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RESEARCH ARTICLE

Novel blood-based proteomic signatures across multiple neurodegenerative diseases

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Abstract

INTRODUCTION: Blood-based biomarkers have the potential to support early and accurate diagnoses of neurodegenerative diseases, which are sensitive to molecular pathology and are predictive of outcome. We evaluated a novel multiplex proteomic method in people with diverse neurodegenerative diseases.

METHODS: Serum from people with Alzheimer's disease ($N = 36$), Lewy body dementia ($N = 34$), frontotemporal dementia ($N = 36$), and progressive supranuclear palsy ($N = 36$) and age-matched controls ($N = 30$) was analyzed with the nucleic acid linked immuno-sandwich assay (NULISA) central nervous system panel (≈ 120 analytes) and inflammation panel (250 analytes). Biomarkers were compared across groups and included as predictors of survival.

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RESULTS: The NULISA panels demonstrated high sensitivity and reliability for detecting multiple biomarkers across neurodegenerative disorders. There were condition-specific proteomic biomarkers, while neurofilament light chain, corticotropin-releasing hormone, CD276, and a data-driven inflammation pattern were significant transdiagnostic outcome predictors.

DISCUSSION: The sensitive NULISA multiplex approach supports differential diagnosis and target identification, with prognostically informative dementia-related biomarkers.

KEYWORDS
blood-based biomarkers, dementia, multiplex assays, non–Alzheimer's disease neurodegenerative diseases, nucleic acid linked immuno-sandwich assay

Highlights

- We tested the novel technology nucleic acid linked immuno-sandwich assay (NULISA) in people with diverse neurodegenerative diseases, which demonstrated high sensitivity and reliability for detecting multiple biomarkers in serum samples.
- We compared the NULISA central nervous system serum results to single molecule array (Simoa) plasma assays for phosphorylated tau (p-tau)217, p-tau231, neurofilament light chain (NfL), and glial fibrillary acidic protein, finding strong correlations.
- Increased levels of serum NfL were identified across all patient groups and most elevated in the frontotemporal dementia (FTD) and progressive supranuclear palsy (PSP) cohorts, while p-tau epitopes were the most significant markers in patients with Alzheimer's disease (AD) and Lewy body dementia.
- Patients with FTD and PSP showed upregulation of many inflammation markers, compared to controls and patients with AD.
- We found condition-specific proteomic biomarkers, while NfL, corticotropin-releasing hormone, CD276, and data-driven immune signatures were significant transdiagnostic predictors of clinical outcomes (survival rates).

1 | BACKGROUND

As the field of neurodegenerative dementias and movement disorders moves into an era of disease-modifying therapies, there is an increasing need to provide early and accurate diagnoses. Current clinically used Alzheimer's disease (AD) biomarkers include cerebrospinal fluid (CSF) measures of amyloid beta (Aβ)40/42 and phosphorylated tau, and amyloid and tau positron emission tomography (PET) scans.[1,2] Both CSF and PET markers have high costs, infrastructure, and patient-tolerance barriers to implementation at scale in "real-world" settings. Seed amplification assays (SAAs) using biofluids and skin biopsies are highly specific to distinct synucleinopathies[3] and tauopathies,[4] but questions regarding clinical interpretation of potential "red herring" positive results from background non-causative co-pathology remain unanswered. The majority of evidence for SAA tools is based upon CSF and skin samples, which potentially limit their scalability to real-world settings, despite recent progress in plasma in Parkinson's disease.[5]

Blood-based proteomic biomarkers offer a promising avenue for early and accurate diagnosis in neurodegenerative diseases that are scalable and deliverable in routine clinical care. Blood tests are very well tolerated, and can be performed repeatedly for an individual, allowing assessment of test–retest reliability and temporal profiles through longitudinal assessment of disease trajectory or in response to treatment.

Blood-based proteomic biomarkers have the potential to support diagnoses based on specific molecular pathologies of disease or concomitant pathophysiological processes such as neuroinflammation and synaptic loss. The clinical and pathological heterogeneity seen within and between diagnostic groups may also be reflected in biomarker signals. Disease-specific fingerprints or disease-agnostic pathways may provide insights into mechanisms of disease and potential therapeutic targets. The revolution of blood-based biomarkers in dementia has been rapid in the last 5 years,[6–12] despite the low concentration of many proteins in circulating blood and the limitation inherent

in the quantification of one marker or a small set of markers at a time.

Recently, proteomic methods have been developed with high sensitivity multiplex approaches. For example, the nucleic acid linked immuno-sandwich assay (NULISA) is a novel method of measuring many molecules in the human plasma and serum proteome, even to attomolar concentrations.[13] The NULISAseq "CNS [central nervous system] disease panel" analyzes > 120 proteins associated with diverse neurodegenerative diseases. Results from this technique have been reported in AD.[14–17] The NULISAseq "inflammation panel" analyzes 250 proteins, many of which have been associated with the inflammatory responses observed in neurodegenerative diseases.[13]

We took a transdiagnostic approach to assess the utility of the NULISAseq method, with two principal aims. First, we compared NULISAseq assays of conventional biomarkers of neurodegeneration (phosphorylated tau [p-tau] isoforms, neurofilament light chain [NfL], and glial fibrillary acidic protein [GFAP]) against results from the Quanterix single molecule array (Simoa)[8–11] with blood samples collected simultaneously from the same participants. Second, we integrated results from both NULISAseq CNS and inflammatory panels to identify differentially expressed diagnostic and prognostic markers in people with well-defined common neurodegenerative diseases, including AD, Lewy body dementia (LBD), frontotemporal dementia (FTD), and progressive supranuclear palsy (PSP).

RESEARCH IN CONTEXT

1. **Systematic review**: The revolution of blood-based biomarkers in dementia has been rapid in the past few years. Recently, proteomic methods have been developed with high sensitivity multiplex approaches. However, only a few studies have applied multiplex panels in neurodegenerative diseases beyond Alzheimer's disease (AD) to quantify markers of amyloid beta peptides, phosphorylated tau, synaptic changes, inflammation, and vascular health.
2. **Interpretation**: High-sensitive multiplex assays, like the nucleic acid linked immuno-sandwich assay, to analyze serum and plasma samples may offer scalable measures and prognostically informative dementia-related biomarkers for clinical trials in AD and non-AD neurodegenerative diseases.
3. **Future directions**: Further investigation is needed to validate markers and targets in larger and more diverse cohorts with longitudinal blood sampling.

2 | METHODS

2.1 | Participants

Patient participants were recruited from specialist clinics for cognitive and movement disorders at the Cambridge University Hospitals National Health Service Trust and collaborating regional psychiatry and neurology services. We included participants with a clinical diagnosis that met clinical consensus diagnostic criteria for mild cognitive impairment (MCI; $n = 20$, amyloid biomarker positive) or AD dementia ($n = 16$),[18,19] probable or possible PSP ($n = 36$),[20] FTD ($n = 36$; 21 with behavioral variant FTD [bvFTD] and 15 with primary progressive aphasia [PPA], consisting of 8 with non-fluent variant PPA [nfvPPA] and 7 with semantic variant PPA),[21,22] and LBD ($n = 34$; 29 with dementia with Lewy bodies [DLB] and 5 with Parkinson's disease dementia).[23] As with previous studies from this patient participant cohort,[24–26] we considered patients with a clinical diagnosis of AD and Aβ-positive MCI as a single cohort (AD/MCI+) as they represent a biological continuum. PET and/or CSF markers for amyloid were available to confirm the presence or absence of Aβ (interpreted as AD -pathology) in conditions with weak clinicopathological correlations and either a high likelihood of a significant fraction with AD as the main or co-pathology (LBD) or a high likelihood of clinical diagnostic false positives (amnestic MCI). All 20 MCI patients included in the study were amyloid positive with PET imaging with the Pittsburgh compound B (PiB tracer at a cut-off of 19 Centiloids[27]) and/or CSF AD biomarkers at lumbar puncture (Aβ1–42/40 ratio < 0.065 as recommended laboratory threshold from University College London Hospitals reference laboratory[28]). These participants were analyzed together with those with early AD dementia as a single AD group (AD/MCI+). Of the LBD cohort, $n = 17/25$ patients with PiB PET were amyloid positive. Amyloid markers have not been assessed systematically for research purposes in patients with a clinical diagnosis of AD dementia. We also included $n = 30$ healthy controls with Mini-Mental State Examination > 26/30, absence of memory symptoms, and no significant medical illnesses.[29] Exclusion criteria for both patient and healthy control cohorts included recent or current acute infection, major concurrent psychiatric illness, other severe physical illness, or a history of other significant neurological illness and/or autoimmune conditions. Participants underwent baseline clinical and neuropsychological assessment, including the revised Addenbrooke's Cognitive Examination (ACE-R, 0–100 points). Survival data were collected up to the census date of November 24, 2024.

2.2 | Blood sample collection and processing

Blood samples were obtained by venipuncture and collected in serum separation and ethylenediaminetetraacetic acid tubes. They were centrifuged to isolate serum and plasma, respectively; aliquoted; and stored at −70°/80°C until further analyses.

Serum samples from all patients and controls were analyzed with NULISA. The CNS panel (see Table S1 in supporting information for panel list), including Aβ peptides, p-tau forms, NfL (or "NEFL" as from the panel nomenclature), and other markers of neurodegeneration, inflammation, and vascular health. In addition to the CNS panel, the

samples were also analyzed with the NULISAseq inflammation panel 250 (full list in Table S2 in supporting information) for simultaneous analysis of 250 proteins associated with inflammatory and immune response processes (e.g., interleukins, chemokines, complement) with attomolar sensitivity to fg/mL units.[13] Sample and data analysis was performed according to kit manufacturer protocols, including log2 transformation of the data and NULISA Protein Quantification (NPQ) on the logarithmic scale (Alamar Biosciences). Specifically, data are first normalized using internal and inter-plate controls. The data are then rescaled by multiplication with a factor of 10^4. After this, +1 is added to all values. The data are then log2 transformed to make the data more symmetrical, forming a more normal distribution and accounting for the effects of outliers in subsequent statistical analyses. These values are defined as NPQ units, which are on a logarithmic scale. Differences in NPQ can be interpreted as log2 (fold change).

The majority (91.8%) of participants (n = 158; control = 29, AD/MCI+ = 34 [MCI = 19, AD = 15], FTD = 33 [bvFTD = 18, PPA = 15], LBD = 31, PSP = 31), also had paired plasma samples that were analyzed at the Clinical Neurochemistry Laboratory in Mölndal (Sweden). Plasma samples were thawed on wet ice and centrifuged at 500 × g for 5 minutes at 4°C. Calibrators (neat) and samples (plasma: 1:4 dilution) were measured in duplicate. The plasma assays performed were the Quanterix Simoa Human Neurology 4-Plex E assay, measuring Aβ40, Aβ42, GFAP, and NfL (Quanterix), p-tau231 (in-house Simoa assay), and the p-tau217 ALZpath assay, as previously described.[6,11] Plasma samples were analyzed at the same time using the same batch of reagents. A four-parameter logistic curve fit data reduction method was used to generate a calibration curve. Two control samples of known concentration of the protein of interest (high control and low control) were included as quality control. Intra-assay coefficients of variation were < 10%.

2.3 | Statistical analysis

Analyses were performed using R (version 4.4.2). For descriptive statistics, chi-square and analysis of variance tests compared variables between groups. Statistical analyses on blood-derived markers were carried out in three steps.

First, we compared NULISA CNS panel results on serum samples to Simoa assays on plasma samples from the same participants. Because NULISA NPQ values are log2-transformed, we applied log2 transformation to the absolute quantification values of plasma markers (as log2[pg/mL + 1]). After transformation, we performed Spearman correlation analyses on like-for-like markers, including p-tau217, p-tau231, NfL, and GFAP. Then, we ran group comparisons with Kruskal–Wallis tests, with Dunn post hoc tests, on NULISA single markers separately.

Second, we implemented linear models for microarray and RNA-seq data (LIMMA models) to compare the differential protein expression on each panel between diagnostic groups and controls. Results were displayed with volcano plots using color schemes to highlight the markers that were significantly different between groups, considering uncorrected p values and after false discovery rate (FDR) correction for multiple comparisons. All LIMMA analyses included age and sex as covariates.

Third, we investigated the relationship between dementia-relevant markers on the CNS panel and survival. Survival status was available for all the patient participants who passed the quality check for the CNS panel (n = 141, 70 deceased and 71 still alive) at the census date. Survival analysis used Cox proportional hazards regression (R function coxph), including all CNS panel markers that were significantly different between controls and patients, with time from blood test to death as the moderating variable of interest. Age, sex, and diagnosis group were added as covariates. We then applied a similar analysis in all the patients who passed the quality check for the inflammation panel (n = 138 patients: 68 deceased and 70 still alive) and included in the model all inflammation panel markers that were significantly different across all patients compared to controls. See Table S3 in supporting information for demographic summaries of survival cohorts.

2.4 | Data availability

Anonymized processed data can be shared upon request with the corresponding author. Raw data may also be requested but are likely to be subject to a data transfer agreement with restrictions required to comply with participant consent and data protection regulations.

3 | RESULTS

3.1 | Cohort characteristics

Participant demographics and clinical characteristics are described in Table 1. Patients with LBD were slightly older than controls and had the highest proportion of male participants. Participants with AD/MCI+ and PSP scored significantly higher on the ACE-R than other groups, while patients with LBD and FTD showed the lowest performance on average.

3.2 | Quality control checks

NULISA markers were detected with high sensitivity in both CNS and inflammation panels (94.1% and 97.3% target detectability, respectively). Two samples (1 control, 1 PSP) failed the quality check for the CNS panel, and four samples (1 DLB, 1 nfvPPA, 2 PSP) failed the quality check for the inflammation panel. In one patient with PSP, both panels failed quality check. These sample failures were excluded from further analyses. On the main CNS markers (p-tau217, p-tau231, NfL, and GFAP), we tested for associations with freezer storage time, as data collection spanned 10 years. Correlation analyses between NULISA CNS protein quantification and years of storage in −70°/−80°C freezers did not identify significant associations (see Figure S1 in supporting information).

TABLE 1 Demographic and clinical characteristics for control and patient groups.

Group	N	Age	Sex (F/M)	ACE-R (score/100)
Control	30	68.3 (6.1)	14/16	–
AD/MCI+	36	71.8 (8.1)	16/20	79.0 (10.7)
LBD	34	73.9 (6.9)[a]	6/28	69.4 (14.8)
FTD	36	64.5 (7.8)	12/24	67.7 (20.4)
PSP	36	69.4 (6.7)	17/19	83.4 (10.2)
Group comparisons	–	$F(4) = 8.55$, $p < 0.001$	$X^2(4) = 9.22$, $p = 0.06$	$F(3) = 9.37$, $p < 0.001$

Abbreviations: ACE-R, revised Addenbrooke's Cognitive Examination; AD, Alzheimer's disease; FTD, frontotemporal dementia; LBD, Lewy body disease; MCI, mild cognitive impairment; PSP, progressive supranuclear palsy.
[a]Significant difference between patient group and controls in post hoc analysis.

3.3 | NULISA versus Simoa biomarker measurements

We compared NULISA serum-based markers to Simoa plasma-based markers. All principal like-for-like proteins significantly correlated (Figure 1A). Specifically, p-tau217 and NfL showed the strongest correlations (rho = 0.798 and rho = 0.881, respectively, $p < 0.0001$), followed by GFAP (rho = 0.762, $p < 0.0001$) and p-tau231 (rho = 0.693, $p < 0.0001$).

3.4 | NULISA CNS panel across diagnostic groups

Group comparisons on dementia-relevant single markers from the NULISA CNS panel revealed diagnostic differences (Figure 1B). Specifically, Kruskal–Wallis tests were significant across all four markers. Post hoc Dunn tests (considering adjusted p values) indicated that p-tau217 levels were significantly increased in patients with AD/MCI+ (AD > MCI+) and LBD compared to controls, while p-tau231 levels were slightly increased also in patients with PSP. Levels of NfL were higher in all patient groups compared to controls, while GFAP levels were increased, especially in patients with AD/MCI+ and LBD, and to a lower extent in patients with PSP.

Comparing each patient cohort to controls (Figure 2), including the whole CNS panel, p-tau epitopes (p-tau217, p-tau231, p-tau181) were significantly increased in AD/MCI+ and LBD. In patients with AD/MCI+, but not LBD, serum levels of tau protein (MAPT) were also significantly increased compared to controls. Increased levels of serum NfL were the most significant marker in comparing FTD and PSP cohorts to controls, followed by multiple cytokines and chemokines. The homeostatic autophagy regulating scaffold protein (SQSTM1) was elevated in PSP versus controls and AD/MCI+, while presenilin 1 (PSEN1), a key protein in Aβ clearance, was elevated in both FTD and PSP cohorts versus controls. Parkinson disease protein 7 and phosphoglycerate kinase-1 (PGK1) levels, important enzymes in glycolysis regulation, were increased in FTD versus controls. All patient groups showed lower levels of corticotropin-releasing hormone (CRH) than controls. This survived FDR correction in the comparison between patients with FTD and controls. See Table S4 in supporting information for all comparisons.

Next, the diagnostic groups were analyzed against the AD/MCI+ group (Figure S2 in supporting information). AD/MCI+ had significantly higher levels of p-tau217, p-tau231, p-tau181, MAPT, and pro-neuropeptide Y (NPY) compared to FTD and PSP cohorts. Comparing FTD and PSP, patients with PSP showed higher levels of inflammation-related markers, including interleukin (IL)-7, fractalkine (CX3CL1), and periostin (POSTN) than FTD. In FTD, higher levels of fibroblast growth factor 2 (FGF2) were observed than in PSP. Compared to the AD/MCI+ cohort (Figure S2), patients with LBD showed higher levels of neuropentraxins (NPTX1/2) than AD/MCI+, but these comparisons did not survive FDR correction.

3.5 | CNS markers for predicting survival

For the NULISA CNS panel, we conducted survival analysis in two steps. First, we applied a model using the eight markers (NfL, p-tau217, p-tau231, p-tau181, GFAP, CRH, PSEN1, and C-X-C motif chemokine 10 [CXCL10]) that were significantly different between all patients and controls at a stringent FDR-corrected level (see Figure S3 and Table S5 in supporting information). We tested these eight markers as predictors of interest for survival alongside days from blood test to death in a Cox proportional hazards regression. Age, sex, and disease groups were included as covariates. We fitted the full model and performed backward stepwise selection, which compared the full model to reduced models based on the Akaike information criterion (AIC) until no further improvement in AIC was achieved, excluding predictors. The model retained NfL, CRH, and diagnosis as predictors in the final model. In this final model post stepwise selection, NfL levels were significantly associated with shorter time to death (hazard ratio 2.12 [1.44–3.15], $p = 0.0017$). CRH levels were inversely associated with time to death (hazard ratio 0.76 [0.60–0.96], $p = 0.021$; see Figure 3A). To illustrate the prognostic effect of NfL (Figure 3C) and CRH (Figure 3D), we plotted Kaplan–Meier curves dividing patients with high and low values of NfL and CRH, as separated by the median. These effects were also plotted by disease groups in Figure S4 in supporting information.

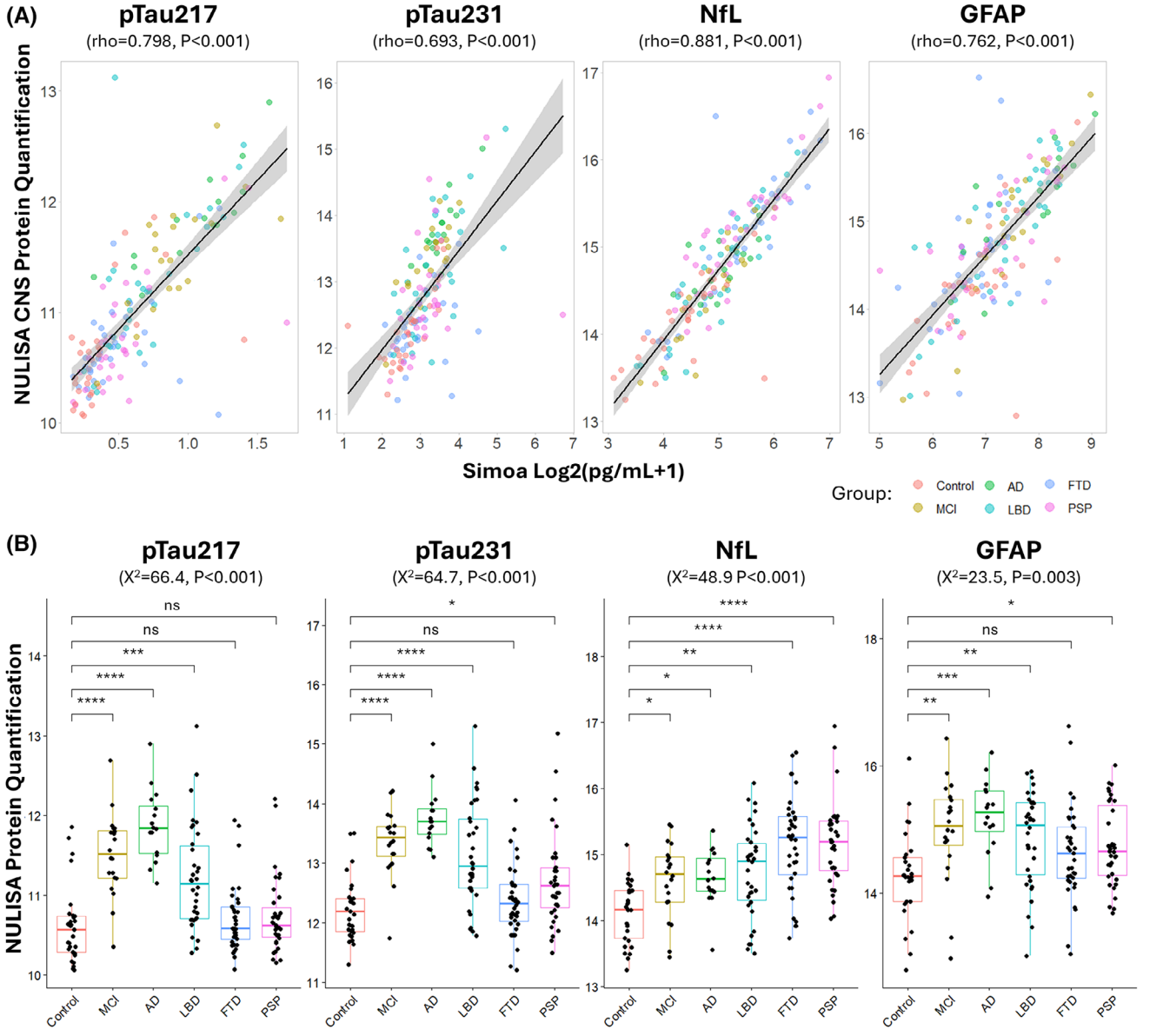

FIGURE 1 Validation of the dementia-relevant single markers on the CNS panel. A, Associations between NULISA serum-based markers and Simoa plasma-based markers, considering like-for-like proteins. B, Group comparisons on dementia-relevant single markers from the NULISA CNS panel. AD, Alzheimer's disease; CNS, central nervous system; FTD, frontotemporal dementia; GFAP, glial fibrillary acidic protein; LBD, Lewy body disease; MCI, mild cognitive impairment; NfL, neurofilament light chain; NULISA, nucleic acid linked immuno-sandwich assay; PSP, progressive supranuclear palsy; ptau, phosphorylated tau; Simoa; single molecule array.

Second, we repeated the analysis including all 24 markers that were significantly different between patients and controls, considering *p* values uncorrected for multiple comparisons to capture the full variance of biomarker signatures (see Figure S3 and Table S5). We included all 24 CNS markers as predictors of interest for survival alongside days from blood test to death in a Cox proportional hazards regression with backward stepwise selection. Age, sex, and disease groups were included as covariates. The final data-driven selected model included NfL, CRH, two additional CNS markers, and diagnosis as significant predictors of survival. The two additional CNS predictors were PSEN1 (hazard ratio 0.28 [0.13–0.58], $p = 0.001$) and PGK1 (hazard ratio 1.36 [1.11–1.67], $p = 0.003$; see Figure 3B, Table S6 in supporting information).

3.6 | NULISA inflammation panel across diagnostic groups

Comparing each patient cohort to controls (Figure 4), increased levels of amphiregulin (AREG) and CD276 (also known as B7-H3) were identified in LBD, FTD, and PSP compared to controls. Patients with FTD and PSP showed higher levels in multiple inflammation markers, while AD/MCI+ and LBD cohorts showed a more limited pattern of upregulated markers. Patients with AD/MCI+ showed increased GFAP levels, but this comparison did not survive FDR correction. Importantly, C-reactive protein levels, a commonly used clinical marker of non-specific systemic inflammation, did not show any significant

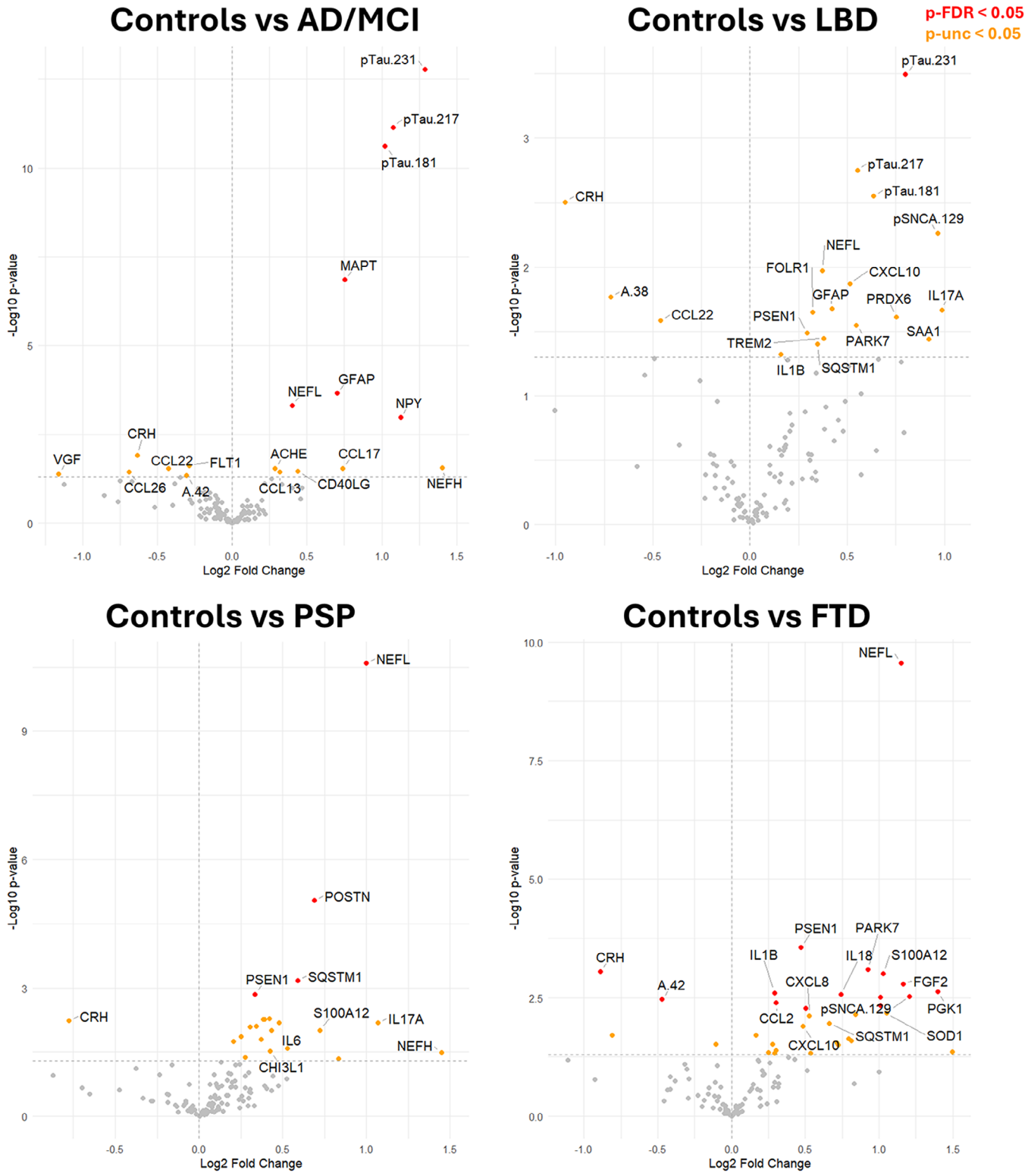

FIGURE 2 Comparisons between each patient group and controls on CNS markers. For each comparison, the panel on the right represents markers that are increased in patients compared to controls, while the panel on the left represents markers reduced in patients. Orange dots represent comparisons with $p < 0.05$, and red dots with $p < 0.05$ corrected for multiple comparisons. AD, Alzheimer's disease; CNS, central nervous system; FDR, false discovery rate; FTD, frontotemporal dementia; LBD, Lewy body disease; MCI, mild cognitive impairment; PSP, progressive supranuclear palsy.

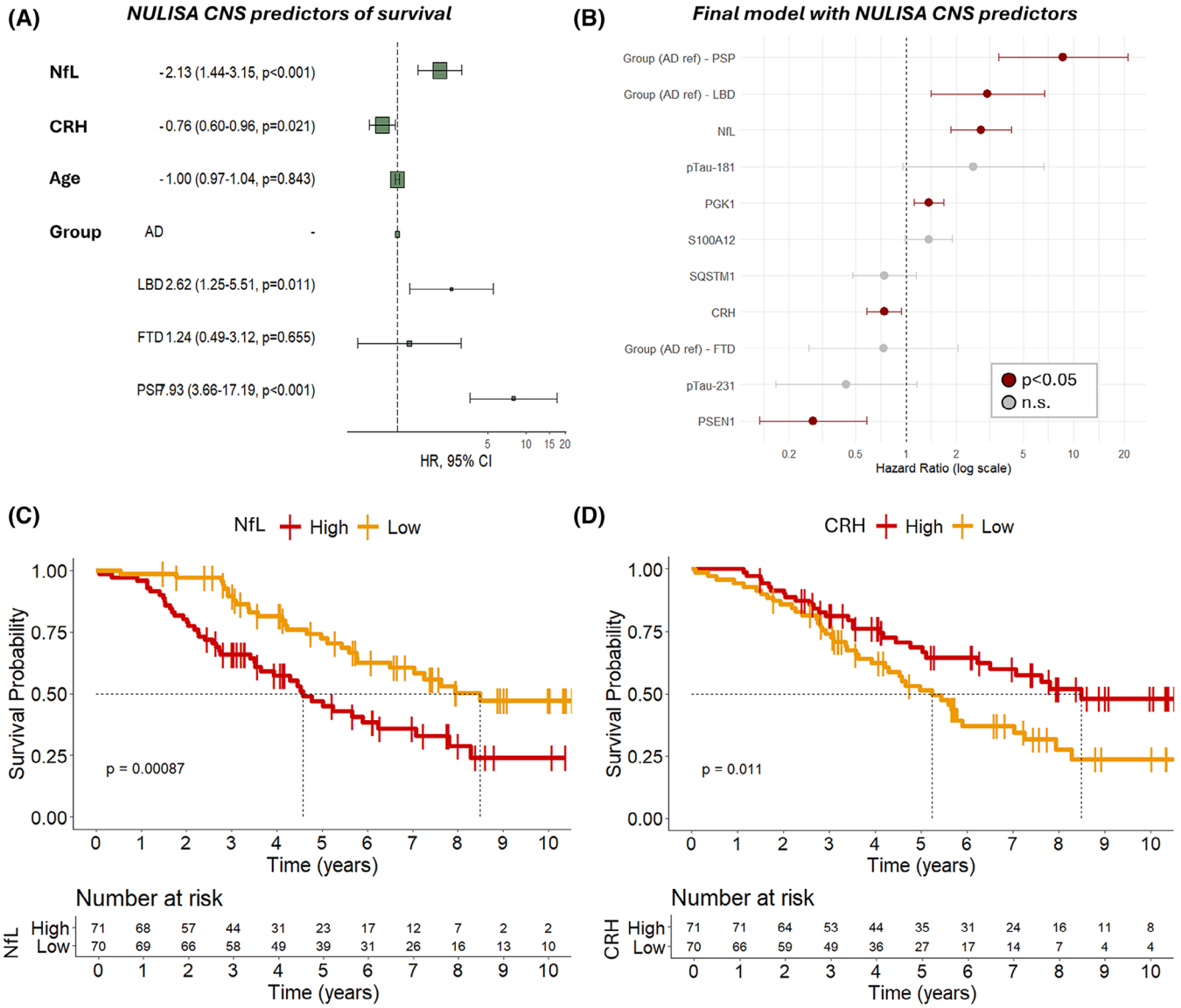

FIGURE 3 Prognostic markers on CNS panel. A, Final model by the backward stepwise selection on predictors in the survival analysis, including eight CNS markers (differentiating patients and controls at FDR-corrected level), age, sex, and diagnosis. B, The final model was selected on survival predictors, including 24 CNS markers (differentiating patients and controls, *p* uncorrected), age, sex, and diagnosis. For each variable, the hazard ratio on survival is plotted. Cand D, Kaplan–Meier survival curves of NfL and CRH, respectively, divided on the median of each marker (C). AD, Alzheimer's disease; CI, confidence interval; CNS, central nervous system; CRH, corticotropin-releasing hormone; FDR, false discovery rate; FTD, frontotemporal dementia; GFAP, glial fibrillary acidic protein; LBD, Lewy body disease; NfL, neurofilament light chain; NULISA, nucleic acid linked immuno-sandwich assay; PSP, progressive supranuclear palsy.

differences between diagnostic groups and controls. See Table S7 in supporting information for all comparisons.

Comparing each patient group to the AD/MCI+ cohort (Figure S5 in supporting information), patients with LBD showed similar levels of inflammation markers as AD/MCI+, with a larger number of markers being upregulated in LBD than AD/MCI+ when FDR correction was not considered. FTD and PSP cohorts had higher levels in several inflammation markers compared to AD/MCI+, including matrix metalloproteinase-9 (MMP9) and hepatocyte growth factor (HGF). Comparing FTD and PSP, no marker was significantly different when FDR correction was considered.

3.7 | Common patterns of inflammation across diseases

When comparing patients (across all groups) to controls, several markers from the NULISA inflammation panel were significantly increased in patients compared to healthy volunteers. Considering FDR-corrected *p* values, these included AREG, CD276, C-C motif chemokine 7 (CCL7), GFAP, inteleukin-1 receptor antagonist (IL1RN), colony stimulating factor-1 (CSF1), oncostatin-M (OSM), colony-stimulating factor-3 (CSF3), cardiotrophin-1 (CTF1), IL-17A and IL-17F, and thymic stromal lymphopoietin (TSLP). In contrast, tumor

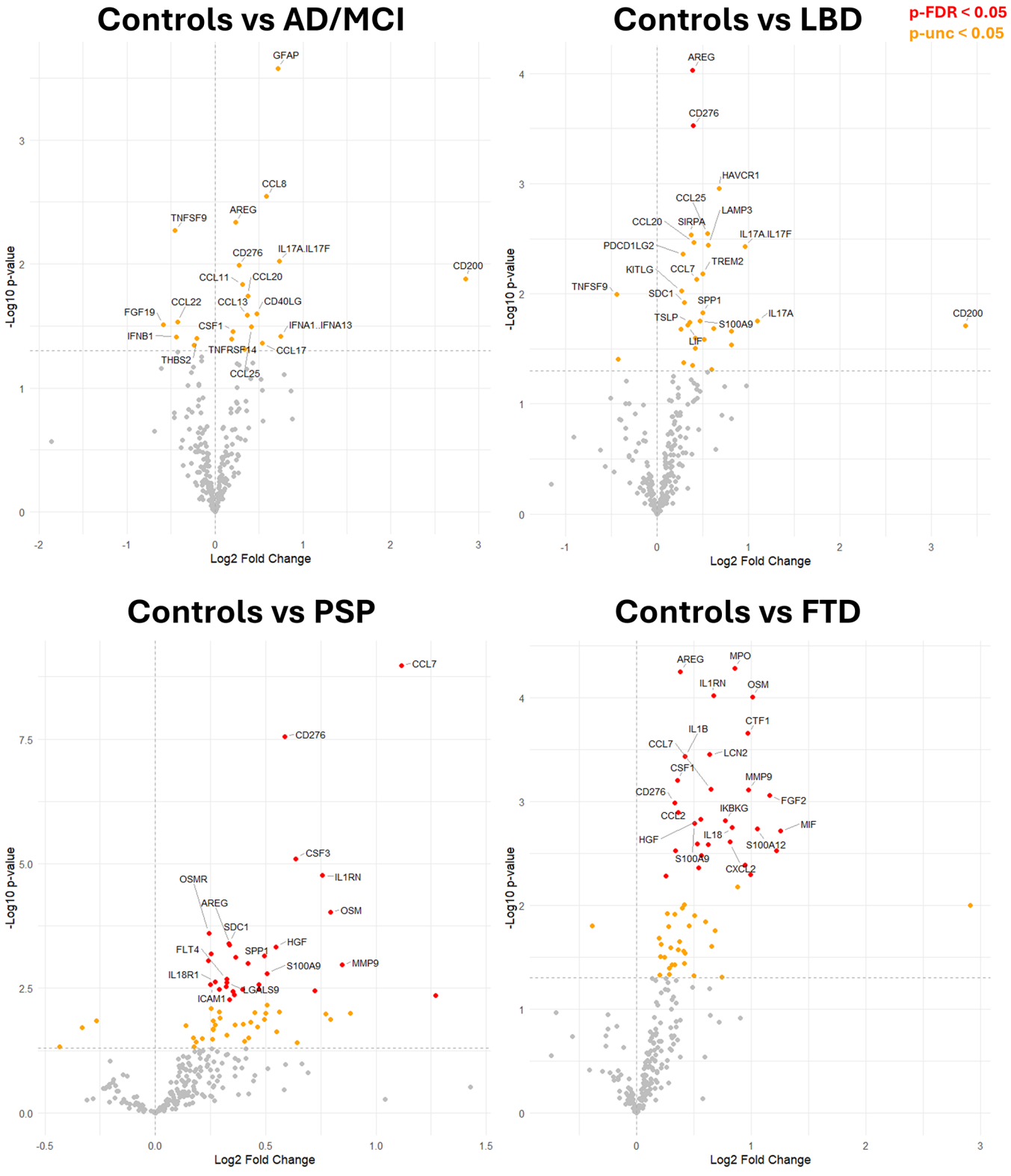

FIGURE 4 Comparisons between each patient group and controls on inflammation markers. For each comparison, the panel on the right represents markers that are increased in patients compared to controls, while the panel on the left represents markers reduced in patients. Orange dots represent comparisons to $p < 0.05$, and red dots with $p < 0.05$ corrected for multiple comparisons. AD, Alzheimer's disease; FDR, false discovery rate; FTD, frontotemporal dementia; LBD, Lewy body disease; MCI, mild cognitive impairment; PSP, progressive supranuclear palsy.

necrosis factor superfamily member 9 (TNFSF9) was decreased in all patient groups. Considering uncorrected *p* values, 45 additional markers were identified as increased in patients compared to controls, including CD200, interferon alpha 1 (IFNα1), nuclear factor kappa beta essential modulator (NEMO), IL-18, IL-1β, lipocalin-2 (LCN2), leukemia inhibitory factor (LIF), MMP9, programmed cell death 1 ligand 2 (PD-L2), S100A12, S100A9, tissue inhibitor of metalloproteinases 2 (TIMP2), tumor necrosis factor receptor superfamily member 14 (TNFRSF14), triggering receptor expressed on myeloid cells 2 (TREM2), IL-6, IL-22, and IL-17A (see Figure S6 and Table S8 in supporting information for the full list).

3.8 | Inflammation markers for predicting survival

Similar to the statistical methods described in Section 3.5, survival analysis on the NULISA inflammation panel was conducted at two levels. First, we applied a conservative model with only those 12 markers that were significantly different between all patient groups and controls at a stringent FDR-corrected level (AREG, CD276, CCL7, GFAP, IL1RN, CSF1, OSM, CSF3, CTF1, IL-17A and IL-17F, TSLP, and TNFSF9—see Figure S6 and Table S8). We included the 12 markers as predictors of interest for survival alongside days from blood test to death in a Cox proportional hazards regression. Age, sex, and disease groups were included as covariates. As with the CNS panel in Section 3.5, we fitted the full model and performed backward stepwise selection, which compared the full model to reduced models based on the AIC until no further improvement in AIC was achieved excluding predictors. The model selection retained CD276, AREG, and diagnosis as predictors in the final model. CD276 (hazard ratio 2.22 [1.25–3.95], $p = 0.007$) levels were significantly associated with time to death (Figure S5B). To illustrate this effect, we plotted Kaplan–Meier curves separating patients with high and low values of CD276 by the marker-specific median (Figure 5A).

Second, we repeated the analyses including all 57 markers that were significantly different between patients and controls, considering *p* values uncorrected for multiple comparisons to capture the full variance of biomarker signatures (see Figure S6 and Table S8). We included all 57 inflammation markers as predictors of interest for survival alongside days from blood test to death in a Cox proportional hazards regression with backward stepwise selection. Age, sex, and disease groups were included as covariates. The final data-driven selected model included 32 inflammation markers (of which 25 were statistically significant predictors of survival), age, sex, and diagnosis (Figure 5C and Table S9 in supporting information). Significant inflammatory predictors of survival included IL1β, CD276, CXCL10 (IP-10), S100A12, IL17A, TREM2, and CD40, among others.

4 | DISCUSSION

Multiplex NULISA biomarkers demonstrated high sensitivity for detecting changes in serum protein levels across clinically and pathologically heterogeneous neurodegenerative disorders. There was high reliability and differential diagnostic sensitivity from the NULISA CNS panel, expanding previous evidence by (1) the inclusion of patients with sporadic FTD and PSP, and (2) the prediction of survival. The NULISA inflammation 250 panel detected changes in inflammatory patterns across multiple neurodegenerative diseases, with anti-inflammatory and proinflammatory changes, highlighting the complexity in interpreting cross-sectional plasma biomarkers in dynamic diseases.

Recent studies have shown the potential utility of the NULISA CNS panel to quantify dementia-relevant markers, including p-tau epitopes, NfL, and GFAP, in the blood of patients with MCI and/or AD,[14–17] genetic FTD, and LBD.[15] In our cohort, increased levels of serum NfL were identified across all patient groups compared to controls and were most elevated in the FTD and PSP cohorts. On the other hand, p-tau epitopes were the most significant markers in separating patients with AD and LBD from controls. These findings align with previous evidence with single-marker assays in similar cohorts[6–11] and suggest common AD co-pathology in the LBD cohort. Across all groups, we compared the NULISA CNS serum results to Simoa plasma assays for p-tau217, p-tau231, NfL, and GFAP, finding strong correlations. This suggests the multiplex panel data can be as informative as more targeted assays, showing good consistency with the advantage of quantifying additional markers, using either plasma or serum samples. This aligns with and expands similar evidence in cohorts of healthy volunteers and patients with AD.[14,16] Importantly, the quantification of the CNS serum proteins in our cohort was not affected by the time they had been stored in freezers at −70°/−80°C.

We also demonstrate that dementia-relevant markers from the CNS panel are prognostic against survival. Serum NfL levels were associated with a subsequent faster decline and shorter time from blood sampling to death, over and above p-tau217, p-tau231, and GFAP levels, and over and above age and diagnosis. People with FTD and PSP show the strongest associations between NfL and survival. Serum and plasma NfL levels are promising markers for disease progression and may be useful trial endpoints in patients with FTD and PSP.[7,30–35] Here, survival rate was used to test for the prognostic utility of these markers, as the high clinical heterogeneity across diagnostic groups makes it difficult to capture disease severity at the time of blood collection.

Beyond the common dementia-relevant markers previously described, the NULISA CNS panel in our cohort identified lower levels of CRH in all patient groups compared to controls. CRH is an important stress hormone that may be expected to be higher in diverse disease states. Impairment of the hypothalamic pituitary adrenal (HPA) axis has long been recognized in neurodegenerative disease. At *post mortem*, diminished CRH is seen across brain regions in AD,[36] while CSF cortisol is elevated in patients with AD versus controls.[37] One hypothesis for reduced levels of CRH in patients is endocrine insufficiency owing to hypothalamus neurodegeneration. Alternatively, high plasma cortisol is known to occur in AD, relating to worse hippocampal atrophy and clinical progression;[38,39] the resultant negative feedback loop may sufficiently explain the pattern of CRH being consistently higher in controls than patients. CRH is the only HPA axis protein examined in the NULISA CNS panel; thus, further investigations are

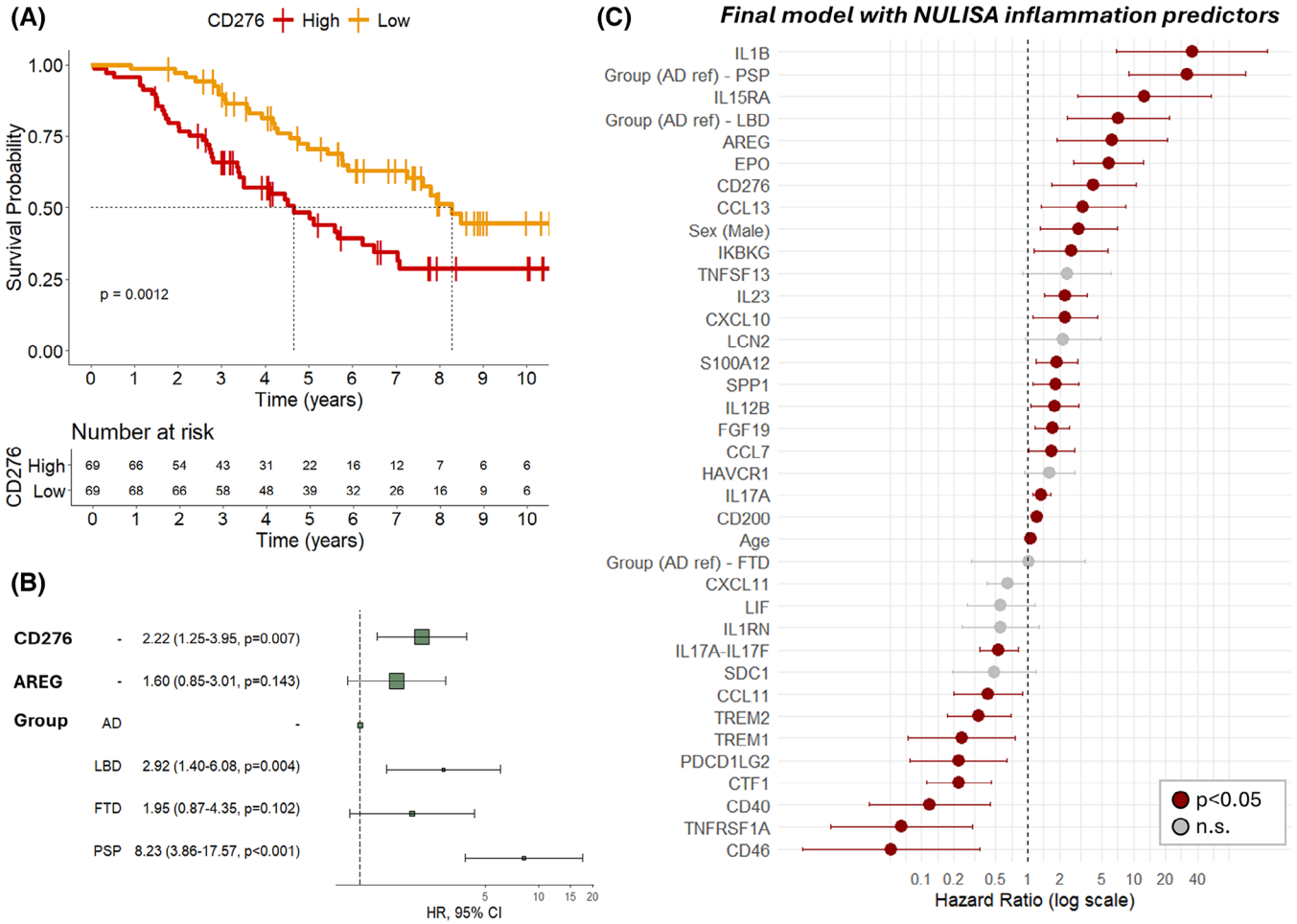

FIGURE 5 Inflammation prognostic markers. A, Kaplan–Meier survival curves of CD276 divided on the median and (B) final model by the backward stepwise selection on predictors in the survival analysis, including 12 inflammatory markers (differentiating patients and controls at FDR-corrected level), age, sex, and diagnosis. C, The final model was selected on survival predictors, including 57 inflammatory markers (differentiating patients and controls, *p* uncorrected), age, sex, and diagnosis. For each variable, the hazard ratio on survival is plotted. AD, Alzheimer's disease; AREG, amphiregulin; CI, confidence interval; FDR, false discovery rate; FTD, frontotemporal dementia; LBD, Lewy body disease; NULISA, nucleic acid linked immuno-sandwich assay; PSP, progressive supranuclear palsy.

required to clarify the biological underpinnings of this finding. Lower CRH levels were also associated with worse survival (Figure 3), with the strongest associations being in the LBD cohort (Figure S4). In line with our findings, CRH has emerged as a biomarker of interest in LBD, but evidence to date has been CSF based.[40,41]

Stepwise selective survival modeling identified PGK1 as a predictor of faster disease progression across this transdiagnostic cohort, while PSEN1 was associated with a longer survival time. PGK1 has an important role in metabolic function and glycolysis, dysregulation of which may potentiate a cycle of oxidative stress and neuroinflammation. This finding of PGK1 elevation may reflect a compensatory response to increased metabolic demand in patients. Treatment to boost PGK1 has shown favorable effects in models of amyotrophic lateral sclerosis (ALS).[42] PSEN1 is a core component of the γ-secretase mediated process through which amyloid precursor protein is cleaved and excreted. PSEN1 being inversely associated with survival time may plausibly reflect failure of the homeostatic clearance system. The potential contributions of PSEN1 to neurodegeneration go beyond amyloid pathology, however, as it has been identified as a potentiator of neuroinflammation and apoptosis.[43,44]

Across the CNS and inflammation panels, patients with FTD and PSP showed upregulation of many inflammation markers, compared to controls and patients with AD. Specifically, we identified proinflammatory proteins OSM, MMP9, and the potent chemo-attractant CCL7 to be elevated in PSP and FTD versus controls. We found significantly increased levels of the anti-inflammatory proteins CD276 and AREG in PSP, FTD, and LBD versus controls. The anti-inflammatory IL-1 receptor antagonist (IL1RN) and the neuroprotective HGF were elevated in FTD and PSP versus controls only. Although previous fluid markers confirmed abnormally high levels of inflammation in patients with FTD and PSP (see Bright et al.[45] for review), they have been largely confined to CSF, small case reports, limited panels, and/or single markers. Our recent study, using Meso Scale Discovery assays on serum samples, identified a pattern of proinflammatory cytokines upregulated in

214 patients with FTD, PSP, and related conditions.[33] This inflammatory pattern, including TNFα, TNFR1, CSF1, IL-17A, IL-12, IP-10, IL-6, and others, differentiated patients (all syndromes) from controls and was associated with significantly lower survival over time. Importantly, proinflammatory cytokines were associated with microglial activation in frontal and brainstem regions, as quantified by translocator protein PET. The NULISA inflammation panel provides simultaneous quantification of a larger number of inflammation markers, with better detectability for specific targets than previously used technologies.[13] In our cohort, patients with PSP or FTD over-expressed proteomic markers of inflammatory/synaptic function compared to controls and patients with AD. The degree of dysregulation across the landscape of inflammatory protein expression supports that FTD and PSP correlate with more peripheral markers of inflammation than in our cohort of AD. Further studies should investigate the inflammatory patterns on the AD spectrum with larger sample sizes that allow the separation of patients in early and late stages of the disease.

Several targets emerged as upregulated across diagnostic groups compared to controls in our cohort. In particular, CD276, also known as B7-H3, is primarily an anti-inflammatory or immunosuppressive molecule. This immune checkpoint plays a complex role in the immune system, but its overall effect tends to inhibit immune responses, particularly in the context of cancer.[46] Although CD276 is predominantly anti-inflammatory, in non–small cell lung cancer, higher CD276 expression has been associated with more CD8+ T cells and plasmacytoid dendritic cells.[46] In our cohort, higher levels of serum CD276 were associated with shorter survival or time to death from blood sampling. However, the role of CD276 in non-cancerous neurological disorders has not been investigated before, and further studies would be needed to elucidate its role in these conditions. When investigating the prognostic values of inflammation markers on survival rates, 25 proteins were found to show significant associations. Shorter time to death was associated with higher levels of IL1β, interleukin 15 receptor subunit alpha (IL15RA), AREG, erythropoietin (EPO), CD276, C-C motif chemokine 13 (CCL13), NEMO/IKBKG, IL-23, CXCL10, osteopontin (SPP1), IL-12 subunit beta (IL-12B), FGF19, CCL7, IL-17A, and OX-2 membrane glycoprotein (CD200). These display complex physiology with a dynamic response to illness and injury but are intrinsically involved in neuro-inflammation and immune modulation. Meanwhile, the predictors of longer survival were membrane cofactor protein (CD46), TNFRSF1A, TNFRSF5 (CD40), CTF1, PD-L2, TREM1, TREM2, eotaxin (CCL11), and IL-17A/IL-17F. While also involved in the neuroinflammatory response, these proteins are involved in apoptosis, tissue repair, and cell survival. The relationship between a biomarker and disease trajectory is unlikely to be linear in such dynamic illnesses, so a static snapshot may not account for disease stage and/or severity. However, it is a testable hypothesis that precision medicine aimed at one or a combination of these potential immune targets may provide true disease modification within or across neurodegenerative diseases. Among other markers, IL-1β and IL-15RA showed particularly strong predictive effects of shorter survival. These cytokines have been extensively described as being involved in the pathophysiology of different neurodegenerative diseases.[45,47–50]

This study has several limitations. First, patient groups were included and defined according to clinical diagnosis rather than definitive pathological confirmation; thus, misdiagnosis and/or co-pathologies may occur. In particular, amyloid markers were available only in patients with MCI and a portion of patients with LBD. As a result, AD pathology could not be consistently confirmed or excluded across the cohort. Second, blood-based inflammatory markers may capture comorbidities that are not directly linked to dementia, such as co-occurring inflammatory conditions or the use of specific anti-inflammatory medication. However, these are unlikely to occur in our cohort as the contributory clinical studies excluded recent systemic infections and concurrent autoimmune diseases. Of note, there was no transdiagnostic difference in C-reactive protein, a commonly used clinical marker of non-specific systemic inflammation. Third, our cohort was predominantly White/Caucasian, reflecting the ethnicity distribution of the > 65-year-old population in the UK (94% White in the 2021 national census). Further studies are needed to test the generalization of results to other groups and to identify environmental, genetic, and socioeconomic factors that might influence proteomic changes in dementia. Fourth, serum markers were measured at a single time point, while longitudinal studies would be needed to establish test–retest reliability and check disease-related dynamics over time. We have not accounted for disease stage or clinical severity at the time of blood collection, which may explain some of the heterogeneity seen across and within disease groups. Fifth, peripheral blood may not be reflective of the environment within the CNS, and patterns discovered may vary depending on blood–brain barrier integrity, which varies across pathologies. Sixth, while there is mixed evidence of temporal stability of p-tau epitopes, NfL, and GFAP, it remains to be seen if the remainder of the proteins highlighted by this multiplex approach are impacted by time-of-day and circadian rhythm or other factors such as posture, exercise, diet, or glymphatic clearance in sleep.[51–53] Seventh, a substantial proportion (approximately half) of patients were still alive at the time of analysis, so predictors of survival time may be characterized only partially (Table S3). Finally, it should be noted that the correlation analyses across analytical platforms were performed using two different sample types (serum for NULISA and plasma for Simoa), which may explain modest correlations for some of the markers.

In conclusion, we have shown that a multiplex approach offers consistent results compared to more targeted Simoas while simultaneously enabling a much broader scope of analysis. The NULISA CNS and inflammation panels identify disease-specific patterns of neurodegeneration and neuroinflammation. This unique disease fingerprint offers insight into disease-specific pathogenesis, with potential inferences that this dysregulation may be deleterious and amenable to drug treatment. Treatment strategies that are highly efficacious at removing amyloid from the brain have shown only moderate clinical efficacy; thus, it appears that alternative or indeed dual treatment strategies may be needed to tackle what are complex, multidimensional pathologies. This multiplex approach identified NfL, CRH, CD276, and inflammation signatures as useful prognostic markers across neurodegenerative diseases. The multiplex assay approach may offer scalable outcome measures for clinical trials in neurodegenerative diseases.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to report related to this work. Unrelated to this work, J.T.O. has received honoraria for work as DSMB chair or member for TauRx, Axon, Eisai, and Novo Nordisk; has acted as a consultant for Biogen and Roche; and has received research support from Alliance Medical and Merck. J.B.R. is a non-remunerated trustee of the Guarantors of Brain, Darwin College, and the PSP Association (UK). He provides consultancy unrelated to the current work to Asceneuron, Astronautx, Astex, Curasen, CumulusNeuro, Wave, and SVHealth and has research grants from AZ-Medimmune, Janssen, and Lilly as industry partners in the Dementias Platform UK. M.M. has acted as a consultant for Astex Pharmaceuticals. W.M. is an academic co-founder and consultant to Trimtech Therapeutics and has a research grant funded by Takeda Pharmaceuticals. H.Z. has served on scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Amylyx, Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, LabCorp, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave; has given lectures in symposia sponsored by Alzecure, Biogen, Cellectricon, Fujirebio, Lilly, Novo Nordisk, and Roche; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). A.H. has served as a consultant for Quanterix and been a paid panel member for Lilly. Author disclosures are available in the supporting information.

CONSENT STATEMENT

Participants with mental capacity gave their written informed consent to take part in the study according to the Declaration of Helsinki. For those who lacked capacity, their participation followed the personal consultee process in accordance with the UK law. The research protocols were approved by the National Research Ethics Service's East of England Cambridge Central Committee (REC references: 07/Q0102/3; 13/EE/0104; 18/EE/0042).

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SUPPORTING INFORMATION

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

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