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ORIGINAL ARTICLE

Neurofilament light chain and profilin-1 dynamics in 30 spinal muscular atrophy type 3 patients treated with nusinersen

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Abstract

Background and Purpose: The aim was to investigate whether neurofilament light chain (NfL) and profilin-1 (PFN-1) might qualify as surrogate disease and treatment-response biomarkers by correlating their concentrations dynamic with clinical status in a cohort of 30 adult spinal muscular atrophy type 3 patients during nusinersen therapy up to 34 months.

Methods: Neurofilament light chain was measured in cerebrospinal fluid at each drug administration with a commercial enzyme-linked immunosorbent assay (ELISA); PFN-1 concentrations were tested in serum sampled at the same time points with commercial ELISA assays. Functional motor scores were evaluated at baseline, at the end of the loading phase and at each maintenance dose and correlated to biomarker levels. The concurrent effect of age and clinical phenotype was studied.

Results: Neurofilament light chain levels were included in the reference ranges at baseline; a significant increase was measured during loading phase until 1 month. PFN-1 was higher at baseline than in controls and then decreased during therapy until reaching control levels. Age had an effect on NfL but not on PFN-1. NfL was partially correlated to functional scores at baseline and at last time point, whilst no correlation was found for PFN-1.

Conclusion: Cerebrospinal fluid NfL levels did not qualify as an optimal surrogate treatment biomarker in adult spinal muscular atrophy patients with a long disease duration, whilst PFN-1 might to a greater extent represent lower motor neuron pathological processes. The observed biomarker level variation during the first 2 months of nusinersen treatment might suggest a limited effect on axonal remodeling or rearrangement.

KEYWORDS
biomarkers, neurofilaments, nusinersen, spinal muscular atrophy

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive disorder due to progressive degeneration of lower motor neurons resulting in muscle atrophy, proximal limbs and axial muscle weakness, and possible respiratory and bulbar involvement. The pathogenic hallmark is the loss of survival motor neuron protein (SMN), encoded by the deleted or mutated *SMN1* gene [1]. *SMN2*, the centromeric paralogous

gene of *SMN1*, produces only a small amount of functional SMN due to the exclusion of exon 7 in mRNA transcript [2].

The *SMN2* gene can be present with various copy numbers and molecular quantification of *SMN2* copy numbers has prognostic implications: less severe phenotypes have generally a higher copy number, although exceptions might be encountered at an individual level [3, 4].

Nusinersen, an *SMN2* splicing modifier able to promote the inclusion of exon 7 in the *SMN2* transcript and to increase the production of functional SMN protein, is the first disease-modifying therapy approved for SMA patients, following the results of clinical trials [5, 6]. This and novel emerging therapies have deeply changed SMA natural history and stressed the need for reliable outcome measures and biomarkers to capture clinical changes and to assess therapy response.

Neurofilaments are intermediate filaments exclusively expressed in neurons and are classified as light chain (NfL), middle chain, heavy chain (NfH), α-internexin and peripherin [7]. Neurofilament levels rise as a result of axonal damage [8, 9] and seem to be promising diagnostic and prognostic biomarkers in motor neuron diseases, particularly amyotrophic lateral sclerosis (ALS) [10–13]. More recently, neurofilaments have been investigated also in SMA patients, with controversial results. Whilst SMA type 1 patients showed elevated levels of NfL and phosphorylated NfH (pNfH) in cerebrospinal fluid (CSF) and serum at baseline, with a decline during nusinersen treatment inversely related to CHOP-INTEND score values [5, 14–16], limited evidence concerning type 2 and 3 patients is currently available. Interestingly, in CSF of SMA type 1 children the concentration of total tau protein (t-tau), a steady biomarker of cortical degeneration in dementias, was also found to be high at baseline and decreasing along with NfL following nusinersen therapy [15, 16].

Profilin-1 (PFN-1) is a small actin-binding protein that promotes actin polymerization; it is required in both the presynaptic and the postsynaptic compartment and has a role in regulating cytoskeletal architecture and the dynamics of neurons [17]. Cytoskeletal defects have been identified in several motor neuron diseases, and mutations in PNF-1 were recently reported in 1%–2% of familial ALS [18]. PFN-1 directly interacts with SMN and a colocalization of PFN-1 and SMN has been demonstrated in cultured cells and in mouse motoneurons [19], suggesting that PFN-1 may be a candidate exploratory biomarker for SMA patients.

This study aims to investigate the role of NfL and PFN-1 as disease and treatment-response biomarkers in a cohort of adult SMA type 3 patients during nusinersen therapy up to 34 months.

METHODS

Study population

Thirty SMA type 3 patients, 19 males and 11 females, with an average age of 36.5 years (range 17–68), who were referred to the Neuromuscular Center of Padua Hospital for nusinersen treatment from February 2018 to September 2021 were recruited. Mean disease duration was 30.8 years; 14 patients lost ambulation, whilst 16 patients were walkers at the time of the study (Table 1).

All the patients had a molecularly defined 5q SMA diagnosis and they were divided into two subgroups, *sitters* and *walkers*. All patients underwent nusinersen intrathecal therapy (12 mg each dose) according to the administration protocol approved by the Italian Medicines Agency, which requires

- a loading phase composed of four administrations: loading dose 1 (L1) at the baseline; loading dose 2 (L2) after 14 days; loading dose 3 (L3) at the 28th day from baseline, and loading dose 4 (L4) at the 63rd day from baseline;
- a maintenance phase in which nusinersen is administered every 4 months.

Data from the CSF analysis of NfL were compared to a control group of 44 patients recruited for a previous study conducted at the Padua Neurological Department [10]. This group includes patients who underwent lumbar puncture to exclude meningitis or inflammatory central nervous system diseases, for mononeuropathies or primary headache and whose diagnostic workup excluded neurological disorders.

Profilin-1 was tested in the serum of a control group of 15 neurologically healthy blood donors (samples classified as diagnostic leftovers) and of 10 ALS patients.

The results on the safety and efficacy of nusinersen treatment in our patients included in a large Italian cohort of adult SMA patients have recently been published [20].

Neuromuscular evaluation

Neuromuscular evaluation was conducted in all patients before the beginning of the therapy (L1), at the end of the loading phase (L4) and thereafter every 4 months at each maintenance dose. The neuromuscular assessment protocol included validated motor scales: the Hammersmith Functional Rating Scale Expanded (HFMSE) (maximum score, corresponding to normal function, 66) [21] and Revised Upper Limb Module (RULM) (maximum score, corresponding to normal function, 37) [22]; 13/16 walker patients performed also the 6 min walk test (6MWT) [23]. Motor scale values at baseline (L1) are shown in Table 2. A correlation analysis between biomarkers and HFMSE was performed by grouping the patients in "responders" and "not-responders" to nusinersen treatment following the criteria previously established (i.e., "responder" is a patient who improves from baseline in at least one of the following outcome measures: 3 points at HFMSE, 2 points at RULM or 30 m in the 6MWT [20]).

Cerebrospinal fluid and serum biomarkers analysis

Cerebrospinal fluid samples were collected within the therapy administration procedure. Just before nusinersen administration (12 mg/5 mL) an equal CSF volume was collected. Patients with severe scoliosis or history of vertebral arthrodesis underwent a

TABLE 1 Demographic and clinical data of the study population at baseline (L1).

	SMA type 3		
	Sitter	**Walker**	**Total**
n	14	16	30
Male/female	12/2	7/9	19/11
Age (range, years)	34.5 years (20–57)	37.18 years (17–68)	36.5 years (17–68)
Age of onset (range, SD)	3.43 years (1–7 ± 2.43)	11.75 years (0.25–28 ± 6.5)	7.9 years (1–28 ± 6.53)
Disease duration (range, years)	33 years (14–52)	28.8 years (10–51)	30.8 years (10–52)
Age at loss of ambulation (years, range, SD)	18.7 years (4–47 ± 13.3)	–	–
SMN1			
Deletion exon 7–8	13	11	24
Deletion exon 7	1	2	3
Compound heterozygosity	–	3	3
SMN2 copies			
2	0	1	1
3	6	5	11
4	8	10	18
NIV			
None	13	15	28
Intermittent	1	1	2
Permanent	0	0	0
Arthrodesis	4	1	5
Fluoroscopy-guided LP	5 + 2 backup	1	8
Salbutamol	2	2	4
Physical therapy	12	11	23

Abbreviations: LP, lumbar puncture; NIV, non-invasive ventilation; SMA, spinal muscular atrophy.

TABLE 2 Baseline (L1) functional data.

	Group	*N*	Mean	SD	Median	25th percentile	75th percentile	Min	Max
HFMSE	SMA type 3 sitters	14	7	8	4	2	8	0	27
	SMA type 3 walkers	16	47	13	49	39	60	27	62
	Total	30	28	23	28	5	49	0	62
RULM	SMA type 3 sitters	14	17	10	17	13	23	0	31
	SMA type 3 walkers	16	34	4	37	33	37	25	37
	Total	30	26	12	30	19	37	0	37
6MWT	SMA type 3 walkers	13	367	119	345	310	463	121	588

Abbreviations: 6MWT, 6 min walk test; HFMSE, Hammersmith Functional Rating Scale Expanded; RULM, Revised Upper Limb Module; SMA, spinal muscular atrophy.

computed tomography scan guided procedure in the Neuroradiology Unit of the University Hospital of Padova.

Cerebrospinal fluid samples were collected at each therapy infusion during the loading dose phase and maintenance phase; CSF was aliquoted and stored in polypropylene vials at −80°C. Paired serum samples were collected at each time point, when available, and stored at −80°C.

Neurofilament light chain quantitative determination was performed at each time point from L1 to M8 (34 months from baseline) with NF-light® enzyme-linked immunosorbent assay (ELISA) in vitro diagnostic kit (UmanDiagnostics, Sweden; reference ranges <290 ng/L, age <30 years; <380 ng/L, age <40 years; <830 ng/L, age <60 years), according to the manufacturer's instructions; the interassay coefficient of variation (CV) calculated with repeated samples from the control group as internal quality control was <15%.

Total tau and p-tau were measured at time points L1 and L3 with a chemiluminescent enzyme immunoassay (Lumipulse G Total Tau

and Lumipulse G pTau 181, Fujirebio, Japan) on a fully automated analyzer (Lumipulse G600 II, Fujirebio, Japan); reference ranges t-tau 146–410 ng/L, p-tau 21.5–59 ng/L. The manufacturer declared CV ≤8.3% for t-tau and CV ≤5% for p-tau 181.

Profilin-1 was measured with a commercial research-use-only manual ELISA kit (Cusabio, China), with a wide detection range of 31.25–2000 ng/L and an interassay precision declared by the manufacturer of CV <10%. PFN-1 was tested in every available serum sample in L1–M3 and then at M6 (26 months from baseline); preliminary testing in CSF samples of six patients (three sitters, three walkers) at time points L1, L3 and M2 found concentrations below the lower limit of detection (<31.25 ng/L). Due to an interassay CV >20% calculated with four additional samples repeated in each batch as internal quality control, linear regression was applied to recalculate results of time point M6.

Statistical analysis

Variables were summarized as mean, standard deviation (SD) or median and range as appropriate. Distributions of quantitative and ordinal variables between groups were compared with the Wilcoxon–Mann–Whitney test or Student's *t* test as appropriate. Correlation between quantitative and/or ordinal variables was tested with the Spearman method. Concurrent effects of age, SMA diagnosis and tau protein levels on the log-transformed concentration of NfL were tested in multivariate ANOVA/ANCOVA models as appropriate. NfL and PFN-1 concentrations were log-transformed for these analyses because of their non-normal distribution. The distribution of outcome measures and CSF and serum biomarkers at different time points was compared to baseline with the Wilcoxon test for paired data. Generalized estimating equations were used to estimate the time-dependent variation of biomarkers in the maintenance phase and a possible correlation between NfL and PFN-1. Statistical significance was set at $p < 0.05$. Analyses were performed with R v.3.5.3.

Ethics

Clinical investigations adhered to the Declaration of Helsinki principles, and written informed consent, approved by Institutional Review Board, was obtained from all participants or their guardians in accordance with ethical requirements. The IRB protocol number is 4947/AO/20.

RESULTS

Cerebrospinal fluid NfL

Baseline CSF NfL levels were overall included in the reference ranges for healthy donors provided by testing assay manufacturers. In one patient aged 68 years old, the NfL measure was minimally above the upper limit of the reference range; this sample was prediluted due to small volume and intra-assay CV was >20%. Mean NfL was 218 ± 188 ng/L in SMA type 3 patients, compared to 809.5 ± 1065 ng/L in controls. No significant difference was found between the sitters group and walkers group and between different *SMN2* copy number (data not shown). Comprehensive results for the entire time points are summarized in Table 3.

Correlation was found between baseline log(NfL) and age (Figure 1) both in SMA patients ($r = 0.70$, $p < 0.0001$) and the control group ($r = 0.75$, $p < 0.0001$). ANCOVA on the entire population showed independent effects of age and SMA versus controls ($p < 0.001$ and $p < 0.001$).

A significant increase in mean NfL concentrations was observed during the loading phase until L3 (Figure 2), with a mean increase of 274 ng/L from the baseline ($p < 0.0001$). From M1 NfL had a monthly increase of 0.9% every month ($p < 0.0001$).

Cerebrospinal fluid t-tau and p-tau

Baseline t-tau and p-tau levels were included in the reference ranges (Table 4); log(t-tau) and log(p-tau) correlated to log(NfL) ($r = 0.57$, $p = 0.0014$ and $r = 0.45$, $p = 0.013$ respectively) (Figure S1). A slight significant increase was found in t-tau ($p = 0.016$) and p-tau ($p = 0.004$) concentrations at time point L3 (Table 4, Figure S2). At this time point value of CSF log(t-tau) and log(p-tau) were not correlated with CSF log(NfL) (Figure S3).

Serum PFN-1

Comprehensive results are summarized in Table 5. No effect of age was found in log(PFN-1) at baseline in the SMA type 3 and control groups (Figure S4).

Profilin-1 at baseline was higher in the SMA group than in healthy controls (mean 1016 ± 472 vs. 618 ± 222 ng/L, $p = 0.016$ pairwise Wilcoxon); no significant differences were found for ALS versus control or SMA (Figure S5).

Profilin-1 showed a complex dynamic during the loading phase, with a significant reduction at L4 compared to baseline (mean 589 ± 271 ng/L, $p = 0.00011$) (Figure 3); in the maintenance phase a monthly decrease of 14.7% ($p < 0.0001$) was calculated and at last follow-up the M6 concentration was not different from controls (mean 593 ± 127 ng/L, $p = 0.921$).

The generalized estimating equations of the entire cohort showed no correlations between NfL concentrations and PFN-1 (Figure S6).

Functional scores

Neurofilament light chain levels were slightly inversely correlated (i.e., concentrations decreased when motor outcome increased) with HFMSE (Spearman's $r = -0.46$, $p = 0.01$) and RULM scores ($r = -0.39$,

TABLE 3 CSF NfL (ng/L) descriptive statistics at each time point.

Group	*N*	Mean	SD	Median	25th percentile	75th percentile	Min	Max
Control group	44	809.5	1065	395.5	320	805	76	5928
L1								
SMA type 3 sitters	14	219	120	181.5	144	247	111	502
SMA type 3 walkers	16	217.5	237	162	120	219.5	41	1068
Total	30	218	188	168	130.5	207.5	41	1068
L2								
SMA type 3 sitters	14	411	259	284	255	568	152	1124
SMA type 3 walkers	16	469	458	325	229	534	97	2025
Total	30	442	373.5	297.5	235	546	97	2025
L3								
SMA type 3 sitters	14	422	259	347	225	573.5	155	1097
SMA type 3 walkers	16	554	526	396	227	680	131	2121
Total	30	492	421	358.5	236.5	602.5	131	2121
L4								
SMA type 3 sitters	14	359	212	283	192	540	147	845
SMA type 3 walkers	16	317	168	237.5	177	471	151	699
Total	30	337	188	264	186	471	147	845
M1								
SMA type 3 sitters	14	270	258	179.5	135.5	299	59	1067
SMA type 3 walkers	16	251	195	210	105	332.5	44	697
Total	30	260	223	198	130	282	44	1067
M2								
SMA type 3 sitters	12	302	250	229	117	423	40	897
SMA type 3 walkers	16	258	172	199	135	356	70	682
Total	28	277	206	206	138.5	365	40	897
M3								
SMA type 3 sitters	12	372	305	276	139.5	608	83	1102
SMA type 3 walkers	16	240	159	187	128	348	85	427
Total	28	296	238	205	139	372	83	1102
M4								
SMA type 3 sitters	12	308	232	249	112.5	508.5	23	742
SMA type 3 walkers	16	241	188	190	93	353	33	758
Total	28	269	207	192	108.5	380	23	758
M5								
SMA type 3 sitters	13	275	199	250	116	451	38	682
SMA type 3 walkers	16	230	177	207	114	307	20	756
Total	29	250	185	208	136	326	20	756
M6								
SMA type 3 sitters	13	354	292	257	187.5	476.5	96	1183
SMA type 3 walkers	16	405	564	243.5	189	354	99	2430
Total	29	382	456	248	199	368	96	2430
M7								
SMA type 3 sitters	13	338	251	234	157	570	82	842
SMA type 3 walkers	16	269	182	227	174	307.5	90	838
Total	29	300	214	228	174	328	82	842
M8								
SMA type 3 sitters	10	640	866	293	155	749.5	106	2920
SMA type 3 walkers	14	247	107	215	173	312	114	484
Total	24	411	582	239	172	357	106	2920

Abbreviations: CSF, cerebrospinal fluid; L1, loading dose 1 at baseline; L2, loading dose 2 after 14 days; L3, loading dose 3 at the 28th day from baseline; L4, loading dose 4 at the 63rd day from baseline; M1 to M8, maintenance dose after four months from previous administration; NfL, neurofilament light; SMA, spinal muscular atrophy.

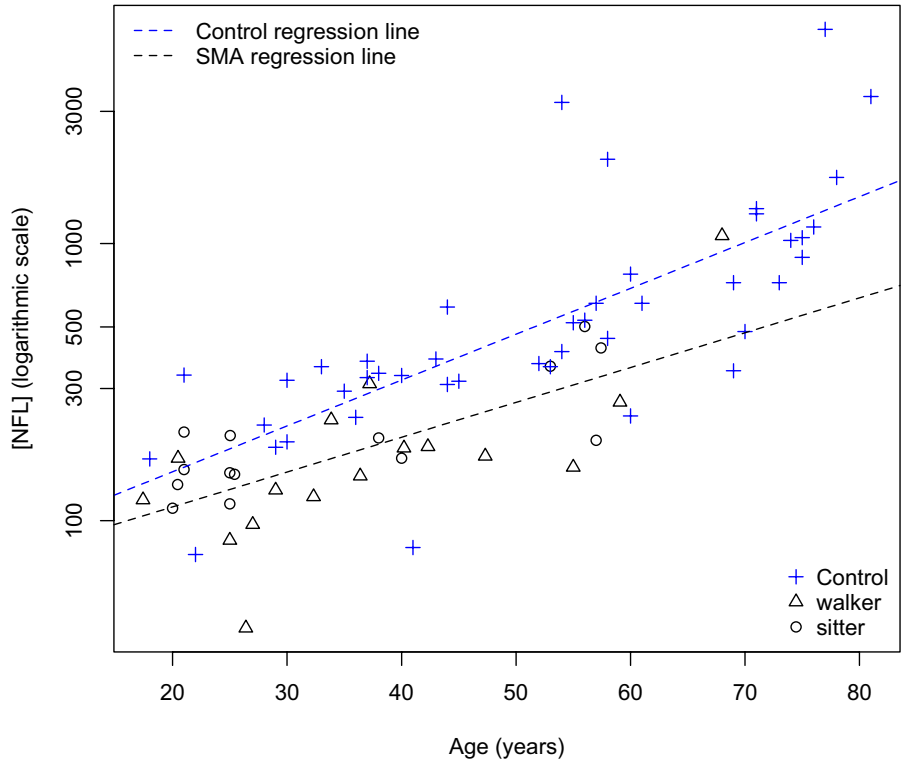

FIGURE 1 Correlation between log(Nfl) and age at baseline in SMA patients at baseline (L1) and controls.

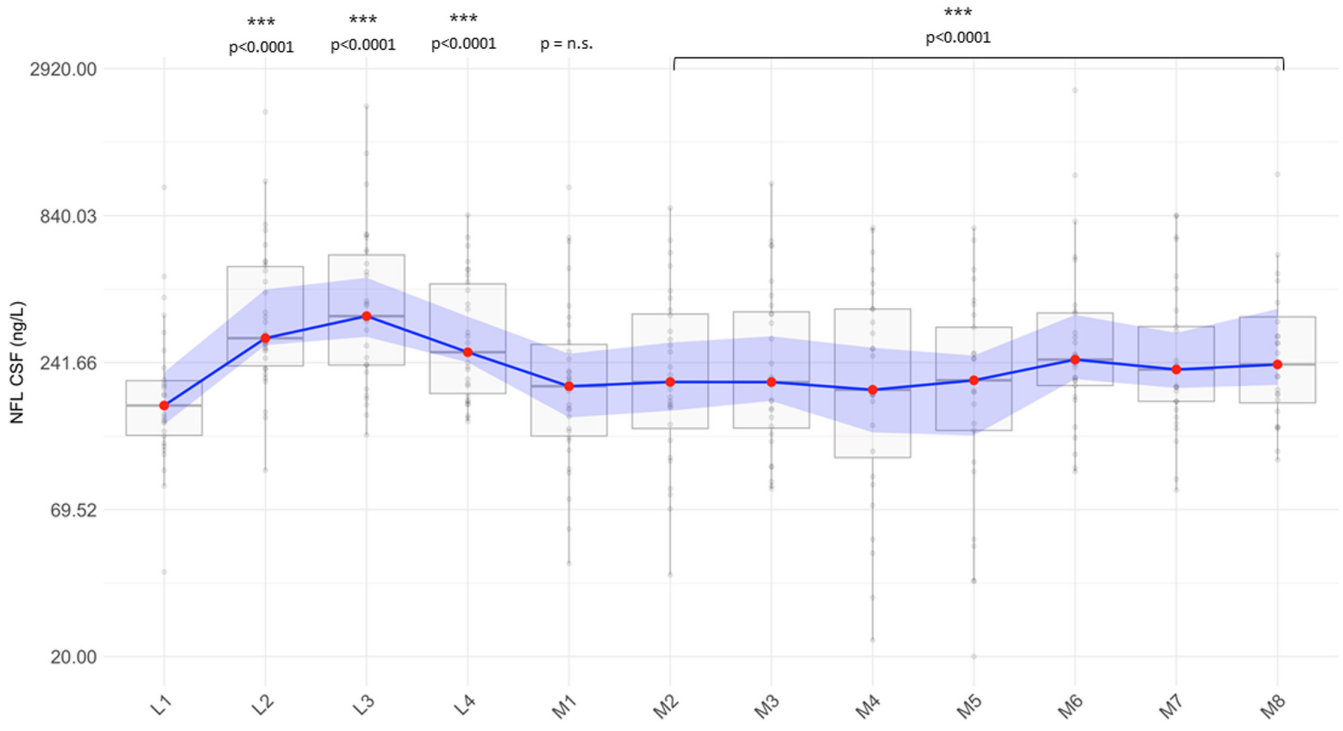

FIGURE 2 Longitudinal analysis of CSF NfL during treatment course. Variation of mean concentrations at each time point compared to baseline. $^{**}p<0.001$; $^{***}p<0.0001$.

$p=0.033$) but not with 6MWT ($r=-0.35$, $p=0.239$) at baseline. Correlations between NfL and HFMSE and RULM were confirmed only at the last follow-up M8 (HFMSE $r=-0.51$, $p=0.019$; RULM $r=-0.46$, $p=0.035$). The 6MWT showed a significant inverse correlation with NfL at time points M3, M4, M7 and M8 (M8 $r=-0.76$, $p=0.016$). NfL levels and HFMSE scores at baseline and last time point (M8) were significantly correlated in the "responders" group only ($r=-0.54$, $p=0.01$).

TABLE 4 Cerebrospinal fluid concentrations (ng/L) of t-tau (top) and p-tau 181 (below) at time points L1 and L3.

Group	Time	Mean	SD	Median	25th percentile	75th percentile	Min	Max
Sitter	L1	210.6	82.5	179	164	244	106	418
	L3	254.1	150.2	179.5	155	361	84	635
Walker	L1	146.9	72.8	118	102	149	75	298
	L3	184.8	67.7	162	135	225.5	103	322
Total	L1	176.6	82.7	162.5	111	202	75	418
	L3	217.1	117.2	176.5	143	233	84	635
Sitter	L1	31	13.3	29.3	22.4	36	14.5	70.2
	L3	35.3	14.1	30.4	25.2	51.7	15.2	59.4
Walker	L1	23.1	11	20.7	14.9	23.2	10	44.3
	L3	29	10	25.8	21.2	37.3	17.5	53.5
Total	L1	26.8	12.6	23.2	18.4	31.6	10	70.2
	L3	32	12.4	29.7	22.9	37.3	15.2	59.4

Abbreviations: L1, loading dose 1 at baseline; L3, loading dose 3 at the 28th day from baseline.

No correlation was found between PFN-1 and HFMSE or RULM or 6MWT (data not shown).

Analysis of the correlation between variation in the loading phase of NfL concentrations (ΔL3–L1) or PFN-1 concentrations (ΔL4–L1) and variation of HFMSE score did not predict a change in HFMSE at M3 or M6 (data not shown).

DISCUSSION

Cerebrospinal fluid NfL and serum PNF-1 were evaluated as possible biomarkers in adult SMA patients treated with nusinersen. In our cohort NfL concentrations were included in accepted reference ranges for healthy controls at baseline and after 34 months of treatment in almost every patient (all except one value at L1 and two at M8); functional status and *SMN2* copy number did not influence NfL levels.

A direct correlation between baseline CSF NfL and age was found both in SMA and control groups. ANCOVA on the entire population showed independent effects of age on SMA versus controls, with SMA patients presenting CSF NfL levels, when age corrected, lower than the control group. The gradual loss of motor neurons (about 5% per annum) as a function of age is a well-known phenomenon in normal aging [24] and the reduced motor neuron pool in SMA may account for the relatively lower level of NfL detected in SMA CSF. The effect of aging on NfL release has been recently reinforced by the extensive results of a prospective study on serum NfL of a community-based neurologically healthy population [25]. Literature data show a direct correlation between CSF NfL and age, with a 2.5-fold increase in NfL levels between the age of 20 years and 50 years [8, 9]. Conversely, studies on early onset SMA patients reported an inverse correlation with age at SMA diagnosis for CSF pNfH [14].

Longitudinal CSF NfL analysis in our cohort during treatment showed a significant increase after 1 month of therapy, but the levels bounced back to baseline levels at 6 months followed by a progressive increase during the maintenance phase that had a rather negligible magnitude (0.9% every month), although being statistically significant. Notably, these are the first data on NfL dynamics during the first month of the loading phase, as the previously published results [26–28] encompassed time points at 2 and 6 months of administration. Interestingly, similar dynamic levels of serum hyperphosphorylated NfH have been observed in the SMA1 mice model treated with antisense oligonucleotide, with a two-fold increase in NfH at day 10 and a steady decline at day 20 [29]. These data may suggest that in response to nusinersen treatment an increase in neurofilament synthesis may trigger neurodegeneration in pools of exhausted motor neurons, allowing rescue in the less damaged ones. This short-lived phenomenon is overcome by a sustained slight decrease of neurofilaments over time indicating a reduction in the neuropathological progression of the disease.

These results are in contrast with the results observed in type 1 SMA [14–16] but showed similar baseline levels to other type 2 and 3 patients [26, 27]. Wurster et al. [28] did not detect elevated NfL and pNfH CSF levels in adolescent and adult patients compared to controls, neither before nor during therapy with nusinersen, and a longitudinal analysis of serum NfL during nusinersen treatment (baseline, 6 months, 10 months, 14 months) revealed almost stable levels [26].

The reasons for these differences between early versus late onset SMA are not completely clear. High neurofilament levels might reflect neuronal plasticity developing early in central nervous system maturation or disease and neurofilament level might tend to normalize later. Indeed, as all type 1 SMA were recruited in the first year of life compared to a much later age in adults, disease duration at the time of CSF sampling might play a pivotal role. As Kariyawasam et al. have recently pointed out in a comprehensive review, plasma pNfH also declined in untreated patients at advancing age, questioning its utility as a biomarker for treatment response and hypothesizing a reduction in motor neuron pool or in disease activity [30].

TABLE 5 Serum PFN-1 (ng/L) descriptive statistics at each time point.

Group	*N*	Mean	SD	Median	25th percentile	75th percentile	Min	Max
Control group	15	618	222	574	487	784	364	1156
ALS	10	735	249	751	640	884	306	1185
L1								
SMA type 3 sitters	10	1151	485	1183.5	619	1631.5	568	1834
SMA type 3 walkers	12	905	450	810	551	1100	340	1977
Total	22	1016	472	944.5	632.5	1273	340	1977
L2								
SMA type 3 sitters	11	542	144	562	428	656	337	764
SMA type 3 walkers	12	588	227	649.5	409	780.5	197	885
Total	23	566	189	588	428.5	692.5	197	885
L3								
SMA type 3 sitters	12	1247	683	1066	654	1932.5	535	2590
SMA type 3 walkers	14	1001	537	919	494.5	1373	316	1912
Total	26	1114	609	1038.5	651	1499	316	2590
L4								
SMA type 3 sitters	13	616	252	534	472	722	252	1192
SMA type 3 walkers	15	565	293	503	377	644	172	1190
Total	28	589	271	518.5	405	680	172	1192
M1								
SMA type 3 sitters	13	795	283	857	635.5	962.5	85	1180
SMA type 3 walkers	16	886	430	797	587.5	929.5	422	2000
Total	29	845	368	841	622	952	85	2000
M2								
SMA type 3 sitters	13	752	250	699	555	983	430	1179
SMA type 3 walkers	15	689	211	688	483	869	387	1018
Total	28	718	228	692	570	918	387	1179
M3								
SMA type 3 sitters	12	739	131	739	622	822	529	987
SMA type 3 walkers	16	703	187	740.5	537	864	327	962
Total	28	718	164	739	610	843	327	987
M6								
SMA type 3 sitters	13	572	111	607	477	651	376	755
SMA type 3 walkers	16	610	140	578	509	714	392	880
Total	29	593	127	581	505	660	376	880

Abbreviation: ALS, amyotrophic lateral sclerosis; L1, loading dose 1 at baseline; L2, loading dose 2 after 14 days; L3, loading dose 3 at the 28th day from baseline; L4, loading dose 4 at the 63rd day from baseline; M1 to M6, maintenance dose after four months from previous administration; PFN-1, profilin-1; SMA, spinal muscular atrophy.

Supporting the recent considerations of Wurster et al. [26], Lombardi et al. [31] found low NfL levels in serum also in spinal and bulbar muscular atrophy patients, in which the prominent lower motor neuron involvement seems to be better related to muscular rather than neurodegenerative biomarkers.

Confirming these results, t-tau and p-tau levels in our SMA group were also within reference range, although longitudinal analysis of CSF t-tau and p-tau over L1–L3 time points showed a statistically significant increase.

Moreover, the exploratory cytoskeletal biomarker PFN-1 changed significantly during the first 2 months of treatment. Whilst cytoskeleton alterations in SMA models have been widely reported [32], the exact pathological process in SMA involving profilins is still unclear, although a loss of actin-binding function hypothetically influenced by SMN protein was noted [32, 33]. In a mouse model of SMA increased levels of the isoform PFN-2a [34] and of phosphorylated PFN-2a were observed [35]. Accordingly, a first report of in vivo profilin concentrations in SMA patients was presented:

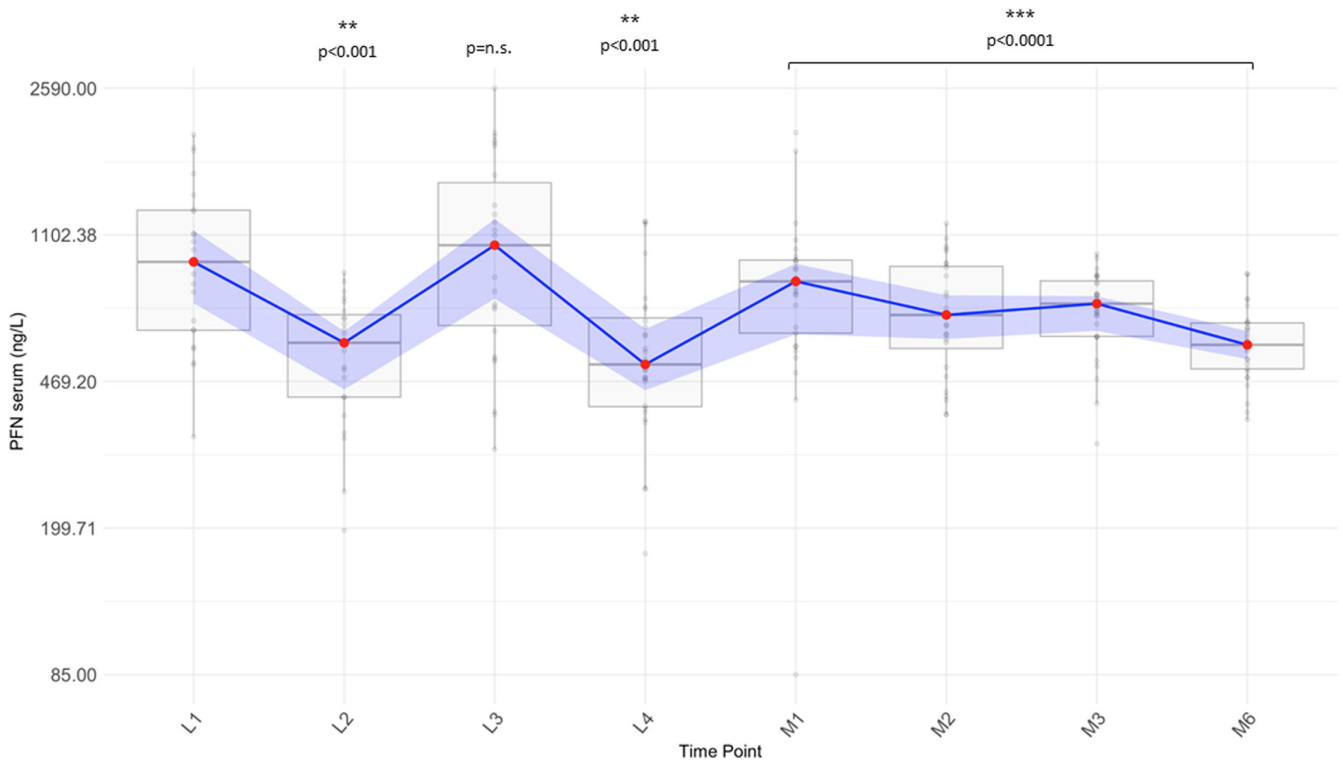

FIGURE 3 Longitudinal analysis of serum PFN-1 during treatment course. Variation of mean concentrations at each time point compared to baseline.

higher concentrations at baseline compared to controls that decreased at L4 and reached control levels at M6. However, it cannot be excluded that this variation, and the monthly decrease of 14.7%, might be due to the natural history of the disease rather than to therapy, as a control group of untreated patients is lacking.

In our cohort NfL levels were significantly inversely correlated with HFMSE and RULM at baseline and at 34 months, but not at other time points, whilst 6MWT was significant starting from M3 to the last time point with an increased magnitude. PFN-1 concentrations did not correlate at any time point with motor scores. HFMSE revealed a statistically significant improvement in our cohort [20], with the "responders" subgroup of patients showing a stronger correlation between NfL levels and HFMSE at baseline and M8. It is well known that nusinersen therapy is less efficient in SMA type 3 adults [20] than in children [6], suggesting that in adults neurodegeneration is a chronic process, as shown by the biomarker concentration. The observed variations in NfL and PFN-1 concentrations during the loading phase did not predict the variation of HFMSE, as De Wel et al. had previously noted in a smaller cohort of SMA type 3–4 treated patients [36].

Our study reinforces the differences observed in SMA type 3 compared to type 1, the latter having a better correlation to NfL levels as a surrogate treatment biomarker. In wider terms, all putative biomarkers studied did not reveal a univocal trend: the most definite neurodegenerative biomarkers (NfL, tau proteins) might inadequately relate to a degenerative disease marked by a long duration, as supported by concentrations mostly comparable to controls. Otherwise, PFN-1 might to a greater extent represent lower motor neuron pathological processes, as suggested by the higher concentrations in SMA patients than in controls and by the concentration decrease during treatment.

This imbalance between clinical and biochemical data should suggest caution in their use as prognostic biomarkers in treated adult SMA patients. Indeed, the relatively low sample size, both for patients and controls, and the measurement method for PFN-1 that is not certified for diagnostic procedures are clear limitations for our study.

It is also worth considering that, with the recent incorporation of SMA testing into newborn screening programs, the need of a reliable biomarker of disease progression is of pivotal relevance not only for therapeutic monitoring but also in asymptomatic carriers to capture the underlying burden of the disease.

Some insights were indeed revealed about the possible biological effects of nusinersen treatment during the first months of treatment, mainly suggested by NfL and PFN-1 concentrations. The results of shorter time points in our cohort might suggest a putative limited axonal remodeling after nusinersen injection, although a transient side effect of lumbar puncture might not be excluded. Additional studies are needed to explore novel candidate biomarkers that might help predict patient prognosis, monitor treatment response and optimize new therapeutic options.

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CONFLICT OF INTEREST STATEMENT

E. P. reports payments or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from Biogen and Roche; support for attending meetings and/or travels from Roche, Biogen, and Alexion; payments for participation on a Data Safety Monitoring Board or Advisory Board from Alexion, UCB Biopharma, and Sanofi. L. B. reports research support from PTC Therapeutics; payments or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from PTC Therapeutics and Pfizer; payments for expert testimony from PTC Therapeutics; payments for participation on a Data Safety Monitoring Board or Advisory Board from PTC Therapeutics, Edgewise Therapeutics, and Roche. G. S. reports research support from ARISLA SYMP-ALS and PNRR-MR1-2022-12375938; payments for participation on a Data Safety Monitoring Board or Advisory Board from Advisory Board Zambon Italia SLA and Advisory Board PHARMALEX Italy S.p.A.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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