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RESEARCH PAPER

Comparison of elevated phosphorylated neurofilament heavy chains in serum and cerebrospinal fluid of patients with amyotrophic lateral sclerosis

Maxim De Schaepdryver,[1,2] Andreas Jeromin,[3] Benjamin Gille,[1] Kristl G Claeys,[4,5] Victor Herbst,[6] Britta Brix,[6] Philip Van Damme,[4,7] Koen Poesen[1,2]

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[1]Department of Neurosciences, Laboratory for Molecular Neurobiomarker Research, KU Leuven (University of Leuven), Leuven, Belgium
[2]Laboratory Medicine, University Hospitals Leuven, Leuven, Belgium
[3]Iron Horse Diagnostics, Inc., Scottsdale, Arizona, USA
[4]Department of Neurology, University Hospitals Leuven, Leuven, Belgium
[5]Department of Neurosciences, Laboratory for Muscle diseases and Neuropathies, KU Leuven (University of Leuven), Leuven, Belgium
[6]Euroimmun AG, Lübeck, Germany
[7]Department of Neurosciences, KU Leuven (University of Leuven) and Center for Brain & Disease Research VIB Leuven, Leuven, Belgium

Correspondence to
Professor Koen Poesen, Department of Neurosciences, Laboratory for Molecular Neurobiomarker Research, KU Leuven (University of Leuven), 3000 Leuven, Belgium; koen.poesen@uzleuven.be

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ABSTRACT

Objective Phosphorylated neurofilament heavy chain (pNfH) levels are elevated in cerebrospinal fluid (CSF) of patients with amyotrophic lateral sclerosis (ALS). Instead of CSF, we explored blood as an alternative source to measure pNfH in patients with ALS.

Methods In this single centre retrospective study, 85 patients with ALS, 215 disease controls (DC) and 31 ALS mimics were included. Individual serum pNfH concentrations were correlated with concentrations in CSF and with several clinical parameters. The performance characteristics of pNfH in CSF and serum of patients with ALS and controls were calculated and compared using receiver operating characteristic (ROC) curves.

Results CSF and serum pNfH concentrations in patients with ALS correlated well (r=0.652, p<0.0001) and were significantly increased compared with DC (p<0.0001) and ALS mimics (p<0.0001). CSF pNfH outperformed serum pNfH in discriminating patients with ALS from DC and ALS mimics (difference between area under the ROC curves: p=0.0001 and p=0.0005; respectively). Serum pNfH correlated inversely with symptom duration (r=−0.315, p=0.0033). CSF and serum pNfH were lower when the disease progression rate was slower (r=0.279, p<0.01 and r=0.289, p<0.01; respectively). Unlike CSF, serum pNfH did not correlate with the burden of clinical and electromyographic motor neuron dysfunction.

Conclusions CSF and serum pNfH concentrations are elevated in patients with ALS and correlate with the disease progression rate. Moreover, CSF pNfH correlates with the burden of motor neuron dysfunction. Our findings encourage further pursuit of CSF and serum pNfH concentrations in the diagnostic pathway of patients suspected to have ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, characterised by progressive degeneration of the upper and lower motor neurons. Although the clinical phenotype is variable, atrophy and weakness of the muscles as well as fasciculations and spasticity are commonly seen in patients with ALS.[1 2] In the majority of the cases, the cause of ALS is unknown, but in about 15% a genetic cause can be identified.[2] ALS remains a relatively rare disorder with a prevalence of five patients per 100 000 people in the USA.[3 4] A limited survival of 2 to 5 years after symptom onset is observed, and the diagnostic delay is around 1 year.[5] Currently, the diagnosis of ALS requires both upper and lower motor neural dysfunction as evidenced by clinical parameters and electrophysiological investigations, as well as the progression of symptoms over time and the exclusion of other conditions that induce the same symptoms. Nonetheless, the diagnosis of ALS remains challenging as for instance upper motor neuron signs are often difficult to recognise if a limb is concurrently affected by lower motor neuron degeneration.[6]

A biochemical diagnostic marker can be instrumental to physicians to speed up the diagnosis of ALS, especially in the early disease stage to differentially diagnose ALS from diseases mimicking ALS (ALS mimics), such as Kennedy disease or multifocal motor neuropathy.[7 8] An early diagnosis of ALS enables rapid initiation of riluzole treatment and early recruitment of patients into clinical trials with disease-modifying drugs. Recently, we and others described phosphorylated neurofilament heavy chain (pNfH) in cerebrospinal fluid (CSF) as a diagnostic biomarker for ALS.[9–11] However, obtaining CSF by lumbar puncture (LP) can be a limiting factor due to the invasiveness and contraindications such as the use of anticoagulants.[12] Therefore, a blood-based biomarker, which can be obtained by a simple venipuncture, would be a valuable alternative if the same diagnostic performance is achieved. Few studies have shown that serum pNfH is increased in patients with ALS as compared with healthy controls or disease controls (DC).[13–16] Yet, it remains elusive whether serum pNfH is an alternative to CSF pNfH as a biomarker for ALS.

In this study, we compared the performance characteristics of CSF and serum pNfH to discriminate patients with ALS from DC and ALS mimics at the time of first visit in an academic reference centre for neuromuscular diseases.

METHODS

Eighty-five patients diagnosed with ALS according to the Awaji and revised El Escorial criteria were enrolled in the study.[17 18] They underwent genetic testing for *C9orf72*, *SOD1*, *TARDBP* and *FUS*. CSF and blood were collected at first visit before a final

Table 1 Demographics and clinical data of the amyotrophic lateral sclerosis mimics

				pNfH concentration (pg/mL)	
Diagnosis	**N (CSF/serum)**	**m/f**	**Age**	**CSF**	**Serum**
Radiculopathy, plexopathy	12 (11/12)	10/2	54 (37–71)	479 (188–1138)	37.6 (6.0–375.4)
Multifocal motor neuropathy	5 (4/4)	4/1	49 (28–68)	385 (36–750)	29.5 (11.5–81.9)
Kennedy disease	4 (0/4)	4/0	71 (51–80)	–	37.2 (10.2–95.7)
Myasthenia gravis	2 (2/2)	1/1	73 (72–74)	630 (546–715)	19.3 (6.0–32.6)
Cervical stenosis	2 (2/1)	2/0	65 (63–66)	391 (377–405)	127.0
Hirayama disease	1 (1/1)	1/0	23	191	6.0
Fewdon syndrome	1 (1/1)	0/1	25	402	11.0
Functional neurological disorder	1 (1/0)	1/0	67	350	–
Motor neuropathy	1 (1/1)	0/1	57	186	6.0
Vascular lesions brainstem	1 (1/1)	0/1	72	422	65.3
Primary progressive MS	1 (1/0)	0/1	64	592	–

Median and ranges are given.
CSF, cerebrospinal fluid; f, female; m, male; MS, multiple sclerosis; pNfH, phosphorylated neurofilament heavy chain.

diagnosis was established at the neuromuscular reference centre of the University Hospitals Leuven. Symptom duration was defined as the time window between symptom onset and date of LP or venipuncture, whereas symptom onset was defined as first signs of muscle weakness or dysarthria. An ALS functional rating scale revised (ALSFRS-R) score was obtained for every patient with ALS. The ALS disease progression rate (ΔALSFRS-R) was calculated as (48 – ALSFRS-R)/(time from symptom onset until time of assessment of the ALSFRS-R score closest to LP). ΔALSFRS-R less than 0.3928 (<25% percentile), between 0.3928 and 1.239, and greater than 1.239 (>75% percentile) points/month was defined as slow, intermediate and fast disease progression, respectively. pNfH concentrations were correlated with the number of regions (bulbar, upper limb and lower limb region) with upper and lower motor neuron involvement. The latter was assessed clinically and by an electromyography (EMG) according to the revised El Escorial and Awaji criteria.[17 18] A control cohort was enrolled consisting of 31 ALS mimics, defined as patients in whom the neurologist was in doubt about the diagnosis of ALS or another (motor neuron) disease at the time of sampling, but in whom ALS was excluded at follow-up (table 1),[8 19] and 215 DC (table 2). If withdrawal of blood and CSF was more than 90 days apart or no paired samples were available, patients were excluded for the correlation analysis between matched CSF and serum samples.

Each patient signed an informed consent before inclusion. A CE marked ELISA, released for in vitro diagnostic (IVD) as required for clinical purposes, was used to measure pNfH concentrations (Euroimmun AG, Lübeck, Germany). The analytical sensitivity of the ELISA in CSF was 20 pg/mL. For serum pNfH quantification, the ELISA was optimised using biotin/streptavidin to improve the sensitivity to 6 pg/mL in serum. The polyclonal capture and monoclonal detection antibodies were provided by Iron Horse Diagnostics (Scottsdale, Arizona, USA). Serum and CSF pNfH concentrations below the analytical sensitivity were nominated 6 and 20 pg/mL, respectively. Samples were measured in duplicate and the mean intra-assay coefficient of variation was 8.7% and 10.5% for CSF and serum pNfH concentrations, respectively. CSF pNfH concentrations, obtained with a research use only (RUO) ELISA from Biovendor (RD191138300R, Brno, Czech Republic), from 83 patients with ALS and 213 controls were used from our previous publication,[9] to perform a paired method comparison with the IVD ELISA from Euroimmun.

Statistical analysis

Normal distribution was assessed with the Shapiro-Wilk test. Both serum and CSF pNfH concentrations followed a left skewed distribution, and logarithmic transformation towards a normal distribution was not successful. Therefore, the non-parametric Kruskal-Wallis test was used at a 5% significance level, corrected for multiple comparisons (Dunn post hoc test). Age and/or gender were used as covariates, when having a significant ($p<0.05$) effect on the performance of pNfH in a logistic regression analysis. The area under the curve (AUC) constructed with the predicted probabilities of the logistic regression analysis and corresponding performance characteristics were reported if the AUC differed significantly from the AUC constructed with the pNfH values. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio and AUC with corresponding 95% CI for serum and CSF pNfH were calculated with receiver operating characteristic (ROC) curves using MedCalc Statistical Software (Medcalc Software bvba, Ostend, Belgium). The highest Youden index was used to calculate the optimal cut-off on a ROC analysis. Prevalence for the predictive values was calculated as the ratio of cases in the positive and negative groups. Correlations were calculated by the Spearman rank correlation coefficient at a 5% significance level.

RESULTS

The demographics of patients with ALS, DC and ALS mimics included in this study are summarised in table 3. Genetic analysis identified seven *C9orf72*, four *SOD1* and one *TARDBP* mutation carriers in our ALS cohort. The composition of the ALS mimic and DC cohort is presented in tables 1 and 2, respectively. pNfH concentrations correlated significantly in matched CSF and serum samples, with lower pNfH concentrations in serum compared with CSF (patients with ALS: $r=0.652$ (CI: 0.509 to 0.652), controls: $r=0.332$ (CI: 0.202 to 0.451), overall: $r=0.575$ (CI: 0.491 to 0.648); $p<0.0001$, respectively) (figure 1). The median time difference between blood and CSF sampling was less than 24 hours for patients with ALS and controls (range: 0–8 days, 0–88 days, respectively).

The CSF and serum pNfH concentration of patients with ALS was significantly increased compared with DC and ALS

Table 2 Demographics and clinical data of the disease controls

				pNfH concentration (pg/mL)	
Diagnosis	**N (CSF/serum)**	**m/f**	**Age**	**CSF**	**Serum**
Guillain-Barré syndrome	55 (55/55)	29/26	52 (7–86)	319 (20–13 669)	52.8 (6.0–2060.9)
Hereditary spastic paraplegia	24 (11/21)	12/12	49 (23–84)	146 (20–1302)	30.4 (6.0–226.0)
CIDP	20 (20/20)	17/3	60 (31–75)	530 (81–5402)	73.5 (6.0–518.2)
Cognitive problems	16 (16/16)	8/8	66 (43–86)	369 (146–1156)	53.7 (6.0–274.2)
Alzheimer's disease	9 (9/9)	6/3	57 (51–74)	362 (284–523)	39.3 (6.0–82.5)
Spinal muscular atrophy	8 (0/8)	3/5	31 (24–64)	–	64.4 (31.4–170.4)
Multiple sclerosis	8 (8/8)	5/3	52 (28–70)	242 (21–886)	13.7 (6.0–47.7)
Leukoencephalopathy	6 (6/6)	1/5	38 (35–82)	226 (106–640)	17.7 (6.0–85.5)
Polyneuropathy	6 (6/6)	3/3	64 (56–77)	336 (138–1216)	23.3 (6.2–345.8)
Epilepsy	4 (4/4)	1/3	67 (49–79)	340 (125–895)	79.4 (12.1–137.4)
Frontotemporal dementia	4 (4/4)	2/2	67 (64–72)	363 (271–673)	15.3 (6.0–70.8)
Myelopathy	4 (4/4)	2/2	43 (38–50)	159 (136–550)	21.6 (5.0–43.1)
Idiopathic intracranial hypertension	3 (3/3)	1/2	36 (33–50)	325 (232–515)	14.9 (6.0–43.4)
Headache	3 (3/3)	1/2	38 (37–45)	192 (122–274)	6.0 (6.0–13.0)
Stroke	3 (3/3)	3/0	49 (33–59)	1134 (137-3247)	155.1 (9.1–236.4)
Inclusion body myositis	3 (1/3)	2/1	63 (48–63)	438	17.5 (11.2–295.5)
Neuralgia	3 (3/3)	1/2	52 (47–66)	243 (201–293)	20.6 (6.0–53.5)
Dementia	2 (2/2)	0/2	74 (67–82)	426 (419–434)	29.9 (21.0–38.8)
Mild cognitive impairment	2 (2/2)	1/1	63 (61–64)	356 (261–450)	119.9 (84.2–155.5)
Primary progressive aphasia	2 (2/2)	1/1	68 (68–68)	731 (518–944)	44.8 (6.0–83.7)
Monomelic atrophy	1 (1/1)	1/0	20	178	6.0
Episodic ataxia type 2	1 (0/1)	1/0	42	–	39.7
Corticobasal degeneration	1 (1/1)	0/1	69	465	55.1
Cerebral palsy	1 (1/1)	1/0	21	140	61.1
Encephalopathy	1 (1/1)	0/1	78	538	401.3
Facial nerve paralysis	1 (1/1)	0/1	17	205	27.1
Glioma tumour	1 (1/1)	0/1	57	185	21.0
Hypersomnolence	1 (1/1)	1/0	51	223	6.0
Hydrocephalus	1 (1/1)	0/1	80	761	43.0
Ulnar neuropathy	1 (1/1)	0/1	44	333	10.3
Myasthenia gravis	1 (1/1)	1/0	67	811	13.2
Papillitis optica	1 (1/1)	0/1	33	205	6.5
Postoperative neuropathic pain	1 (1/1)	1/0	40	156	25.5
Sarcoidosis	1 (1/1)	0/1	51	243	15.2
Syncope	1 (1/1)	1/0	71	734	251.3
Vogt-Koyanagi-Harada disease	1 (1/1)	0/1	57	341	64.9
Normal control	14 (14/14)	7/7	49 (27–72)	225 (112–560)	10.5 (6.0–45.9)

Median and ranges are given.
CIDP, chronic inflammatory demyelinating polyneuropathy; CSF, cerebrospinal fluid; f, female; m, male; normal control, patient without any underlying neurological condition after examination by the neurologist; pNfH, phosphorylated neurofilament heavy chain.

mimics ($p<0.0001$, respectively) (table 3, figures 2A, B). To evaluate pNfH concentrations at an earlier stage of the disease, a subset of patients with ALS with a symptom duration at LP below or equal to 7.8 months were selected as it represents the first 25th percentile of the symptom duration of our total ALS cohort. Within this subset, both median CSF and serum pNfH concentrations were significantly elevated compared with DC and ALS mimics combined ($p<0.0001$) (table 4). The majority of patients with ALS at this early disease stage cohort showed a fast disease progression (55%). Nonetheless, two patients with ALS had a slow disease progression with a median serum and CSF pNfH concentration of 344 pg/mL (range: 106–582 pg/mL) and 2470 pg/mL (range: 1593–3347 pg/mL), respectively.

Selectivity of serum and CSF pNfH towards motor neuron disease

Elevated CSF pNfH in patients with ALS versus DC was associated with a sensitivity of 88.2% (CI: 79.4% to 94.2%), a specificity of 85.3% (CI: 79.5% to 90.0%), a PPV of 72.8% (CI: 65.3% to 79.2%) and a NPV of 94.2% (CI: 90.1% to 96.7%). Logistic regression with age as a covariate decreased the AUC significantly from 0.943 (CI: 0.914 to 0.972) to 0.926 (CI: 0.893 to 0.960) (figure 2C). Serum pNfH was able to distinguish patients with ALS from DC with a sensitivity of 71.8% (CI: 61.0% to 81.0%), a specificity of 78.3% (CI: 72.1% to 83.7%), a PPV of 57.0% (CI: 49.8% to 63.9%), a NPV of 87.4% (CI: 83.0% to 90.7%). The corresponding AUC of serum pNfH was 0.782 (CI: 0.720 to 0.845) (figure 2C).The AUC of CSF pNfH to

Table 3 Patients' demographics, clinical characteristics and median biomarker concentration

	ALS	Disease controls	ALS mimics
N (CSF/serum)	85 (85/85)	215 (191/212)	31 (25/27)
Age	62 (38–84)	54 (8–86)	57 (23–80)
Gender (m/f)	52/33	113/102	23/8
pNfH (pg/mL)			
CSF	2451 (314-17 247)	281 (20–13 669)*	479 (163–1137)*
Serum	173.9 (6.0–1024.2)	36.3 (6.0–2060.9)*	37.6 (6.0–375.4)*
Site of onset (bulbar/spinal)	26/59	–	–
Diagnostic delay (months)	9.8 (2.9–40.2)	–	–
Symptom duration at LP (months)	11.8 (2.4–45.9)	–	–
ALSFRS-R score	39 (16–47)	–	–
ΔALSFRS-R (points/month)	0.64 (0.04–5.00)	–	–

Median values and ranges are given.
Kruskal-Wallis with Dunn post hoc correction (*vs ALS: p<0.0001).
ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; f, female; LP lumbar puncture,m, male; pNfH, phosphorylated neurofilament heavy chain.

discriminate patients with ALS from DC was significantly better compared with the AUC of serum pNfH (p=0.0001, figure 2C).

Discriminative ability of serum and CSF pNfH in patients with ALS towards ALS mimics

The optimal cut-off value of CSF pNfH to discriminate ALS from ALS mimics was 750 pg/mL, associated with a sensitivity of 92.9% (CI: 85.3% to 97.4%), a specificity of 96.0% (CI: 79.6% to 99.9%), a PPV of 98.7% (CI: 92.0% to 99.8%) and a NPV of 80.0% (CI: 64.8% to 89.7%). The corresponding AUC was 0.971 (CI: 0.943 to 0.999), with a likelihood ratio for the positive result of 23.2 (CI: 3.4 to 158.7) (figure 2D and online supplementary figure S1). For serum pNfH, the optimal cut-off value of 81.9 pg/mL differentiated patients with ALS from ALS mimics with a sensitivity of 71.8% (CI: 61.0% to 81.0%), a specificity of 85.2% (CI: 66.3% to 95.8%), a PPV of 93.8% (CI: 85.9% to 97.4%), a NPV of 48.9% (CI: 39.7% to 58.2%) and a likelihood ratio for the positive result of 4.8 (CI: 1.9 to 12.1) (online supplementary figure S1). The corresponding AUC of serum pNfH was 0.812 (CI: 0.728 to 0.897) (figure 2D). The AUC of CSF pNfH to discriminate patients with ALS from ALS mimics was significantly better compared with the AUC of serum pNfH (p=0.0005, figure 2D).

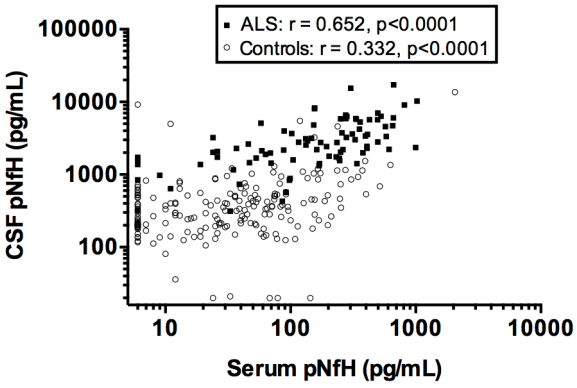

Figure 1 Correlation of matched cerebrospinal fluid (CSF) and serum phosphorylated neurofilament heavy chain (pNfH) concentrations. pNfH concentrations correlated significantly between matched CSF and serum samples in patients with amyotrophic lateral sclerosis (ALS) and controls (disease controls and ALS mimics).

Correlation of pNfH concentrations with clinical parameters

The median time difference between sampling and the ALSFRS-R score and the EMG was 15 days (range: 0–262 days, 90th percentile: 69 days) and 34 days (range: 0–877 days, 90th percentile: 94 days), respectively. Both serum and CSF pNfH correlated significantly with the disease progression rate (r=0.279 (CI: 0.0639 to 0.4697); p<0.01, r=0.289 (CI: 0.0744 to 0.478); p<0.01, respectively). CSF pNfH concentration correlated with the extent of upper and lower motor neuron degeneration (Kruskal-Wallis, p=0.0084), whereas no such correlation was found for serum pNfH (figure 3A). In contrast to CSF pNfH, serum pNfH inversely correlated with the symptom duration (r=−0.315 (CI: −0.498 to −0.103); p=0.0033) (figure 3B,C).

Paired method comparison of CSF pNfH concentrations

To investigate whether previous findings with the RUO Biovendor assay on CSF pNfH can be confirmed by using the CE marked Euroimmun assay for clinical use, we performed a paired method comparison between both assays. A strong correlation was found between the Biovendor and the Euroimmun assay for CSF pNfH concentrations, with higher concentrations measured with the Euroimmun assay (r=0.9232 (CI: 0.9038 to 0.9388); p=0.0001, figure 3D).

pNfH concentrations for screening purposes or study inclusion

For screening purposes at first consultation with complaints of muscle weakness or dysarthria, a biomarker at a fixed sensitivity level of 95% is preferred to avoid false negative results. At a 95% sensitivity for CSF pNfH, a specificity of 88.0% was reached, whereas for serum pNfH a specificity of 40.7% was achieved (table 5). This indicates that the use of serum pNfH can rule out ALS in about 40.7% of patients with a disease mimicking ALS.

To enrol patients into clinical trials, objective biomarkers for the disease at a fixed specificity of 95% are employed. For CSF and serum pNfH concentrations, a corresponding sensitivity of 92.9% and 51.8% was achieved, respectively (table 5).

DISCUSSION

This study supports our previous results of increased pNfH concentrations in CSF of patients with ALS, now using a CE marked ELISA from Euroimmun that can be used for clinical diagnostics and management (IVD).[9] Also, we now demonstrate that serum pNfH concentrations correlate well with CSF pNfH

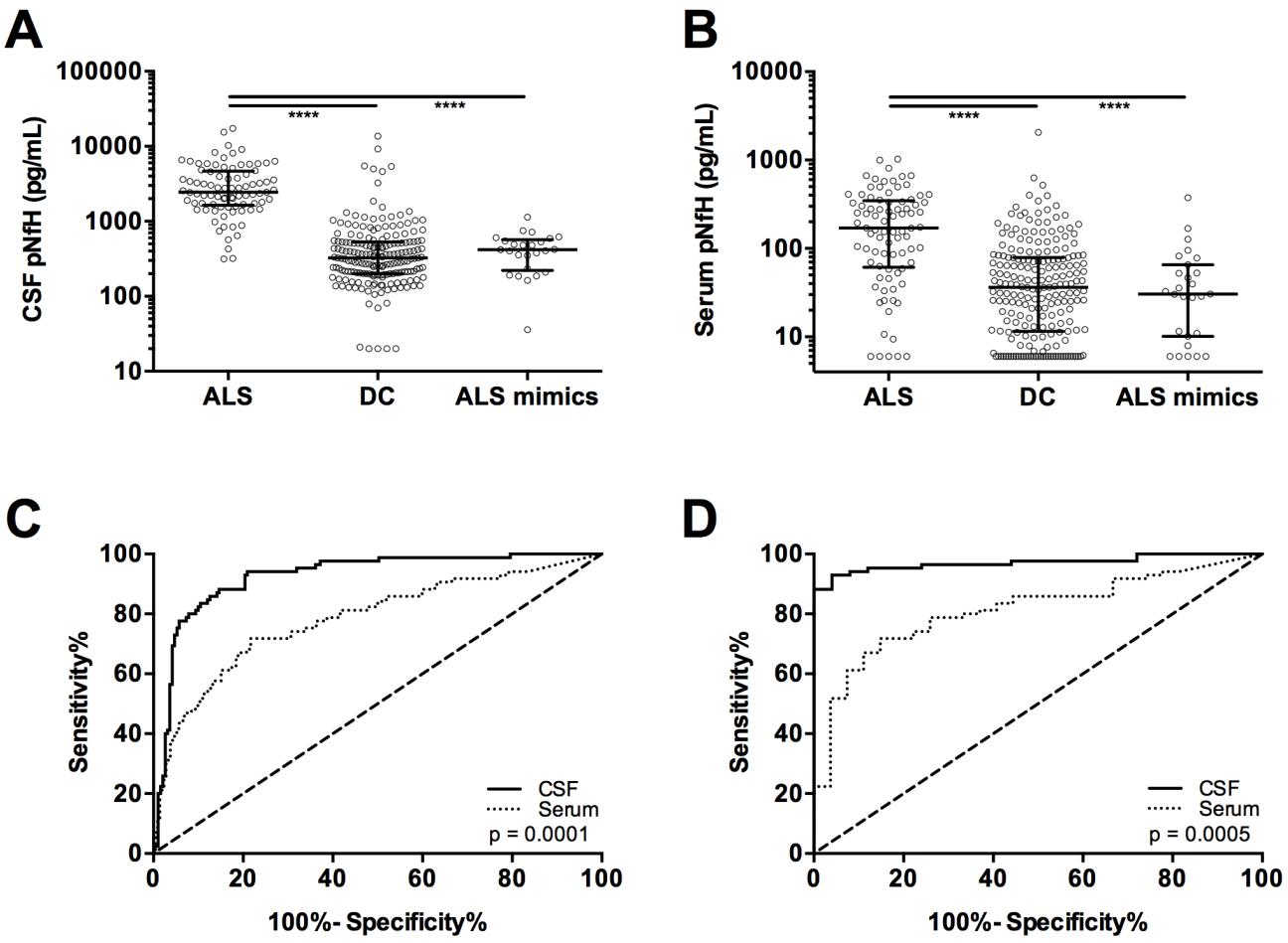

Figure 2 Performance characteristics of phosphorylated neurofilament heavy chain (pNfH) concentrations in cerebrospinal fluid (CSF) and serum. Scatter plot showing pNfH concentrations in CSF (A) and serum (B) of patients with amyotrophic lateral sclerosis (ALS), disease controls (DC) and ALS mimics. Median and IQR are presented on top of the plot. Kruskal-Wallis test with Dunn post hoc correction for multiple testing (****p<0.0001). Receiver operating characteristic (ROC) curves to discriminate patients with ALS from disease controls (C) and ALS mimics (D) based on pNfH concentrations in CSF (solid line) and serum (dotted line). p Values represent the significant difference of the area under the ROC curve between CSF and serum for disease controls (C) and ALS mimics (D).

concentrations, especially in patients with ALS.[14 16 20] Furthermore, we show that CSF and serum pNfH concentrations of ALS mimics like Kennedy disease and multifocal motor neuropathy are significantly lower compared with pNfH concentrations of patients with ALS at the time of first visit. However, serum pNfH concentrations show a larger overlap between patients with ALS and disease controls or ALS mimics than CSF pNfH concentrations. As a consequence, CSF pNfH performs better than serum pNfH to discriminate patients with ALS from those with an ALS mimic. Additionally, serum pNfH is not associated with the burden of upper and lower motor neuron dysfunction assessed by clinical and EMG evaluation in comparison to CSF pNfH.[9] These findings indicate that CSF pNfH reflects the core features of ALS. We can speculate that the possible formation of aggregates in serum might explain the worse diagnostic performance and the absence of a correlation with the burden of motor

Table 4 CSF and serum pNfH concentration at early disease stage

	Amyotrophic lateral sclerosis		Controls
Symptom duration at LP (months)	≤7.8	>7.8	–
CSF pNfH (pg/mL)	2793 (570–17 247)*	2284 (314–8 100)*	337 (20–13 669)
Serum pNfH (pg/mL)	276.4 (45.8–1024.2)*	153.2 (6.0–1000.7)*	35.7 (6.0–2060.9)
ΔALSFRS-R (points/month)	1.32 (0.14–5.00)	0.53 (0.04–2.34)	–
Slow (n)	2	19	–
Intermediate (n)	7	35	–
Fast (n)	13	9	–

Median values and ranges are given.
Kruskal-Wallis with Dunn post hoc correction (*vs ALS: p<0.0001).
ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; f, female; LP, lumbar puncture; m, male; pNfH, phosphorylated neurofilament heavy chain.

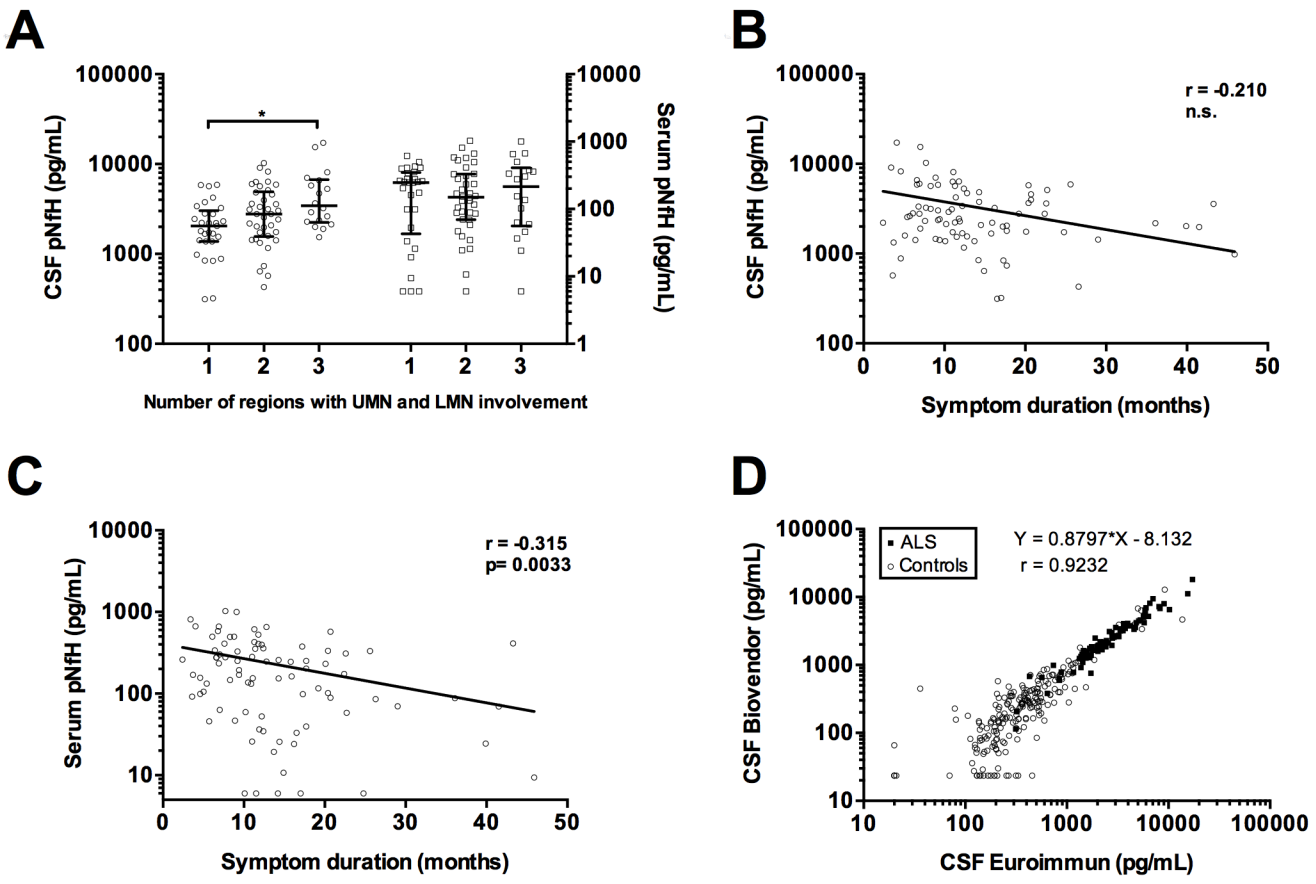

Figure 3 Correlation of pNfH concentrations with clinical parameters and a paired method comparison. CSF pNfH concentrations correlated with the extent of motor neuron disease (left Y-axis, indicated with circles on the plot) or serum pNfH (right Y-axis, indicated with rectangles on the plot) with the number of regions (X-axis) showing upper and lower motor neuron (UMN and LMN) involvement (the latter assessed clinically and with an EMG according to the revised El Escorial and Awaji criteria). Kruskal-Wallis test (p=0.0084) with Dunn post hoc correction for multiple testing was used for all comparisons (*p=0.0064; A). Unlike for CSF pNfH (B), serum pNfH concentrations (C) inversely correlated with symptom duration. A paired method comparison was performed between the research use only ELISA from Biovendor and the in vitro diagnostic, CE marked ELISA from Euroimmun. A significant correlation of CSF pNfH concentrations was found between both assays (D). ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; pNfH, phosphorylated neurofilament heavy chain.

neuron dysfunction.[15] As previously reported, spiking serum samples with an urea-calcium chelator buffer could solubilise serum pNfH aggregates which could improve the diagnostic performance of serum pNfH.[21]

Furthermore, our results suggest that in 40% of the patients with a disease mimicking ALS seen at a neuromuscular reference centre, ALS can be ruled out by means of serum pNfH measurement when applying a higher sensitivity of 95%. Also, CSF pNfH might be used as a biomarker for inclusion of patients with ALS into clinical trials given the high sensitivity of 92% at a fixed specificity of 95%.

To date, most studies have investigated the role of serum pNfH as a biomarker to assess disease progression and survival of patients with ALS.[15 20 22] We found a weak but significant correlation between the disease progression rate on the one hand and both serum and CSF pNfH concentrations on the other hand. These findings are in line with previous investigations that report an increase of both serum and CSF pNfH in patients with a rapid disease progression.[9 15] Serum pNfH inversely correlated with symptom duration, most likely because patients with a fast disease progression, who have high serum pNfH concentrations, are referred faster to the neuromuscular reference centre.

A limitation to our study is that for some patients with ALS the clinical data were not obtained on the same day as the sampling of CSF and serum. Therefore, the lack of contemporaneous measurement of clinical parameters and sampling must be

Table 5 Performance of pNfH at fixed 95% sensitivity and specificity

	CSF pNfH		Serum pNfH	
	Disease controls	ALS mimics	Disease controls	ALS mimics
95% Sensitivity	68.1% (60.9% to 74.6%)	88.0% (68.8% to 97.5%)	45.3% (38.5% to 52.2%)	40.7% (22.4% to 61.2%)
95% Specificity	72.9% (62.2% to 82.0%)	92.9% (85.3% to 97.4%)	34.1% (24.2% to 45.2%)	51.8% (40.7% to 62.7%)

The specificity and sensitivity for each fixed 95% sensitivity and specificity level, respectively. Between brackets the 95% CI.
ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; pNfH, phosphorylated neurofilament heavy chain.

considered in the more aggressive subgroup of patients with fast disease progression.

Our results show that pNfH is elevated in serum and CSF of patients with ALS. We now defined cut-offs for serum and CSF pNfH, which will allow us to further investigate the diagnostic added value of this biomarker in comparison with other diagnostic techniques. Furthermore, we now provide preliminary evidence that patients with ALS with a short symptom duration display elevated concentrations of serum and CSF pNfH. Therefore, our observations warrant further prospective studies on pNfH concentrations closer to the symptom onset to shorten the diagnostic delay of patients with ALS.

Contributors Concept and design: MDS, AJ, PVD, KP; drafting of the manuscript and figures: MDS, BG, AJ, VH, BB, PVD, KP; acquisition, analysis or interpretation of data: MDS, BG, KGC, PVD, KP; critical revision of the manuscript for important intellectual content: all authors. PVD and KP contributed equally.

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Competing interests MDS, BG, KGC, PVD: Nothing to report. KP: ELISA kits were provided by Euroimmun AG (Lübeck, Germany) VH, BB: full time employees of Euroimmun AG (Lübeck, Germany) AJ: Paid employee and stock holder of Iron Horse Diagnostics, Inc. (Scottsdale, AZ, USA) with a pending patent for biomarkers in neuronal injury

Ethics approval Ethical Committee of the University Hospitals Leuven.

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Data sharing statement The data used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Comparison of elevated phosphorylated neurofilament heavy chains in serum and cerebrospinal fluid of patients with amyotrophic lateral sclerosis

Maxim De Schaepdryver, Andreas Jeromin, Benjamin Gille, Kristl G Claeys, Victor Herbst, Britta Brix, Philip Van Damme and Koen Poesen

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