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RESEARCH ARTICLE

Longitudinal biomarkers in amyotrophic lateral sclerosis

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Abstract

Objective: To investigate neurodegenerative and inflammatory biomarkers in people with amyotrophic lateral sclerosis (PALS), evaluate their predictive value for ALS progression rates, and assess their utility as pharmacodynamic biomarkers for monitoring treatment effects. **Methods**: De-identified, longitudinal plasma, and cerebrospinal fluid (CSF) samples from PALS (n = 108; 85 with samples from ≥2 visits) and controls without neurological disease (n = 41) were obtained from the Northeast ALS Consortium (NEALS) Biofluid Repository. Seventeen of 108 PALS had familial ALS, of whom 10 had *C9orf72* mutations. Additional healthy control CSF samples (n = 35) were obtained from multiple sources. We stratified PALS into fast- and slow-progression subgroups using the ALS Functional Rating Scale-Revised change rate. We compared cytokines/chemokines and neurofilament (NF) levels between PALS and controls, among progression subgroups, and in those with *C9orf72* mutations. **Results**: We found significant elevations of cytokines, including MCP-1, IL-18, and neurofilaments (NFs), indicators of neurodegeneration, in PALS versus controls. Among PALS, these cytokines and NFs were significantly higher in fast-progression and *C9orf72* mutation subgroups versus slow progressors. Analyte levels were generally stable over time, a key feature for monitoring treatment effects. We demonstrated that CSF/plasma neurofilament light chain (NFL) levels may predict disease progression, and stratification by NFL levels can enrich for more homogeneous patient groups. **Interpretation**: Longitudinal stability of cytokines and NFs in PALS support their use for monitoring responses to immunomodulatory and neuroprotective treatments. NFs also have prognostic value for fast-progression patients and may be used to select similar patient subsets in clinical trials.

Introduction

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative illness. Motor neuron loss leads to progressive weakness, with average survival 2–5 years after diagnosis. ALS is heterogeneous for onset age, site of onset, progression rate, cognition and behavior changes, and survival duration.[1,2] Most cases are sporadic, whereas familial ALS exhibits an inheritance pattern or a clear monogenic cause, such as mutations in *C9orf72* or other genes.[3,4] Proposed pathogenic mechanisms include excitotoxicity via glutamate receptors, mitochondrial dysfunction, oxidative stress, protein aggregation, and neuroinflammation,[2–6] but symptom onset triggers and disease progression drivers remain unknown.

Only two drugs have U.S. Food and Drug Administration (FDA) approval for ALS. Riluzole is a glutamate-release inhibitor that improves survival ~3 months.[7] Edaravone, a free radical scavenger that inhibits neuronal death in animal models by reducing oxidative stress, initially failed in a broadly defined group of people with ALS (PALS).[8] A later trial showed a 33% slower decline in the ALS Functional Rating Scale-Revised (ALSFRS-R) over 6 months, specifically in edaravone-treated fast progressors versus placebo,[9] leading to edaravone approval in the United States in 2017. These studies highlighted the importance of trial designs that enrich for PALS with more homogeneous pathogenesis or disease progression rates.

Given the modest effect of current therapies, finding better treatments by improving trial design is vital. Biomarkers may strengthen trial design by (1) allowing selection of likely responders, (2) predicting disease progression, (3) reflecting target engagement, and (4) reflecting treatment effects. Currently, there are no validated biomarkers for ALS drug development.[10] Promising candidates include neurofilaments (NFs), essential structural components of neuronal axons. Mutations in phosphorylated neurofilament heavy chain (pNFH) are linked to ALS,[11] and elevated NFs in cerebrospinal fluid (CSF) and blood indicate motor neuron dysfunction and axonal injury in ALS and other neurodegenerative diseases.[1,11–16]

Early evidence suggests that neurofilament levels rise in the year prior to symptom onset in presymptomatic people carrying a mutation in the *SOD1* gene.[17] In symptomatic PALS, both neurofilament light chain (NFL) and phosphorylated neurofilament heavy chain (pNFH) levels have been shown to be elevated in people with ALS and have been correlated with patient survival; prompting investigations into their use for stratifying PALS into prognostic subsets.[14,18–21] While NFL and pNFH have been reported to be essentially stable over time,[14,17] some uncertainty remains. Better characterization of this longitudinal stability will help set the stage for the use of neurofilaments as markers of treatment response in early phase ALS trials.

Both nonclinical studies of transgenic rodents and clinical studies of patients with familial ALS implicate neuroinflammation and immune dysregulation in pathogenesis and heterogeneity.[6,22] Activated astrocytes, microglia, and MCP1-CCR2–mediated infiltration of monocytes have been detected in the motor cortex of ALS patients and TDP-43 mouse models.[23] Lu et al. demonstrated higher levels of creatine kinase, ferritin, TNF-α, and interleukins in plasma samples from PALS compared to controls, indicating that systemic inflammatory biomarkers acting on T-cell responses affected neuromuscular ALS pathology.[24] In addition, C-reactive protein (CRP), a general biomarker of inflammation, has been shown to be elevated in the serum of PALS and to correlate with more rapid disease progression.[25] Mutations in triggering receptor expressed on myeloid cells 2 (TREM2), a receptor of the innate immune system expressed on microglia, macrophages, dendritic cells, and osteoclasts, are associated with ALS, Alzheimer's Disease (AD), and frontotemporal dementia (FTD).[26] Soluble TREM2 (sTREM2), a proteolytic product of TREM2, may indicate activated myeloid cells in both the central nervous system (CNS) and the periphery.[27]

Despite mounting evidence supporting the role of neuroinflammation in ALS, results from immunosuppressive approaches have been discouraging; they have not slowed disease progression, which suggests nuanced immune dysregulation.[28] This failure of immune suppression may be attributable to the complexity of the inflammatory response in ALS. Future efforts should aim for careful immunomodulation, rather than broad immunosuppression, a goal more readily achieved if guided by a clear understanding of the specific inflammatory responses within individual ALS patients.

To better characterize biomarkers of neurodegeneration and inflammation in ALS, we obtained longitudinal plasma and CSF samples that had been collected prospectively from PALS and controls and stored in the Northeast ALS (NEALS) Consortium biorepository. We first modeled individuals' disease progression, identifying fast- and slow-progressing subgroups. We then examined inflammatory cytokine and NF levels in CSF and plasma, comparing PALS progression among subgroups and to controls without neurological disease. Then, we assessed the stability of these candidates over time. Finally, we evaluated the use of NF levels for patient selection and sample size calculations for clinical trials.

Methods

Sources of CSF and plasma samples

De-identified plasma and CSF samples from PALS ($n = 108$) and controls without neurological disease ($n = 41$) were obtained from the NEALS Biofluid Repository. Eighty-five of the 108 PALS had samples available from two or more visits. Longitudinal CSF and blood samples and accompanying clinical information were collected between 2011 and 2016 in a prospective, centrally coordinated, multicenter study to establish a source for longitudinal biomarker studies (ClinicalTrials.gov: NCT01495390). Briefly, participants were enrolled at six centers; detailed clinical information and biofluid samples (CSF, serum, and plasma) were obtained at baseline and at follow-up visits, approximately every 4 months for up

to 2 years. Samples were collected with standardized protocols and processed immediately. Briefly, CSF was centrifuged, aliquoted into cryovials, and frozen at −80°C. Blood was collected using K2EDTA tubes and centrifuged at 1750 ***g*** for 10 min. Supernatants were aliquoted into cryovials and frozen at −70°C to −80°C. Repeat-primed PCR testing for C9 hexanucleotide repeat expansion mutations was conducted on all samples at the Cecil B. Day Laboratory for Neuromuscular Research (University of Massachusetts Medical School, Worcester, MA). All visits included characterization of a battery of clinical outcomes including: the Fronto-Temporal Dementia (FTD) Assessment to assess any subtle cognition and behavior dysfunction; the ALSFRS-R to measure bulbar, motor, and respiratory functions; and slow vital capacity (SVC) to measure pulmonary function. Raters for these measures were trained by the NEALS Outcomes Training Center at the Barrow Neurological Institute.

In addition to the non-neurological controls from NEALS, we included CSF samples from aged, healthy controls obtained from multiple sources, and included baseline samples from Denali-sponsored clinical studies (n = 15) or that were purchased commercially from Innovative Research (Novi, MI, USA; n = 17) and Sanguine Biosciences (Sherman Oaks, CA, USA; n = 3). These samples were collected using protocols similar to those used for the PALS cohort and were centrifuged, aliquoted, and stored at −80°C.

Written informed consent was obtained from all study participants who contributed information or samples.

Multiplex cytokine immunoassay

We measured levels of a large panel of cytokines in CSF (see Table S1 for a list) using the Luminex multiplex immunoassay platform at Eve Technologies (Calgary, Canada) with the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a Milliplex Human Cytokine kit (Millipore, St. Charles, MO, USA) according to manufacturer protocols. The reported lower limit of detection (LLOD) for the 65 markers ranged from 0.1–55.8 pg/mL (intra-assay coefficient of variation [CV]: 2.58% ± 1.06%; interassay CV: 10.73% ± 4.02% [mean, SD]). Eve Technologies provided assay quality control (QC) data. Each Luminex sample was measured by two technical replicates. To ensure consistency, samples were excluded for analytes with more than 50% of measurements outside the range of quantification, a technical replicate CV greater than 80%, or with one technical replicate missing. Although this method may bias against cytokines that have low values for which variation is likely higher, we aimed to prioritize cytokines that could be robustly detected with current assay. Sample results were summarized as the arithmetic mean of the two technical replicates.

Meso Scale Discovery (MSD) cytokine assays

For clinical biomarker development, singleplex immunoassays have the advantage of ensuring the optimal detection of each target in different matrices with optimal dilutions and minimal interference from other antibody pairs,[29] in addition to integration with automation systems for operational precision. We thus selected a panel of various cytokines of interest including MCP-1, eotaxin-1, IL-18, TNF-α, CRP, and IL-15 and measured them in plasma using a well-characterized MSD V-Plex assay system (Meso Scale Discovery, Rockville, MD, USA) following manufacturer instructions. MCP-1, IL-15 and CRP in CSF were also measured using MSD V-Plex with optimal dilution factors that were determined at Denali Therapeutics to confirm the Luminex data.

Soluble triggering receptor expressed on myeloid cells 2 (sTREM2) assay

sTREM2 levels in CSF and plasma were measured with an MSD plate-based immunoassay developed at Denali Therapeutics using a biotinylated goat anti-human TREM2 antibody (BAF1828; R&D Systems, Minneapolis, MN, USA) and a sulfo-conjugated rat anti-human TREM2 antibody (MAB 17291; R&D Systems, Minneapolis, MN, USA) for capture and detection, respectively. sTREM2 CSF and plasma concentrations were determined by interpolating from a standard curve generated using recombinant human sTREM2.

Neurofilament light chain (NFL)

NFL measurements in CSF and plasma were performed at Quanterix (Billerica, MA, USA). The Simoa NF-LIGHT® assay used an optimized dilution factor for each matrix. Technical replicates were run for all samples, and all values were within the assay's linear range. Concentrations were interpolated from the standard curve and adjusted by dilution factors.

Phosphorylated neurofilament heavy chain (pNFH)

pNFH was measured in a subset of all CSF samples with an MSD immunoassay employing a mouse anti-human pNFH antibody and a sulfo-tagged polyclonal anti-pNFH antibody for capture and detection, respectively. The assay was analytically validated as a laboratory-developed test at Iron Horse Diagnostics, Inc. (Scottsdale, AZ, USA), in a

Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. Samples were tested in duplicate, with a CV below 8%. Intra- and interassay CVs were <10%.

Statistical analysis

Categorization of fast/slow progressors using modeled progression rates

Of 108 ALS patients, we used data from 85 patients with at least two ALSFRS-R measurements to model progression rates. A multivariate linear mixed-effects model of ALSFRS-R, adjusting for age at disease onset, disease duration at the time of reported visit, sex, ALS family history, and all pairwise interactions with disease duration, along with a subject-specific random intercept and slope, were fit to the data.

A linear mixed-effects model of ALSFRS-R was used to model progression of patients who had at least two observed time points. Specifically,

$$Y_{ijk} = a_0 + a_1X_{1i} + a_2X_{2ij} + a_3X_{3i} + a_4X_{4i} + a_5X_{1i}X_{2ij} + a_6X_{3i}X_{2ij} + a_7X_{4i}X_{2ij} + b_{0i} + b_{1i}X_{2ij} + \epsilon_{ijk}$$

where Y_{ijk} is the modeled ALSFRS-R score for patient i at time j at some repeated measure k, X_{1i} is the age of onset of disease for patient i, X_{2ii} is the recorded disease duration in years for patient i at time j, X_{3i} is the categorical variable representing the gender of patient i, X_{4i} is the categorical variable representing whether patient i had a family history of ALS, a_0–a_7 are estimated model coefficients, b_{0i} and b_{1i} are patient-specific random effects, and ε_{ijk} is the resulting error associated with model residual error modeled as a Gaussian distribution with variance σ_ϵ^2. Furthermore, we model a correlation structure between the patient specific random effects b_{0i} and b_{1i}. To complete specification of the model, we note that

$$\begin{pmatrix} b_{0i} \\ b_{1i} \end{pmatrix} \sim N\begin{pmatrix} 0 \\ 0 \end{pmatrix}, \begin{pmatrix} \sigma_0 & \sigma_2 \\ \sigma_2 & \sigma_1 \end{pmatrix}$$

and

$$\epsilon_{ijk} \sim N(0, \sigma_\epsilon)$$

where $\sim N(0, \sigma_\epsilon)$ represents normally distributed with model-estimated mean and variance/covariance matrix.

The fitted model was used to estimate the predicted rate of progression as defined by $Y_{iL} - Y_{iM}$ where times L and M are separated by 1 year. To confirm the validity of the model in predicting rates of progression, empirically observed progression rates were estimated for each patient as well. To calculate observed progression rates, a linear decline was assumed from age of onset when the ALSFRS-R score was assumed to be the maximum of 48 points to the last observed ALSFRS-R score. This can be expressed as

$$\text{observed progression rate for patient } i = \left(Y'_{i\text{Last}} - 48\right)/\left(X_{2i\text{Last}}\right)$$

where $Y'_{i\text{Last}}$ is the last observed ALSFRS-R score and $X_{2i\text{Last}}$ is the recorded disease duration at this last observed ASLFRS-R score.

A summary figure (Fig. 1A) comparing observed and modeled progression rates shows that the model accurately predicts progression rates while helping to smooth some of the extreme progression rates that would be empirically observed. To ensure sufficient distinction between fast and slow progressors for comparing subgroups, we proposed leaving a gap and excluding subjects with modeled ALSFRS-R declines between 4 and 9.6 points per year. This separation is arbitrarily chosen but helps to clearly distinguish differences in biomarker levels between the two extremes of the ALS population in terms of progression.

The fitted model was used to predict each patient's progression, and PALS were stratified by the model-estimated rate of disease progression. Patients with a model-estimated drop in the ALSFRS-R of greater than 9.6/year (0.8/month) or less than 4.0/year (0.33/month) were categorized as slow (Fig. 1B) or fast (Fig. 1C) progressors, respectively. Visual inspection showed that fast progressors were uniformly captured in this model. Patients with *C9orf72* fell mainly into the fast-progression group (Fig. 1D). The goal of modeling progression was to borrow population information to robustly categorize observed patients as fast or slow progressors. As such, the specified model was designed as an ALSFRS-R prediction model for this specific population and was not intended as an inferential model for the covariates.

Observed progression rates

Observed progression rates were computed as a linear decline for each subject from the last observed time point based on length of disease, assuming an ALSFRS-R score of 48 at disease onset. This empirical estimate is more prone to extreme observations than the modeled progression discussed above but is useful as a validity check to the modeled progression rates and for discussions relating to the selection (inclusion/exclusion) of a trial population where all individuals must be categorized.

Modeling differential effects

All measured analyte concentrations were log transformed and modeled using a linear mixed-effects model on

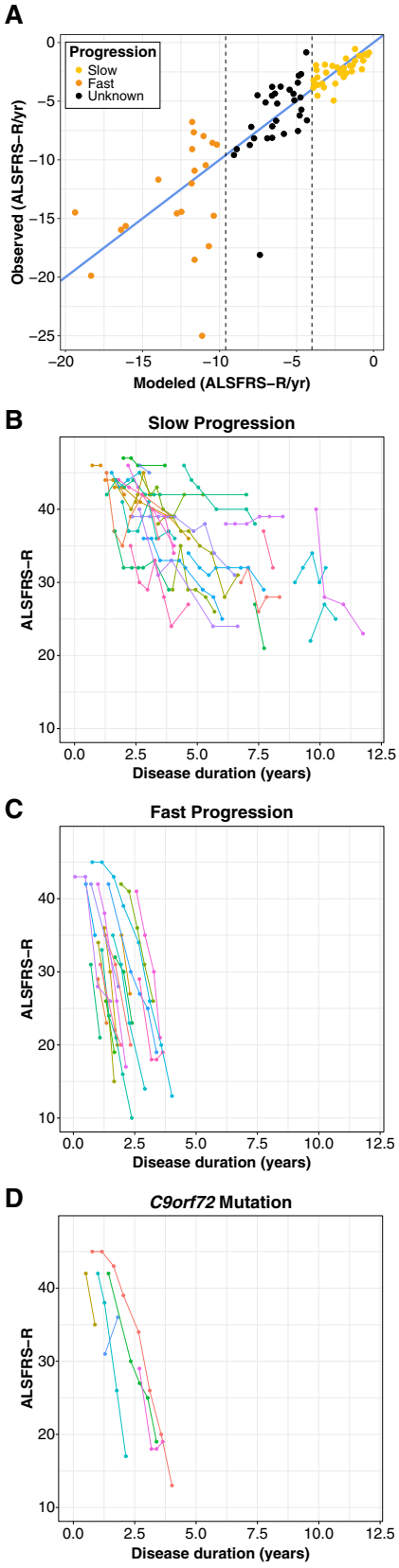

Figure 1. (A) Comparison of observed versus modeled progression rates. ALSFRS-R trajectories over the course of disease for (B) slow-progression, (C) fast-progression, and (D) *C9orf72* subgroups. Slow and fast progressors were defined as those patients showing a drop in the ALSFRS-R of less than 0.33/month or greater than 0.8/month, respectively

progression subgroups with a subject-specific random intercept to account for the intrasubject correlation structure of multiple measurements over time. All models were adjusted for age and sex. Where appropriate, a Benjamini–Hochberg procedure was used to adjust for comparison across multiple hypotheses.

Modeling longitudinal stability of NFs and cytokines

Average longitudinal trends in NFL, pNFH, and selected cytokines were modeled as a constant rate of change over time. To account for potential differences across individuals, a linear mixed-effects model of the independent variable, analyte log concentration, was modeled on the disease duration with a subject-specific random intercept and slope. An unstructured covariance matrix was assumed between the subject-specific random intercept and slope parameters.

Correlation analysis

To estimate correlations, Pearson's correlation was computed between associated analytes.

Survival analysis

Subjects were divided into equal groups of "high" and "low" by baseline analyte concentrations, separated by the median among all measured PALS. We then fit a Cox proportional hazards model to the data, adjusting for site of onset, gender, and age of disease onset. We performed a statistical test of significance based on Wald's method.

Estimating impact of neurofilaments on clinical trial design

We briefly explored two potential uses for NFs in ALS clinical trials. First, we examined the potential benefit of using NF levels for trial selection. Because slow progressors contribute less information, including large percentages of slow progressors in clinical trials can reduce statistical power. We therefore evaluated the effect of using an NF threshold for trial inclusion to reduce the number of slow progressors. We chose a threshold based

on our plasma NFL analyses and examined the impact of using this threshold on patient selection. Second, we performed exploratory sample size calculations for clinical trials based on the CV of NFL in CSF and in plasma. For these calculations, we assumed a power of 80% at an alpha level of 0.2. We chose an alpha level of 0.2 because for this use-case, NF would support early phase proof-of-concept studies.

Statistical software

All data processing and analysis was done using R software, version 3.6.1 (https://rstudio.com/products/rstudio/download/). Modeling of mixed effects models was done using the *lme4* package, whereas figures were generated using the *ggplot2* package.

Results

Demographics

Demographic characteristics of the PALS and non-ALS control groups were similar, except that more PALS were Caucasian, and PALS were older (PALS, mean [SD] age at diagnosis 56.9 [10.0] years); Table 1). Twenty PALS were fast progressors and 34 were slow progressors, as defined by our model. Seventeen of 108 PALS had familial ALS, of whom 10 had *C9orf72* mutations; nine of those 10 were rapid progressors.

Cytokine analyses

Of 65 cytokines measured using multiplex panels, PALS had significant increases of cytokine/chemokines including MCP-1 (128.8% of controls, adjusted $P < 0.01$], IL-18 (148.7% of controls, adjusted $P < 0.01$), and MIP-1α (120.5% of controls, adjusted $P = 0.01$) and significant decreases of 6CKine (adjusted $P = 0.03$), CTACK (adjusted $P < 0.01$) and PDGF-AA (adjusted $P < 0.01$) compared with non-ALS controls (Fig. 2A; Table S1). Based on the CSF cytokine data, we used MSD assays to measure a selected group of cytokines in plasma, and found plasma MCP-1 (125.7% of controls, adjusted $P = 0.02$) and IL-18 (124.8% of controls, adjusted $P = 0.02$) levels in PALS were also significantly increased (Fig. 2B). However, the association between central (CSF) and peripheral (plasma) levels of these cytokines was minimal (MCP-1, Pearson *r*: 0.06 [−0.05, 0.18]; IL-18, Pearson *r*: 0.29 [0.19, 0.39]). We also examined the effect of age on cytokine levels, appropriately adjusting for modeling differential effects. We found that IL-15 and Flt-3L in CSF were positively correlated with age (IL-15, Pearson *r*: 0.61, 95% CI [0.49, 0.7]; Flt-3L, Pearson *r*: 0.59, 95% CI [0.48, 0.69]).

Table 1. Demographics and clinical data.

	Non-ALS control (n = 79)	ALS (n = 108)
Sex, male, no. (%)	52 (65.8)	60 (55.6)
Ethnicity, no. (%)		
Asian	3 (3.8)	1 (0.9)
Black	2 (2.5)	3 (2.8)
Caucasian	53 (67.1)	100 (92.6)
Hawaiian	0	1 (0.9)
Hispanic/Latino	3 (3.8)	2 (1.9)
Multiracial	1 (1.3)	1 (0.9)
Unknown	6 (7.6)	0
NA	11 (13.9)	0
Age, mean (SD), years	43.4 (11.8)	56.9 (10.0)
Site onset, no. (%)		
Bulbar	0	22 (20.4)
Limb	0	86 (79.6)
NA	79 (100.0)	0
Genetics, no. (%)		
C9orf72	0	10 (9.3)
Others	0	7 (6.5)
ALSFRS-R, 1st visit, mean (SD)[1]	NA	36.7 (6.9)
SVC, 1st visit, mean (SD), % predmax	NA	89.3 (21.9)
Source, no. (%)		
DNLI-A-0001 baseline[2]	3 (3.8)	0
DNLI-B-0001 baseline[2]	12 (15.2)	0
Innovative research[2]	17 (21.5)	0
NEALS	41 (51.9)	108 (100.0)
Sanguine[2]	6 (7.6)	0
Disease progression subgroups[3]		
Fast (ALSFRS-R change >9.6/year)	NA	20
Slow (ALSFRS-R change <4.0/year)	NA	34
C9orf72[4]	NA	10
Others		
ALSFRS-R change >4.0/year, <9.6/year	NA	31
Subjects with only one visit	NA	23

ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; NA, not applicable; predmax, predicted maximum; SVC, slow vital capacity.

[1]ALSFRS-R scores and SVC values were determined during the first patient visit.

[2]Only CSF samples.

[3]Disease progression subgroups were defined according to the model-estimated rate of disease progression described in the Methods.

[4]*C9orf72* subjects overlap with the other progression subgroups; nine were fast progressors.

MCP-1 and IL-18 were more elevated in CSF from the *C9orf72* and fast-progression groups compared to slow-progression and control groups (Table 2; Fig. 3); plasma levels did not differ between subgroups (Table 2; Fig. 3). While CSF 1L-15 was not elevated in the PALS group

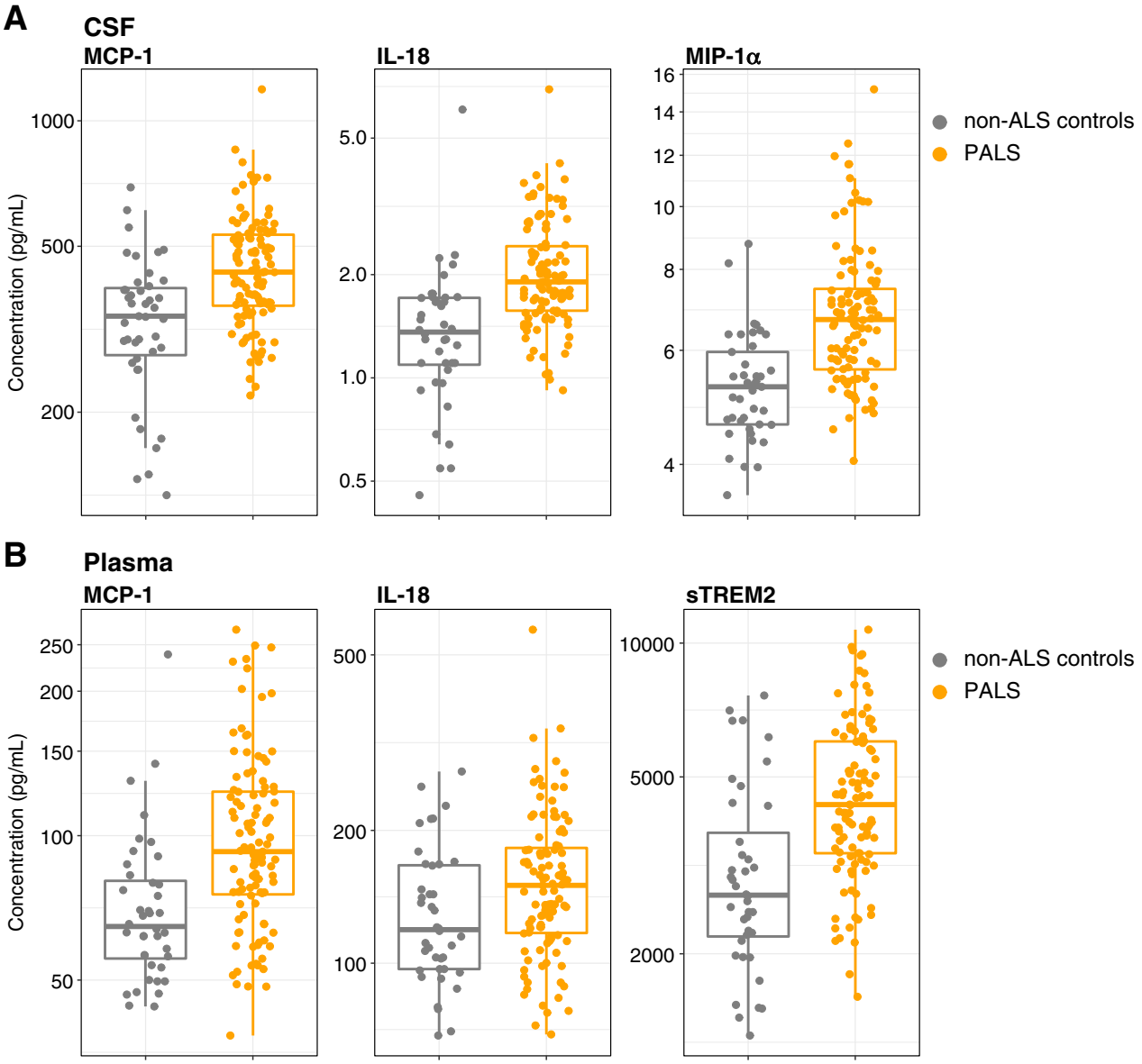

Figure 2. Inflammatory cytokines and glial cell markers in (A) CSF and (B) plasma with significant differences between PALS and non-ALS controls. Each dot represents an individual patient visit and box plots indicate median ± interquartile range (IQR).

overall, it was elevated in the fast-progression and *C9orf72* groups (Fig. 3A). In plasma, however, IL-15 showed the opposite trend when comparing subgroups (Fig. 3B), indicating potentially different mechanisms for central and peripheral regulation of this cytokine. CRP was not increased in PALS, but there was a trend toward elevated CRP in CSF and plasma in *C9orf72* patients (Table 2). Interestingly, tumor necrosis factor α (TNF-α) was significantly increased in CSF and plasma from *C9orf72* patients (Table 2; Figure S1A, B), consistent with data indicating a potential role for autoimmunity particularly in *C9orf72* ALS individuals. Other inflammatory cytokines (IL-6, IL-23, and IL-17) were not significantly different from controls in CSF (IL-6, Table 2; data not shown).

In the PALS group, sTREM2 levels were elevated in plasma (Fig. 2B), but not in CSF (data not shown), compared with controls. However, sTREM2 was significantly increased in both CSF and plasma of the fast-progression and *C9orf72* groups (Table 2; Fig. 3), suggesting activation of both central microglial cells and peripheral macrophages. In CSF, sTREM2 showed high correlation with IL-15 (Pearson $r = 0.68$), modest correlation with IL-18 (Pearson $r = 0.38$), and MIP-1α (Pearson $r = 0.44$), but little correlation with MCP-1 (Pearson $r = 0.17$).

Table 2. CSF cytokine and NF levels in disease progression subgroups.

	All PALS vs. controls	ALS-slow vs. controls	ALS-fast vs. controls	ALS-*C9orf72* vs. Controls	*P*-value (PALS)	*P*-value (*C9orf72*)	*P*-value (fast)
CSF analyte							
MCP-1	128.8%	121%	139%	177%	<0.01	<0.01	<0.01
IL-18	148.7%	135%	201%	174%	<0.01	<0.01	<0.01
MIP-1α	120.5%	111%	137%	135%	0.01	<0.01	<0.01
CRP	138.9%	112%	137%	266%	0.56	0.08	0.50
IL-15	101.9%	95%	111%	122%	0.75	0.09	0.28
TNFα	111.1%	102.4%	120.6%	135.5%	0.15	<0.01	0.01
IL-6	97.5%	90.5%	92.9%	92.6%	0.70	0.65	0.60
sTREM2	93.5%	83%	128%	136%	0.56	0.04	0.05
NFL	712.2	320.3%	1414.6%	1642.5%	<0.01	<0.01	<0.01
pNFH	485.1%	251.3%	799.5%	1155.2%	<0.01	<0.01	<0.01
Plasma analyte							
MCP-1	125.7%	141.7%	130.9%	136.6%	0.02	0.04	0.03
IL-18	124.8%	129.4%	127.%	142.7%	0.02	0.02	0.05
NFL	412.7%	247.7%	683.6%	977.6%	<0.01	<0.01	<0.01
IL-15	94.6%	95.2%	92.5%	83.7%	0.44	0.04	0.28
TNF-α	103.5%	93.4%	91.2%	229.5%	0.81	<0.01	0.65
sTREM2	123.6%	116.5%	134.2%	146.8%	0.02	0.01	0.02
CRP	116.2%	87.6%	89.5%	181.1%	0.76	0.02	0.77

Estimates and *P*-values are based on a linear mixed-effects model that accounts for repeated longitudinal measures structure within subject. Note that *P*-values are reported as adjusted *P*-values.

In longitudinal samples from PALS ($n = 85$; follow-up duration range: 4 months to 2 years), MCP-1 and IL-18 levels in CSF showed an estimated rate of annual change in 1.4% for MCP-1 [95% CI: −0.8%, 3.6%] and −3.4% for IL-18 [95% CI: −6.7%, 0.0%]), and in plasma of 1.1% for MCP-1 [95% CI: −1.7%, 4.0%] and −3.4% for IL-18 [95% CI: −7.2%, 0.6%] (Fig. 4A, B). sTREM2 levels were similarly stable (Figure S1C).

Neurofilaments

NFL was elevated in PALS in both CSF (712.2% of controls, $P < 0.01$) and plasma (412.7% of controls, $P < 0.01$) (Fig. 5A, B). NFL levels in plasma and CSF correlated highly (Pearson $r = 0.69$, Fig. 5C). pNFH was elevated in PALS in CSF (485.1% of controls, $P < 0.01$). NFL and pNFH levels correlated highly in CSF (Pearson $r = 0.85$, Fig. 5D), but showed only modest correlation in plasma (Pearson $r = 0.47$, data not shown).

When stratifying PALS by disease progression rate, NFL in CSF and plasma and pNFH in CSF were significantly higher in fast-progression and *C9orf72* mutant groups compared to the slow-progression group and controls (Table 2; Fig. 5E–G). We performed additional analyses to examine the impact of NFL (in CSF), along with the cytokines MCP-1 (in CSF) and sTREM2 (in CSF), on predicting survival. When subjects were stratified into "high" and "low" groups by the median baseline concentration of each analyte, we found that NFL was a significant prognostic factor for survival, but MCP-1 and sTREM2 were not (Figure S2).

In samples from our study population, levels of NFL were stable over time, with an estimated rate of annual change in only 3% in CSF [95% CI: −3.3%, 9.8%]), and −3.8% in plasma [95% CI: −8.4%, 1.1%]). Levels of pNFH also remained stable with an estimated rate of annual change in 0.8% in CSF [95% CI: −5.3%, 7.4%] (Fig. 4C, D).

Based on our analysis of neurofilaments, we chose a cutoff of 40 pg/mL for plasma NFL as a threshold for inclusion into a hypothetical ALS trial based on the current assay. This plasma NFL threshold considerably reduced the percentage of slow progressors from 41% to 13% (Fig. 5H). Our exploratory sample size calculations for clinical trials based on an NFL CV in CSF and plasma demonstrated that, with 10 participants per arm, we would be able to detect NFL changes in 35% in CSF and 44% in plasma to reach nominal statistical significance ($P < 0.2$) with 80% power (Table 3). Increasing the sample size to 30 patients per arm allows the statistically significant detection of 21% and 27% changes in NFL in CSF and plasma, respectively.

Discussion

Drug development for ALS has been hindered by the lack of understanding of ALS pathogenesis and effective biomarkers. It is clear that neuroinflammatory responses

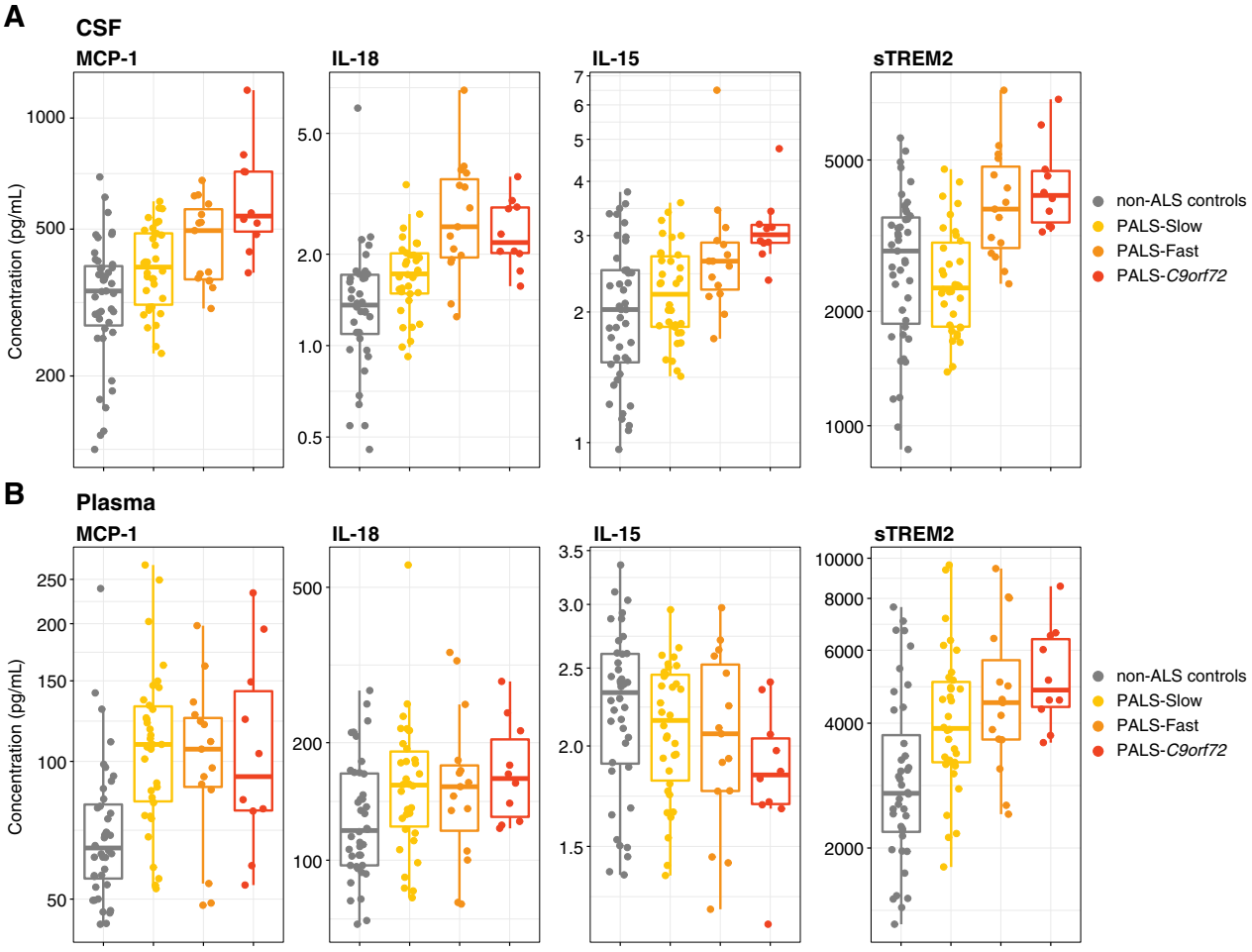

Figure 3. Cytokine levels in (A) CSF and (B) plasma in disease progression subgroups. Box plots indicate median ± IQR.

are present and that there may be important biomarkers reflecting these changes, but rarely have markers of inflammation been explored in large longitudinal cohorts. NFs appear to be reliable, though nonspecific, markers of ALS. They may be useful in predicting rate of progression, stratifying participants, and/or demonstrating a treatment effect. While a great deal of elegant work has been done to evaluate NFs in ALS, and some work has explored markers of neuroinflammation in plasma and CSF from people with ALS, our study bolsters and extends the existing evidence using cutting-edge techniques to quantify and correlate a large set of biomarkers in a large, longitudinally collected set of plasma and CSF samples from carefully characterized people with ALS.

Extending the findings of previous reports that found activated central and peripheral immune cells in ALS,[6,15,27,30] we found significant elevations of the proinflammatory cytokines MCP-1, MIP-1α, and IL-18 in CSF of PALS compared with non-ALS controls. Specifically, elevated MCP-1 (CCL2) and MIP-1α (CCL3), both CC chemokines, implicate macrophage and microglia chemotaxis, leading to microgliosis activation in the CNS.[28,30-32] Increased IL-18 in both CSF and plasma support inflammasome-mediated mechanisms.[27,30] Elevated plasma MCP-1 and IL-18 in PALS also suggests peripheral immune activation, but we saw no significant correlation between central and peripheral levels, indicating differential cytokine regulation in these compartments.

The biology determining the heterogeneity in the rapidity of ALS progression is poorly understood. Our study begins to address this knowledge gap by demonstrating important differences in inflammatory cytokines between PALS with slow and fast disease progression. Both fast-progression and *C9orf72* PALS exhibited robust increases of MCP-1, IL-18, IL-15, and the microglial cell marker, sTREM2, in CSF. In general, PALS with *C9orf72* mutations tend not to be slow progressors.[13] Indeed, in our cohort, all but one of the *C9orf72* patients were fast

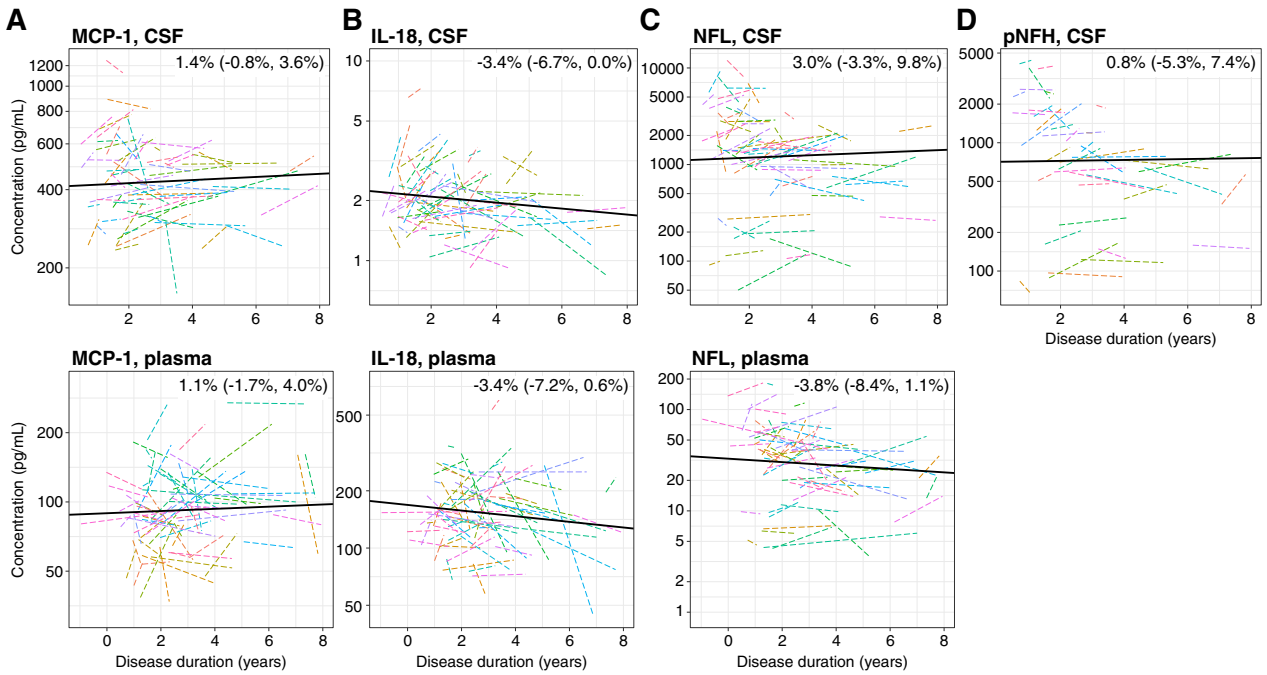

Figure 4. Longitudinal analysis of (A) MCP-1, (B) IL-18, (C) NFL, and (D) pNFH in CSF and plasma of PALS (n = 85). Solid lines are the average trajectory of log MCP-1, IL-18, NFL, and pNFH levels from the multilevel model analysis, and dotted lines represent the analyte trajectories of individual PALS. For better visualization, only subjects in the first 8 years of disease are included. Values within each plot indicate the estimated rates of annual change with 95% confidence intervals (CIs).

progressors, so it remains unclear if the elevated cytokines in this group are due to the effects of the specific genotype or simply reflect the faster disease progression. Recent research in *C9orf72* ALS individuals and preclinical animal models with human *C9orf72* mutations increasingly suggests a potential role for autoimmunity.[33,34] Indeed, TNF-α seems to be specifically higher in the *C9orf72* group in both CSF and plasma. Elevations in inflammatory cytokines and sTREM2, markers of activated microglial cells and macrophages in fast progressors and *C9orf72* ALS patients, suggests activation of glial cells and peripheral immune cells at variable magnitudes depending on the disease progression rate. Our data substantiate previous studies showing activation of microglia and astrocytes, the appearance of lymphocytes and infiltrating monocytes in postmortem tissue of PALS and in the spinal cord of transgenic mice expressing a mutant form of human SOD1,[32,35] and the systemic regulation of inflammatory biomarkers mostly active on T-cell immune responses.[24] While plasma MCP-1, IL-18, and sTREM2 are higher in the fast-progression groups, IL-15 in plasma shows the opposite trend, likely reflecting the complexity of adaptive immune system regulation depending on disease stage.[6,32,36]

This report is the first to analyze a comprehensive panel of cytokines in longitudinal PALS CSF samples. We demonstrate the general stability of MCP-1 and IL-18 levels in both CSF and plasma over the course of disease. Differences in cytokine levels between patient subgroups and their longitudinal stability suggest that these markers could be used to select subsets of PALS for a clinical trial or to evaluate treatment response.

Our observations of robust increases in NFL in CSF and plasma and pNFH levels in CSF from PALS supports prior evidence for their suitability as disease biomarkers.[11,15] Importantly, central and peripheral NFL levels were well correlated, which would allow for sample collection using plasma instead of CSF, improving the feasibility of implementation and reducing patient burden and cost. pNFH levels in CSF and plasma were correlated, although not as strongly as CSF and plasma levels of NFL. While pNFH and NFL were correlated strongly in CSF, their correlation in plasma was less robust. This finding could be because of assay variability, biological variability, or they may contain slightly nonoverlapping information about the biology of ALS. For trialists selecting an assay, either pNFH or NFL from CSF could be evaluated, but NFL appears to have an advantage over pNFH when measured in the plasma.

Consistent with previous reports,[18,37] we confirmed that NFL in CSF and plasma and pNFH in CSF were significantly elevated in PALS and essentially stable when sampled repeatedly over time. We also confirmed significantly higher levels of NFL and pNFH in fast-progression

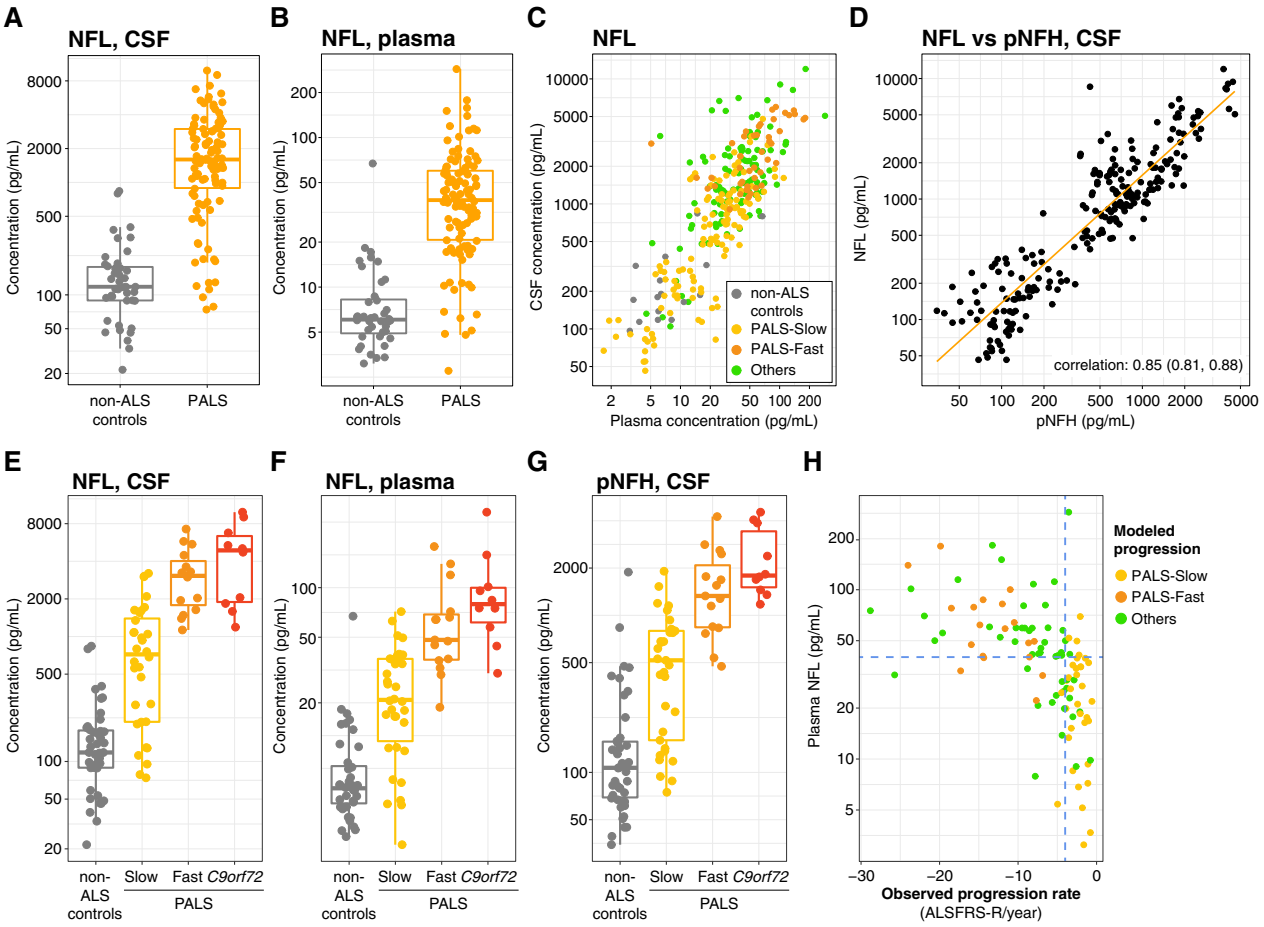

Figure 5. NF levels in CSF and plasma in PALS, PALS subgroups, and non-ALS controls. NFLs in PALS and non-ALS controls in (A) CSF and (B) plasma. (C) Correlation of CSF and plasma NFL levels. (D) Correlation of CSF NFL and pNFH levels. Values within the plot indicate the Pearson r and 95% CI. NFL levels in (E) CSF and (F) plasma, and (G) pNFH in CSF in the non-ALS cohort, the slow- and fast-progression PALS, and the *C9orf72* group. (H) Observed progression rates versus plasma NFL levels, modeled using an ALSFRS-R score of 48 at disease onset and an NFL cutoff of 40 pg/mL in 105 PALS. Nonslow progressors were defined by an observed decline of ≥4 points/year in the ALSFRS-R score. "Others" include PALS with an ALSFRS-R change in 4–9.6/year and those with single timepoint sampling. Box plots indicate median ± IQR.

Table 3. Sample size calculation for a phase 2a study design using plasma and CSF NFL as primary biomarker endpoints.

Sample Size	MDB in CSF (%)	MDB in plasma (%)
$N = 10$	35	44
$N = 15$	29	37
$N = 20$	25	32
$N = 25$	23	29
$N = 30$	21	27

Table provides MDB of treatment versus placebo under various sample size options (N per arm). Assumptions: analyte: NFL CSF ESDDL-2 = 0.56, 15% dropout, alpha = 0.2; analyte: NFL plasma ESDDL-2 = 0.76, 15% dropout, alpha = 0.2. ESDDL-2 = estimated standard deviation of the delta difference between 2 log base 2 measurements. MDB = minimally detectable benefit: the smallest percent reduction needed to reach statistical significance ($P < 0.2$) with 80% power.

patients, consistent with prior reports of NF level correlations with disease progression and patient survival.[11,14] In addition, we confirmed previous studies that NFL was a significant prognostic factor for survival.[11,14] Cytokines did not have a similarly robust prognostic value for survival, but these findings might be attributed to the small magnitude of changes, intersubject variations, and potential comorbidity factors in cytokine regulation. *C9orf72* patients also had higher levels of NFs, which appears to simply reflect their faster disease progression in our cohort, although it is challenging to discern whether the mutation itself impacts NF levels, because all but one of our participants with *C9orf72* mutations were fast progressors.

NFs could be used as prognostic biomarkers to enrich a trial population and improve ALS clinical trial efficiency. We present a hypothetical plasma NFL cutoff that

illustrates a sample size benefit to using NFs as selection criteria. Still, further NF assay characterization and validation with an independent cohort of patients are required to establish applicable cutoffs for trials. Unfortunately we did not have pNFH data on all longitudinal plasma samples and could not perform a similar analysis for pNFH, nor could we perform a correlation analysis between the two NF proteins in longitudinal plasma samples.

In addition, our model of longitudinal data showed relatively stable NF levels during the course of disease, highlighting their potential use as pharmacodynamic markers in short-term, proof-of-concept phase 2 ALS studies. Preclinical studies are encouraging, showing that NF levels respond to disease-modifying therapies in multiple sclerosis and ALS models.[38,39] Furthermore, clinical studies in multiple sclerosis have also shown that NF levels respond to treatment,[38,40] and in infants with spinal muscular atrophy (SMA), nusinersen treatment reduced plasma pNFH levels (ENDEAR trial).[41] Additionally, treatment-dependent NFL changes have been observed in a small phase I trial of people with SOD1-mediated ALS treated with antisense oligonucleotides.[42] The reliability and stability of NFL and pNFH (CSF) in our study bolsters the proposal that NF levels can act as useful tools for ALS trial design. While the degree of expected and clinically meaningful NF changes in response to different therapies is still unknown, this study provides a starting point for determining the sample sizes and power required to detect NF changes in different magnitudes, although the actual utility of NFs as pharmacodynamic biomarkers will only be verified once we have truly effective treatments for ALS.

Because ALS progresses rapidly, limitations of our study include potential biomarker sampling biases. Collecting multiple measurements in the fast-progression group was more challenging, so higher numbers of slow progressors were included in the study cohort. In addition, the fast-progression group defined here may not reflect patient groups enrolled in clinical studies. For example, PALS in the edaravone phase 3 trial showed an average ALSFRS-R change in >1.2/month,[8] which is faster than our definition. Our population also excluded patients who only had samples from a single visit from our disease progression analysis, and some may have been fast progressors. Indeed, we observed high baseline cytokine and NF levels in subjects with sampling from a single timepoint and disease onset of less than 3 years. Another limitation is that the controls were not all healthy subjects and were not perfectly age- and race-matched to the PALS population. However, we ensured that control samples were collected with the same protocols used to collect PALS samples and were corrected for age, gender, race, and sample resources during the data analysis. We also acknowledge that comorbidities may have been confounding factors in our analysis, but we did not include them in the model given the challenges in data interpretation that would have arisen with the small sample sizes for each comorbidity category. Future analysis with a large dataset is warranted to investigate this aspect.

In summary, our data support a role for inflammatory mechanisms in ALS pathogenesis, especially in patients with fast disease progression. Furthermore, our data support the rationale for patient stratification depending on the mechanism of action of the therapy (inflammation or neuroprotection) and enrichment of a more homogeneous population. Specifically, the inflammatory markers MCP-1, MIP-1α, and IL-18 in CSF and MCP-1 and IL-18 in plasma appear to be markers of inflammation. NFs are reliably elevated in ALS, stable over time, and predictive of the rate of progression and survival. Ultimately, these data may improve the design of future clinical trials and increase the probability of identifying more effective treatments for ALS.

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Author Contributions

F.H. and Y.Z. contributed equally to this manuscript. F.H. designed the study, analyzed the data, and wrote and revised the manuscript. Y.Z. analyzed and interpreted the data, generated the figures, and wrote and revised the manuscript. J.H-N., X.T., and J.C.D. performed assays and collected data. J.M.H. contributed to the study design, data interpretation, and writing of the manuscript. K.S-L. was involved in the study design, data analysis and interpretation, and editing of the manuscript. C.H. was involved in the study design and data interpretation. J.D.G., R.H.B., S.S.L., and D.L., contributed to clinical data collection, sample collection, data interpretation, and editing of the manuscript. M.M.L., J.D.B., and R.B., contributed to the study design, sample distribution, clinical data collection, data interpretation, and writing and editing of the manuscript.

Conflict of Interest

F.H., Y.Z., J.H-N., X.T., J.C.D., J.M.H., K.S-L., and C. H. are employees and stockholders of Denali Therapeutics. M.M.L. has no conflicts to declare. J.D.G. has received funding unrelated to this work from the National Institutes of Health, the ALS Association, and the Muscular Dystrophy Association and has consulting agreements with Biogen, Voyager Therapeutics, Apellis Pharmaceuticals, Apic Bio, Clene Nanomedicine, Aruna Bio, Rapa Therapeutics, and Vida Ventures. R.H.B. has no conflicts relevant to this manuscript. S.S.L. has received consulting fees and honoraria unrelated to this work or ALS from Biogen and Sanofi-Genzyme. D.L. has no conflicts to declare. R.B. is the founder and President of Iron Horse Diagnostics, Inc., a company commercializing diagnostic and prognostic tests for neurologic diseases. J.D.B. has been a consultant to Denali Therapeutics, Clene Nanomedicine, Biogen, Alexion and Anelixis Therapeutics. He has received research support from Anelixis Therapeutics, Amylyx Therapeutics, Biogen, Brainstorm Cell Therapeutics, Cytokinetics, Genentech, the ALS Association, the Muscular Dystrophy Association, ALS Finding A Cure, Project ALS and the National Institute of Neurological Disorders and Stroke.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. CSF cytokine comparison in PALS versus non-ALS control subjects.

Figure S1. TNF-α in (A) CSF and (B) plasma in disease progression subgroups. (C) Longitudinal sTREM2 in CSF from PALS.

Figure S2. Summary of survival analyses in PALS with CSF NFL and cytokines. Distinct curves representing cumulative survival in PALS with different CSF baseline levels. Estimated hazard ratios between "high" versus "low" analytes: (A) NFL: 9.99 (95% CI: 4.75–21.0), $P < 0.001$; (B) MCP-1: 1.36 (95% CI: 0.80–2.32), $P = 0.260$; (C) sTREM2: 1.36 (95% CI: 0.77–2.41), $P = 0.295$; $N = 102$ with 54 observed deaths.

