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ORIGINAL ARTICLE

Performance of serum neurofilament light chain in a wide spectrum of clinical courses of amyotrophic lateral sclerosis—a cross-sectional multicenter study

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Abstract

Background and purpose: The objective was to assess the performance of serum neurofilament light chain (sNfL) in amyotrophic lateral sclerosis (ALS) in a wide range of disease courses, in terms of progression, duration and tracheostomy invasive ventilation (TIV).

Methods: A prospective cross-sectional study at 12 ALS centers in Germany was performed. sNfL concentrations were age adjusted using sNfL *Z* scores expressing the number of standard deviations from the mean of a control reference database and correlated to ALS duration and ALS progression rate (ALS-PR), defined by the decline of the ALS Functional Rating Scale.

Results: In the total ALS cohort ($n = 1378$) the sNfL *Z* score was elevated (3.04; 2.46–3.43; 99.88th percentile). There was a strong correlation of sNfL *Z* score with ALS-PR ($p < 0.001$). In patients with long (5–10 years, $n = 167$) or very long ALS duration (>10 years, $n = 94$) the sNfL *Z* score was significantly lower compared to the typical ALS duration of <5 years ($n = 1059$) ($p < 0.001$). Furthermore, in patients with TIV, decreasing sNfL *Z* scores were found in correlation with TIV duration and ALS-PR ($p = 0.002$; $p < 0.001$).

Conclusions: The finding of moderate sNfL elevation in patients with long ALS duration underlined the favorable prognosis of low sNfL. The strong correlation of sNfL *Z* score with ALS-PR strengthened its value as progression marker in clinical management and research. The lowering of sNfL in correlation with long TIV duration could reflect a reduction either in disease activity or in the neuroaxonal substrate of biomarker formation during the protracted course of ALS.

KEYWORDS
amyotrophic lateral sclerosis (ALS), long disease duration, serum neurofilament light chain (sNfL), sNfL *Z* score, tracheostomy invasive ventilation (TIV)

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive and often fatal degenerative disorder of motor neurons. Symptoms and individual prognosis are very variable. Diagnosis, management and therapeutic trials are hampered by a lack of informative biomarkers [1, 2]. A wide range of efforts are being made to develop new disease-modifying therapies to expand survival that is typically still restricted to 2–4 years after symptom onset. However, subgroups of ALS patients survive more than 5 years (20%) or even 10 years (5%–10%) [3, 4]. Neurofilament light chain (NfL) in cerebrospinal fluid (CSF) and serum (sNfL) emerged as a biomarker for supporting the diagnosis and, remarkably, as a prognostic marker [5–17]. Specifically, NfL concentrations in CSF and serum were significantly correlated with disease progression as measured by the ALS Functional Rating Scale revised (ALSFRS-R) and, most importantly, with survival [5–15]. Furthermore, NfL has been introduced as a secondary end-point in clinical trials as an early indicator of treatment response. Correspondingly, NfL was significantly lowered in patients treated with the anti-sense oligonucleotide tofersen [18, 19].

Despite the potential of NfL, important unsolved issues limit the interpretation of the biomarker as previous studies were based on small sNfL sample sizes and included low numbers of individuals in the longer course of the disease [10, 14]. The few available long-term studies offer rather inconsistent results of stable or moderately increasing NfL concentrations during disease progression [5, 9, 20–22]. Long-term survivors and ventilated patients offer a unique opportunity to evaluate NfL levels in late stages of the disease. Conversely, NfL may provide further insights in the protracted course of neurodegeneration when studying patients with long disease duration.

The characteristics of NfL in the long course of ALS are decisive for the value of this biomarker in advanced disease stages. To address the conclusiveness of sNfL in different disease stages of ALS, a multicenter cross-sectional study was performed. The aims of the present study were (i) to generate a broad database on sNfL in a large cohort of ALS patients, (ii) to determine sNfL levels in patients with a long (5–10 years) or very long (>10 years) ALS duration, (iii) to elucidate sNfL concentrations in tracheostomy invasive ventilation (TIV) and (iv) to correlate the biomarker level with the functional deficit and ALS progression rate (ALS-PR).

METHODS

Study design

The observational study was conducted as a prospective, multicenter, cross-sectional cohort study. The investigation was reported according to the STROBE criteria [23].

Participants and definition of cohorts

All participants met the diagnostic criteria of ALS including the clinical variants of progressive muscle atrophy and primary lateral sclerosis [24, 25]. The diagnosis of ALS referred to the revised El Escorial criteria which were recently updated [24]. Diagnostic classification was made by experienced neurologists at the participating study centers. The total cohort of ALS patients was divided into two main cohorts based on the criteria of tracheostomy invasive ventilation (TIV): "ALS without TIV" and "TIV-ALS". For both cohorts, three patient groups were defined, differing in ALS duration. The TIV-ALS cohort was additionally divided into three groups differing in TIV duration. A definition of studied cohorts and subgroups is provided in Figure 1.

Serum NfL analysis

Sample collection and sNfL measurement

Blood samples were collected from all participating patients at the respective study centers and shipped to the lead study center at the ALS center in Berlin (Germany), where the core facility for NfL analysis was located. Blood samples were centrifuged, aliquoted and stored at −80°C following a standard operating procedure. The determination of sNfL was realized with the single molecule array (Simoa) technology (HD-X instrument, Quanterix Inc.) using the commercially available NfL Advantage Kit (Quanterix Inc.).

Setting

Recruitment

Following informed consent, patients were recruited at 12 multidisciplinary ALS centers in Germany between March 2019 and August 2022.

Data collection

Demographic data and clinical characteristics were captured in the electronic medical records of the respective participating multidisciplinary center. The rating of ALSFRS-R was performed by a certified evaluator at the time of blood sampling for sNfL analysis. Additional ALSFRS-R data were assessed by self-rating on a digital platform and mobile application ("ALS-App") that have been described elsewhere [26, 27].

Protocol approvals and registrations

The study protocol was approved by the Medical Ethics Committee of Charité—Universitätsmedizin Berlin, Germany, under numbers EA2/168/20 and EA1/219/15 (for the sub-study of platform-based sNfL data management). A signed patient information and informed consent form was obtained from all the participating patients.

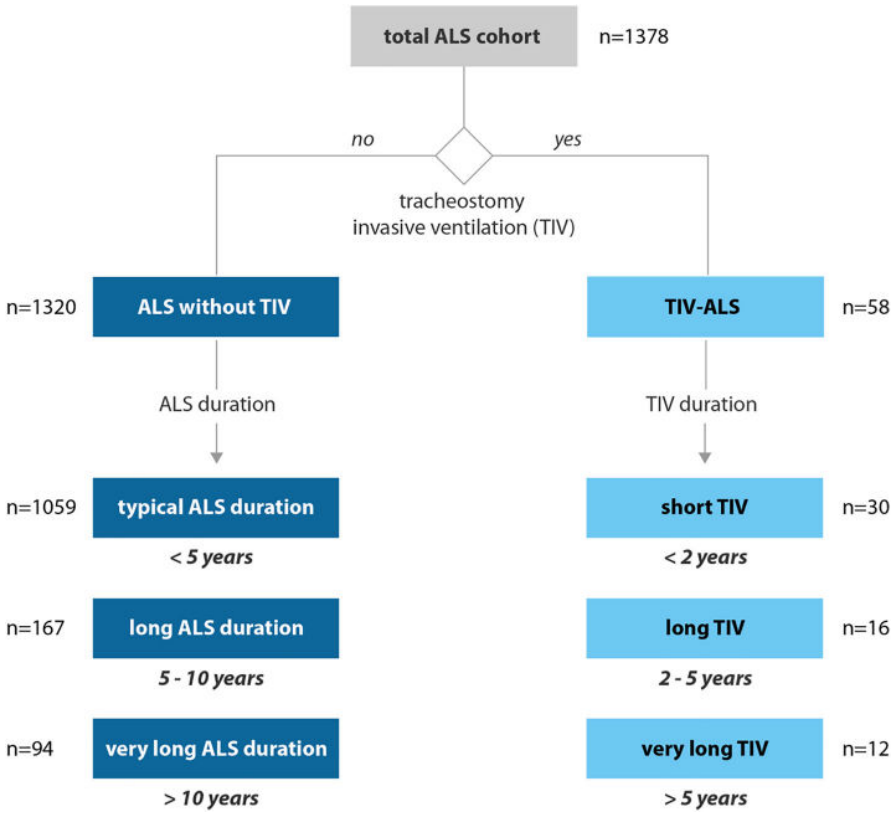

FIGURE 1 Studied amyotrophic lateral sclerosis (ALS) cohorts. The total cohort of ALS patients was divided into two main cohorts based on the criteria of tracheostomy invasive ventilation (TIV): ALS without TIV and TIV-ALS. Both cohorts were subdivided into patient groups of typical ALS duration (up to 5 years), long ALS duration (5–10 years) and very long ALS duration (more than 10 years). The TIV-ALS cohort was analyzed in three patient groups: short TIV (TIV up to 2 years), long TIV (TIV duration of 2–5 years) and very long TIV (TIV more than 5 years).

Variables

Demographic and clinical characteristics

The following demographic and clinical characteristics were collected: age at disease onset, sex, ALS duration (defined as the number of months between disease onset and time of sNfL sampling), non-invasive ventilation (NIV) or TIV, duration of ventilation (number of months between initiation of TIV or NIV and time of sNfL sampling).

Serum concentration of neurofilament light chain

Serum NfL concentrations were analyzed in all participants and referred to the time of sNfL sampling [28, 29]. The reference value was related to the age of the patient (in years) at the time blood sampling for sNfL analysis. An open-access reference database was used to derive reference values corrected for age, and to calculate age-adjusted *Z* scores and percentiles for each individual patient [29]. The resulting *Z* score indicated how strongly, in terms of number of standards deviations from the mean, the sNfL concentration in ALS deviated from age-adjusted sNfL levels in neurologically healthy controls. The percentiles describe the percentage of the age-adjusted general population expected to have an sNfL concentration lower than the given one.

Amyotrophic Lateral Sclerosis Functional Rating Scale revised (ALSFRS-R)

The ALSFRS-R is a 12-item disease-specific instrument that measures bulbar, limb and trunk functions, respiratory symptoms [30]. In total, 0–48 points can be achieved.

Age-related referencing of sNfL (sNfL *Z* score)

Given the physiological, nonlinear increase of sNfL in relation to age, a comparison of sNfL results to age-adjusted reference values was performed [28, 29]. The reference value was related to the age of the patient (in years) at the time of blood sampling for sNfL analysis. An open-access reference database was used to derive reference values corrected for age, and to calculate age-adjusted *Z* scores and percentiles for each individual patient [29]. The resulting *Z* score indicated how strongly, in terms of number of standard deviations from the mean, the sNfL concentration in ALS deviated from age-adjusted sNfL levels in neurologically healthy controls. The percentiles describe the percentage of the age-adjusted general population expected to have an sNfL concentration lower than the given one.

Amyotrophic lateral sclerosis progression rate

The ALS-PR was calculated using the following formula: 48 minus ALSFRS-R divided by disease duration (months). A classification of patients with slower (<0.5 ALSFRS-R/month), intermediate (≥0.5 and ≤1.0 ALSFRS-R/month) and faster (>1.0 ALSFRS-R/month) progression was applied as in most biomarker studies in ALS [5, 9, 22, 31, 32]. Additionally, a general distinction between faster progressing ALS (≥0.9 ALSFRS-R/month) and slower progressing ALS (<0.9 ALSFRS-R/month) according to the phase 3 study of tofersen was investigated [19].

Serum NfL in correlation with ALS progression rate

The sNfL *Z* score was analyzed in the above-mentioned groups of progression. ALS-PR was calculated at the time of sNfL sampling. Moreover, in 51 TIV patients, the ALS-PR before the initiation of TIV was identified. These data allowed a comparison of ALS-PR before the start of TIV with ALS-PR during ongoing TIV.

Serum NfL in correlation with ALS duration

Amyotrophic lateral sclerosis duration was defined by the number of months between disease onset and the date of blood collection for sNfL analysis. The sNfL *Z* score was analyzed for ALS duration in the cohorts of ALS without TIV and TIV-ALS. Both cohorts were subdivided into patient groups of typical disease duration (<5 years), long disease duration (5–10 years) and very long disease duration (>10 years).

Serum NfL in correlation with TIV duration

Tracheostomy invasive ventilation duration was defined by the number of months between the start of TIV and the time of blood collection for sNfL analysis. The TIV-ALS cohort was analyzed in three patient groups being defined according to TIV duration: "short TIV" (TIV duration <2 years), "long TIV" (TIV duration of 2–5 years) and "very long TIV" (TIV duration >5 years).

Statistical methods

Statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, Version 27.0). For graphical representation of data GraphPad Prism (Version 9.0.0 for Windows, GraphPad Software) was used. Continuous variables were assessed for normality using the Shapiro–Wilk test and described accordingly as mean ± standard deviation (SD) or median and interquartile range (IQR). Categorical variables were provided as absolute number (*n*)

and percentage (%). Differences in sNfL *Z* scores between two groups were analyzed using the Mann–Whitney *U* test. Differences in sNfL *Z* scores between more than two groups were analyzed by analysis of variance (ANOVA). Correlations between two non-parametric variables were analyzed using Spearman's correlation. A linear regression analysis was performed to assess the effect of the ALSFRS-R on the sNfL *Z* score. Within the TIV-ALS cohort the ALS-PR before TIV and after a period of TIV was compared by the Wilcoxon matched-pairs signed rank test. Statistical significance was defined as $p < 0.05$.

RESULTS

Characteristics of studied cohorts and patient groups

A total cohort of 1378 ALS patients was analyzed (Figure 1). The ALS cohort without TIV consisted of 1320 patients, including 1059 with typical ALS duration, 167 with long ALS duration and 94 with very long ALS duration. The TIV-ALS cohort consisted of 58 patients including 30 patients with short TIV, 16 with long TIV and 12 with very long TIV. Clinical characteristics are provided in Table 1.

Amyotrophic lateral sclerosis Functional Rating Scale

In the total cohort, the ALSFRS-R showed a median value of 36 (28–42) (Table 1). In the cohort of ALS without TIV, the median ALSFRS-R was 37 (30–42). In the cohort of TIV-ALS the ALSFRS-R was 3 (1–10) reflecting the severe functional deficit.

Amyotrophic lateral sclerosis progression rate

In the total ALS cohort, the median ALS-PR was 0.47/month (0.24–0.96). In the groups of long ALS duration and very long ALS duration, the median ALS-PR was low, both fulfilling the criteria of slow progressing ALS (0.21 [0.14–0.32] and 0.11 [0.07–0.15], respectively). In the group of short TIV, ALS-PR was 0.86 (0.43–1.43). In contrast, in the groups of long TIV and very long TIV ALS-PR was decreased (0.51 [0.40–0.66]; 0.28 [0.20–0.35], respectively). However, in both groups a higher ALS-PR was found before the start of TIV. In long TIV and very long TIV, the ALS-PR before TIV was 0.70 (0.54–1.05) and 0.57 (0.43–1.35), respectively. There was a significant secondary decrease of ALS-PR in the patient groups of long TIV and very long TIV ($p < 0.001$; Table 1).

Serum NfL in correlation with ALSFRS-R

In the ALS without TIV cohort, linear regression analysis showed no significant association between the ALSFRS-R and the sNfL *Z* score ($F(1, 1318) = 2.223$, $p = 0.1362$; $R^2 = 0.002$; Figure 2a).

Serum NfL in correlation with ALS progression rate

In the main cohort of ALS without TIV, there was a strong positive correlation of ALS-PR with the sNfL *Z* score (Spearman's $\rho = 0.564$, $p < 0.001$). The sNfL *Z* score differed significantly between the three progression groups (Welch's $F(2, 819.46) = 171.44$, $p < 0.001$, $\eta^2 = 0.17$), with highest values in the fast progression group (sNfL *Z* score 3.31 ± 0.50; $n = 286$), somewhat lower values in the intermediate progression group (sNfL *Z* score 3.00 ± 0.74; $n = 362$) and lowest values in the slow progression group (sNfL *Z* score 2.37 ± 1.07; $n = 672$). Games–Howell post hoc analysis revealed significant differences between sNfL *Z* scores of all groups ($p < 0.001$ each; Figure 2b). Likewise, the sNfL *Z* score was significantly higher in faster progressing ALS (as defined by ALS-PR ≥ 0.9; sNfL *Z* score 3.43 [3.12–3.54]; $n = 357$) compared to slower progressing ALS (ALS-PR < 0.9; sNfL *Z* score 2.88 [2.14–3.24]; $n = 963$; $U = 79256.5$, $Z = -15.07$, $p < 0.001$; Figure 2c). Also, in the TIV-ALS cohort, there was a strong positive correlation of the ALS-PR with the sNfL *Z* score (Spearman's $\rho = 0.681$, $p < 0.001$).

Serum NfL in correlation with ALS duration

In ALS without TIV, there was a moderate negative correlation between the ALS duration and the sNfL *Z* score (Spearman's $\rho = -0.467$, $p < 0.001$). The sNfL *Z* score differed significantly between the patient groups of typical, long and very long ALS duration (Welch's $F(2, 176.135) = 104.107$, $p < 0.001$, $\eta^2 = 0.20$). Games–Howell post hoc analysis revealed significant differences between sNfL *Z* scores of all groups ($p < 0.001$ each). More specifically, the highest sNfL *Z* score ($2.94 \pm 0.0.80$) was found in patients with typical ALS duration (<5 years), whilst in long ALS duration (5–10 years) and very long ALS duration (>10 years) the sNfL *Z* scores were lower (2.22 ± 1.09 and 1.42 ± 1.17, respectively; Figure 2d). Also, in the cohort of TIV-ALS, a strong negative correlation between the ALS duration and the sNfL *Z* score was demonstrated (Spearman's $\rho = -0.58$, $p < 0.001$). The highest sNfL *Z* score was found in TIV-ALS patients with typical ALS duration (3.19 ± 0.59), whilst the sNfL *Z* scores were declining in long and very long ALS duration, respectively (2.96 ± 0.57 and 2.33 ± 0.66; $(2, 55) = 10.27$, $p < 0.001$, $\eta^2 = 0.27$).

Serum NfL in correlation with TIV duration

In the overall TIV-ALS cohort, an increased sNfL *Z* score was found (2.99 [2.46–3.35]). When analyzing groups of different duration of TIV, the sNfL *Z* score differed significantly ($F(2, 55) = 6.85$, $p = 0.002$, $\eta^2 = 0.20$). Tukey post hoc analysis revealed significant differences in sNfL *Z* scores between short TIV (3.08 ± 0.64) and very long TIV (2.30 ± 0.72; $p < 0.002$) and between long TIV (2.97 ± 0.52) and very long TIV ($p = 0.019$) but not between short TIV and long TIV ($p = 0.833$; Figure 3a).

TABLE 1 Demographic and clinical characteristics

		ALS without TIV			ALS with TIV		
	Total cohort (*n* = 1378)	**Typical ALS duration (*n* = 1059)**	**Long ALS duration (*n* = 167)**	**Very long ALS duration (*n* = 94)**	**Short TIV (*n* = 30)**	**Long TIV (*n* = 16)**	**Very long TIV (*n* = 12)**
Demographic data							
Age (years)	65 (57–72)	65 (57–72)	64 (56–74)	68 (60–76)	61 (49–72)	59 (50–69)	62 (52–73)
Male/female	823 (60%)	628 (59%)	102 (61%)	54 (57%)	21 (70%)	8 (50%)	10 (83%)
	555 (40%)	431 (41%)	65 (39%)	40 (43%)	9 (30%)	8 (50%)	2 (17%)
Disease characteristics							
ALS duration (months)	21 (11–49)	15 (9–27)	78 (68–91)	169 (139–204)	44 (31–64)	86 (62–105)	167 (128–234)
ALSFRS-R	36 (28–42)	38 (32–42)	31 (23–38)	29 (23–34)	7 (2–16)	1 (2–7.5)	2 (1–4)
ALS-PR at sampling	0.47 (0.24–0.96)	0.60 (0.34–1.10)	0.21 (0.14–0.32)	0.11 (0.07–0.15)	0.86 (0.43–1.43)	0.51 (0.40–0.66)	0.28 (0.20–0.35)
Ventilation status							
None	1188 (86%)	982 (93%)	132 (79%)	74 (79%)			
TIV	58 (4%)						
Duration of TIV (months)					9 (4–11)	36 (28–43)	107 (102–160)
ALS-PR before TIV[a]					0.63 (0.44–1.05)	0.70 (0.54–1.05)	0.57 (0.43–1.35)
NIV	132 (10%)	77 (7%)	35 (21%)	20 (21%)			
Duration of NIV (months)	14 (4–32)	8 (3–17)	25 (12–48)	57 (35–89)			
Onset type							
Limb onset	963 (70%)	715 (68%)	136 (81%)	73 (78%)	20 (67%)	11 (70%)	8 (67%)
Bulbar onset	339 (25%)	287 (27%)	25 (15%)	14 (15%)	8 (27%)	4 (25%)	1 (8%)
Axial onset	50 (4%)	47 (4%)	0	0	2 (7%)	1 (6%)	0
Unknown	26 (2%)	10 (1%)	6 (4%)	7 (7%)	0	0	3 (25%)
Serum neurofilament light chain (sNfL)							
sNfL (pg/ml)	64 (35–110)	74 (45–122)	35 (20–63)	21 (15–41)	82 (46–128)	55 (35–95)	31 (19–62)
sNfL *Z* score	3.04 (2.46–3.43)	3.16 (2.75–3.43)	2.58 (1.66–3.04)	1.6 (0.69–2.42)	3.32 (2.93–3.46)	2.93 (2.60–3.28)	2.42 (1.73–2.78)
sNfL percentile	99.88 (99.31–99.97)	99.92 (99.7–99.97)	99.51 (95.15–99.88)	94.52 (75.49–99.22)	99.95 (99.83–99.97)	99.83 (99.53–99.95)	99.22 (95.82–99.73)

Note: The cohort of patients without tracheostomy invasive ventilation (ALS without TIV) was divided according to ALS duration into groups with typical ALS duration (<5 years), long ALS duration (5–10 years) and very long ALS duration (>10 years). Patients with tracheostomy invasive ventilation (TIV) were subdivided into three groups, with either short TIV (TIV duration <2), long TIV (TIV duration 2–5 years) or very long TIV (TIV duration >5 years). ALS duration refers to the period (in months) from the onset of ALS to the date of blood sampling for sNfL analysis. Likewise, TIV duration refers to the time interval (in months) from the start of TIV to the date of blood collection for sNfL measurement. The age-adjusted sNfL *Z* scores and percentiles were derived from an open-access reference database with sNfL measurements of a large number of neurologically healthy control persons. Categorical variables are given as number and percentages. Continuous variables are given as median (interquartile range).

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale revised; ALS-PR, ALS progression rate; NIV, non-invasive ventilation; sNfL, serum neurofilament light chain; TIV, tracheostomy invasive ventilation.

[a]ALS-PR before TIV data were available for 51 TIV-ALS patients (28 short TIV, 14 long TIV, nine very long TIV).

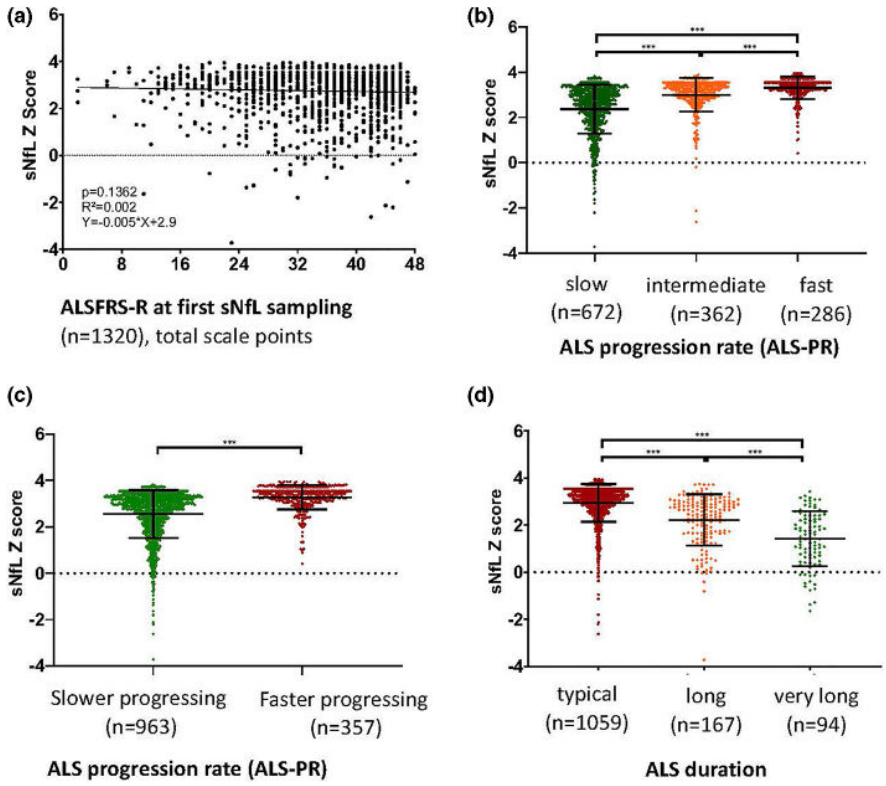

FIGURE 2 Amyotrophic lateral sclerosis (ALS) without TIV cohort. sNfL in correlation with ALSFRS-R, ALS-PR and ALS duration. (a) Linear regression analysis showed no significant association between the ALSFRS-R and the sNfL *Z* score ($F(1, 1318) = 2.223$, $p = 0.1362$; $R^2 = 0.002$). (b) The sNfL *Z* score differed significantly between patients with slow (2.37 ± 1.07; $n = 672$), intermediate (3.00 ± 0.74; $n = 362$) and fast progression (3.31 ± 0.50; $n = 286$; $p < 0.001$). (c) When dichotomizing patients at an ALS-PR cut-off of 0.9 points/month, the sNfL *Z* score was significantly higher in patients with faster progressing ALS (3.43 [3.12–3.54]; $n = 357$) compared to slower progressing ALS (2.88 [2.14–3.24]; $n = 963$; $p < 0.001$). (d) The sNfL *Z* score was significantly lower in patients with long and very long ALS duration ($p < 0.001$). Bars indicate mean and SD. ALSFRS-R, ALS Functional Rating Scale revised; ALS-PR, ALS progression rate; sNfL, serum neurofilament light chain; TIV, tracheostomy invasive ventilation. Significance levels are indicated as $^{ns}p > 0.05$; $^{*}p \le 0.05$; $^{**}p \le 0.01$; $^{***}p \le 0.001$.

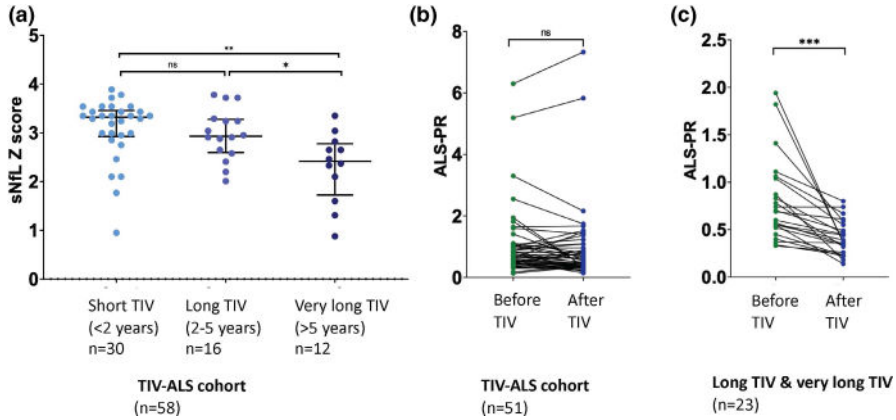

FIGURE 3 TIV-ALS cohort. (a) The sNfL *Z* score differed significantly between patient groups of short, long and very long TIV duration ($F(2, 55) = 6.85$, $p = 0.002$, $\eta^2 = 0.20$). (b) ALS-PR before TIV data were available for 51 TIV-ALS patients. There was no difference in ALS-PR before and after TIV ($p = 0.1815$). (c) ALS-PR before TIV data were available for 14 long TIV and nine very long TIV patients ($n = 23$). In long TIV and very long TIV, the ALS-PR before tracheotomy was 0.70 (0.54–1.05) and 0.57 (0.43–1.35), respectively. The ALS-PR in these patients was lower after TIV compared to their respective ALS-PR before TIV ($p < 0.001$). ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale revised; ALS-PR, ALS progression rate; sNfL, serum neurofilament light chain; TIV, tracheostomy invasive ventilation. Significance levels are indicated as $^{ns}p > 0.05$; $^{*}p \le 0.05$; $^{**}p \le 0.01$; $^{***}p \le 0.001$.

DISCUSSION

Neurofilament light chain in CSF and serum has been previously proposed as a diagnostic and prognostic biomarker in ALS [16, 17]. Reportedly, increased NfL was correlated with disease progressiveness and reduced survival in ALS [5–14]. However, few data are available on NfL in the longer course of ALS. This issue is of relevance as there were conflicting reports of stable, decreasing or increasing NfL concentrations over time [5, 9, 20–22]. In this multicenter study, sNfL was investigated in a large ALS cohort that might broaden the data basis for the use of sNfL in clinical management and research. Moreover, this cohort included patients with long ALS duration who provided some of the needed data on sNfL in the long trajectory of ALS.

Studied cohorts—expanded data on long ALS duration and tracheostomy

In this study, sNfL was investigated in two different ALS cohorts, patients without TIV and patients receiving TIV, each offering insights into different aspects of NfL research. In both cohorts, patients over a wide range of disease duration were included: from the early phase until a very long ALS duration of more than 10 years. These cohorts served to extend the knowledge on sNfL in correlation with the functional deficit and aggressiveness at large scale. Furthermore, patient groups with survival of more than 5 or even 10 years—encompassing 183 and 106 patients, respectively—were of particular interest to learn about the performance of sNfL in the protracted course of the disease. The second studied cohort encompassed 58 ALS patients with TIV including 12 with TIV of more than 5 years—a patient population that has not been specifically investigated before. Despite the achievements of extensive and diversified recruitment, the findings of this study must be considered in the context of the limitations. One limitation is the cross-sectional design of this study. Therefore, longitudinal sNfL data during natural history as well as concomitant with NIV, TIV and other interventions such as percutaneous endoscopic gastrostomy would be of interest. Furthermore, the relation of NfL to phenotypic variants (e.g., primary lateral sclerosis, progressive muscle atrophy), distinct genotypes and clinical characteristics such as weight loss and respiratory function need to be further investigated. Despite the limitations, the present study can provide initial insights into the performance of NfL over the time course of ALS, which can be derived from the inter-cohort comparison of patient groups with different ALS durations. Other potential factors affecting sNfL, such as cognitive changes and different genotypes, were not analyzed in the current study and should be considered in future investigations.

Introduction of sNfL *Z* score for age adjustment of sNfL

As sNfL increases with physiological ageing in normal controls, age adjustment is necessary [33]. sNfL concentrations from our cohort were compared to sNfL levels of age-adjusted neurologically healthy controls using an external database [29]. From this source, the sNfL *Z* score was derived, expressing the deviation of sNfL from the control population as the number of standard deviations from the mean. The sNfL *Z* score was previously introduced in the context of multiple sclerosis and hereby applied to ALS. Beyond this study, the sNfL *Z* score may be used to correct for the physiological increase of sNfL with ageing (Figure S1). However, further studies are needed to confirm the sNfL *Z* score concept in ALS.

Confirmation of sNfL *Z* score as ALS progression marker

The correlation of sNfL with disease aggressiveness has been described before [5–14]. This study demonstrated a strong correlation of the sNfL *Z* score with the ALS-PR confirming the feasibility of sNfL as a progression marker. This is of relevance as previous reports were mainly based on the investigation of CSF or smaller serum sample sizes [5–13]. Here, sNfL was correlated to the previously suggested three-strata classification of disease progression [31] that is increasingly applied for patient stratification in biomarker studies and clinical trials (Figure 2b) [5, 9, 22, 31, 32]. These results demonstrated an alignment of sNfL to distinct strata of ALS progression. Also, when applying a stratification of faster and slower progressing patients that was used in controlled studies of tofersen, sNfL was significantly correlated with ALS progression (Figure 2c) [18, 19]. Previous reports described conflicting data on the association of NfL to functional status in terms of ALSFRS-R [7, 11, 12, 34–37]. In this study, no significant association of sNfL with the ALSFRS-R was found. This finding underlined that sNfL has limited performance to reflect disease severity whereas the main value proposition of sNfL lies in the association with ALS progression, and potentially treatment response.

Performance of sNfL *Z* score in long and very long ALS duration

A remarkable number of patients with very long ALS was investigated (110 patients with ALS duration of more than 10 years) allowing insights into the performance of sNfL in the protracted course of ALS. In patients with long and very long ALS duration the sNfL *Z* score was significantly lower compared to the group of typical ALS duration. Presumably, these results reflected the slow progression rate and intrinsically long disease duration. The lower sNfL was probably present already in the early stage of disease and prevailed at the long course of ALS. However, a major limitation of this study was the cross-sectional design that refers any conclusions on the longitudinal slope of sNfL to the realm of the hypothetical. Another limitation is that there is no consensus definition of typical, long and very long ALS duration. An analogous limitation also exists for the definition of short, long and very long TIV. Therefore, an arbitrary

definition was used in this study based on survival data with and without NIV and TIV, respectively, previously reported from one of the participating study centers [38].

Considerations on sNfL *Z* score in clinical trials and treatment evaluation

The use of sNfL as a stratification criterion in clinical trials or a response marker for disease-modifying drugs is of major interest [18,19]. The robust association between ALS progression and sNfL supports the concept that the sNfL *Z* score is an additional criterion for inclusion or stratification in clinical trials. Thus, sNfL, in addition to the pre-study slope of ALSFRS-R, could help to better define study groups at baseline. The results of this study reinforce the use of sNfL as an important end-point in clinical trials, at least for drugs that aim to reduce neuroaxonal degeneration and go beyond symptomatic therapy. A heterogeneous situation exists in expanded access programs, such as with tofersen, in which also patients with low progression and moderate pre-treatment sNfL are included. In these patients, the analysis of the intra-individual slopes of sNfL before and during treatment might be considered, an approach of personalized interpretation of sNfL that needs further research [18,19].

Decline of sNfL in ALS with tracheostomy invasive ventilation

The group of short TIV showed an elevated sNfL *Z* score, indicating a continued disease aggressiveness after TIV. Remarkably, sNfL significantly decreased in the groups of long TIV and very long TIV. This observation can be discussed in two ways: (1) the decreased sNfL values result from the depletion of the neuroaxonal substrate leading to a biomarker floor-effect in the protracted course of ALS, (2) the lowering of sNfL reflects a reduction in actual disease activity in terms of a slowing of neurodegeneration. In fact, the phenomenon of plateauing in advanced courses of TIV-ALS can also be observed at the clinical level. Thus, a secondary decrease of ALS progression in long TIV (ALS-PR 0.51 vs. 0.70 before TIV) and very long TIV was found (ALS-PR 0.28 vs. 0.57 before TIV). This observation can also be explained by different constellations: either by the previously reported methodical floor-effect of the ALSFRS-R [39] or a clinical floor-effect resulting from loss of quantifiable motor function or an actual slowing of the degenerative process [40–43]. This open discussion was symbolized by the patient with 21 years of TIV, a clinical floor-effect (incomplete locked-in syndrome) and low sNfL *Z* score (1.31; 90th percentile). At this point, there is an interesting cross-link to 5q-associated spinal muscle atrophy, where sNfL was found to be elevated in the early infantile course of spinal muscle atrophy and normal in the adult phases of the disease [44]. Although the observation of decreasing sNfL in ALS after long TIV may suggest an attenuation of neurodegeneration, this interpretation warrants caution. It was beyond the scope of this study to clarify the neurobiological basis of declining sNfL in the long-term course of ALS. Longitudinal studies, preferably combining NfL analyses and pathoanatomical studies, are needed to expand the knowledge base on the protracted course of neurodegeneration, to which NfL studies in ALS have already made a valuable contribution.

AUTHOR CONTRIBUTIONS

TM, PK and MD designed and conceptualized the study, analyzed and interpreted the data and drafted the manuscript for intellectual content. ES, DK, JN, YC, JG and AM had a major role in data acquisition, interpreted the data and revised the manuscript for intellectual content. TG, UW, DK, PW, RG, PL, JK, SP, AH, PB, PB, MB, MM, JN, IC, JW, JD, AL, CM and SSP had a major role in data acquisition and revised the manuscript for intellectual content. BW had a major role in data aggregation and preparation of data.

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CONFLICT OF INTEREST STATEMENT

TM has received grants, personal fees, non-financial support and research support from AL-S Pharma, Amylyx, Cytokinetics, Ferrer, Sanofi, Mitsubishi Tanabe, Apellis Pharmaceuticals, Orphazyme, and served on the advisory boards of Amylyx, Biogen and ITF Pharma outside of the submitted work. TM and CM are founders and shareholders of the Ambulanzpartner Soziotechnologie APST GmbH, which makes the mobile application "ALS-App". TG has received personal fees from ITF Pharma and served on the advisory boards of Amylyx and ITF Pharma outside of the submitted work. PW has served on advisory boards of Biogen, ITF Pharma and Novartis outside of the submitted work. RG has received grants, personal fees, non-financial support and research support from Biogen and served on the advisory boards of Biogen, Roche, Sanofi and ITF Pharma outside of the submitted work. JCK has received consulting fees and compensation for talks from Biogen, Roche and AbbVie and has served on advisory boards for Biogen and Roche. PL has received consulting fees from AbbVie, Alexion, BIAL, Desitin, ITF Pharma, STADA Pharm, Woolsey Pharmaceuticals and Zambon outside of the submitted work. He is co-inventor on patents EP 2825175 B1, US 9.980,972 B2 for the use of Fasudil in ALS. SP has received speaker fees, non-financial support and research support from Biogen, Roche, AL-S Pharma, Amylyx, Cytokinetics, Ferrer, ITF-Pharma and Sanofi and served on advisory boards of Amylyx, Biogen, Roche, Zambon and ITF Pharma outside of the submitted work. AH has received funding from the European Social Funds, the Federal Ministry of Education and Research and the Hermann und Lilly Schilling-Stiftung für medizinische Forschung im Stifterverband. He has received honoraria for presentations/advisory boards from Amylyx, Desitin and IFT Pharma, and royalties from Elsevier Press and Kohlhammer. All of

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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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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