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# Phosphorylated neurofilament heavy chain: a biomarker of survival for *C9ORF72*-associated amyotrophic lateral sclerosis

**Tania F. Gendron, PhD**[1,2,*], ***C9ORF72* Neurofilament Study Group**, **Lillian M. Daughrity, BS**[1], **Michael G. Heckman, MS**[3], **Nancy N. Diehl, BS**[3], **Joanne Wuu, ScM**[4], **Timothy M. Miller, MD, PhD**[5], **Pau Pastor, MD, PhD**[6,7], **John Q. Trojanowski, MD, PhD**[8], **Murray Grossman, MD, EdD**[9], **James D. Berry, MD, MPH**[10], **William T. Hu, MD, PhD**[11], **Antonia Ratti, PhD**[12,13], **Michael Benatar, MD, PhD**[4], **Vincenzo Silani, MD**[12,13], **Jonathan D. Glass, MD**[11,14], **Mary Kay Floeter, MD, PhD**[15], **Andreas Jeromin, PhD**[16], **Kevin B. Boylan, MD**[17], and **Leonard Petrucelli, PhD**[1,2,*]

[1]Department of Neuroscience, Mayo Clinic, Jacksonville, FL 32224, USA

[2]Mayo Clinic Graduate School of Biomedical Sciences, Mayo Clinic, Jacksonville, FL 32224, USA

[3]Division of Biomedical Statistics and Informatics, Mayo Clinic, Jacksonville, FL 32224, USA

[4]Department of Neurology, University of Miami, Miami, FL 33136, USA

[5]Department of Neurology, Washington University School of Medicine, St Louis, MO 63110, USA

[6]Department of Neurology, University Hospital Mútua de Terrassa, and Research Foundation Mútua de Terrassa, University of Barcelona, Terrassa, Barcelona, Spain

[7]Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain

[8]Department of Pathology and Laboratory Medicine, Center for Neurodegenerative Disease Research, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

[9]Department of Neurology and the Penn Frontotemporal Degeneration Center, University of Pennsylvania, Philadelphia, PA 19104, USA

[10]Neurological Clinical Research Institute, Massachusetts General Hospital, Boston, MA 02114, USA

[11]Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA

[*]To whom correspondence should be addressed: Leonard Petrucelli, PhD, Department of Neuroscience, Mayo Clinic Florida, Telephone number: +1 904-953-2855, Fax number: +1 904-953-6276, Petrucelli.Leonard@mayo.edu, Tania Gendron, PhD, Department of Neuroscience, Mayo Clinic Florida, Telephone number: +1 904-953-6414, Fax number: +1 904-953-6276, Gendron.Tania@mayo.edu.

**Author contributions**

K.B.B., T.F.G., A.J., and L.P. contributed to the conception and design of the study. T.F.G., L.M.D., M.G.H., N.N.D., J.W., T.M.M., P.P., J.Q.T., M.G., J.D.B., W.T.H., A.R., M.B., V.S., J.D.G., M.K.F., and K.B.B. contributed to the acquisition and analysis of data. T.F.G. and M.G.H. drafted the text and prepared figures. Members of the *C9ORF72* Neurofilament Study Group participated in the collection of patient samples and data (Supplementary Table 1).

**Potential conflicts of Interest**

A.J. receives a salary from, and holds stock options with, Iron Horse Diagnostics, Inc.. T.F.G. and L.P. have a US patent on methods and materials for detecting *C9ORF72*-associated ALS and FTD using poly(GP) proteins (European patent filed).

[12]Department of Neurology-Stroke Unit and Laboratory of Neuroscience, IRCCS Istituto Auxologico Italiano, Milan, Italy

[13]Department of Pathophysiology and Transplantation, 'Dino Ferrari' Centre – Università degli Studi di Milano, Milan, Italy

[14]Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322, USA

[15]Motor Neuron Disorders Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA

[16]Iron Horse Diagnostics, Inc. Scottsdale, AZ 85255, USA

[17]Department of Neurology, Mayo Clinic, Jacksonville, FL 32224, USA

## Abstract

As potential treatments for *C9ORF72*-associated amyotrophic lateral sclerosis (c9ALS) approach clinical trials, the identification of prognostic biomarkers for c9ALS becomes a priority. We show that levels of phosphorylated neurofilament heavy chain (pNFH) in cerebrospinal fluid (CSF) predict disease status and survival in c9ALS patients, and are largely stable over time. Moreover, c9ALS patients exhibit higher pNFH levels, more rapid disease progression, and shorter survival after disease onset than ALS patients without *C9ORF72* expansions. These data support the use of CSF pNFH as a prognostic biomarker for clinical trials, which will increase the likelihood of successfully developing a treatment for c9ALS.

## Introduction

Despite more than 50 large clinical trials in the past half-century, there is only one minimally effective treatment for amyotrophic lateral sclerosis (ALS), a devastating motor neuron disease. Nevertheless, since the discovery of *C9ORF72* $G_4C_2$ repeat expansions as the most common genetic cause of ALS,[1, 2] significant advances have been made towards elucidating the mechanisms by which this mutation causes *C9ORF72*-associated ALS (c9ALS), and devising therapeutic strategies to combat them. Multiple lines of evidence place $G_4C_2$ repeat RNA and dipeptide repeat (DPR) proteins synthesized from these transcripts at the crux of c9ALS. Indeed, therapeutic strategies that target $G_4C_2$ RNA, such as antisense oligonucleotides (ASOs) and small molecules, reduce DPR protein levels, and mitigate other abnormalities caused by $G_4C_2$ transcripts in c9ALS models.[3–6]

As therapeutics for c9ALS are sought, we must address barriers in moving a treatment from bench to bedside, such as the lack of biomarkers to forecast disease progression and confirm target engagement in clinical trials. We recently established poly(GP) DPR proteins as a promising pharmacodynamic biomarker for $G_4C_2$ RNA-targeting ASOs (c9ASOs),[6] but forthcoming clinical trials for c9ASOs will also benefit from biomarkers that predict disease course. Phosphorylated neurofilament heavy chain (pNFH) and neurofilament light chain, which are released into the interstitial fluid during axonal injury and neurodegeneration, have emerged as putative prognostic biomarkers.[7] Levels of pNFH are elevated in cerebrospinal fluid (CSF) and blood from patients with ALS,[8–14] and some studies show that pNFH levels associate with survival and/or indicators of disease progression.[8, 9, 11–13, 15]

The prognostic potential of pNFH, however, has yet to be specifically evaluated in c9ALS patients, a population that differs clinically and pathophysiologically from ALS patients without a *C9ORF72* repeat expansion.[16–19] We thus investigated pNFH as an urgently needed prognostic biomarker for c9ALS.

## Methods

### Participants

An international sampling of CSF from *C9ORF72* expansion carriers (N=135) and non-carriers with no known ALS- or frontotemporal dementia (FTD)-linked mutation (N=107) was used. Samples were obtained from asymptomatic *C9ORF72* expansion carriers, healthy individuals, and clinically symptomatic patients diagnosed with ALS, ALS with comorbid FTD (ALS-FTD), or FTD (Table 1). ALS patients met El Escorial criteria for this diagnosis.[20] Diagnosis of FTD was obtained through established guidelines[21–24] and supported by neuropsychological testing and, in autopsied patients, by pathologically verified frontotemporal lobar degeneration. Our cohort comprised a collection of existing samples from multiple biobanks and included samples collected specifically for studies on neurofilaments. Longitudinally collected CSF was available from 37 *C9ORF72* expansion carriers and 17 non-carriers. Written informed consent was obtained from all participants or their legal next of kin if they were unable to give written consent, and biological samples were obtained with ethics committee approval.

### Sample collection

The standard operating procedures for the collection, processing and storage of CSF were generally consistent among sites. In brief, CSF was collected in polypropylene tubes by lumbar puncture (LP) and immediately placed on ice. With the exception of samples from three groups, the CSF was spun at low speed at 4°C within 30 minutes of collection to pellet any cellular debris. Samples were aliquoted before storing at −80°C.

### pNFH analysis

The previously described Meso Scale Discovery immunoassay used for this study employs a mouse anti-human pNFH antibody and a sulfo-tagged polyclonal anti-pNFH antibody as the capture and detection antibodies, respectively, and a purified bovine pNFH calibrator.[25] The assay has been analytically validated as a laboratory-developed test in the Iron Horse CLIA-certified laboratory. Samples tested in duplicate have a coefficient of variation below 10%. The intra- and inter-day precision of the assay is also less than 10%. Reagents and quality control samples were transferred to Mayo Clinic Jacksonville, where all CSF samples were tested at an 8-fold dilution, to establish commutability between the two sites.

### Statistical analysis

ALS patient functional status was determined using the Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R). For cross-sectional studies, the disease progression score was calculated using the equation: (48 - ALSFRS-R score at baseline)/ disease duration in months from disease onset to baseline LP.[8] For longitudinal studies, we

used: (48 - ALSFRS-R score at last LP)/disease duration in months from disease onset to last LP.

Comparisons of pNFH levels across disease group, and associations of pNFH with disease progression score or survival since disease onset were conducted separately for *C9ORF72* expansion carriers and non-carriers, as described below. Our primary analyses also included comparing pNFH levels, disease progression scores, and survival after disease onset between *C9ORF72* expansion carriers and non-carriers. Given our nine primary analyses, a Bonferroni adjustment was made and P≤0.0056 was considered statistically significant.

For regression analyses, pNFH values were log-scaled, and a square root transformation was applied to the disease progression scores, due to their skewed distributions.

pNFH levels were compared among disease groups (asymptomatic/healthy, ALS/ALS-FTD, FTD) using multivariable linear regression (MLR) models adjusted for age at LP and gender. Given a statistically significant (P≤0.0056) difference among groups, post-hoc pair-wise comparisons were made, with P≤0.0167 considered significant after Bonferroni correction. The ability of pNFH to discriminate between disease groups was examined by estimating the area under the receiver operating characteristic (ROC) curve.

Associations of pNFH levels in ALS/ALS-FTD patients with disease progression scores were evaluated using MLR models adjusted for age at disease onset, gender, and onset site. Additional adjustment for disease group (ALS or ALS-FTD) was made only in *C9ORF72* mutation carriers as only two non-carriers had ALS-FTD.

Associations of pNFH levels in ALS/ALS-FTD patients with survival after disease onset were examined using multivariable Cox proportional hazards regression models. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated, and censoring occurred at the date of last follow-up. The multivariable model for *C9ORF72* mutation carriers was adjusted for age at disease onset, gender, disease group, and onset site. For non-mutation carriers, we adjusted only for age at disease onset and onset site due to the smaller number of deaths in this subgroup.[26] We additionally estimated the concordance index (c-index) with and without pNFH in a given Cox model to provide an alternative measure of the predictive ability of pNFH; a c-index of 0.5 indicates predictive ability equal to that obtained by chance, and a c-index of 1.0 indicates perfect predictive ability.

Comparisons of pNFH between ALS patients with a *C9ORF72* mutation and those without were made using MLR models adjusted for age at LP, gender, onset to LP, and onset site. Disease progression scores were compared using MLR models adjusted for age at disease onset, gender, and onset site. Comparisons of survival after disease onset were made using multivariable Cox regression models adjusted for age at disease onset, gender, and onset site.

For our secondary analyses, P≤0.05 was considered statistically significant. The association of pNFH with poly(GP) levels in *C9ORF72* repeat expansion carriers, the latter measured as part of a recently published study,[6] was examined using a linear regression model. We also evaluated whether pNFH levels change over time in subjects with longitudinally collected CSF. The slope from a linear regression model (where pNFH was the response and time

after the baseline pNFH measure was the predictor variable) was calculated separately for each patient, and these slopes were tested for difference from a value of zero (indicating no change in pNFH levels over time) using a one-sample t-test.

All statistical tests were two-sided, and analyses were performed using SAS (version 9.2; SAS Institute, Inc., Cary, North Carolina) and R Statistical Software (version 2.14.0; R Foundation for Statistical Computing, Vienna, Austria)

## Results

### Evaluating CSF pNFH in *C9ORF72* mutation carriers

To investigate the prognostic utility of pNFH in c9ALS patients with or without comorbid FTD, the prevalence of which is increased in *C9ORF72* expansion carriers,[16, 17] we evaluated associations between pNFH levels and disease status, progression, and survival after disease onset using CSF from 86 c9ALS patients [14 with comorbid FTD (c9ALS-FTD)] and 28 asymptomatic individuals. Included for comparison was CSF from 21 c9FTD patients (Table 1).

Compared to asymptomatic *C9ORF72* expansion carriers, CSF pNFH levels were higher in patients with c9ALS/c9ALS-FTD (P<0.0001) or c9FTD (P=0.0004, Fig 1A). Indeed, pNFH levels discriminated between c9ALS/c9ALS-FTD patients and asymptomatic individuals with an almost perfect area under the ROC curve of 0.996 (95% CI: 0.989 – 1.000, Fig 1B). A cut-off value of 176 pg/ml produced a sensitivity of 98.8% and a specificity of 96.4%. In addition, pNFH levels were higher in c9ALS/c9ALS-FTD compared to c9FTD (P<0.0001; Fig 1A), with pNFH levels discriminating between c9ALS/c9ALS-FTD patients and c9FTD patients with an area under the ROC curve of 0.899 (95% CI: 0.808–0.990, Fig 1B).

In c9ALS/c9ALS-FTD patients, there was a positive correlation between pNFH levels and disease progression score calculated using the ALSFRS-R (P=0.012, N=63, Fig 1C), but this did not reach statistical significance (set at P≤0.0056) after correction for multiple testing.

Survival data was available for 81 of the 86 c9ALS/c9ALS-FTD patients; the median length of follow-up after disease onset was 3.3 years (Range: 1.0 – 11.7 years), and 59 patients (72.8%) died. A strong association was observed between higher pNFH levels and shorter survival after disease onset (HR [per each doubling of pNFH]: 2.16, 95% CI: 1.47 – 3.16, P<0.0001, Fig 1D). Further illustrating the ability of pNFH levels to predict survival after disease onset, the model c-index was 0.66 when adjusting for age at disease onset, gender, disease group, and onset site; it improved to 0.72 when pNFH was additionally included in the model.

### Evaluating CSF pNFH in non-*C9ORF72* mutation carriers

We examined the same associations as above in individuals without a *C9ORF72* mutation: 37 healthy subjects, 45 ALS patients (2 with comorbid FTD) and 25 FTD patients (Table 1).

CSF pNFH levels were significantly higher in ALS/ALS-FTD patients compared to healthy individuals or FTD patients (P<0.0001, Fig 1E), but not significantly different between FTD

patients and controls (P=0.054). pNFH levels discriminated between ALS/ALS-FTD patients and healthy individuals with an area under the ROC curve of 0.926 (95% CI: 0.861 – 0.991); a cut-off value of 245 pg/ml produced a sensitivity of 89.0% and a specificity of 100.0% (Fig 1F). pNFH levels discriminated between ALS/ALS-FTD patients and FTD patients with an area under the ROC curve of 0.867 (95% CI: 0.776–0.957, Fig 1F).

pNFH levels in ALS/ALS-FTD patients did not associate with disease progression score (P=0.12, N=36, Fig 1G).

Survival data was available for 40 of the 45 ALS/ALS-FTD patients; the median length of follow-up after disease onset was 5.0 years (Range: 1.4 – 20.0 years), and 18 patients (45.0%) died. A strong association was observed between higher pNFH levels and shorter survival after disease onset (HR [per each doubling in pNFH]: 3.04, 95% CI: 1.58 – 6.59, P=0.002, Fig 1H). Again demonstrating the ability of pNFH to predict survival after disease onset, the model that was adjusted for only age at disease onset and onset site had a c-index of 0.61, which increased to 0.77 when pNFH was also included in the model.

## c9ALS patients exhibit higher pNFH levels and disease progression scores, and shorter survival compared to ALS patients without a *C9ORF72* expansion

We next compared indicators of disease progression between ALS patients with and without *C9ORF72* expansions. Given the increased prevalence of FTD in ALS patients with a *C9ORF72* expansion [14 of 86 patients (16.3%)] versus those without [2 of 45 patients (4.4%)], ALS-FTD patients were omitted from these analyses.

CSF pNFH levels (P<0.0001, Fig 1I) and disease progression scores (P=0.003, Fig 1J) were significantly higher in c9ALS patients compared to patients without the expansion. Similarly, there was strong evidence of shorter survival after disease onset for patients with c9ALS compared to those without the expansion (HR: 4.08, 95% CI: 2.24 – 7.43, P<0.0001, Fig 1K).

We recently reported that CSF poly(GP) levels, while a promising pharmacodynamic biomarker, do not associate with survival after disease onset in c9ALS/c9ALS-FTD.[6] Consistent with these findings, poly(GP) levels did not associate with pNFH levels (P=0.21, Fig 1L).

## Longitudinal pNFH measurements

Longitudinally collected CSF was available from 37 *C9ORF72* expansion carriers and 17 non-carriers. The median length of time between the first and last pNFH measurement was 12.9 months (Range: 4.4– 22.6 months) and 5.8 months (Range: 3.0 – 6.7 months) for *C9ORF72* expansion carriers and non-carriers, respectively. There was no evidence of a change in CSF pNFH levels over time in *C9ORF72* expansion carriers (P=0.75), and the rate of change in CSF pNFH did not differ between asymptomatic individuals or patients with c9ALS or c9ALS-FTD (P=0.83, Fig 2A). A similar lack of change in CSF pNFH over time was observed in ALS patients without a *C9ORF72* expansion (P=0.80, Fig 2B). There was no significant difference in the rate of change in CSF pNFH between faster and slower

progressors in patients with a *C9ORF72* expansion (P=0.33), without an expansion (P=0.16), or combined (P=0.094).

## Discussion

Clinical trials for ALS are hampered by the lack of biomarkers to monitor target engagement and predict disease course. The latter frequently results in clinical trial treatment groups with significantly different proportions of fast and slow progressors thereby greatly increasing the number of patients needed to detect a treatment effect. With therapeutics for c9ALS being intensely investigated, and clinical trials for c9ASOs approaching, we evaluated pNFH as a prognostic biomarker for c9ALS to overcome this barrier.

Our study revealed a strong association between higher CSF pNFH levels and shorter survival in ALS patients with or without a *C9ORF72* expansion, which is consistent with previous reports.[9, 13] Given that the clinical course of disease can vary substantially – one study reports survival ranges of 0–7 years for c9ALS, and 3–38 years for ALS[16]– the ability of pNFH levels to predict survival could facilitate the assessment of treatment on prolonging life, and be used to stratify patients into more homogenous groups to improve clinical trial efficiency and the estimation of treatment outcomes.

We also noted a positive correlation between pNFH levels and disease progression score in c9ALS and c9ALS-FTD patients, but this was not significant after adjustment for multiple testing, perhaps because of lower power due to missing data for some patients, or a lack of precision in the disease progression score which depends on a functional rating that is subject to bias.

That ALS patients with *C9ORF72* expansions had significantly higher pNFH levels than patients without this mutation presumably reflects increased neuronal injury. c9ALS patients develop greater brain atrophy, particularly in extramotor regions, compared to ALS patients without a *C9ORF72* expansion.[17, 18] This diffuse spread of degeneration may account for the more rapid disease progression and shorter survival of c9ALS patients, as observed herein and by others.[16–19]

We show that CSF pNFH levels are largely stable over time. These findings from our relatively large cohort of ALS patients are consistent with those from a study examining 11 ALS patients longitudinally.[9] This stability of pNFH could facilitate its use as a pharmacodynamic biomarker for drugs that mitigate axonal injury and neurodegeneration. Additional longitudinal studies spanning asymptomatic and symptomatic phases of disease would be useful in determining at what point pNFH levels begin to rise – information that could inform when best to initiate treatment.

Overall, we established the prognostic potential of CSF pNFH for c9ALS. These findings, together with our discovery of a pharmacodynamic biomarker for $G_4C_2$ RNA-targeting therapies,[6] will increase the likelihood of successfully developing a treatment for c9ALS.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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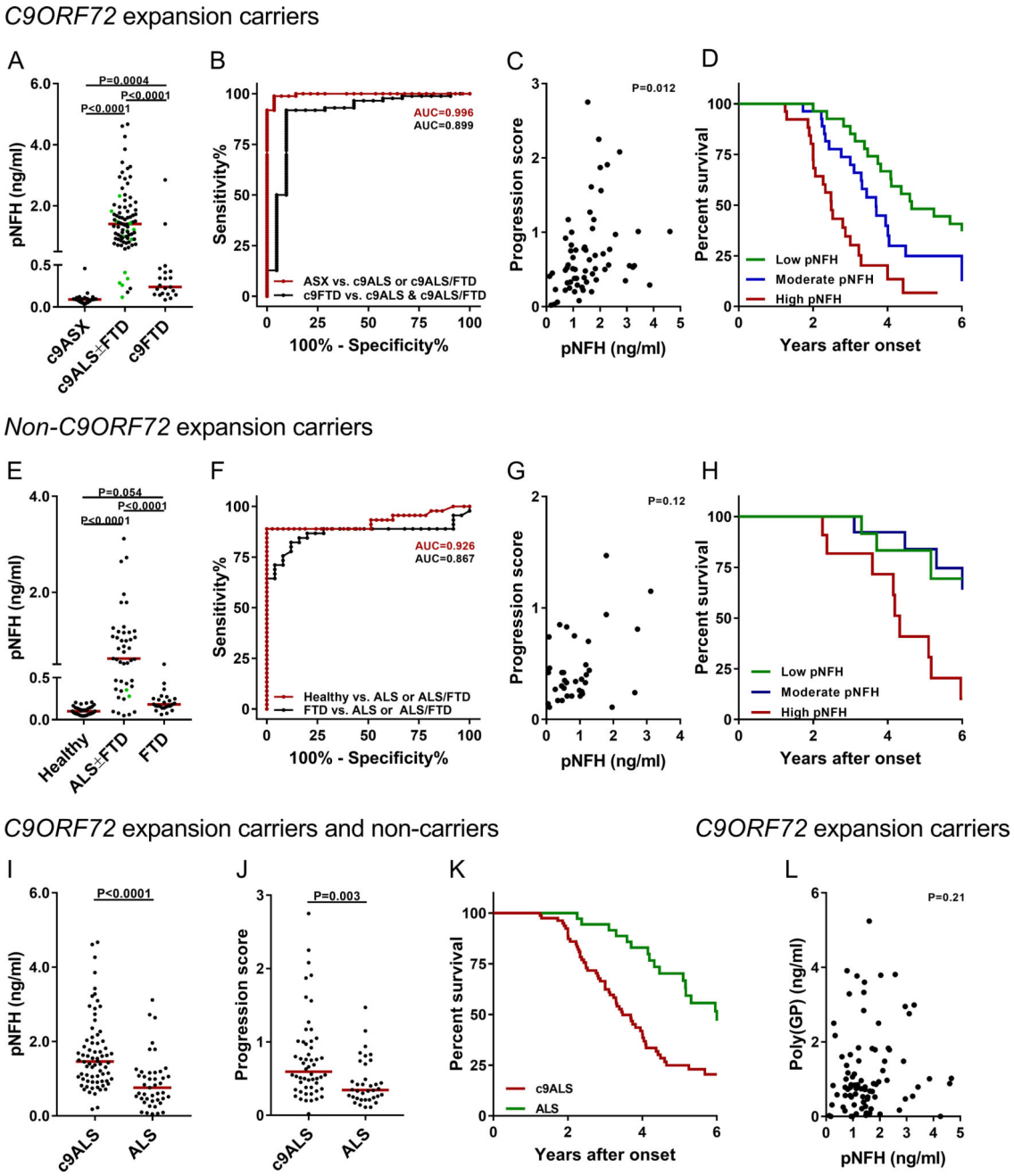


**Figure 1. Evaluation of CSF pNFH in *C9ORF72* repeat expansion carriers and non-carriers**

pNFH levels were measured in CSF from individuals with a *C9ORF72* repeat expansion (panels **A–D**), and those without an expansion (panels **E–H**). (**A**) Scatter plot showing that CSF pNFH levels were higher in patients with c9ALS with or without comorbid FTD (c9ALS±FTD, N=86), or patients with c9FTD (N=21), compared to asymptomatic *C9ORF72* repeat expansion carriers (c9ASX, N=28). Patients with c9ALS-FTD are represented by green circles. (**B**) ROC curves showing the ability of CSF pNFH levels to discriminate between patients with c9ALS or c9ALS-FTD (N=86) and either asymptomatic *C9ORF72* repeat expansion carriers (N=28) or patients with c9FTD (N=21). (**C**) Association

of CSF pNFH levels and disease progression scores in patients with c9ALS or c9ALS-FTD (N=63). (**D**) Survival after disease onset according to CSF pNFH levels for c9ALS and c9ALS-FTD patients (N=81). For ease of presentation, pNFH levels were divided into a three-level categorical variable based on sample tertiles (Low: ≤1.0980 ng/ml, Moderate: 1.0981–1.6860 ng/ml, High: >1.6860 ng/ml). (**E**) Scatter plot showing that CSF pNFH levels among individuals without a *C9ORF72* repeat expansion were higher in ALS patients with or without comorbid FTD (N=45) compared to healthy individuals (N=37) or patients with FTD (N=25). Patients with ALS-FTD are represented by green circles. (**F**) ROC curves showing the ability of CSF pNFH levels to discriminate between patients with ALS or ALS-FTD (N=45) and either healthy individuals (N=37) or patients with FTD (N=25) without a *C9ORF72* expansion. (**G**) CSF pNFH levels did not associate with disease progression scores in patients with *C9ORF72*-negative ALS or ALS-FTD (N=36). (**H**) Survival after disease onset according to CSF pNFH levels for ALS and ALS-FTD patients without a *C9ORF72* expansion (N=40). For presentation purposes, pNFH levels were divided into a three-level categorical variable based on sample tertiles (Low: ≤0.4660 ng/ml, Moderate: 0.4661–1.0540 ng/ml, High: >1.0540 ng/ml). (**I**) Comparison of CSF pNFH levels between patients with c9ALS (N=72) and *C9ORF72*-negative ALS (N=43). (**J**) Comparison of disease progression scores between patients with c9ALS (N=54) and *C9ORF72*-negative ALS (N=36). (**K**) Comparison of survival after disease onset between patients with c9ALS (N=68) and *C9ORF72*-negative ALS (N=38). (**L**) CSF pNFH levels did not associate with CSF poly(GP) levels in patients with c9ALS or c9ALS-FTD (N=86). Straight lines in panels **A**, **E**, **I** and **J** represent the median.

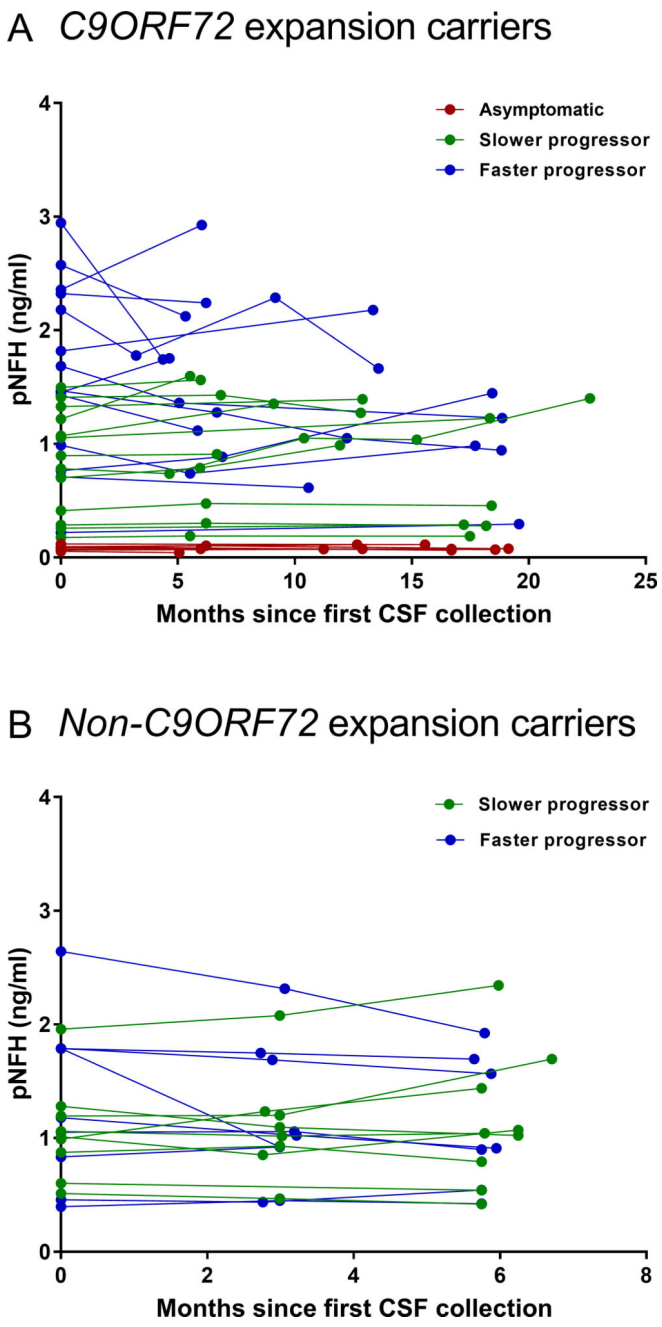


**Figure 2. Longitudinal evaluation of CSF pNFH in *C9ORF72* repeat expansion carriers and non-carriers**

pNFH levels were measured in CSF collected longitudinally from *C9ORF72* repeat expansion carriers and non-carriers. Subjects are color-coded based on their disease progression score, with patients having a score higher than the median (0.54 and 0.45 for *C9ORF72* expansion carriers and non-carriers, respectively) considered faster progressors, while those with a score equal to or lower than the median considered slower progressors. (**A**) Among the expansion carriers, 10 were asymptomatic, 20 were patients with c9ALS, and 7 were patients with c9ALS and comorbid FTD. For these 37 subjects, the median length of time between the first and last pNFH measurement was 12.9 months (Range: 4.4–

22.6 months); 25 subjects had 2 measurements, 9 subjects had 3 measurements, 2 subjects had 4 measurements, and 1 subject had 5 measurements. (**B**) For the 17 ALS patients without a *C9ORF72* expansion, the median length of time between the first and last pNFH measurement was 5.8 months (Range: 3.0 – 6.7 months), and CSF pNFH was measured either 2 times (N=2) or 3 times (N=15).

**Table 1**

Patient characteristics according to *C9ORF72* repeat expansion status and disease group

| | ***C9ORF72* repeat expansion carriers** | | | **Non-*C9ORF72* repeat expansion carriers** | | |
|---|---|---|---|---|---|---|
| | **Asymp-tomatic (N=28)** | **c9ALS or c9ALS-FTD (N=86)** | **c9FTD (N=21)** | **Healthy (N=37)** | **ALS or ALS-FTD (N=45)** | **FTD (N=25)** |
| Age at LP (years) | 43 (28, 63) | 59 (36, 76) | 63 (45, 77) | 60 (23, 85) | 60 (26, 90) | 67 (59, 84) |
| Age at disease onset (years) | N/A | 57 (34, 74) | 61 (44, 76) | N/A | 57 (25, 87) | 65 (39, 80) |
| Gender (male) | 8 (28.6%) | 53 (61.6%) | 11 (52.4%) | 13 (35.1%) | 30 (66.7%) | 17 (68.0%) |
| Disease onset to LP (months) | N/A | 23 (0, 132) | 47 (0, 103) | N/A | 27 (6, 204) | 51 (6, 243) |
| Onset site | | | | | | |
| Bulbar | N/A | 28 (33.7%) | N/A | N/A | 10 (23.3%) | N/A |
| Limb | N/A | 51 (61.4%) | N/A | N/A | 31 (72.1%) | N/A |
| Other | N/A | 4 (4.8%) | N/A | N/A | 2 (4.7%) | N/A |
| ALSFRS-R score | N/A | 36 (7, 46) | N/A | N/A | 37 (19, 45) | N/A |
| Disease progression score | N/A | 0.5 (0.0, 2.8) | N/A | N/A | 0.3 (0.1, 1.5) | N/A |
| pNFH level (pg/ml) | 89 (35, 459) | 1406 (117, 4671) | 238 (86, 2848) | 100 (45, 209) | 621 (50, 3119) | 181 (61, 502) |

The sample median (minimum, maximum) is given for continuous variables. LP=lumbar puncture; Information was unavailable regarding age at LP (N=1 c9ALS, N=1 ALS, N=1 FTD), age at disease onset (N=1 c9FTD), disease onset to LP (N=1 c9FTD, N=1 FTD), onset site (N=1 c9ALS, N=2 c9ALS-FTD, N=2 ALS-FTD), ALSFRS-R score (N=17 c9ALS, N=5 c9ALS-FTD, N=7 ALS, N=2 ALS-FTD), disease progression score (N=18 c9ALS, N=5 c9ALS-FTD, N=7 ALS, N=2 ALS-FTD). CSF samples were collected at the following institutes: Emory University School of Medicine, National Institutes of Health, Mayo Clinic Jacksonville, University of Miami, IRCCS Istituto Auxologico Italiano - Università degli Studi di Milano and Ospedale Maggiore Crema, Massachusetts General Hospital, University of Massachusetts Medical School, Barrow Neurological Institute, University of Pittsburgh Medical Center, University of Pennsylvania, Washington University School of Medicine, and University Hospital Mútua de Terrassa, Terrassa, Barcelona, Spain.