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RESEARCH ARTICLE

Neurodegenerative biomarkers outperform neuroinflammatory biomarkers in amyotrophic lateral sclerosis

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Abstract

Objective: To describe the diagnostic and prognostic performance, and longitudinal trajectories, of potential biomarkers of neuroaxonal degeneration and neuroinflammation in amyotrophic lateral sclerosis (ALS). *Methods*: This case-control study included 192 incident ALS patients, 42 ALS mimics, 114 neurological controls, and 117 healthy controls from Stockholm, Sweden. Forty-four ALS patients provided repeated measurements. We assessed biomarkers of (1)neuroaxonal degeneration: neurofilament light (NfL) and phosphorylated neurofilament heavy (pNfH) in cerebrospinal fluid (CSF) and NfL in serum, and (2)neuroinflammation: chitotriosidase-1 (CHIT1) and monocyte chemoattractant protein 1 (MCP-1) in CSF. To evaluate diagnostic performance, we calculated the area under the curve (AUC). To estimate prognostic performance, we applied quantile regression and Cox regression. We used linear regression models with robust standard errors to assess temporal changes over time. *Results*: Neurofilaments performed better at differentiating ALS patients from mimics (AUC: pNfH 0.92, CSF NfL 0.86, serum NfL 0.91) than neuroinflammatory biomarkers (AUC: CHIT1 0.71, MCP-1 0.56). Combining biomarkers did not improve diagnostic performance. Similarly, neurofilaments performed better than neuroinflammatory biomarkers at predicting functional decline and survival. The stratified analysis revealed differences according to the site of onset: in bulbar patients, neurofilaments and CHIT1 performed worse at predicting survival and correlations were lower between biomarkers. Finally, in bulbar patients, neurofilaments and CHIT1 increased longitudinally but were stable in spinal patients. *Conclusions*: Biomarkers of neuroaxonal degeneration displayed better diagnostic and prognostic value compared with neuroinflammatory biomarkers. However, in contrast to spinal patients, in bulbar patients neurofilaments and CHIT1 performed worse at predicting survival and seemed to increase over time.

Keywords: *Amyotrophic lateral sclerosis, biomarker, survival, neurodegeneration, neuroinflammation*

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1. Introduction

In amyotrophic lateral sclerosis (ALS) there are no established biomarkers to definitely diagnose or to reliably predict prognosis (1). However, there are potential candidates including the most widely used neurofilament light chain (NfL) as well as phosphorylated neurofilament heavy chain (pNfH) (2–4). Neurofilaments (NFs) are biomarkers of neuroaxonal degeneration and elevated levels are found in the cerebrospinal fluid (CSF) and blood of ALS patients compared with ALS mimics, patients with other neurological disorders, and healthy individuals (3, 5–8). Higher levels of NFs have been linked to faster disease progression and shorter survival in ALS patients (6, 9–12). In longitudinal studies, NFs have been reported to be stable over time since ALS diagnosis (13–17). Decreasing NfL levels have, in contrast, been used as an indicator of effect in clinical trials (18, 19), and tofersen was recently approved merely based on decreases in NfL (20).

In recent years, evidence of neuroinflammation in ALS has emerged (21, 22). Proteomics of CSF have led to the identification of proteins involved in neuroinflammatory processes, with CHIT1, a biomarker of microglia activation, being the most studied (17, 23). The chemokine monocyte chemoattractant protein-1 (MCP-1) is another protein related to myeloid activation that is also likely elevated in the CSF of ALS patients (9, 16). Few studies have however described CHIT1 and MCP-1 trajectories over time since ALS diagnosis.

Most previous studies on ALS biomarkers have relied on analyses of CSF, but the introduction of more sensitive methods that allow much lower concentrations to be measured in blood, such as single-molecule array (Simoa), now facilitate tracking of dynamic changes over time (24). Still, variable estimates of how well NfL correlates between CSF and blood from ALS patients have been reported (Spearman's rho: 0.62 to 0.84) (15,25–28).

In this study we aimed to compare the diagnostic and prognostic performance, as well as the longitudinal trajectories, of potential biomarkers reflecting neuroaxonal degeneration (NfL and pNfH) and neuroinflammation (CHIT1 and MCP-1) in both CSF and blood of ALS patients and controls.

2. Materials and methods

2.1. Study design and participants

We conducted a case-control study, and a longitudinal study of ALS patients, in Stockholm, Sweden. Study participants were prospectively recruited between March 2016 and March 2021 at the Karolinska ALS Clinical Research Center (ALS CRC), a tertiary clinic that cares for all ALS patients in Stockholm. Of all 381 individuals diagnosed with motor neuron disease (MND) during this time period, 192 ALS patients consented and were included in the study (Figure S1). Of these, 44 patients provided repeated measurements, and were included in the analysis of longitudinal trajectories of the biomarkers. Survival status was noted from the date of diagnosis until the date of death or invasive ventilation, or until 8 February 2023, whichever came first.

Individuals evaluated at the ALS CRC with suspicion of ALS, but who were diagnosed with other diseases (not MND), were recruited as ALS mimics ($n = 42$) (Table 1). We also included 114 patients with other neurological diseases as neurological controls, grouped as patients with inflammatory diseases (multiple sclerosis [MS], chronic inflammatory demyelinating polyneuropathy [CIDP], or paraproteinemic demyelinating polyneuropathy [PDN]) (INC; $n = 80$) or non-inflammatory diseases (normal pressure hydrocephalus, headache, spinal stenosis, hypersomnia, etc.) (NINC; $n = 34$) (Table S1). Siblings and partners of the ALS patients were included as healthy controls ($n = 117$).

2.2. Clinical data

Functional disability was assessed using the revised ALS Functional Rating Scale (ALSFRS-R). Baseline progression rate was calculated as 48 minus the ALSFRS-R score at diagnosis divided by the number of months from onset to diagnosis. Longitudinal progression rate was calculated as the ALSFRS-R score at diagnosis minus the ALSFRS-R score at last visit divided by number of months between the two measurements (time between diagnosis and last ALSFRS-R score; median: 16.3 months; interquartile range: 8.1–27.0 months). At diagnosis, patients were also evaluated using the revised El Escorial Criteria (29) and classified using King's clinical staging system (King's) (30). Signs of frontotemporal degeneration (FTD) at diagnosis were based on a combination of clinical observations by the neurologist and cognitive screening tools (Edinburgh Cognitive and Behavioral ALS Scale and Montreal Cognitive Assessment).

Among the ALS patients, there were no missing data on clinical characteristics including site of onset, ALSFRS-R, progression rate, King's, revised El Escorial criteria, body mass index (BMI), and signs of FTD at diagnosis. However, there were missing data on *C9orf72* status (missing $n = 44$; 22.9%).

Table 1. List of ALS mimics.

Mimic number	Diagnosis
1	Progressive multifocal leukoencephalopathy.
2	Motor neuropathy.
3	Inclusion body myositis, multiple system atrophy (parkinsonian type) and polyneuropathy.
4	Spinal stenosis and neuroborreliosis.
5	Dysarthria and dysphagia of unknown origin.
6	Isolated unilateral hypoglossal nerve paralysis, possibly due to atlanto-occipital dislocation.
7	Polyneuropathy.
8	Vasculitic neuropathy.
9	Degenerative spine (cervical and especially lumbar).
10	Dysarthria and dysphagia due to radiation therapy after base of tongue cancer.
11	Neuroborreliosis.
12	Dysarthria and dysphagia. Halted progression. Unknown cause.
13	Paraneoplastic process resulting in lower motor neuron involvement in one arm. Colon cancer with liver metastases and prostate cancer.
14	Cervical spinal stenosis and polyneuropathy.
15	Spastic paraparesis.
16	Spastic paraparesis.
17	Dysarthria and dysphagia due to base of tongue cancer.
18	Parkinson's disease.
19	Progressive supranuclear palsy.
20	Polyneuropathy and dementia.
21	Benign fasciculations, axonal polyneuropathy and lumbar degeneration.
22	Familial prion disease (probably fatal familial insomnia).
23	Neuroborreliosis, cervical spondylosis and polyneuropathy.
24	Myelopathy, neuropathy and myopathy partly explained by excessive alcohol consumption.
25	Pronounced degenerative cervical spine.
26	Polymyositis.
27	Myelopathy due to radiation therapy after breast cancer.
28	Parkinson's disease.
29	Lumbar spondylosis.
30	Drug induced polyneuropathy.
31	Cervical radiculopathy.
32	Benign fasciculations.
33	Multiple system atrophy (parkinsonian type).
34	Benign fasciculations.
35	Unknown myopathy.
36	Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP).
37	Psychological stress and fatigue.
38	Multiple sclerosis.
39	Benign fasciculations.
40	Benign fasciculations.
41	Bulbar dystonia.
42	Suspected mitochondrial disease (motor neuropathy and leukodystrophy).

Mimics were evaluated at the time of the diagnostic work-up, and their medical records were reviewed again in November 2022, to ensure that they did not convert to ALS or to other motor neuron diseases.

2.3. Sample collection and measurements

Blood and CSF were collected from ALS patients within 90 days after diagnosis. Samples were collected from ALS mimics and some neurological controls (CIDP, PDN and all NINCs) during the diagnostic work-up, and from MS controls at variable times during the disease course. CSF was obtained through lumbar puncture using polypropylene tubes and centrifuged for ten minutes at 400 g at room temperature. Blood was drawn into serum separator tubes and EDTA tubes (for plasma) and centrifuged for ten minutes at 2000 *g* at room temperature. All samples were aliquoted within two hours, directly frozen and then stored in −80 °C.

The measurements of NFs, CHIT1 and MCP-1 in CSF were based on sandwich enzyme-linked immunoassay (ELISA) techniques. We used commercially available ELISA kits: pNfH from Euroimmun, Lübeck, Germany; NfL from UmanDiagnostics, Sweden; CHIT1 from CircuLex, MBL, Japan; MCP-1 from R&D Systems, Minneapolis, MN, USA. Serum and plasma NfL were measured using a Simoa assay (Quanterix, Billerica, MA). The CSF was diluted 2-10-fold depending on the assay. Twenty-four readings of pNfH (one mimic, 10 INC, nine NINC, and four healthy controls) and 15 readings of CHIT1 (13 ALS patients, one mimic, and one healthy control) were below the detection limits of the assays. These were assigned the value of the lower limit of

Table 2. Characteristics of study participants and biomarker concentrations at diagnosis (in ng/L).

Characteristics	ALS patients	ALS mimics	Inflammatory neurological controls	Non-inflammatory neurological controls	Healthy controls
n (CSF)	192	42	80	34	23
n (serum)	157	30	80	34	117
Female, n (%)*	86 (44.8%)	10 (23.8%)	41 (51.2%)	20 (58.8%)	9 (39.1%)
Age at first sample, median (IQR)*	67.6 (59.1–74.0)	71.2 (63.1–75.4)	50.0 (46.0–57.0)	51.0 (41.0–63.0)	50.5 (41.0–60.2)
pNfH, median (IQR)	2,124 (1,350–3,092)	411 (258–568)	198 (157–339)	177 (62–330)	194 (143–225)
CSF NfL, median (IQR)	6,300 (3,764–10,234)	1,373 (881–2,407)	706 (404–1,305)	536 (354–990)	455 (314–662)
Serum NfL, median (IQR)	106.0 (74.5–167.6)	24.6 (15.7–52.4)	14.4 (10.4–21.8)	11.3 (7.3–19.5)	20.4 (14.6–27.3)
CHIT1, median (IQR)	7,290 (3,038–17,398)	2,320 (1,277–4,745)	NA	NA	1,295 (631–1,837)
MCP-1, median (IQR)	601 (514–700)	592 (436–685)	NA	NA	423 (347–499)
Site of onset, n (%)	Spinal: 114 (59.4%)				
	Bulbar: 70 (36.5%)				
	Other†: 8 (4.2%)				
Months from onset to diagnosis, median (IQR)	11.7 (7.6-16.6)				
ALSFRS-R at diagnosis, median (IQR)	39.5 (35.0-43.0)				
Progression rate at diagnosis, median (IQR)	0.7 (0.4-1.3)				
Longitudinal progression rate, median (IQR)	1.2 (0.7-2.0)				
No. of deaths during follow-up, n (%)‡	165 (85.9%)				

Note: * Sex and age based on the study participants providing CSF samples, not serum. † 4 patients with frontal lobe onset and 4 patients with respiratory onset. ‡ Death or invasive ventilation. n: number of study participants. IQR: interquartile range. pNfH: phosphorylated neurofilament heavy. NfL: neurofilament light. CHIT1: chitotriosidase-1. MCP-1: monocyte chemoattractant protein-1.

quantification (pNfH 62.5 ng/L and CHIT1 141 ng/L). Intra- and inter-assay variability was below 10%.

Neurofilaments were measured in all participants. CHIT1 and MCP-1 were measured in all ALS patients, all ALS mimics, and 13 healthy controls. In 83 of 114 neurological controls and nine of 117 healthy controls, plasma was analyzed instead of serum. To enable comparisons between plasma and serum measurements, a separate cohort of 126 controls was used as reference to compare serum and plasma concentrations of NfL, concluding that serum concentration was 11% higher than the corresponding value in plasma (Figure S2). Therefore, serum NfL for these neurological and healthy controls was estimated by calculating the plasma NfL concentrations times 1.11.

2.4. Statistical analysis

Pearson's χ^2-test was used to determine differences in proportions of categorical variables between groups. Due to non-normality of the continuous variables, Mann-Whitney U test was applied to test differences between groups whereas Spearman's rank correlation (rho) was used to calculate correlations between continuous variables. Diagnostic performance of the biomarkers was evaluated with Area Under the Curve (AUC) and Receiver Operating Characteristics (ROC) curves, with adjustment for age at measurement. To compare the AUCs, tests for equality between AUCs were performed (31). Youden index was used to derive the optimal cutoff for each biomarker from the ROC curves, giving equal weight to sensitivity and specificity (32). Because data were non-normally distributed, quantile regression was applied to make inferences on the median longitudinal progression rates, instead of the more common linear model. Cox proportional hazard regression was used to analyze the risk of death in relation to biomarker levels, with time since measurement as underlying time scale. The proportional hazards assumption was met for all Cox models. For both quantile regression and Cox model, biomarker concentrations were divided into tertiles, and adjustments were made for sex, age at diagnosis, site of onset, classification according to the revised El Escorial criteria, diagnostic delay, baseline ALSFRS-R, baseline progression rate, BMI, and signs of FTD at diagnosis (33, 34). Due to incompleteness in *C9orf72* status, it was adjusted for in a separate sensitivity analysis. A genotype of the CHIT1 gene causes lower levels of CHIT1 in CSF and serum (35, 36). The prevalence of homozygous carriers of this genotype is estimated at 3–9% in European populations (11, 35, 37, 38), i.e. in the same range as the number of study participants with undetectable CHIT1 concentrations in our

study ($n = 16$; 6.5%). Therefore, CHIT1 values <200 ng/L ($n = 13$ ALS patients, one mimic, and two healthy controls) were dropped in separate sensitivity analyses. Repeated measurements of the biomarker concentrations were analyzed among 44 ALS patients, using a linear regression model with cluster-robust standard errors. In all analyses, we set statistical significance at 5%. We performed the analyses in Stata software, version 16 (StataCorp, College Station, TX).

2.5. Protocol approvals and registrations

This study was approved by the Swedish Ethical Review Authority (Dnrs 2014/1815-31/4, 2017/1895-31/1, 2009/2107-31/2, 2017/952-31). The study followed the ethical principles of the Declaration of Helsinki.

3. Results

3.1. Participant characteristics

ALS patients differed from ALS mimics with regard to sex (female 44.8% vs. 23.8%; $p = 0.01$) but not age (median 67.6 years vs. 71.2 years; $p = 0.20$), and from neurological and healthy controls in age (median 51.0 years and 50.5 years, respectively; $p < 0.001$ for both) but not sex (53.5% and 39.1%, respectively; $p = 0.14$ and 0.61, respectively) (Table 2). The characteristics of the ALS patients in our study were similar to the characteristics of all ALS patients diagnosed at ALS CRC during the same period (Table S2).

3.2. Biomarker concentrations and correlations

All biomarker concentrations were higher among ALS patients at time of diagnosis compared with all other groups, except for MCP-1 (Table 2; Figure 1; Table S1). Comparing spinal and bulbar patients, there was no difference in any biomarker at time of diagnosis ($p = 0.57$, 0.79, 0.19, 0.13, and 0.61 for pNfH, CSF NfL, serum NfL, CHIT1, and MCP-1, respectively).

Among ALS patients, NfL and pNfH concentrations correlated strongly with each other, whereas inflammatory biomarkers demonstrated weaker correlations with each other and with NFs (Table 3; Figure S3). Correlations were generally weaker in bulbar than in spinal patients.

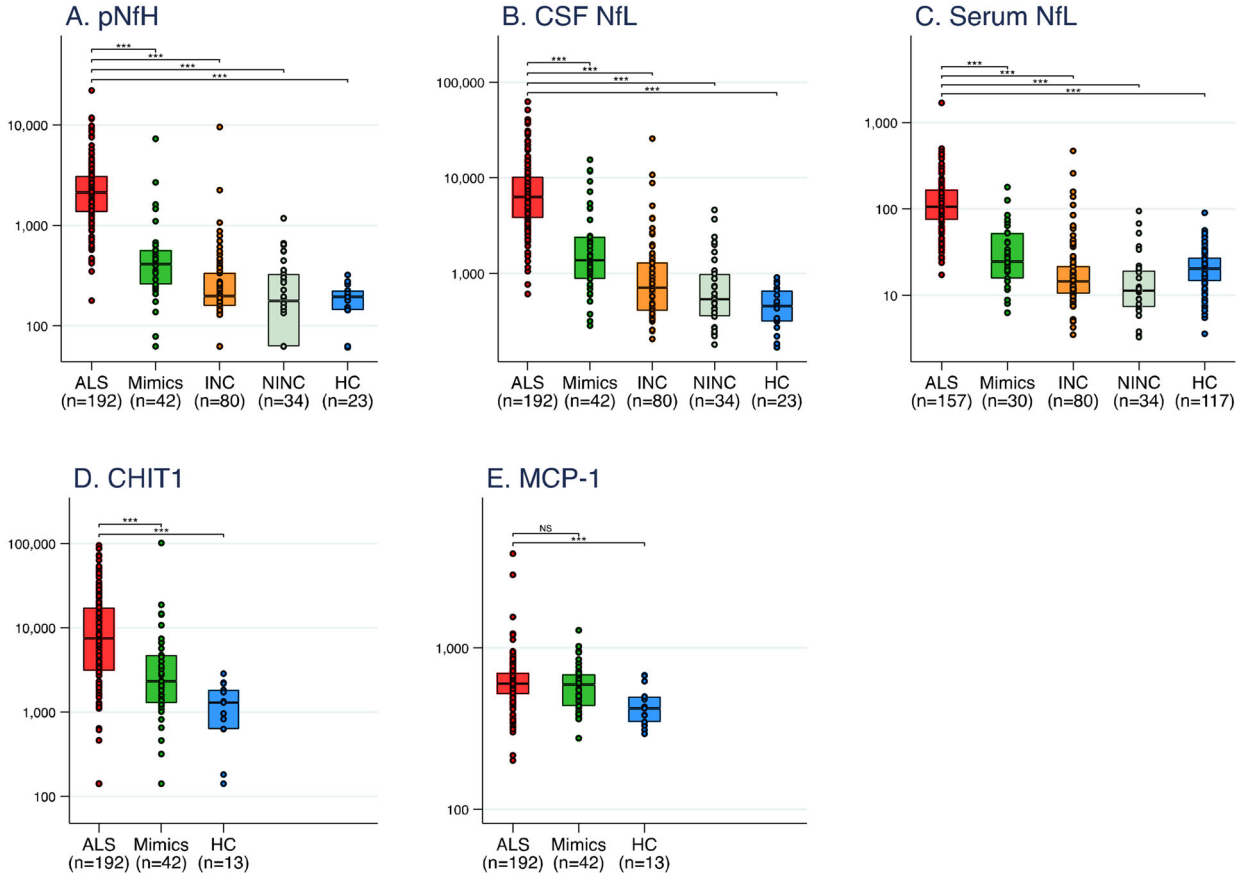

Figure 1. Box plots for biomarker concentrations in ALS patients, ALS mimics, and different control groups. Note: Biomarker concentrations are shown on a logarithmic scale in ng/L. Differences are calculated using Mann–Whitney *U* test. *** $p < 0.001$. NS: Not statistically significant. INC: Inflammatory neurological controls. NINC: Non-inflammatory neurological controls. HC: Healthy controls. pNfH: phosphorylated neurofilament heavy. NfL: neurofilament light. CHIT1: chitotriosidase-1. MCP-1: monocyte chemoattractant protein-1.

Table 3. Correlation matrix of biomarkers measured in ALS patients at the time of diagnosis.

	pNfH	CSF NfL	Serum NfL	CHIT1	MCP-1
CSF NfL	**0.84*****	**1.00**			
- Spinal patients	0.91***				
- Bulbar patients	0.73***				
Serum NfL	**0.75*****	**0.78*****	**1.00**		
- Spinal patients	0.82***	0.83***			
- Bulbar patients	0.57***	0.65***			
CHIT1	**0.43*****	**0.44*****	**0.28****	**1.00**	
- Spinal patients	0.54***	0.57***	0.44***		
- Bulbar patients	0.22	0.18	−0.14		
MCP-1	**0.22****	**0.28*****	**0.19**	**0.28*****	**1.00**
- Spinal patients	0.28**	0.36***	0.27*	0.21*	
- Bulbar patients	0.19	0.20	0.01	0.37**	

Note: Correlations are estimated using Spearman rank order correlation (rho). For paired CSF: $n = 192$, including 114 onset spinal patients and 70 bulbar onset patients. For paired CSF-serum: $n = 105$, including 69 spinal onset patients and 31 bulbar onset patients. Paired biomarkers were sampled on the same day. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. pNfH: phosphorylated neurofilament heavy. NfL: neurofilament light. CHIT1: chitotriosidase-1. MCP-1: monocyte chemoattractant protein-1.

Table 4. Area under the curve (AUC) for differentiating ALS patients from ALS mimics, adjusted for age at measurement, together with optimal cutoffs (estimated with Youden index) and their sensitivity and specificity.

Biomarker	AUC (95% CI)	Cutoff (ng/L)	Sensitivity	Specificity
pNfH	0.92 (0.86–0.98)	726	94.3%	83.3%
CSF NfL	0.86 (0.78–0.94)	2,588	90.6%	76.2%
serum NfL	0.91 (0.84–0.97)	56.4	91.7%	76.7%
CHIT1	0.71 (0.63–0.80)	3,379	75.5%	64.3%
MCP-1	0.56 (0.44–0.67)	535	84.4%	28.6%

Note: pNfH, CSF NfL, CHIT1 and MCP-1 were measured in CSF of the 192 ALS patients and 42 mimics. Serum NfL was measured in 157 ALS patients and 30 mimics. CI: Confidence interval. pNfH: phosphorylated neurofilament heavy. NfL: neurofilament light. CHIT1: chitotriosidase-1. MCP-1: monocyte chemoattractant protein-1.

3.3. Diagnostic performance

AUC was higher for pNfH compared with CSF NfL ($p = 0.007$) for differentiating ALS patients from ALS mimics (Table 4; Figure S4). When assessing study participants who provided both CSF and serum measurements, there was no difference between serum NfL and pNfH ($p = 0.81$), or between serum NfL and CSF NfL ($p = 0.27$). AUCs for NFs were higher than for CHIT1 and MCP-1 ($p < 0.001$ for all comparisons). Combining pNfH, CSF NfL, CHIT1 and MCP-1 did not improve the diagnostic performance compared with pNfH alone ($p = 0.53$). AUCs comparing bulbar and spinal patients separately were similar for all biomarkers.

3.4. Prognostic performance

Comparing low to high concentrations of NFs, there was an increase in predicted median longitudinal progression rates, and risk of death, in both simple and multivariable models, although the multivariable progression rate model was not significant for pNfH (Figure 2; Table S3; Kaplan-Meier curves in Figure S5). Serum NfL performed particularly well, both for progression rate and survival. For CHIT1 and MCP-1, there was an increased risk of death by comparing low to medium concentrations. Adding all CSF biomarkers into a multivariable Cox model and using backward elimination, pNfH and CHIT1 emerged as strongest predictors of death (Table S4). Only minor changes were detected in the models additionally adjusted for *C9orf72* status (Table S5).

When comparing bulbar and spinal patients, NFs and CHIT1 seemed to predict survival better for spinal patients (Table S6). In spinal patients, serum NfL performed particularly well (hazard ratio [HR] in the multivariable analysis comparing low to medium concentrations 4.39 [95% CI 2.35–8.20], and HR comparing low to high 3.29 [95% CI 1.55–6.97]). The only biomarker with a statistically significant hazard ratio for bulbar patients after multivariable adjustment was pNfH (HR comparing low to high 2.47 [95% CI 1.11–5.35]). The longitudinal progression rate could not be predicted by any biomarker when stratifying the analysis by site of onset.

3.5. Longitudinal analysis

In a longitudinal analysis with a median follow-up of 1.1 year (range: one month to 3.9 years), including ALS patients with at least two measurements, there was no clear temporal change in any biomarker after controlling for age at diagnosis (Table 5; Figure 3). However, when analyzing bulbar and spinal patients separately, NFs and CHIT1 increased over time in those with bulbar onset. Levels were stable among spinal patients, except for pNfH that decreased over time, but with a low coefficient of determination. There were two patients with follow-up time longer than three years. Restricting the follow-up time to three years did not change the results. In a post-hoc analysis, applying a non-parametric bootstrap quantile regression model on the median change, the results were similar to the above analysis (Table S7).

3.6. Sensitivity analysis of CHIT1

In a sensitivity analysis, study participants with very low CHIT1 concentrations (<200 ng/L; $n = 16$) were removed. This yielded slightly stronger results for CHIT1 in all analyses except for temporal trends (Tables S8-S11).

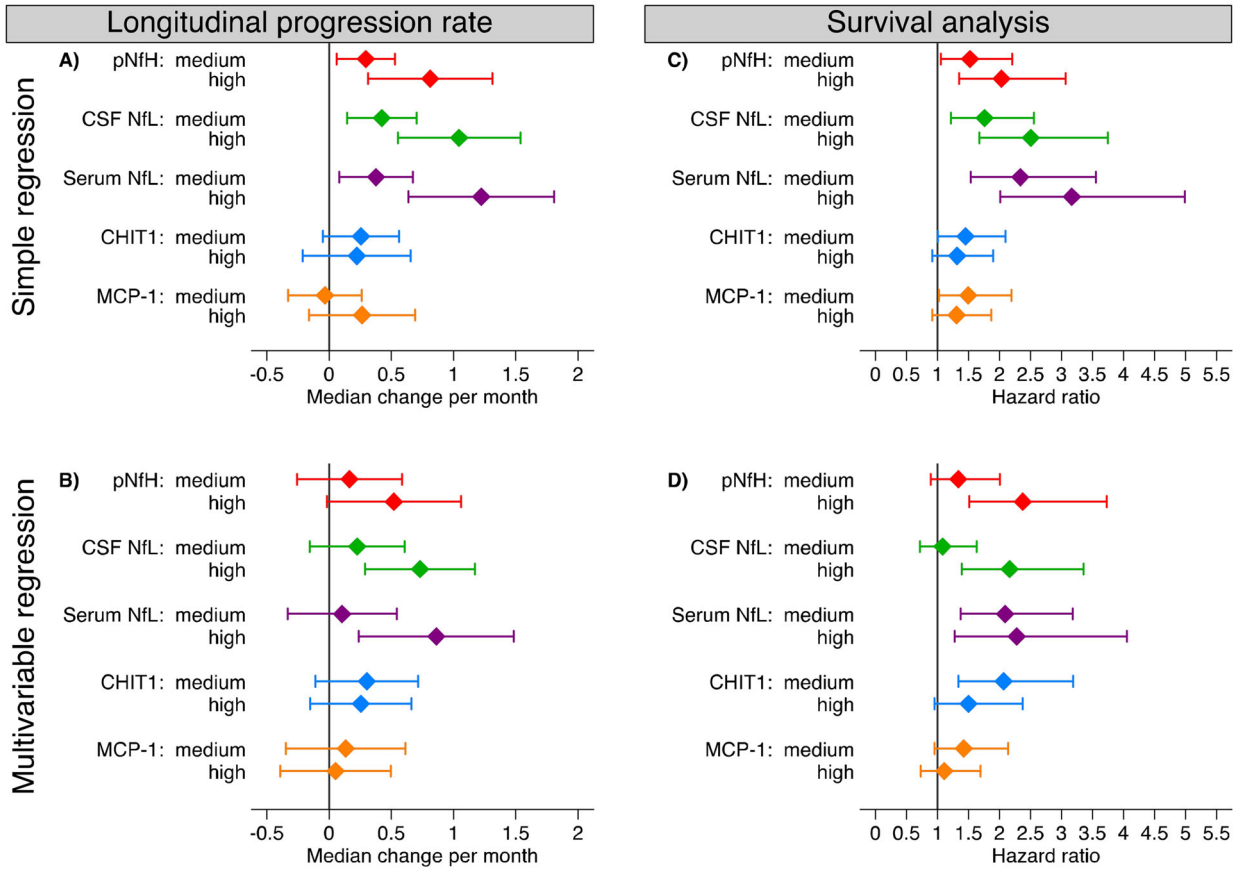

Figure 2. Predicting longitudinal progression rate and survival among ALS patients. (A-B) Quantile regression models predicting median longitudinal progression rate in relation to biomarker levels (medium and high versus low), in simple (A) and multivariable (B) analyses. (C-D) Cox proportional hazards regression models predicting survival with biomarker levels (medium and high versus low), in simple (C) and multivariable (D) analyses. Note: 192 patients with CSF and 157 patients with serum. In multivariable analyses the following variables were adjusted for: sex, age at diagnosis, BMI at diagnosis, site of onset, El Escorial diagnostic criteria, diagnostic delay, baseline ALSFRS-R, baseline progression rate, and sign of FTD at diagnosis. Biomarker concentrations were divided into tertiles, with low/medium/high concentrations. pNfH: phosphorylated neurofilament heavy. NfL: neurofilament light. CHIT1: chitotriosidase-1. MCP-1: monocyte chemoattractant protein-1.

4. Discussion

In this case-control study including 192 incident ALS cases from a tertiary clinic in Stockholm, Sweden, biomarkers of neuroaxonal degeneration performed better than biomarkers of neuroinflammation as potential diagnostic and prognostic biomarkers, and the performance of serum NfL was comparable to that of CSF NfL. However, there were substantial differences between bulbar and spinal patients.

Diagnostic performance was better for pNfH compared with CSF NfL, as reported by some studies (26, 39–41), although others have shown similar performance (6, 11, 28, 42). Importantly, serum NfL and CSF NfL showed comparable performance, as previously reported (25, 26, 28, 42). CHIT1 could also discriminate ALS patients from mimics, but performed less well than NFs and worse than previously reported, even when excluding CHIT1 concentrations <200 ng/L (9, 43–45). We failed to show elevated concentrations of MCP-1 in ALS patients compared with mimics, and MCP-1 could not differentiate ALS patients from mimics, contrasting one previous (and smaller) study (9). Combining biomarkers did not improve diagnostic performance, which has also been reported before (44).

We present an optimal cutoff in differentiating ALS patients from ALS mimics for pNfH of 726 ng/L, which is in the upper range of previously reported cutoffs (460-768 ng/L) (6, 7, 39–41). Our cutoff for CSF NfL of 2,588 ng/L is in concordance with many previous studies (6, 25, 26, 39). For serum NfL, our cutoff was 56.4 ng/L. Earlier studies on NfL in blood found cutoffs between 62 and 97 ng/L in serum (14, 42, 46), and between 32.7 and 95.4 ng/L in plasma (25, 26). Such wide range is possibly dependent on the diagnoses included in the mimics groups.

As previously reported (6, 9–12), we found that NFs were good predictors for both progression rate and survival. Serum NfL was found to perform particularly well, consistent with findings of two earlier studies (12, 26). CHIT1 and MCP-1 did not reliably predict survival or progression rate. However, when adding all CSF biomarkers in a multivariable Cox model and using backward

Table 5. Number of samples (a) and temporal changes of biomarker concentrations per year after ALS diagnosis (b), for all patients and for spinal and bulbar onset patients (mean annual change in ng/L per year).

4 (a) Number of samples	pNfH	CSF NfL	Serum NfL	CHIT1	MCP-1
Sample 1, *n*	41	43	44	41	41
Sample 2, *n*	41	43	44	41	41
Sample 3, *n*	10	11	6	10	10
Sample 4, *n*	3	3	1	3	3

4 (b)	pNfH			CSF NfL			Serum NfL			CHIT1			MCP-1		
Linear regression	Mean annual change	95% CI	R^2	Mean annual change	95% CI	R^2	Mean annual change	95% CI	R^2	Mean annual change	95% CI	R^2	Mean annual change	95% CI	R^2
All patients	−194	−473 – 85	0.02	−163	−1,288 - 962	0.002	1.96	−16.43 – 20.35	0.05	848	−1,964 – 3,659	0.03	23	−19 - 66	0.02
Spinal	−397*	−700 - −95	0.10	−854	−2,080 – 371	0.08	−8.90	−23.87 – 6.08	0.03	−1,071	−4,073 – 1,930	0.01	16	−35 - 67	0.01
Bulbar	494*	85 – 903	0.28	2,329*	558 – 4,100	0.31	43.54*	8.42 – 78.66	0.34	7,932**	2,219 – 13,645	0.18	80	−18 – 178	0.05

Note: Linear regression using cluster-robust standard errors, adjusted for age at diagnosis. R^2: coefficient of determination. * $p < 0.05$, and ** $p < 0.01$. pNfH: phosphorylated neurofilament heavy. NfL: neurofilament light. CHIT1: chitotriosidase-1. MCP-1: monocyte chemoattractant protein-1.

elimination, pNfH and CHIT1 emerged as the strongest predictors. Also, combining pNfH and CHIT1 provided lower Akaike Information Criterion (AIC) (47) than with pNfH alone, indicating that both biomarkers of neurodegeneration and neuroinflammation carry predictive information. Additionally, when merely analyzing spinal patients CHIT1 performed well in the survival analysis. Previous studies on the predictive value of CHIT1 show inconsistent results, probably due to small sample size, different adjustment for covariables, and inclusion of a low proportion of deceased patients (9, 11, 12, 43–45, 48, 49).

In the longitudinal analysis of 44 ALS patients, we found no overall temporal change in any biomarker, in line with earlier studies[13–17].

Interestingly, we found substantial differences between bulbar and spinal patients in several of our analyses. Compared with spinal patients, in bulbar patients, 1) NFs and CHIT1 performed worse at predicting survival (with a particular advantage of serum NfL in spinal patients, and of pNfH in bulbar patients), 2) correlations between biomarkers were slightly weaker at the time of diagnosis, and 3) NFs and CHIT1 seemed to increase over time. All biomarkers seemed stable over time since diagnosis in spinal patients, except for pNfH, which showed decreasing concentration over time but with a low R^2. This suggests a difference in how these biomarkers are expressed and/or leaked into the CSF and blood depending on site of onset.

The difference in serum NfL and CSF NfL correlation between spinal and bulbar patients could be explained by lower BMI in bulbar patients. Lower BMI is associated with higher serum NfL concentrations (50, 51), also in our study (serum NfL and BMI: Spearman's rho −0.25; $p = 0.001$; posthoc analysis). Upon generating a NfL/BMI ratio and running Spearman's rank-order correlation, Spearman's rho in spinal patients stayed nearly unchanged on 0.82, but increased from 0.65 to 0.77 in bulbar patients, i.e. close to that in spinal patients.

4.1. Strengths and limitations

The strengths of this study are a relatively large sample size with incident ALS cases only, and almost complete data on patient characteristics. Also, there were no differences in clinical characteristics between our patient sample and all incident ALS patients in Stockholm during the same period, indicating good generalizability. Furthermore, the very high number of deceased patients during follow-up strengthens the validity of the survival analysis.

There are several limitations in our study. We did not test for the specific genotype associated with very low CHIT1 levels. However, we explored the

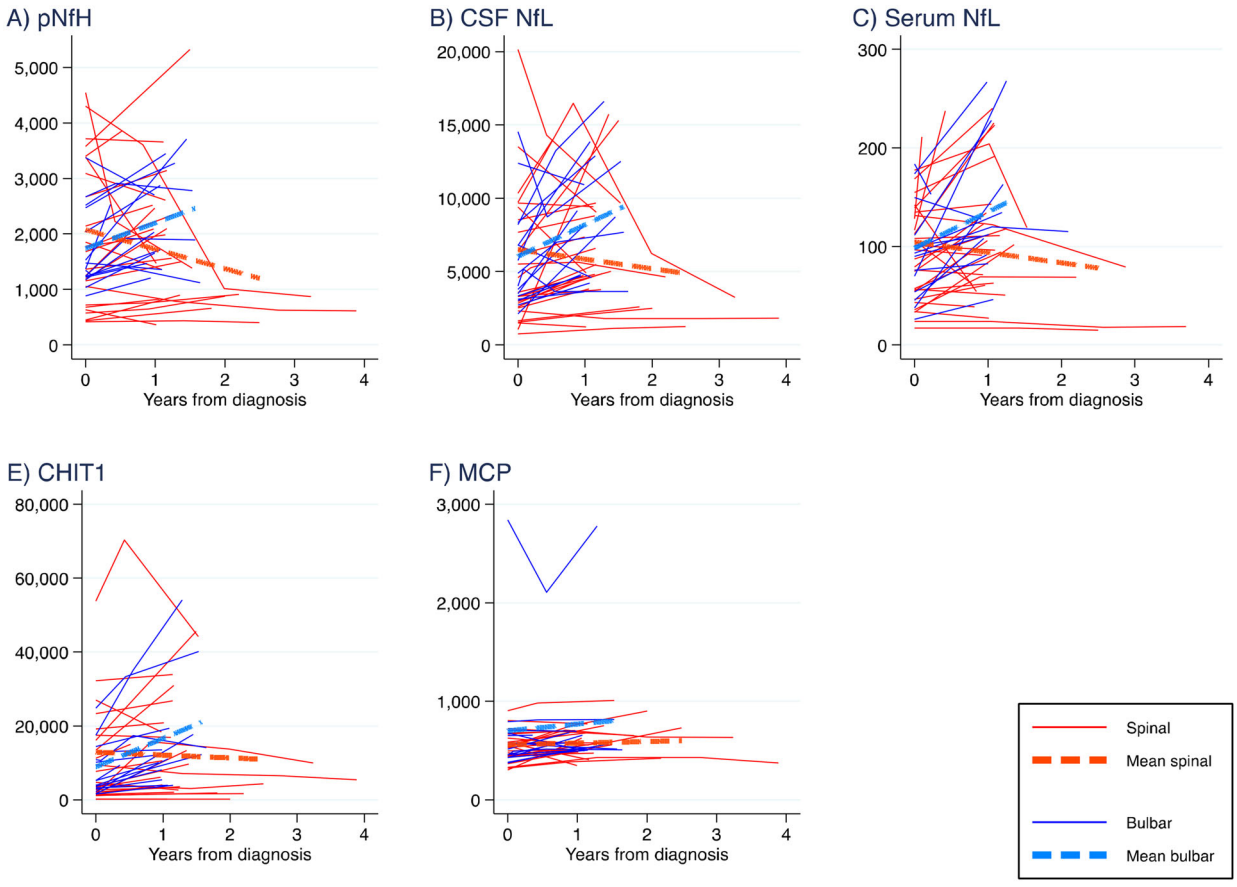

Figure 3. Temporal patterns per biomarker for each ALS patient from date of first sampling and with mean change (assuming a linear model, from the longitudinal analysis in Table 5), for spinal and bulbar onset patients separately (all measured in ng/L). pNfH: phosphorylated neurofilament heavy. NfL: neurofilament light. CHIT1: chitotriosidase-1. MCP-1: monocyte chemoattractant protein-1.

potential bias by performing a sensitivity analysis removing all patients with CHIT1 concentrations <200 ng/L. Results were in general more striking after removal of these samples. *C9orf72* status is often reported as predictor of ALS survival (9, 33, 52). We had a high degree of incompleteness in *C9orf72* status (22.9%), but in sensitivity analyses further adjusting for *C9orf72* status, we did not find evidence of strong confounding effect. For some of our neurological (85% of INCs; 26.9% of NINCs) and healthy (7.8%) controls, NfL was analyzed in plasma instead of serum, although we had serum analyzed for all ALS patients and all ALS mimics. Plasma levels were converted to serum levels using a separate cohort as reference. This could potentially have affected the analyses of INCs and NINCs. Our primary comparison group was ALS mimics. In this group, we included all persons who were were evaluated at the ALS CRC with the suspicion of ALS, but who were diagnosed with other diseases or symptoms unrelated to MND. Therefore, the comparison group contains a wide range of diagnoses, which possibly impedes a comparison to other studies. However, our cutoffs are similar to other studies, indicating that this might not be a large limitation. ALS patients were generally older than the neurological and healthy controls, and because neurofilament concentrations correlate positively with age (although this relationship is possibly not so strong among ALS patients) (2, 51, 53–55), comparisons with neurological and healthy controls could have been affected. However, ALS patients were similar in age to the ALS mimics, which was the primary comparison group of this study. Patients included in the longitudinal analysis of biomarkers had lower progression rate, lived longer, and displayed lower NF concentrations compared with patients in the entire cohort (Table S12). As a result this analysis does not pertain to all ALS patients. Also, the longitudinal analysis had low sample size, generating wide confidence intervals, but this is to some extent alleviated by the stable results across biomarkers. Finally, due to difficulties in collecting CSF, the number of healthy controls with CSF was very limited.

5. Conclusions

In conclusion, biomarkers of neuroaxonal degeneration outperformed biomarkers of neuroinflammation as potential diagnostic and prognostic biomarkers in ALS. Importantly, the diagnostic performance of serum NfL was comparable to CSF NfL, indicating that serum NfL could be analyzed instead of CSF NfL in the diagnostic work-up of ALS. Also, the differences in NFs in bulbar versus spinal patients suggest that NFs should possibly be

approached differently in these groups in clinical practice, research, and clinical trials.

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Data availability statement

Anonymized data will be made available upon reasonable requests to the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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