time in SP/PP MS patients,[46] with additional support from brain imaging[47] and epidemiological studies.[27]

Secondly, a correlation between CSF NfH^{SMI35} and 3 clinical scales was shown. This finding indirectly confirms two previous reports on a different neurofilament subunit (the 68 kDa light chain, NfL).[11,12] In the study of Lycke *et al.* CSF NfL correlated with the EDSS at baseline (R=0.27) and follow–up (R=0.34) in RR MS patients.[11] In the study by Semra *et al.* CSF NfL correlated with the EDSS (R=0.41) in progressive MS patients. However, no such correlations were found at baseline in either our original study,[17] the present follow–up cohort or a recent study on RR MS and SP MS patients.[48] Because clinically some of our SP/PP patients improved a degree of disability at baseline, it is likely that conduction block and demyelination which are, in contrast to axonal loss, reversible, contributed to the deficit.

Thirdly, a tendency for higher median CSF NfH$^{SMI35}_{baseline}$ and NfH$^{SMI34}_{baseline}$ levels

were observed in those MS patients who progressed clinically on the EDSS scale. This was most marked for patients with RR disease and suggests that axonal damage during the course of MS is a poor prognostic feature. However, the study failed to show statistical difference. The high positive predictive value and sensitivity suggest that this might be due to small sample size. It is important to note that axonal loss is not the dominant pathological feature in MS compared to other neurological diseases.[22,49] Nevertheless the demonstrated slow accumulation of axonal loss seems a logical explanation for the development of sustained disability in the progressive course of the disease.

The interpretation of the present data needs to consider that the patient group is population based and numbers are small. Clinically there was no significant change in the EDSS of the SP/PP MS patients within 3 years. This represents a benign course compared to the more rapid progression observed in other cohorts of patients selected from hospital populations. Additionally the low median CSF NfH^{SMI35} levels in the RR MS cohort at follow–up would suggest that these patients might have a benign disease course. The results must be interpreted with caution and will need to be cross–validated in other longitudinal studies with different cohort of patients.

Taking all these observations together, the results of this study are in accordance with the current concept of progressive axonal degeneration in MS which is based on evidence from animal,[50,51] human post-mortem,[2,3,5,35,46,52] magnetic resonance spectroscopy,[53] magnetic resonance imaging[7–9,54] and epidemiological studies.[27,55]

The results of this prospective 3–year study support the idea that CSF NfH phosphoforms might be valuable surrogate markers which have the

potential to be used as a new secondary outcome measures in MS treatment trials.

Note During the review process of this paper a seperate study on the cerebrospinal neurofilament phosphoforms (NfH^{SMI34} and NfH^{SMI35}) has been published by Ee Tuan Lim *et al.* "Cerebrospinal fluid levels of brain specific proteins in optic neuritis". Multiple Sclerosis 2004;10:261–265.

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	OND			Multiple sclerosis		
	NfH^{SMI35}	NfH^{SMI34}	Ratio	MS (all)	SP/PP	RR
Female:Male	155:116	61:58	32:28	15:19	11:12	4:7
Age (years)	44.0 (1–77.9)	45.4 (34.9–52.9)	47.2 (42.5–51.5)	46.5 (42.5–51.5)	48.5 (42.5–51.5)	39. (34
Disease duration (years)	N/A	N/A	N/A	14.0 (8.0–19.9)	16.0 (11.8–21.8)	8.1 (3.
Relapse–free interval (months)	N/A	N/A	N/A	38.0 (8.0–96.0)	83.0 (23.5–144.5)	8.0 (3.
Number	271	119	60	34	23	11

Table 1: *Baseline characteristics of the patients. The median (IQR) are shown. Patients with SP/PP disease had a longer disease duration (p<0.05) and relapse-free interval (p<0.05).*

(A)

Baseline	CTRL	MS (all)	Clinical subgroup	
			SP/PP	RR
NfH^{SMI35} pg/mL	260 (0–3990)	78 (80–610)	95 (25–163)	53 (11–139)
	—— p<0.001 ——			
NfH^{SMI34} pg/mL	10 (0–12000)	9 (7–13)	11 (7–14)	7 (5–9)
			—— p<0.05 ——	
Ratio	1.52 (0.36–5.49)	1.5 (0.6–5.4)	1.7 (0.6–5.8)	1 (0.42–3.5)
EDSS	N/A	3.25 (2.0–6.5)	6.0 (3.0–7.0)	1.5 (1.0–2.0)
			—— p<0.001 ——	
AI	N/A	2 (1–7)	6 (2–8)	1 (1–1)
			—— p<0.01 ——	
9HPT	N/A	24.5 (20.5–28.5)	26.0 (24.0–30.0)	20.0 (18.0–22.0)
			—— p<0.01 ——	
Number	271/119	29	19	10

(B)

Follow–Up	CTRL	MS (all)	Clinical subgroup	
			SP/PP	RR
NfH^{SMI35} pg/mL	N/A	113 (0–178)	129 (0–209)	0 (0–120)
			—— p<0.05 ——	
NfH^{SMI34} pg/mL	N/A	50 (9–129)	30 (10–114)	51 (3–120)
Ratio	N/A	3 (1–10.1)	3 (1–10.1)	5 (0–10)
Δ NfH^{SMI35} pg/mL	N/A	4 (-59–98)	82 (-38–115)	-49 (-38–104)
Δ NfH^{SMI34} pg/mL	N/A	37.0 (-1–123)	22.0 (-3–95)	51 (1–112)
EDSS	N/A	4.5 (3.5–6.0)	5.5 (4.0–6.5)	3.0 (2.5–4.0)

			——— p<0.01 ———	
AI	N/A	2 (2–5)	4 (2–7)	1 (1–2)
			——— p<0.01 ———	
9HPT	N/A	23.5 (20.0–28.5)	24.5 (21.5–31.0)	20.0 (18.0–21.0)
			——— p<0.05 ———	
Number	—	29	19	10

Table 2: *(A) Baseline CSF levels and ratio of NfH phosphoforms, EDSS, AI and 9HPT (median, IQR) in OND and MS patients. (B) Follow–up CSF levels, ratio and change over time (△NfH), NfH phosphoforms, EDSS, AI and 9HPT.*

	MS (all)		SP/PP		RR	
	Stable	Progressive	Stable	Progressive	Stable	Progressive
NfH^{SMI35} (pg/mL)	38 (17–155)	107 (49–163)	42 (24–173)	104 (78–173)	5 (0–53)	115 (43–165)
NfH^{SMI34} (pg/mL)	11 (5–13)	8 (5–14)	11 (7.5–13.0)	9 (5–16)	2 (0–5)	7.5 (6–11)
Ratio	3.2 (0.6–5.8)	1.0 (0.4–3.5)	3.2 (0.6–6.0)	1.1 (0.5–1.7)	2.5 (0.9–4.0)	0.9 (0.4–3.5)
Number	19	15	16	7	3	8

Table 3: *CSF levels and ratio of NfH phosphoforms (median, IQR) for clinically progressive versus stable patients at baseline.*
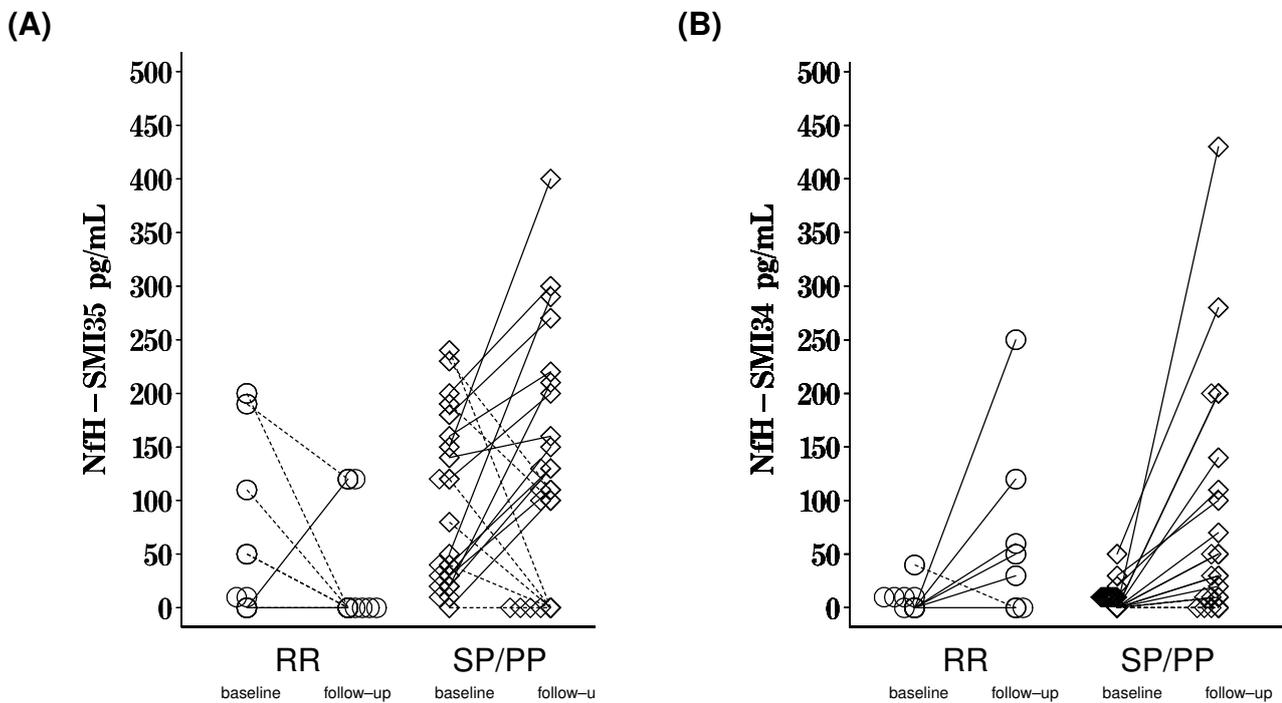
(A)

(B)

Figure 1: *(A) CSF NfH^{SMI35} levels in patients RR (open circles) and SP/PP (diamonds) forms of MS. A significantly higher proportion of SP/PP MS patients (13/22) had increased CSF NfH^{SMI35} levels between baseline and follow–up (straight lines) when compared to RR MS patients (1/7, p<0.05, Fisher's exact test). (B) CSF NfH^{SMI34} levels in patients RR (open circles) and SP/PP (diamonds) forms of MS.*
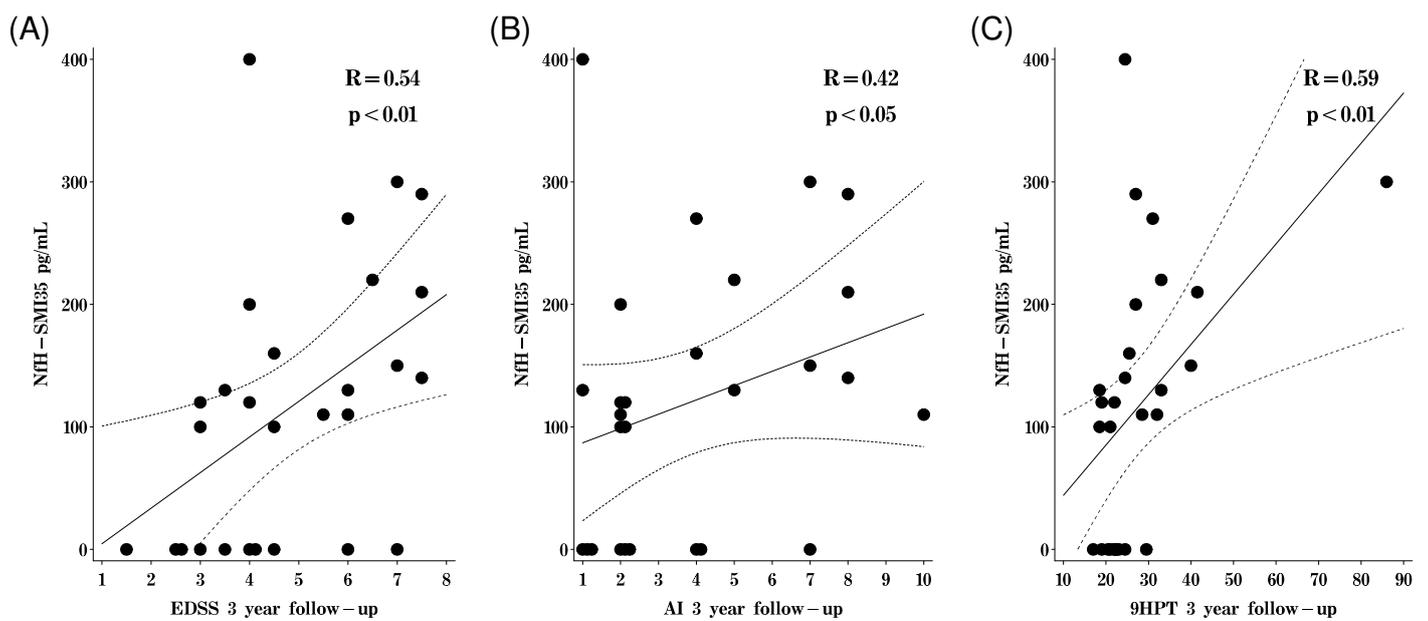
Figure 2: *Correlation between the CSF NfH^{SMI35} levels and (A) the EDSS, (B) the AI and (C) the 9HPT (log transformed scale) at follow–up.*