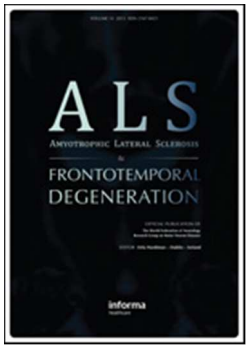

Multicenter validation of CSF neurofilaments as diagnostic biomarkers for ALS

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Multicenter validation of CSF neurofilaments as diagnostic biomarkers for ALS

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Abstract

OBJECTIVE: Neurofilaments are leading neurochemical biomarkers for amyotrophic lateral sclerosis (ALS). Here, we investigated the effect of preanalytical factors on neurofilament concentrations in cerebrospinal fluid (CSF) in a “reverse” round-robin with 15 centers across Europe/U.S..

METHODS: Samples from ALS and control patients (5/5 each center, n=150) were analyzed for phosphorylated neurofilament heavy chain (pNfH) and neurofilament light chain (NfL) at two laboratories.

RESULTS: CSF pNfH was increased (p<0.05) in ALS in 10 out of 15 centers and NfL in 5 out of 12 centers. The coefficient of variation (CV%) of pNfH measurements between laboratories was 18.7±19.1%. We calculated a diagnostic cut-off of >568.5 pg/mL for pNfH (sensitivity 78.7%, specificity 93.3%) and >1,431pg/mL for NfL (sensitivity 79.0%, specificity 86.4%).

CONCLUSION: Values in ALS patients are already comparable between most centers, supporting eventual implementation into clinical routine. However, continuous quality control programs will be necessary for inclusion in the diagnostic work-up.

Keywords: Cerebrospinal fluid, neurofilament, biomarker, amyotrophic lateral sclerosis, round robin

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects approximately 1.5-2.5 in 100,000 persons and the median disease duration after first clinical symptoms is 30 months [1]. New biomarkers are urgently needed to facilitate earlier diagnosis and therapy control in future therapeutic trials. Several potential biomarker candidates have been investigated in recent years (e.g. [2,3]). Neurofilament (Nf) concentrations, i.e. phosphorylated Nf heavy chain (pNfH) and Nf light chain (NfL) in cerebrospinal fluid (CSF) and blood have been shown to be increased in ALS patients in several studies [4–21] and are the most promising neurochemical diagnostic and prognostic biomarker candidates to date. Although blood is more easily accessible than CSF, sensitivity and specificity of Nf concentrations in CSF are higher compared with blood [4]. Thus, CSF is still the preferred source of Nf measurements for diagnostic purposes.

CSF pNfH and NfL concentrations have consistently been shown to be increased in CSF of ALS patients but concentrations among studies can vary considerably indicating preanalytical, analytical and assay-related factors which influence Nf determination. Standardization of these factors in combination with regular quality control and training of personnel is a prerequisite for the successful implementation of Nf determination into clinical routine and diagnostic criteria of ALS [22]. In this context, the SOPHIA-project (Sampling and biomarker OPtimization and Harmonization In ALS and other motor neuron diseases) in the EU Joint Programme – Neurodegenerative Disease Research (JPND) was initiated to support and promote the optimization, standardization and quality assurance of ALS biomarker determination between laboratories. Similar attempts have successfully been performed in the past for the biomarkers tau, phosphorylated tau and Aβ42 in Alzheimer´s disease [23,24]. A common approach here was to ship samples from a single center to different laboratories for sample analysis which gives information about sources of variation from assay type and performance. To identify preanalytical factors that influence Nf concentrations in CSF the SOPHIA project group decided to design a “reverse round-robin” where samples from different centers are analyzed in a single laboratory. In a first pilot study

with six contributing centers we analyzed different biomarker candidates in CSF of ALS and control patients [6]. In this study, concentrations of pNfH were consistently increased in ALS patients in all centers but also disclosed concentration differences between centers which have already been observed by comparison of other, single-center studies. On this basis standard operating procedures (SOPs) for CSF collection and processing were discussed and exchanged in the SOPHIA project group to identify confounding factors and harmonize protocols for the reduction of preanalytical variation in Nf determination.

The aim of the present study was to investigate the effects of SOP harmonization on Nf concentration differences between 15 centers across Europe and the U.S.. In addition, CSF NfL and pNfH were determined in two different laboratories to investigate the robustness and inter-laboratory comparability of measurements. Diagnostic cut-off values were calculated and compared with values from previously published (single-center) studies.

2. Material and Methods

2.1. Patients

Patients were recruited at the following 15 centers across Europe and the U.S.: Department of Neurology, Ulm University Hospital (Germany), Department of Neurology, University of Umea (Sweden), Divisions of Neurology and Neurobiology, Barrow Neurological Institute, Phoenix (U.S.), Faculty of Medicine - Instituto de Medicina Molecular, University of Lisbon (Portugal), Department of Neurology, University Hospitals Leuven (Belgium), Department of Clinical Neurosciences, University of Oxford (UK), Department of Neurology, Jena University Hospital (Germany), Institute of Clinical Medicine / Neurology, University of Eastern Finland (Finland), Department of Neuroscience, Sheffield Institute for Translational Neuroscience, University of Sheffield (UK), Department of Neurology, Medical University of Warsaw (Poland), U.O. Neurologia, IRCCS Istituto Auxologico Italiano, Milano (Italy), Neurologische Klinik mit Klinischer Neurophysiologie, Hannover (Germany), FIB Hospital Carlos III, Madrid, Spain, Hôpitaux Universitaires Pitié Salpêtrière-Charles Foix, Paris (France), Neuroscience Department, Institute of Experimental Medical Research, Istanbul University (Turkey).

Characteristics of patients are described in table 1. ALS was diagnosed according to revised El Escorial criteria [25]. Neurological control patients included the following diagnoses and/or symptoms: **center 1**: exclusion inflammatory CNS disease (n=3), polyneuropathy (n=2); **center 2**: healthy control (n=5); **center 3**: polyneuropathy (n=2), paranoid schizophrenia, exclusion inflammatory CNS disease, epilepsy; **center 4**: mild cognitive impairment (n=3), herpes simplex caused neuropathy, mild obstructive sleep apnea; **center 5**: healthy control (n=5); **center 6**: Alzheimer´s disease (AD, n=2), normal pressure hydrocephalus (n=2), polyneuropathy; **center 7**: polyneuropathy (n=2), brachial plexitis, undetermined spinal cord lesion, diabetic neuropathy; **center 8**: idiopathic lumbar plexopathy, non-progressive foot drop, multiple sclerosis (n=2), AD; **center 9**: chronic inflammatory demyelinating polyradiculoneuropathy (n=3), chronic ataxic sensorimotor neuropathy, CANOMAD syndrome; **center 10**: headache (n=2), non organic (n=2), not specified; **center 11**: benign paroxysmal positional vertigo, transient global amnesia, opticus neuropathy, ocular myasthenia, exclusion brain stem ischemia; **center 12**: diabetic polyneuropathy, polyneuropathy (n=2), mitochondrial myopathy, multifocal motoric neuropathy, benign fasciculation syndrome; **center 13**: disorders of the optic nerve, sciatic neuralgia, vertigo – exclusion of stroke, herniated disc, headache; **center 14**: herniated disc, polyneuropathy, double vision, headache (n=2); **center 15**: no neurological symptoms (n=5).

The study was approved by the local ethics committees for all participating centers, including the sharing of samples among research collaborators of the principal investigators. All participants gave written informed consent to participate in the study.

2.2. CSF collection

CSF was collected by lumbar puncture according to local SOPs. Information about CSF processing is provided in table 2. From each center, two CSF aliquots from each ALS and control patient (n=5) were shipped on dry ice to the Department of Neurology, Ulm University Hospital and stored at -80°C until analysis (two aliquots were not available for all centers). In total 150 patients were analyzed.

2.3. *Determination of pNfH and NfL in CSF*

Nf were measured in CSF using commercially available ELISAs (pNfH, BioVendor GmbH; NfL, IBL International) according to the manufacturer´s instructions. For samples with values below the limit of detection (LOD) of the ELISAs the LOD value (i.e. 187.5 pg/mL for pNfH and 100 pg/mL for NfL) was used for statistical analysis. The first aliquot of samples was analyzed at the Neurochemical Laboratory at the Department of Neurology in Ulm and the second aliquot was analyzed at the department of metabolic biochemistry, Hôpitaux Universitaires Pitié Salpêtrière-Charles Foix, Paris. Measurements in Ulm and Paris were performed with the same lot of ELISA kits. For measurements in Paris, CSF samples and ELISA kits were transferred from Ulm to Paris personally under optimal storage conditions.

2.4. *Statistics*

Statistical analysis was performed using GraphPad Prism 5.0 and JMP 11.1.1 software. The ALS and respective control groups were compared by the Mann-Whitney test. Differences of ALS or control groups between centers were analyzed with a Kruskal-Wallis test and Dunn´s posthoc test. Sensitivity and specificity was calculated using receiver operating characteristic (ROC) curve analysis and cut-offs were selected using the Youden index. Correlations were analyzed by Spearman's rank correlation coefficient and nonlinear regression analysis. Data with $p<0.05$ were regarded as statistically significant different.

3. Results

3.1. *Phosphorylated neurofilament heavy chain (pNfH)*

The CSF concentration of pNfH was significantly increased in ALS patients in 10 out of 15 centers (Fig. 1).

Comparison between centers showed significant differences between control groups ($p=0.0046$) but not ALS groups ($p=0.053$). The inter-laboratory variation of pNfH values (CV%) measured in Ulm and Paris was 18.7±19.1%. We combined all control and ALS

groups (n=75) to calculate the diagnostic sensitivity, specificity and a cut-off value of pNfH concentration using ROC curves and the Youden index (Fig. 2). The area under the curve (AUC) was 0.92 (95% CI 0.88-0.97) and sensitivity and specificity were 78.7% (95% CI 67.7% - 87.3%) and 93.3% (95% CI 85.1% - 97.8%) at a cut-off of 568.5 pg/mL. ~~Using the cut off of 568.5 pg/mL, the positive predictive value (PPV) was 90.8% in the cumulative dataset and the mean PPV in the 15 centers was 92.7±13.6% whereas it was 100% in 11 out of 15 centers.~~

~~Negative predictive value (NPV) for the cut-off at 568.5 pg/mL was 81.4% in the cumulative cohort and the mean NPV of single centers was 76.8±24.8% (100% in 5 out of 15 centers).~~

Using pNfH concentrations measured in Paris, we calculated a cut-off of 472.5 pg/mL (sensitivity 81.1%, specificity 83.6%).

3.2. Neurofilament light chain (NfL)

The NfL concentration in CSF was significantly increased in ALS patients in 5 out of 12 centers (Fig. 3).

ALS groups did not differ between centers (p=0.52) but there was a significant difference in NfL concentrations between control groups (p=0.03). As a coating problem with the ELISA plates become obvious during this analysis, which finally was solved by the manufacturer, there was not enough material available to perform measurements in Ulm and Paris with the same samples. ROC analysis in the cumulative dataset (Con: n=66, ALS: n=57) revealed an AUC of 0.86 (95% CI 0.79 - 0.93) and a sensitivity and specificity of 79.0% (95% CI 66.1% - 88.6%) and 86.4% (95% CI 75.7% - 93.6%) at a cut-off of 1,431 pg/mL (Fig. 4). ~~With this cut-off we calculated a PPV of 83.3% in the cumulative cohort and a mean PPV of 86.7±12.5% in single centers (100% in 4 out of 11 centers).~~

~~NPV was 82.6% in the cumulative cohort and the mean NPV in the 12 centers was 83.9±15.7% (100% in 5 centers).~~

With NfL concentrations measured in Paris, we calculated a cut-off of 2521 pg/mL (sensitivity 81.1%, specificity 88.3%).

4. Discussion

This study confirmed previous reports of increased pNfH and NfL concentrations in CSF of ALS patients from 15 centers across Europe and the U.S.. Furthermore, concentrations in ALS patients were comparable between most centers and inter-laboratory variation of measurements was acceptable for pNfH. Although samples were slightly differently treated in the different centers, a high diagnostic performance was reached for pNfH and NfL.

The calculated cut-off value of 568.5 pg/mL for pNfH supported our recently suggested cut-off value of 560 pg/mL from a large single-center cohort with over 450 patients [21]. In contrast, our calculated cut-off value for CSF NfL (1,431 pg/mL) was lower than values published in recent single-center studies (2,200 pg/mL [21], 1,781 pg/mL [4] and 1,981 pg/mL [8]) which might be due to stability issues (see discussion below).

The observed consistency of Nf concentrations in ALS patients between centers indicates that previous efforts of optimization and standardization of CSF collection and processing have already been successful, although the pNfH assay seems to be more robust against preanalytical variables than the NfL assay used here. However, there was no significant difference of Nf concentrations between ALS groups.

Although the data in our study are consistent in most centers, there are some limitations and confounding factors that must be mentioned including the small sample size from each center, different preanalytical conditions and patient characteristics as well as deviations in the NfL determination. These points and their potential influence on the data are discussed in the following paragraphs.

Nf concentrations tended to be lower in some centers (center 8). This might be due to the slightly longer disease duration of ALS patients in these centers which negative correlates with Nf concentrations as shown in the present (Fig. 2C and 4C) and previous studies [5,15]. However it is unlikely that this holds also true for all centers (e.g. center 13 and 14). Here the clinical data set in ALS was quite similar to the other centers. Another known factor, which can influence biomarker levels in CSF, is the material of collecting and storage tubes. Most

centers followed the recommendation of using polypropylene (PP) tubes for CSF collection and storage [26]. As there is still a great difference between suppliers and tube types this might be investigated in more detail in the future, especially as it has been shown for other biomarkers that different PP tubes can significantly affect biomarker concentrations [27]. While it cannot be completely ruled out that the type of tubes is responsible for lower Nf levels in ALS patients of centers 13 and 14, this seems unlikely as a similar storage procedure was used to that in center 3 or 4. Contrarily, Nf seems to be quite independent from the storage procedure, compared to Abeta-peptides, as even similar Nf levels occurred even when glass tubes were used for storage (e.g. center 1).

Nf autoantibodies have been detected in CSF of ALS patients [28–30] and discussed as a source of inter-individual and between-center variation. They might contribute to the differences between ALS groups seen in our study. Stability of Nf in CSF has been addressed in a few studies. pNfH seems to be very stable against delayed storage in the freezer, blood contamination and freeze-thaw cycles [31,32]. Since time from LP to storage was less than three hours in all centers, pNfH degradation should not be a prominent source for concentration differences. Stability data for NfL are conflicting showing unaffected concentrations for storage at room temperature for several days, several freeze-thaw cycles and low blood contamination (0.05%) [31] but another study observed a fast decline of NfL concentrations in CSF [32]. In the present study, the samples for NfL measurement in Ulm were freeze-thawed two to three times due to technical issues with the ELISA whereas this was not the case for the samples measured in Paris. The NfL values measured in Ulm were considerably lower than values from Paris (Fig. 3) supporting stability issues with NfL in CSF.

The differences between Nf concentrations in ALS and control patients did not reach the significance level across all centers in our study. This may result from the small group size from each center, however, some centers had high levels of Nf in the control group, e.g. center 6. This center included a patient with polyneuropathy showing a CSF pNfH concentration of 7,983 pg/mL and NfL of 8,831 pg/mL which might be indicative of a misdiagnosed ALS patient or a rare form of a polyneuropathy since Nf are usually not

increased in polyneuropathies [21]. In addition, centers with a great overlap of the control and ALS group (centers 6 and 12) did not centrifuge CSF samples before storage but recent findings [31] and the fact, that other centers with no centrifugation did not show this overlap, argues against a significant effect of low blood contamination on Nf concentrations in CSF.

Beyond all the (pre-)analytical aspects discussed here as a reason for Nf concentration variability between centers, we cannot rule out effects of clinical differences especially of the control patients which comprise a variety of syndromes.

In conclusion, this study confirmed the general applicability of the monocentric obtained cut-off values for Nf in ALS, especially for pNfH. Although the measured Nf values are already comparable between most centers, our study revealed that there is a need for a continuous quality control program. This should include regular round robins, qualification and education programs of staff, but also this kind of multicenter studies (reverse round robin). The availability of a reference cohort of patients would further strengthen the significance of the studies.

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6. Disclosure of interest

PVD holds a senior clinical investigatorship of FWO-Vlaanderen. The other authors report no conflicts of interest.

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8. Tables

Table 1. Characteristics of patients

	N (m/f)	Age* (years)	Onset of ALS (S/B)	Disease Duration* (month)	Total protein* (mg/mL)	pNfH Ulm* (pg/mL)	pNfH Paris* (pg/mL)	NfL Ulm* (pg/mL)	NfL Paris* (pg/mL)
Center 1 Control	5 (2/3)	48 (31-60)			0.338 (0.299-0.422)	187.5 (187.5-187.5)	187.5 (187.5-1579)	200 (171-278)	542 (200-7928)
ALS	5 (3/2)	61 (40-71)	5/0	12 (5-24)	0.446 (0.336-0.673)	1635 (1035-7968)†	2163 (187.5-10805)	6565 (1242-21342)‡	11080 (628-40640)‡
Center 2 Control	5 (3/2)	45 (32-52)			0.757 (0.591-0.832)	187.5 (187.5-187.5)	187.5 (187.5-266)	200 (100-523)	632 (308-1338)
ALS	5 (4/1)	43 (37-48)	5/0	30 (19-53)	0.759 (0.573-0.889)	1143 (579-2700)†	1211 (726-2855)‡	5291 (3530-5816)‡	9484 (3778-15950)‡
Center 3 Control	5 (2/3)	47 (26-74)			0.354 (0.301-0.500)	187.5 (187.5-558)	187.5 (187.5-743)	271 (166-1975)	1010 (532-4832)
ALS	5 (5/0)	50 (36-74)	5/0	12 (6-19)	0.43 (0.328-0.479)	1989 (873-3960)†	1899 (1167-4569)	2968 (1719-15992)‡	13100 (5218-21600)‡
Center 4 Control	5 (2/3)	63 (39-80)			0.365 (0.295-0.470)	187.5 (187.5-225)	187.5 (187.5-319)	605 (201-3095)	1526 (412-3972)
ALS	5 (4/1)	60 (47-65)	5/0	14 (4-20)	0.515 (0.291-0.602)	3339 (471-5715)‡	2581 (473-2608)‡	9440 (6905-10000)‡	19280 (2437-21910)
Center 5 Control	5 (2/2)[4]	58 (31-72)§			0.510 (0.400-0.670)	187.5 (187.5-187.5)	187.5 (187.5-187.5)	200 (200-284)	840 (346-20000)
ALS	5 (3/2)	51 (34-72)	5/0	15 (6-72)	0.435 (0.310-0.610)	1047 (420-6498)†	1215 (628-6305)†	(-)	24700 (8648-44100)‡
Center 6 Control	5 (3/2)	75 (71-81)			0.396 (0.287-0.640)	447 (201-7983)	(-)	330 (195-8831)	(-)
ALS	5 (2/3)	59 (57-68)‡	3/2	7 (5-72)	0.300 (0.030-0.410)	2346 (687-8145)	(-)	4761 (433-9573)	(-)
Center 7 Control	5 (4/1)	64 (40-76)			0.490 (0.370-0.860)	486 (187.5-801)	714 (280-1062)	426 (272-2462)	2386 (848-4184)
ALS	5 (4/1)	50 (38-62)	4/1	10 (5-48)	0.480 (0.340-0.680)	1938 (187.5-11337)	2197 (294-10600)	3453 (200-13848)	7896 (776-25840)
Center 8		54			0.479	187.5	(-)	300	(-)

Control	5 (2/3)	(46-75)			(0.047-1.220)	(187.5-267)		(200-1811)	
ALS	5 (2/3)	64 (55-69)	5/0	85.3 (38.8-247.4)	0.880 (0.670-1.150)	891 (222-1116)‡	(-)	1194 (200-12871)	(-)
Center 9									
Control	5 (4/1)	72 (56-79)			0.59 (0.380-0.800)	453 (187.5-711)	561 (252-1001)	1340 (232-3954)	1792 (540-2462)
ALS	5 (3/2)	60 (55-65)	3/2	13 (6-60)	0.555 (0.330-0.610)	3402 (420-4959)	3624 (502-6090)	4266 (1806-12000)‡	18586 (3750-30445)‡
Center 10									
Control	5 (3/2)	50 (39-77)			(-)	405 (187.5-540)	(-)	516 (334-3863)	(-)
ALS	5 (3/2)	65 (38-72)	4/1	16 (15-25)	(-)	1542 (531-4950)‡	(-)	3305 (2064-9045)	(-)
Center 11									
Control	5 (3/2)	67 (51-76)			0.549 (0.512-0.633)	330 (187.5-435)	325 (297-472)	815 (421-1326)	1784 (956-3130)
ALS	5 (5/0)	58 (45-68)	4/1	8.9 (5.7-36.1)	0.500 (0.314-0.540)	2733 (387-6585)‡	3117 (564-6155)‡	4734 (645-12390)	11324 (1640-30445)
Center 12									
Control	6 (5/1)	65 (41-72)			0.268 (0.241-0.464)	347 (187.5-4000)	264 (187.5-12890)	583 (116-18062)	1736 (730-45430)
ALS	5 (4/1)	47 (43-75)	3/2	33 (16-92)	0.366 (0.287-0.414)	1110 (234-3651)	792 (187.5-2550)	2745 (100-11983)	6274 (1504-26930)
Center 13									
Control	5 (3/2)	45 (23-66)			0.440 (0.410-0.470)	187.5 (187.5-315)	187.5 (187.5-240)	130 (114-880)	600 (372-2162)
ALS	5 (3/2)	56 (30-62)	4/0¶	24 (18-30)	0.575 (0.395-0.650)	312 (187.5-1770)	234 (187.5-1425)	1062 (115-3820)	2334 (448-7534)
Center 14									
Control	5 (0/5)	45 (31-46)			0.410 (0.230-0.490)	187.5 (187.5-239)	187.5 (187.5-234)	(-)	818 (418-1562)
ALS	5 (3/2)	54 (46-83)‡	4/0#	24 (13-48)	0.380 (0.260-0.690)	458 (200-1291)‡	273 (187.5-1077)	(-)	3296 (1114-9040)‡
Center 15									
Control	5 (2/3)	67 (53-77)			(-)	296 (187.5-407)	273 (187.5-306)	(-)	1476 (976-2508)
ALS	5 (2/3)	61 (50-65)	(-)	(-)	(-)	3743 (1669-4648)‡	(-)	(-)	(-)

f: female; m: male; S: spinal; B:bulbar

*median and range

[†]p<0.01 vs control

[‡]p<0.05 vs control

[§]information available for 4 patients only

[¶]Information only for 4 patients available

[#]One case with both spinal and bulbar onset

Table 2. CSF collection, processing and storage

	Cell count (per µL)*	Time from LP to storage (h)	Tubes (sampling/storage)	Freeze/Thaw cycles	Centrifugation	Number of blood contaminations
Center 1						
Control	1.0 (0.3-2.3)	1	PP/glass	1	No	0
ALS	0.7 (0.7-1.3)	1	PP/glass	1	No	0
Center 2						
Control	(-)	<2	PP/PP (low binding)	1	Yes	3
ALS	(-)	<2	PP/PP (low binding)	1	Yes	3
Center 3						
Control	1 (1-2)	0.5	PP/PP	0	Yes	2
ALS	1 (1-6)	0.5	PP/PP	0	Yes	1[†]
Center 4						
Control	1.4 (0.2-3.4)	<1	PP/PP	0	Yes	0
ALS	0.6 (0.2-2.4)	<1	PP/PP	0	Yes	0
Center 5						
Control	2.6 (0.3-378)	<1	Glass/PP	1	Yes	0
ALS	1.0 (0.3-12.3)	<1	Glass/PP	1	Yes	0
Center 6						
Control	(-)	<1[‡]	PP/PP	0	No[§]	2
ALS	(-)	<1[¶]	n.a./PP	0	No	1
Center 7						
Control	<2	0.5	PP/PP	1	No	0
ALS	<2	0.5	PP/PP	1	No	0
Center 8						
Control	(-)	<1	PP/PP	1	Yes	4
ALS	(-)	<1	PP/PP	1	Yes	4
Center 9						
Control	(-)	2	PET/PP	1	Yes	0
ALS	(-)	2	PET/PP	1	Yes	0
Center 10						
Control	(-)	<1	PS/PP	0	Yes	0
ALS	(-)	<1	PS/PP	0	Yes	0
Center 11						
Control	2 (0-2)	<2	PP/PP	0	Yes	0
ALS	0 (0-4)	<2	PP/PP	0	Yes	0
Center 12						
Control	2 (2-2)[#]	<0.5	PP/PP	1	No	0
ALS	2 (0-4)[‡]	<0.5	PP/PP	1	No	0
Center 13						
Control	1.6 (0.3-3.0)	1	PP/PP	1	Yes	0
ALS	1.6 (0.3-3.6)	1	PP/PP	1	Yes	0
Center 14						
Control	1 (0-75)	2	PP/PP	0	No	0
ALS	6 (2-32)	2	PP/PP	0	No[§]	0
Center 15						
Control	(-)	3	PP/PP	0	Yes	0
ALS	(-)	3	PP/PP	0	Yes	0

LP: lumbar puncture, n.a.: not available, PET: polyethylene terephthalate, PP: polypropylene,

PS: polystyrene

*median and range

[†]not determined in one sample

[‡]information available for 2, [¶]1 and [#]3patients only

[§]except one sample

9. Figure legends

Fig 1: CSF pNfH concentration is increased in ALS patients in multiple centers. pNfH concentration was determined in CSF with ELISA in neurological control (Con) and ALS patients (n=5) from multiple centers across Europe and the U.S. (center number in brackets). Control (white) and ALS (grey) groups were compared by the Mann-Whitney test. Left columns show values measured in Ulm (clear) and right columns values from Paris (dotted). The dotted lines are the suggested cut-off values (Ulm: 568.5 pg/mL; Paris: 472.5 pg/mL) calculated from the cumulative cohort. Data are median and interquartile range and whiskers are min and max values. *p<0.05, **p<0.01.

Fig 2. Cumulative CSF pNfH concentrations of 15 centers, ROC analysis and correlation with disease duration. (A) pNfH concentrations of neurologic control and ALS patients (n=75) were compared by the Mann-Whitney test. (B) Receiver operating characteristic (ROC) curve of CSF pNfH concentration. (C) Correlation analysis of pNfH concentration and disease duration of ALS at lumbar puncture (LP). Bars are median and dots are individual values.

Fig 3. CSF NfL concentration is increased in ALS patients in multiple centers. NfL concentration was determined in CSF with ELISA in neurological control (Con) and ALS patients (n=5) from multiple centers across Europe and the U.S. (center number in brackets). Control (white) and ALS (grey) groups were compared by the Mann-Whitney test. Left columns show values measured in Ulm (clear) and right columns are values from Paris (dotted). The dotted lines are the suggested cut-off values (Ulm: 1,431 pg/mL; Paris: 2,521 pg/mL) calculated from the cumulative cohort. Data are median and interquartile range and whiskers are min and max values. *p<0.05.

Fig 4. Cumulative CSF NfL concentrations of 13 centers, ROC analysis and correlation with disease duration. (A) NfL concentrations of neurologic control (n=66) and ALS patients (n=57) were compared by the Mann-Whitney test. (B) Receiver operating characteristic (ROC) curve of CSF NfL concentration. (C) Correlation analysis of NfL concentration and disease duration of ALS at lumbar puncture (LP). Bars are median and dots are individual value

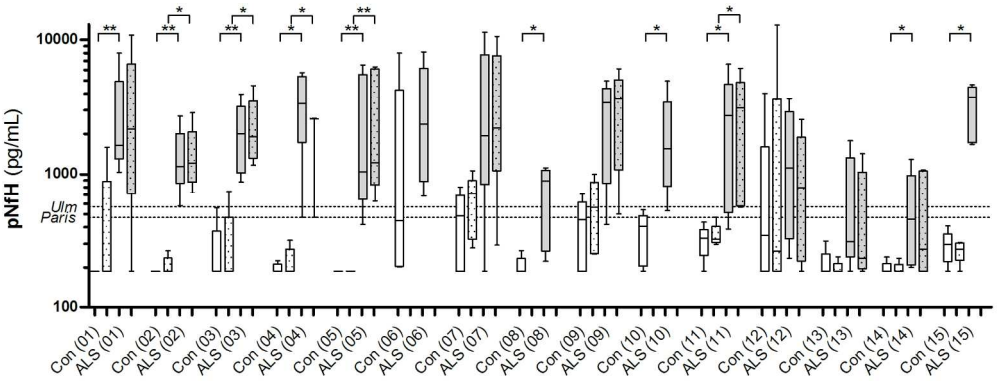

Fig 1: CSF pNfH concentration is increased in ALS patients in multiple centers.
176x67mm (300 x 300 DPI)

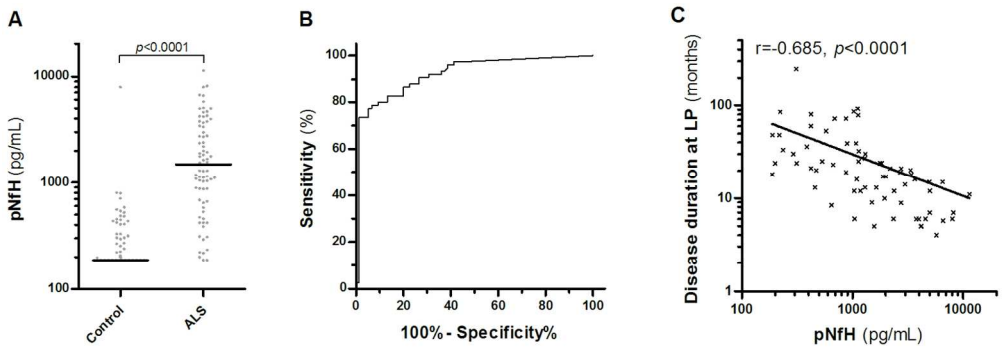

Fig 2. Cumulative CSF pNfH concentrations of 15 centers, ROC analysis and correlation with disease duration.
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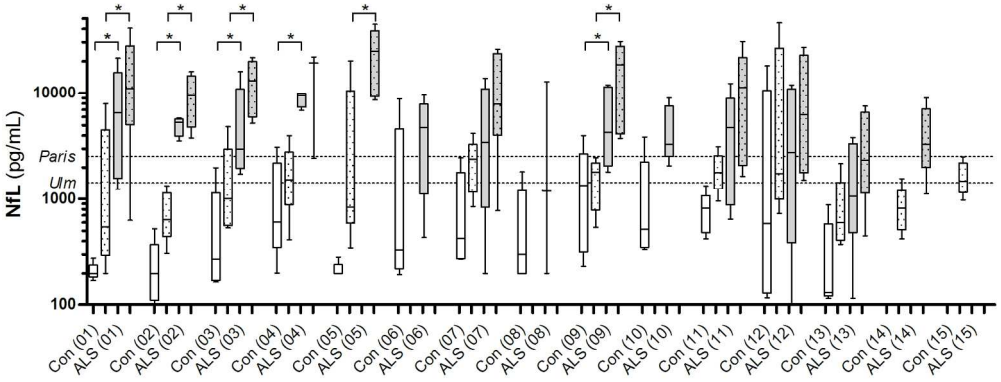

Fig 3. CSF NfL concentration is increased in ALS patients in multiple centers.
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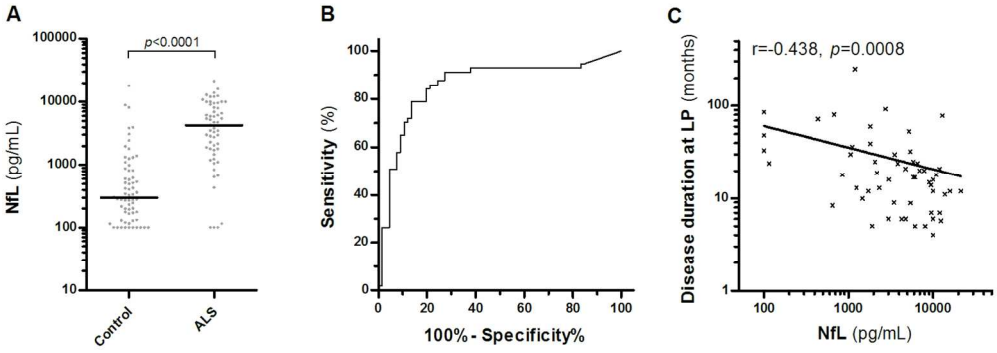

Fig 4. Cumulative CSF NfL concentrations of 13 centers, ROC analysis and correlation with disease duration.
132x46mm (300 x 300 DPI)