


## ORIGINAL ARTICLE

# Plasma neurofilament light chain levels predict improvement in late phase after stroke

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## Abstract

**Background and purpose:** Although functional recovery is most pronounced in the first 6 months after stroke, improvement is possible also in the late phase. The value of plasma neurofilament light chain (NfL), a biomarker of axonal injury and secondary neurodegeneration, was explored for the prediction of functional improvement in the late phase after stroke.

**Methods:** Baseline plasma NfL levels were measured in 115 participants of a trial on the efficacy of multimodal rehabilitation in the late phase after stroke. The association between NfL levels, impairment in balance, gait and cognitive domains, and improvement 3 and 9 months later was determined.

**Results:** Plasma NfL levels were associated with the degree of impairment in all three domains. Individuals with meaningful improvement in balance and gait capacity had higher plasma NfL levels compared with non-improvers ( $p = 0.001$  and  $p = 0.018$ , respectively). Higher NfL levels were associated with improvement in balance (odds ratio [OR] 2.34, 95% confidence interval [CI] 1.35–4.27,  $p = 0.004$ ) and gait (OR 2.27, 95% CI 1.25–4.32,

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$p = 0.009$ ). Elevated plasma NfL levels showed a positive predictive value for cognitive improvement, and this effect was specific for the intervention targeting the cognitive domain. The association of NfL levels with cognitive improvement withstood correction for baseline impairment, age and total years of schooling (OR 7.54, 95% CI 1.52–45.66,  $p = 0.018$ ).

**Conclusions:** In addition to its established role as a biomarker in the acute phase, elevated circulating NfL levels may predict functional improvement in the late phase after stroke. Our results should prompt further studies into the use of plasma NfL as a biomarker in the late phase after stroke.

#### KEYWORDS

biomarkers, cerebrovascular disease, intermediate filaments, late phase after stroke, nanofilaments, neurofilament light chain, stroke recovery

## INTRODUCTION

Stroke survivors often suffer from long-term and often permanent impairment involving different aspects of cognition, motor function and balance. Whilst functional recovery is most pronounced in the first 6 months after the stroke event [1,2], additional improvement is possible also in the late phase and can be enhanced through targeted rehabilitation programmes [1,3,4]. Stroke-induced secondary neurodegeneration in remote cortical and subcortical regions develops over the course of months and years, contributes to cognitive decline and can impede long-term functional recovery [5–8].

Circulating neurofilament light chain (NfL), a neuron-specific intermediate filament protein, is a blood and cerebrospinal fluid biomarker of axonal damage in multiple sclerosis [9,10] and neurodegenerative disorders such as Alzheimer's disease, frontotemporal dementia, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis [11]. Serum NfL levels positively correlate with infarct volume [12,13] and neurological impairment [13,14] in the acute phase after ischaemic stroke. High serum NfL levels in the first 3 months after ischaemic stroke show a strong association with poor short-term and long-term clinical outcome [12,14,15], and at 3–5 months after stroke serum NfL levels are negatively correlated with performance in activities of daily living [13]. Six months after stroke, serum NfL levels positively correlate with secondary neurodegeneration determined by diffusion tensor magnetic resonance imaging (MRI) [12].

In a three-armed randomized trial, it was previously found that multimodal interventions can lead to improvement in balance, motor function, cognition and long-term perception of recovery in

individuals in late phase ( $\geq 10$  months and  $\leq 5$  years) after stroke [4,16]. Here the potential association between circulating levels of NfL and the degree of impairment in balance, gait and cognition in the late phase after stroke was addressed. Moreover, the value of circulating NfL as a predictor of functional improvement was explored.

## MATERIALS AND METHODS

### Study population and study design

Data and plasma samples from a prospective single-blinded three-armed randomized trial on the efficacy of rehabilitation by rhythm and music-based therapy and horse-riding therapy in late phase recovery after stroke were used, trial registration ClinicalTrials.gov NCT01372059 [17]. Briefly, 123 participants who suffered from stroke 10 months to 5 years prior to inclusion were randomly allocated to one of three parallel intervention groups: rhythm and music-based therapy, horse-riding therapy or delayed intervention group. Participants in the delayed intervention group received the rhythm and music-based therapy a year later. Randomization included stratification according to sex and hemispheric location of the stroke [4].

### Standard protocol approvals, registrations and patient consents

The study was conducted according to the CONSORT guidelines and approved by the Regional Ethical Review Board in Gothenburg

(reference number 698-09). Written informed consent was received from participants prior to inclusion in the study.

## Outcome assessment

Observer-assessed outcome measures were evaluated at baseline (trial entry), as well as 3 and 9 months later, that is, directly after completing the 12-week long intervention and at 6-months follow-up, respectively, using validated tests within the gait, balance, hand function and cognitive domains. The focus was on objective outcome parameters that were previously shown to improve in response to rhythm and music-based therapy or horse-riding therapy interventions in this study population [4,16]. Gait capacity was assessed by the 10-m walk test in which participants were asked to walk at a self-selected speed and then as fast as they could. Balance was assessed with the Berg Balance Scale. Cognitive function (working memory) was evaluated with the Letter Number Sequence test. Nominal change in each outcome measure was converted into a binary improvement score, that is, a score of 1 was given for  $\geq 10\%$  improvement, considered as a clinically meaningful change [4], whereas a score of 0 was given for  $< 10\%$  improvement, no change or worsening. Due to very low baseline impairment in balance, a cutoff of  $\geq 1$  point improvement was used for the Berg Balance Scale. Alternatively, changes in outcome parameters were converted to a binary worsening score, that is, a score of 1 was given for  $\geq 10\%$  or  $\geq 1$  point deterioration, respectively, and 0 for  $< 10\%$  or  $< 1$  point deterioration, respectively, no change or improvement.

## Blood sampling and NfL measurement

Blood samples collected at study inclusion (baseline) were available for 115 of the 123 study participants. Blood was drawn between 8:00 and 10:00 AM after an overnight fasting and EDTA plasma was prepared and stored in aliquots at  $-70^{\circ}\text{C}$  until the analysis. NfL concentration was measured using the commercially available NF-Light kit on the single-molecule array (Simoa) HD-1 Analyzer as described by the manufacturer (Quanterix, Billerica, MA, USA). The analysis was performed by board-certified laboratory technicians blind to the clinical information; all samples were analysed on the same occasion with the same batch of reagents. The lower limit of detection was 0.17 pg/ml. The intra-assay coefficient of variation was 1.9% and 13.4% for quality control samples with concentrations of 7.2 pg/ml and 139.5 pg/ml, respectively.

## Statistics

The sample size required to satisfy power criteria of 80% with an alpha level of 0.05 (i.e., 40 participants per intervention group) was determined with regard to the trial primary outcome measure (Stroke Impact Scale) as described previously [17]. A post hoc power analysis in the present study revealed that, when aiming for 80% power at

the alpha level of 0.05, the minimum absolute detectable difference in plasma NfL levels between improvers and non-improvers was 25% and 36% for the pooled and individual intervention groups, respectively. Data distribution was assessed by the Shapiro–Wilk test. As the data were not normally distributed, non-parametric tests were used. The Mann–Whitney  $U$  and Kruskal–Wallis tests were used for comparison between two and three groups, respectively. Bonferroni's correction for multiple testing was applied as indicated. Associations between continuous variables were examined using Pearson's and Spearman's correlation. Associations between plasma NfL levels and baseline impairment were analysed with univariate and multivariable linear regression. To evaluate the associations with clinically meaningful improvement or worsening in given functional parameters, participants were dichotomized into improvers ( $\geq 1$  point improvement for the Berg Balance Scale,  $\geq 10\%$  improvement for the 10-m walk test and Letter Number Sequence score compared to baseline) and non-improvers, and univariate or multivariable binary logistic regression was used. Unless stated otherwise,  $\log_2$  transformed values of NfL plasma concentration were used for the linear and logistic regression analyses to improve compliance with linearity, normality and the homoscedasticity assumption, as assessed by inspection of residual plots. The significance of the effect size of a predictor in a given model was assessed by the Wald test. Model improvement, that is, the added value of the inclusion of NfL concentration (or baseline impairment) in the predictive models, was compared by McFadden's pseudo  $R^2$  [18] for the model with or without the predictor in question and the significance of the difference was assessed by the likelihood-ratio test. The relative importance of each predictor in determining improvement in outcome was estimated by comparing the standardized coefficient ( $\beta$ ) in the fully adjusted regression models, that is, using z-scores for each predictor instead of the original values. Missing data for outcome at 3 or 9 months were replaced using the last observation carried forward (Berg Balance Scale score, rhythm and music-based therapy,  $n = 1$ ; 10-m fast walking speed, rhythm and music-based therapy,  $n = 2$ ; horse-riding therapy,  $n = 2$ ; Letter Number Sequence score, delayed intervention,  $n = 1$ ; rhythm and music-based therapy,  $n = 2$ ; horse-riding therapy,  $n = 6$ ). Participants with missing relevant baseline values for outcome measures were excluded from the respective analysis (one participant from each intervention group, different for different domains). The National Institutes of Health Stroke Scale (NIHSS) score was missing for two participants in the rhythm and music-based therapy group. Statistical analyses were performed in the R package (ver. 3.3.2) [19]. Power calculation was performed in G\*Power software [20] using the test for means with asymptotic relative efficiency correction. All tests were two-sided and a  $p$  value  $< 0.05$  was considered significant.

## RESULTS

The baseline characteristics of the study population are presented in Table 1. There were no differences between the intervention groups

in plasma NfL levels ( $\chi^2(H) = 0.535$ ,  $p = 0.766$ ,  $df = 2$ ) or any other parameter (data not shown).

When data for all three groups were pooled, plasma NfL levels were positively correlated with age ( $r = 0.4089$ ,  $p < 0.001$ ). There was no association between plasma NfL levels and NIHSS score ( $r = -0.087$ ,  $p = 0.349$ ), time since the stroke event ( $r = -0.11$ ,  $p = 0.216$ ) or any other baseline characteristic of the study population (data not shown).

### In late phase after stroke, stroke severity, age and baseline degree of impairment in balance and cognition are associated with longitudinal improvement

Based on the change between baseline and the follow-up 3 and 9 months later, respectively, the participants were dichotomized into improvers ( $\geq 1$  point improvement for the Berg Balance Scale,  $\geq 10\%$  improvement for the 10-m walk test and Letter Number Sequence score compared to baseline) and non-improvers ( $< 1$  point or  $< 10\%$  improvement, respectively, no change or worsening) within each outcome measure (Table 2). In the univariate analysis of data from all participants, that is, regardless of the intervention group, stroke severity (baseline NIHSS score) was positively associated with

improvement in the Berg Balance Scale at both 3 and 9 months (odds ratio [OR] 1.20, 95% confidence interval [CI] 1.04–1.43,  $p = 0.018$ , and OR 1.19, 95% CI 1.03–1.41,  $p = 0.027$ , respectively). Higher baseline Berg Balance Scale score (indicating better balance and coordination) was negatively associated with improvement at both 3 and 9 months (OR 0.89, 95% CI 0.82–0.95,  $p = 0.002$ , and OR 0.90, 95% CI 0.82–0.96,  $p = 0.003$ , respectively). Higher baseline speed in the 10-m walk test was negatively associated with improvement at 9 months but not at 3 months (OR 0.43, 95% CI 0.19–0.91,  $p = 0.034$ , and OR 1.29, 95% CI 0.64–2.70,  $p = 0.483$ , respectively). Higher age, but not total years of schooling, showed an association with lower odds of improvement in the Letter Number Sequence score at 3 months but not at 9 months (OR 0.92, 95% CI 0.87–0.97,  $p = 0.005$ , and OR 1.02, 95% CI 0.96–1.07,  $p = 0.533$ , respectively; and data not shown). However, a higher Letter Number Sequence score (indicating better working memory) at baseline was associated with lower odds of improvement at 3 and 9 months (OR 0.82, 95% CI 0.70–0.96,  $p = 0.016$ , and OR 0.66, 95% CI 0.53–1.79,  $p < 0.001$ , respectively). No associations between baseline characteristics and worsening in any of the outcome measures were found. Time since the stroke event, stroke type and sex did not show significant association with improvement in any of the univariate analyses (data not shown) and were therefore not included as covariates in multivariable analyses.

**TABLE 1** Baseline characteristics of the study population

	Delayed intervention (n = 36)	R-MT (n = 39)	H-RT (n = 40)
Sex: men, n (%)	19 (50.0)	22 (52.4)	23 (57.5)
Age, years, median (IQR)	66 (61–70)	64 (59–68)	63 (59–67)
Total years of schooling, median (IQR)	14 (4)	14 (4)	13 (4)
Time since stroke onset, days, median (IQR)	1082 (796–1444)	854 (686–1363)	1074 (669–1371)
Stroke type: haemorrhage, n (%)	12 (31.6)	11 (26.2)	14 (35.0)
Modified Rankin Scale score grade 2/grade 3, n (%)	25 (66)/13 (34)	23 (55)/19 (45)	23 (57.5)/17 (42.5)
NIHSS score, median (IQR)	2.0 (1.0–3.0)	2.5 (1.0–4.0)	2.0 (0–4.0)
Berg Balance Scale score, median (IQR)	55 (49–56)	52 (50–55)	52 (47–54)
10-m fast walk test, speed, m/s, median (IQR)	1.59 (0.96–1.78)	1.55 (1.29–1.79)	1.32 (0.90–1.62)
Letter Number Sequence score, median (IQR)	6 (4–7)	6 (4–8)	6 (4–7)
NfL, pg/ml, median (IQR)	15.7 (12.9–24.3)	20.0 (12.0–25.0)	18.9 (12.8–26.2)

Note: Data are shown as median and interquartile range (IQR,  $Q_1$ – $Q_3$ ) or number and percentages (%) for each intervention group. No differences between groups were found for any variable by Kruskal–Wallis test for continuous variables and  $\chi^2$  for proportions.

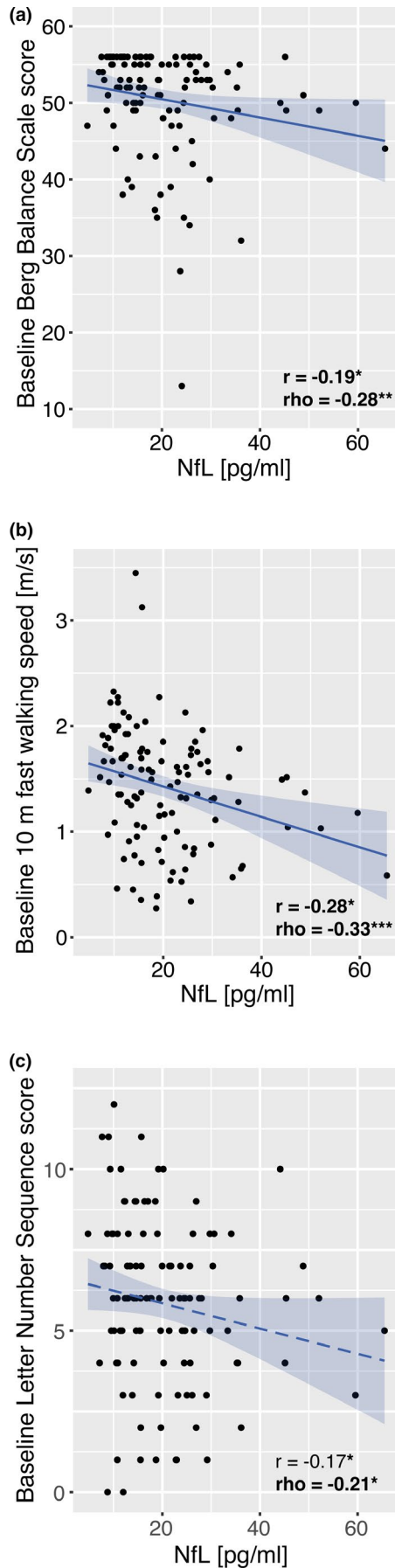
Abbreviations: H-RT, horse-riding therapy; NfL, neurofilament light chain; NIHSS, National Institutes of Health Stroke Scale; R-MT, rhythm and music-based therapy.

**TABLE 2** Distribution of the study participants and the extent of change in the outcome variables between improvers and non-improvers

Test	Time point	Parameter	All		R-MT		H-RT		Delayed intervention	
			Improvers	Non-improvers	Improvers	Non-improvers	Improvers	Non-improvers	Improvers	Non-improvers
Berg Balance Scale score (points)	3 months	n	57	58	23	16	23	17	11	25
		Median $\Delta$ (IQR)	2 (1; 3.25)	0 (-1; 0)	2 (1; 3)	0 (-1; 0)	3 (2; 4)	0 (-1; 0)	1 (1; 3)	0 (-1; 0)
		$\Delta$ min; max	1; 9	-10; 0	1; 9	-10; 0	1; 7	-8; 0	1; 6	-7; 0
10-m fast walk test, speed (m/s)	6 months	n	56	59	23	16	20	20	13	23
		Median $\Delta$ (IQR)	2 (1; 4)	0 (-1; 0)	2 (1; 3.75)	0 (-1; 0)	2 (2; 4)	0 (-1; 0)	1 (1; 4)	0 (-1; 0)
		$\Delta$ min; max	1; 13	-17; 0	1; 13	-17; 0	1; 7	-15; 0	1; 8	-6; 0
10-m fast walk test, speed (m/s)	3 months	n	36	78	12	27	17	22	7	29
		Median $\Delta$ (IQR)	0.25 (0.18; 0.41)	-0.05 (-0.08; -0.01)	0.38 (0.25; 0.42)	-0.05 (-0.13; 0.01)	0.21 (0.15; 0.30)	-0.01 (-0.10; 0.02)	0.24 (0.18; 0.29)	0 (-0.07; 0.05)
		$\Delta$ min; max	0.05; 0.61	-1.13; 0.17	0.19; 0.61	-1.13; 0.17	0.05; 0.57	-0.24; 0.10	0.17; 0.41	-0.23; 0.18
Letter Number Sequence score (points)	6 months	n	32	82	11	28	12	27	9	27
		Median $\Delta$ (IQR)	0.22 (0.18; 0.24)	-0.09 (-0.23; 0.04)	0.22 (0.20; 0.23)	-0.14 (-0.31; 0.02)	0.21 (0.15; 0.24)	-0.04 (-0.17; 0.04)	0.24 (0.20; 0.32)	-0.04 (-0.26; 0.04)
		$\Delta$ min; max	0.06; 0.73	-1.20; 0.17	0.07; 0.40	-1.20; 0.17	0.05; 0.73	-0.82; 0.17	0.08; 0.43	-1.12; 0.11
Letter Number Sequence score (points)	3 months	n	45	68	21	17	15	25	9	26
		Median $\Delta$ (IQR)	3 (1; 4)	-1 (-1; 0)	2 (1; 3)	0 (-1; 0)	3.0 (1; 4.5)	-1 (-1; 0)	3 (3; 4)	-1 (-1; 0)
		$\Delta$ min; max	1; 6	-6; 0	1; 6	-6; 0	1; 6	-5; 0	1; 6	-3; 0
Letter Number Sequence score (points)	6 months	n	51	63	19	19	18	22	14	22
		Median $\Delta$ (IQR)	3 (1; 4)	-1.0 (-3.0; -0.75)	3 (1; 4)	-2 (-5; 0)	3 (2; 4)	-1 (-2; -1)	1.5 (1; 4)	-2 (-2; -1)
		$\Delta$ min; max	1; 10	-8; 1	1; 8	-8; 1	1; 10	-7; 0	1; 8	-4; 0

Note: Distribution and the extent of nominal change in the outcome variables for participants classified as improvers ( $\geq 1$  point in the Berg Balance Scale or  $\geq 10\%$  in the 10-m fast walking speed and Letter Number Sequence score) or non-improvers, in all the study participants and in the three intervention groups.

Abbreviations: H-RT, horse-riding therapy; IQR, interquartile range (Q1; max, range of change; median  $\Delta$  (IQR), median change; Q3); R-MT, rhythm and music-based therapy;  $\Delta$  min.



**FIGURE 1** Plasma NfL levels show association with the degree of impairment in balance, gait and cognitive domains in the late phase after stroke. (a) Associations of plasma NfL levels with baseline Berg Balance Scale score, (b) 10-m fast walking speed and (c) Letter Number Sequence score. Solid line represents model with a significant linear association, dashed line indicates lack of significant linear association. Blue-shaded zone indicates 95% confidence interval for the estimated regression line.  $r$ , Pearson's correlation coefficient;  $\rho$ , Spearman's correlation coefficient; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  for the respective correlation; values in bold indicate significant correlations [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### In late phase stroke survivors, plasma NfL levels are associated with more pronounced physical and cognitive impairment

Plasma NfL levels correlated positively with the baseline degree of impairment in balance, gait and cognitive domains (Figure 1).

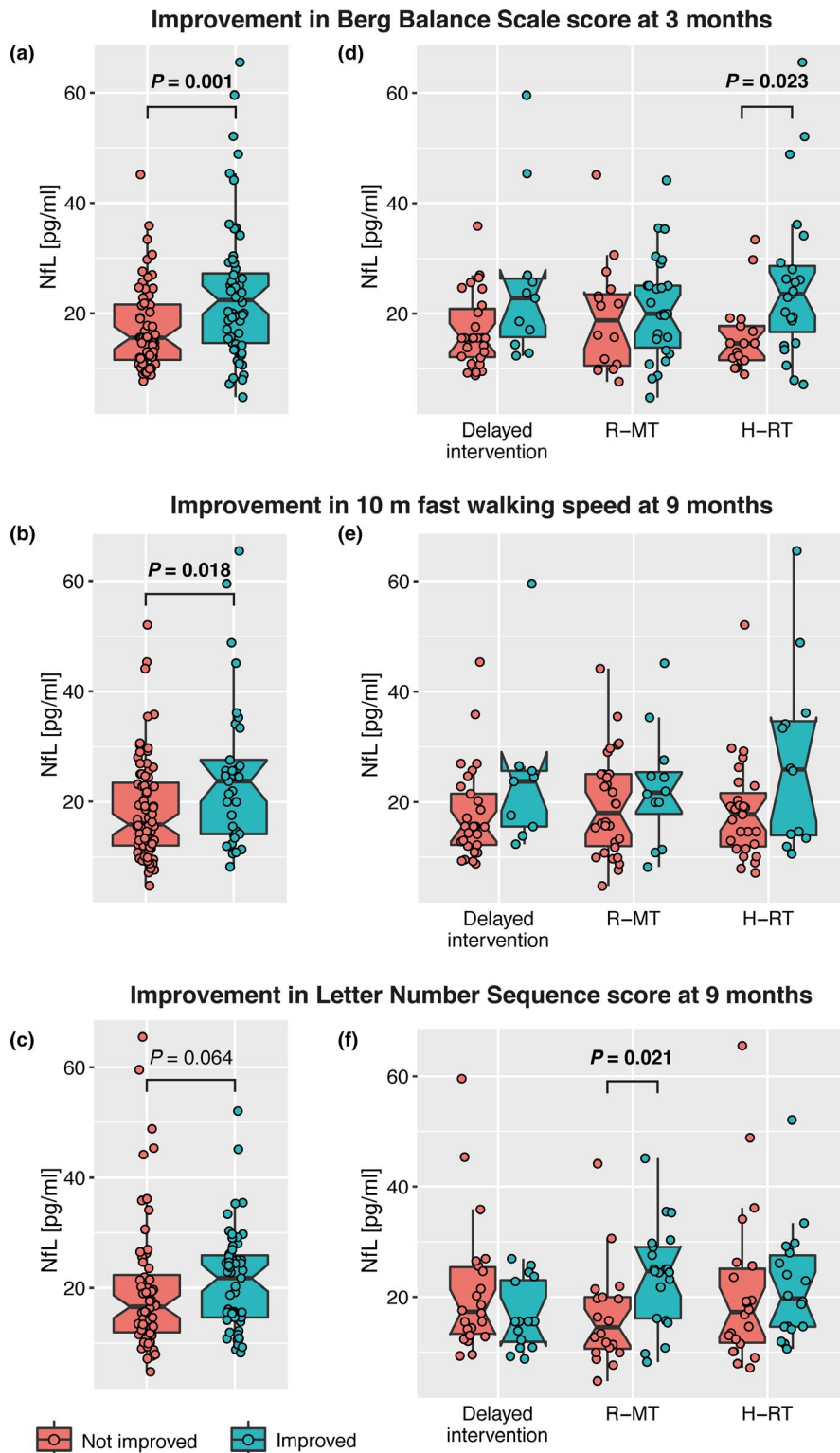
In the linear regression analysis, the association of higher plasma NfL levels (on a  $\log_2$  scale) with lower baseline Berg Balance Scale score ( $B = -2.3$ , 95% CI  $-3.95$  to  $-0.40$ ,  $p = 0.017$ ) withstood adjustment for age ( $B = -2.59$ , 95% CI  $-4.65$  to  $-0.54$ ,  $p = 0.014$ ) or baseline NIHSS score ( $B = -1.73$ , 95% CI  $-3.32$  to  $-0.10$ ,  $p = 0.037$ ). High plasma NfL levels were associated with lower baseline speed in the 10-m fast walk test ( $B = -0.24$ , 95% CI  $-0.37$  to  $-0.10$ ,  $p = 0.009$ ) and withstood adjustment for age ( $B = -0.21$ , 95% CI  $-0.34$  to  $-0.08$ ,  $p < 0.001$ ) or baseline NIHSS score ( $B = -0.30$ , 95% CI  $-0.46$  to  $-0.14$ ,  $p = 0.001$ ). Similarly, high plasma NfL levels were associated with lower baseline Letter Number Sequence score ( $B = -0.72$ , 95% CI  $-1.37$  to  $-0.082$ ,  $p = 0.028$ ). These associations remained significant after adjustment for age ( $B = -0.75$ , 95% CI  $-1.50$  to  $-0.01$ ,  $p = 0.048$ ) or baseline NIHSS score ( $B = -0.68$ , 95% CI  $-1.29$  to  $-0.06$ ,  $p = 0.036$ ).

### Even late phase stroke survivors with high plasma NfL levels can improve both physically and cognitively

High plasma levels of NfL were positively associated with improvement in the Berg Balance Scale at 3 months ( $U = 1196$ ,  $p = 0.001$ ) and improvement in the 10-m fast walking speed at 9 months ( $U = 1020$ ,  $p = 0.018$ ; Figure 2a,b; Table 3). These associations were independent of age, baseline NIHSS score and baseline degree of impairment within the respective domain, and the predictive value of the model improved when plasma NfL levels were added to these variables (Table 3).

When assessing the efficacy of the specific interventions, it was found that both rhythm and music-based therapy and horse-riding therapy were associated with improvement in the Berg Balance Scale at 3 months (OR 3.99, 95% CI 1.59–10.51,  $p = 0.004$ ; OR 3.32, 95% CI 1.32–8.75,  $p = 0.012$ , respectively); in the horse-riding therapy group, median NfL levels were higher amongst participants who





**FIGURE 2** Even late phase stroke survivors with high plasma NfL levels can improve both physically and cognitively. (a) Baseline plasma NfL levels in participants who achieved  $\geq 1$  point improvement in balance, (b)  $\geq 10\%$  improvement in gait and (c)  $\geq 10\%$  improvement in cognition at follow-up compared to those who did not improve. (d)–(f) Plasma NfL levels in participants grouped based on intervention type. Box plots: midline represents median; notches denote 95% confidence interval for the median; upper and lower edges of the boxes define interquartile range, IQR; whisker ends define the inner fences (i.e., minimum/maximum or distance of  $1.5 \times$  IQR, whichever is smaller); individual points beyond whiskers show outliers.  $p$  values refer to the Mann–Whitney  $U$  test, adjusted for multiple comparisons in (d) and (f). R-MT, rhythm and music-based therapy; H-RT, horse-riding therapy [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

improved in the Berg Balance Scale compared to those who did not ( $U = 99$ , multiplicity adjusted  $p = 0.023$ ; Figure 2a). In the rhythm and music-based therapy group but not the other groups, NfL levels were positively associated with improvement in the Letter Number Sequence score at 9 months (median  $U = 106$ , multiplicity adjusted  $p = 0.021$ ; Figure 2c and Table 3). Rhythm and music-based therapy was the only intervention that was associated with improvement in

the Letter Number Sequence at 3 months (OR 3.24, 95% CI 1.28–8.64,  $p = 0.015$ ). The association of NfL levels with long-term improvement in this test withstood correction for baseline Letter Number Sequence score, age (Table 3) and total years of schooling (OR 7.54, 95% CI 1.52–45.66,  $p = 0.018$ ).

There was no association between plasma NfL levels and worsening in any of the domains (OR 0.59, 95% CI 0.32–1.05,  $p = 0.080$

**TABLE 3** Association between baseline plasma NfL levels, or baseline level of impairment, and the improvement in balance, gait and cognition

Predictor	Odds ratio (95% confidence interval)	Effect of the predictor, <i>p</i> value	Model improvement, $\Delta$ pseudo $R^2$	Model improvement, <i>p</i> value
Berg Balance Scale score—improvement at 3 months				
$\log_2$ NfL	2.34 (1.35–4.27)	0.004	0.06 vs. 0	0.002
Adjusted for age and NIHSS score	2.13 (1.11–4.33)	0.028	0.09 vs. 0.06	0.023
Adjusted for age, NIHSS score and baseline score	1.92 (1.05–3.94)	0.030	0.12 vs. 0.10	0.018
Baseline score	0.89 (0.82–0.95)	0.002	0.08 vs. 0	<0.001
Adjusted for age and NIHSS score	0.91 (0.85–0.98)	0.025	0.10 vs. 0.06	0.012
Adjusted for age, NIHSS score and $\log_2$ NfL	0.92 (0.84–1.00)	0.056	0.12 vs. 0.09	0.012
10-m fast walking speed—improvement at 9 months				
$\log_2$ NfL	2.27 (1.25–4.32)	0.009	0.05 vs. 0	0.007
Adjusted for age and NIHSS score	2.66 (1.32–5.73)	0.009	0.07 vs. 0.01	0.006
Adjusted for age, NIHSS score and baseline speed	2.37 (1.14–5.22)	0.025	0.08 vs. 0.04	0.003
Baseline speed	0.42 (0.18–0.91)	0.038	0.03 vs. 0	0.028
Adjusted for age and NIHSS score	0.35 (0.13–0.84)	0.061	0.04 vs. 0.01	0.054
Adjusted for age, NIHSS score and $\log_2$ NfL	0.58 (0.23–1.40)	0.238	0.08 vs. 0.04	0.054
Letter Number Sequence score—improvement at 9 months				
$\log_2$ NfL	1.47 (0.88–2.53)	0.148	0.01 vs. 0	0.143
$\log_2$ NfL $\times$ intervention (R-MT)	7.54 (1.74–39.43)	0.010	0.06 vs. 0	0.023
Adjusted for age	7.54 (1.74–39.61)	0.011	0.06 vs. <0.01	0.023
Adjusted for age and baseline score	7.92 (1.59–48.58)	0.016	0.20 vs. 0.15	0.039
Baseline score	0.66 (0.53–0.79)	<0.001	0.15 vs. 0	<0.001
Adjusted for age	0.65 (0.53–0.79)	<0.001	0.15 vs. <0.01	<0.001
Adjusted for age and $\log_2$ NfL $\times$ intervention	0.65 (0.52–0.79)	<0.001	0.20 vs. 0.15	<0.001

Note: Results of multivariable logistic regression analysis between baseline plasma NfL levels or baseline level of impairment and binary improvement variable ( $\geq 1$  point in Berg Balance Scale at 3 months and  $\geq 10\%$  in 10-m fast walking speed at 9 months), irrespective of intervention type; and Letter Number Sequence score at 9 months, including interaction with intervention type.

Effect of the predictor, *p* value in Wald test for *B* coefficient; Model improvement, the change ( $\Delta$ ) in McFadden's pseudo  $R^2$  for model including versus model excluding the primary predictor and the corresponding *p* value in the likelihood-ratio test.

Abbreviations: NfL, neurofilament light chain; NIHSS, National Institutes of Health Stroke Scale; R-MT, rhythm and music-based therapy.

for 10-m fast walking speed at 9 months; OR 0.68, 95% CI 0.15–2.82, *p* = 0.604 for Berg Balance Scale at 3 months; OR 0.65, 95% CI 0.37–1.10, *p* = 0.110 for Letter Number Sequence score at 9 months).

### Plasma NfL is a robust predictor of functional improvement in the late phase after stroke

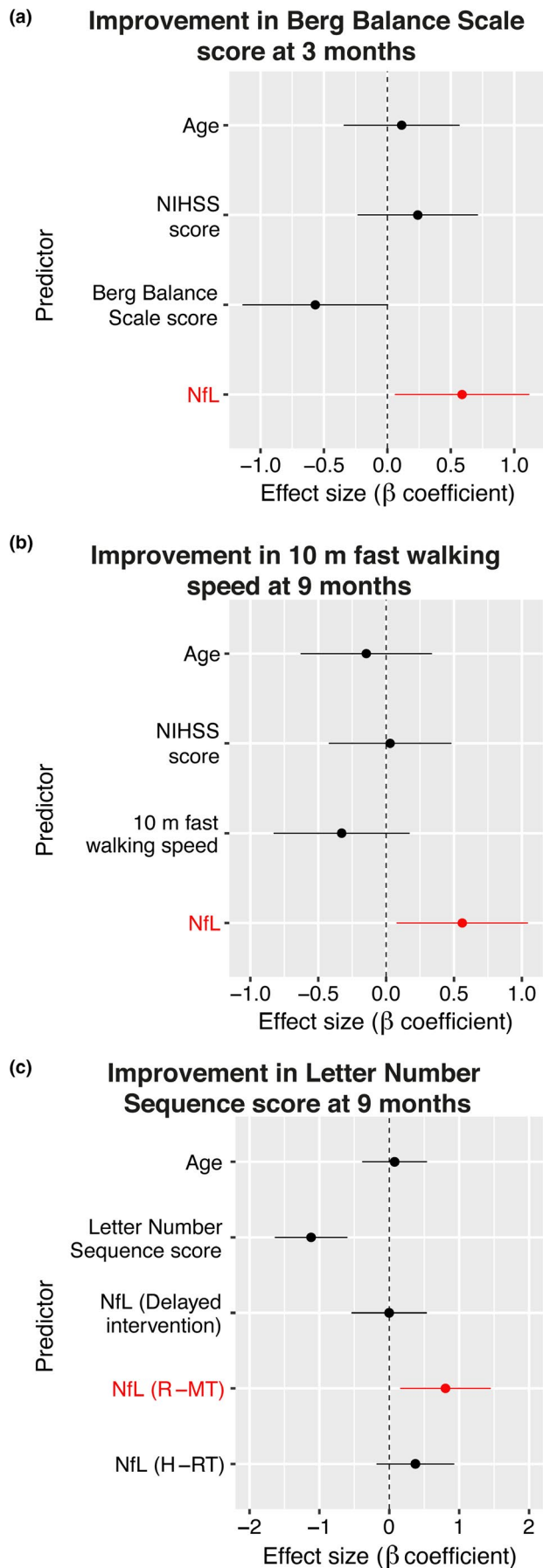
To compare the relative importance of plasma NfL with other relevant cofactors for the prediction of improvement, analysis was performed using the standardized variables. In the relevant fully adjusted regression models (as defined in Table 3), change in plasma NfL levels (on a linear scale) by one standard deviation showed the

largest effect on the probability of functional improvement in balance and gait (Figure 3a,b). In the cognitive domain, plasma NfL levels in combination with the rhythm and music-based intervention showed the second largest effect, after the baseline Letter Number Sequence score, on the probability of functional improvement of all the covariates tested (Figure 3c).

### DISCUSSION

Using plasma samples and data from a three-armed randomized trial on the efficacy of multimodal rehabilitation, it is shown that in the late phase after stroke plasma levels of NfL, a neuronal intermediate





**FIGURE 3** Plasma NfL levels predict functional improvement in the late phase after stroke. Forest plots showing estimated standardized  $\beta$  coefficients and their 95% confidence interval for the predictors in fully adjusted logistic regression analysis, modelling functional improvement in (a) balance, (b) gait and (c) cognition as a measure of relative variable importance. R-MT, rhythm and music-based therapy; H-RT, horse-riding therapy [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

filament (nanofilament) protein and a biomarker of both stroke-induced primary axonal injury and secondary neurodegeneration [12], are associated with the degree of functional impairment in the balance, gait and cognitive domains. Previously, serum NfL levels were shown to negatively correlate with performance in activities of daily living at 3–5 months after stroke [13]. Ours is the first study that demonstrates a positive correlation between circulating NfL levels and functional impairment in late phase stroke survivors. It was also found that higher plasma NfL levels were associated with improvement in balance, gait and cognition, and the type of intervention was an important factor in determining the specific outcome domain in which the improvement was achieved. Notably, there was no association between plasma NfL levels and worsening in any of the domains. These results indicate that, in the late phase after stroke, elevated plasma NfL levels may serve as a positive predictor of further functional recovery and improvement.

Neurofilament light chain levels in the blood of healthy individuals increase with age [9]. In line with this finding and reports on the correlation between serum NfL levels in the acute phase after ischaemic stroke and age [12], or age-related white matter changes [14], it was found that plasma NfL levels in late stroke survivors were positively correlated with age. However, whereas age on its own was a negative predictor of improvement in the Letter Number Sequence score, it was not associated with improvement in the other domains.

Stroke leads to neurodegeneration in remote cortical and sub-cortical regions; the extent of secondary neurodegeneration in the thalamus positively correlates with infarct volume and can impede the long-term outcome [7]. Elevated serum NfL levels at 6 months after stroke positively correlate with an MRI-based quantitative measure of secondary neurodegeneration [12]. As an association between plasma NfL levels and time since stroke was not found, the elevated plasma NfL levels in our study population conceivably reflect stroke-induced secondary neurodegeneration, as well as age-related changes in the white matter and other ongoing neurodegenerative processes such as those leading to Alzheimer's disease [21], or small vessel disease [22,23].

In the acute phase, serum NfL levels positively correlate with infarct volume [12,24], as well as unfavourable clinical outcome [12,15]. In sharp contrast, our results show that, in the late phase after stroke, individuals with higher plasma NfL levels were more likely to achieve functional improvement. The observed negative associations between baseline performance and improvement indicate that level of impairment was also an improvement predictor, arguably due to the ceiling effect. Given the observed correlation between circulating NfL levels and functional impairment in late phase stroke survivors, it is important

to note that all associations between plasma NfL levels and improvement withstood the correction for the baseline level of impairment. Thus, the associations between plasma NfL levels and improvement were not driven solely by the effect of baseline level of impairment. The contention that plasma NfL levels are a robust predictor of improvement in the late phase after stroke is further supported by our finding that, in the predictive models of improvement in gait and balance, the effect of baseline performance was lost when plasma NfL levels were included.

The NIHSS score at admission is a good predictor of clinical outcome at 3 months after ischaemic stroke [25]. In our study, baseline NIHSS score was positively associated with improvement in balance score in the univariate analysis, but importantly, no correlation between plasma NfL levels and NIHSS score in the late phase after stroke or between improvement and time since stroke was found. Baseline plasma NfL levels in the late phase were a more important predictor of improvement in balance and gait than baseline NIHSS score. Thus, the predictive value of NIHSS with respect to secondary neurodegeneration and late phase improvement is limited; in contrast, plasma NfL levels may be a more robust predictor of improvement at this stage.

Using the dichotomization approach, it was shown that the rhythm and music-based therapy was associated with improvement in Letter Number Sequence at 3 months. These findings are in line with our previous report based on the analysis of nominal change in outcome [4] and point to the highly specific effects of this intervention in the cognitive domain. Plasma NfL levels showed a positive predictive value for cognitive improvement, and this effect was specific for the cognitive-domain targeting intervention. An explanation for this highly unexpected finding could be that the circulating NfL levels reflect both the ongoing neuropathology, that is, neurodegenerative processes, and the neurodegeneration-induced adaptive neural plasticity. Such plasticity is known to share many characteristics of structural and functional changes associated with normal learning and constitutes the basis for functional improvement [26]. NfL plays a role in the regulation of synaptic function, and its close association with synapses raises the possibility that synaptic turnover can be a major contributor to NfL release [27]. The negative correlation between baseline NfL levels and cognitive performance, together with our finding that cognitive performance was a negative predictor of improvement, indicate that late stroke survivors with more pronounced cognitive impairment and higher plasma NfL levels are those that would most likely benefit from this type of rehabilitative intervention.

The strength of this study lies in the fact that it was conducted in a rather heterogeneous population of late phase stroke survivors with a range of dysfunctions in both cognitive and physical domains. An additional strength is the randomization into three groups with different interventions including a delayed intervention group. However, the relatively low number of participants and the strict eligibility criteria of the trial (modified Rankin Scale score 2–3) limit the generalizability of our findings to individuals with minor (modified Rankin Scale score 0–1) or more severe (modified Rankin Scale score 4–5) chronic stroke-related disability. Whilst larger studies with a wider range of impairment are

needed to address these weaknesses, plasma biomarkers indicative of the improvement potential would aid in designing individually tailored and cost effective rehabilitation plans for stroke survivors.

In summary, in late phase stroke survivors, elevated plasma levels of NfL are associated with a more pronounced impairment of physical and cognitive performance. Intriguingly, late phase stroke survivors with high plasma NfL levels and moderate levels of disability can improve both physically and cognitively, and, in sharp contrast to the acute phase, elevated circulating NfL levels may serve as a positive predictor of functional improvement and the effectiveness of neurorehabilitative interventions in the late phase after stroke. Larger longitudinal studies assessing the value of plasma NfL as a biomarker of improvement of late phase stroke survivors are required.

### CONFLICT OF INTEREST

HZ has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. KB has served as a consultant or at advisory boards for Abcam, Axon, Biogen, Lilly, MagQu, Novartis and Roche Diagnostics and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program.

### AUTHOR CONTRIBUTIONS

Anna Stokowska: Data curation (equal); formal analysis (lead); visualization (lead); writing original draft (equal); writing review and editing (equal). Lina Bunketorp Käll: Project administration (lead); writing review and editing (equal). Christian Blomstrand: Conceptualization (equal); funding acquisition (equal); writing review and editing (equal). Joel Simren: Data curation (equal); writing review and editing (equal). Michael Nilsson: Conceptualization (equal); funding acquisition (equal); writing review and editing (equal). Henrik Zetterberg: Data curation (equal); funding acquisition (equal); writing review and editing (equal). Kaj Blennow: Data curation (equal); funding acquisition (equal); writing review and editing (equal). Milos Pekny: Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); writing original draft (equal); writing review and editing (equal). Marcela Pekna: Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); writing original draft (equal); writing review and editing (equal).

### DATA AVAILABILITY STATEMENT

The raw data used in the preparation of the figures and tables will be shared in anonymized format on request by a qualified investigator to the corresponding author for purposes of replicating procedures and results.

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