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Plasma Synaptic Biomarkers and Clinical Progression in Mild-to-Moderate Alzheimer Disease

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Abstract

Background

Blood-based biomarkers of amyloid and tau pathology are transforming Alzheimer disease (AD) diagnosis. However, biomarkers reflecting neurobiological processes associated with severity and progression remain less well established.

Methods

We measured 129 plasma proteins using NULISA-Seq™ in 307 individuals with mild-to-moderate AD. Clinical Dementia Rating – Sum of Boxes (CDR-Sb), Alzheimer Disease Assessment Scale – Cognitive Subscale (ADAS-Cog) and Disability Assessment for Dementia (DAD) were assessed at baseline, 12 and 18 months. Baseline and longitudinal associations were examined using linear and mixed-effects regression with multiple testing correction and adjustment for important clinical covariates. Complementary elastic-net analysis incorporated bootstrap stability selection and Bayesian Information Criterion based model refinement. Prioritised proteins identified were then evaluated in addition to established AD biomarkers including brain-derived phosphorylated tau-217 (BD-pTau217), glial fibrillary acidic protein (GFAP) and neurofilament light (NfL).

Results

Forty-four proteins were associated with baseline severity and 22 with progression. Synaptic and neuronal network proteins emerged consistently across analyses, in particular Neuronal Pentraxin Receptor (NPTXR). The highest vs lowest NPTXR tertile was associated with lower baseline CDR-Sb (β: -1.83; 95% confidence interval − 2.70 to -0.96; $p < 0.001$) and slower CDR-Sb progression (β: -0.51; 95% confidence interval − 0.99, -0.03; $p = 0.04$ per year). NPTXR increased explained variance beyond clinical variables, BD-pTau217, GFAP and NfL by up to 8.0% at baseline and 6.0% longitudinally. In addition to NPTXR, Neuronal Pentraxin 2 (NPTX2) was consistently associated with baseline disease severity whilst Fatty acid binding protein 3 (FABP3) provided additional information on clinical progression. Associations between β-synuclein and clinical progression were more modest and attenuated in sensitivity analyses.

Conclusions

Synaptic and neuronal network proteins were consistently associated with AD severity and progression. NPTXR provided complementary information beyond established biomarkers, supporting NPTXR as a marker of synaptic dysfunction in the clinical expression of AD. These findings warrant confirmation in independent cohorts and further evaluation of their potential to inform prognosis in established AD.

Background

The development of fluid biomarkers accurately reflecting the pathological hallmarks of Alzheimer Disease (AD) has transformed the clinical-biological diagnosis of AD. High-sensitivity Blood-Based Biomarkers (BBM) have now been approved for clinical use. (1–4) Plasma phosphorylated tau-217 (p-tau217) has demonstrated high accuracy in identifying AD pathological changes, particularly amyloid-β pathology. (5–8) Emerging BBMs more closely reflecting tau tangle pathology, such as plasma endogenously cleaved, microtubule-binding region containing residue 243 (eMTBR-tau243) (9) are increasingly being evaluated alongside p-tau217 in clinical studies (10) and may enable more precise biological staging of AD. (11) These BBMs are associated with future clinical decline and neurodegeneration, and may therefore provide valuable prognostic information in established AD. (12–15)

However, established AD is characterised by marked heterogeneity in clinical progression. There is thus an unmet need for additional BBMs reflecting neurobiological processes beyond amyloid and tau pathology that may capture the downstream clinical expression of AD. These may include BBMs of synaptic integrity, neuronal network function, neuroinflammation, vascular and glial biology. (16–24) Developing such BBMs is challenging as many candidate proteins occur at very low concentrations in plasma, and established platforms have historically offered limited multiplexing capacity. (25) Recent advances in ultrasensitive multiplex proteomic technologies, including Nucleic Acid Linked Immuno-Sandwich Assay (NULISA™), enable the simultaneous measurement of large multiplex panels of low-abundance proteins across multiple neurobiological pathways relevant to AD. (26)

Studies using NULISA-Seq™ have demonstrated that, alongside p-tau217, glial fibrillary acidic protein (GFAP) and neurofilament light (NfL), the platform can detect plasma alterations across a broader range of AD-relevant biological processes, including synaptic, inflammatory, microglial and vascular pathways. (27) In preclinical and at-risk AD cohorts, NULISA-Seq™-derived p-tau217 accurately identifies AD pathology, whilst additional inflammatory and synaptic proteins showed associations with cognitive change, clinical severity and neuroimaging measures. (28–32) In a large NULISA-Seq™ study involving over 3,000 individuals across AD and other neurodegenerative conditions, 81 proteins were associated with AD diagnosis and 72 with clinical AD severity. (33) These findings implicated synaptic and neuronal dysfunction, glial activation, inflammatory signalling and cytoskeletal pathways. (33) Most recently, multiplex NULISA-Seq™ signatures have shown utility in discriminating dementia aetiologies, identifying co-pathological signatures, and even estimating neocortical tau burden in individuals with established amyloid pathology. (34–36) Such panels may have an important role beyond diagnosis in characterising biological heterogeneity in AD.

Comparatively fewer studies have examined the prognostic utility of NULISA-derived plasma signatures reflecting different processes beyond amyloid and tau in established AD. A recent study from the BALTAZAR cohort examined paired cerebrospinal fluid (CSF) and plasma NULISA-Seq™ profiles and conversion to dementia over three years in 173 individuals with mild cognitive impairment. (37) In CSF,

markers of neurodegeneration, including NfL, and synaptic dysfunction, particularly neuronal pentraxin 2 (NPTX2), outperformed p-tau217 for predicting conversion to dementia. In contrast to CSF, plasma combinations provided only modest incremental prognostic information, highlighting the continued need to identify robust BBMs of biological processes associated with clinical progression (37). Another recent NULISA-Seq™ study has demonstrated a role for both plasma acetylcholinesterase (ACHE) and plasma neuronal pentraxin receptor (NPTXR) beyond p-tau217 in risk stratification for conversion to symptomatic AD. (38) Together, these findings suggest that emerging BBMs of synaptic dysfunction and neuronal network biology may provide complementary information beyond core AD pathology. However, their relationship with subsequent clinical progression in individuals with established symptomatic AD remains less well characterised.

In the current study, we took advantage of the ultrasensitive nature and high-multiplexing capability of NULISA-Seq™ to measure 129 plasma proteins in a well-characterised cohort of individuals with mild-to-moderate dementia due to AD, with repeated assessments of global disease severity, cognition and functional ability over 18 months. We aimed to identify BBMs associated with both baseline AD severity and subsequent clinical progression, and to determine whether prioritised candidate biomarkers provided incremental explanatory information beyond more established and well-studied BBMs including plasma p-tau217, GFAP and NfL.

Methods

Study Setting & Design

The current analysis used biological samples and clinical data from the NILVAD Study. NILVAD was a phase 3 investigator-led clinical trial of the antihypertensive Nilvadipine, which did not demonstrate an effect of Nilvadipine on its primary endpoint of change in cognitive function/dementia severity in individuals with mild-to-moderate dementia due to AD. (39) For inclusion, participants had a Neurological and Communicative Disorders and Stroke - Alzheimer's Disease (NINCDS-ADRA) diagnosis of AD with a baseline Mini-Mental State Examination score of 12–26. (40) Exclusion criteria included significant comorbid neurological, cardiovascular or psychiatric disorder, active substance misuse or significant liver/renal impairment. As Nilvadipine had no effect on the primary outcome, participants from both treatment groups were included in the present analysis, with treatment allocation included as a covariate in longitudinal models. The NILVAD study was powered to detect a treatment effect of *Nilvadipine* on AD progression over 18 months. No separate sample-size calculation was undertaken for this secondary biomarker analysis and all available plasma samples from participants taking part in the blood and genetic substudy of NILVAD were included.

Clinical and Dementia Severity Assessment

Baseline assessment ascertained sociodemographic characteristics (age, sex, body mass index, years of education), AD characteristics (diagnosis duration, symptom duration) and medical comorbidity (adapted version of the Charlson Comorbidity Index for individuals with dementia). (41) Sex was recorded as either male or female based on clinical records and no gender identity/ethnicity data were collected in the NILVAD study. AD severity was assessed using the Clinical Dementia Rating Scale – Sum of Boxes (CDR-Sb), the Alzheimer's Disease Assessment Scale – Cognitive Subscale (ADAS-Cog) and the Disability Assessment for Dementia (DAD). (42–44) Assessments of dementia severity took place at baseline, 12 and 18 months.

Blood Sampling

All participants in NILVAD were eligible to participate in the NILVAD sub-studies which included the blood and genetic sub-study from which biological samples for the current study were used. (45) Briefly, for those participating in the blood and genetic sub-study, 30 mL of blood was donated at baseline, 12-month and 18-month study visits using standard aseptic venepuncture. Blood was processed immediately on site. For plasma sample processing, 2 x 10 mL EDTA tubes were obtained and centrifuged for *5 min at 1380 x g*. Plasma was then stored at -80^{O}C in 1 mL aliquots. Samples were stored locally on processing, and subsequently transferred on temperature-monitored dry ice shipments to a central biobank repository in Centre Hospitalier Universitaire de Lille in Lille, France in monitored –80^{O}C freezers. For the current analysis, samples from all individuals with available plasma at baseline (before randomisation to Nilvadipine or placebo) were obtained and sent to the Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn for analysis using NULISA-Seq™ technology. Fresh sample aliquots which had not undergone any prior freeze-thaw cycles were used for this analysis.

NULISA-Seq™ Measurements

Plasma samples were thawed and plated onto 96-well plates. The NULISA CNS 120 panel was run on an ARGO™ HT system according to manufacturer's instructions at the Institute of Clinical Chemistry and Chemical Pathology, University of Bonn, Germany. Baseline samples were randomly distributed across 4 plates in addition to pooled controls to estimate inter- and intra-assay coefficients of variation (CVs). Laboratory personnel were blinded to clinical data and outcomes. Libraries were sequenced using the AVITI Sequencing Cloudbreak FS Medium (AVITI) from Element Biosciences. Output files were processed to generate NULISA Protein Quantification (NPQ) for each biomarker. Assay precision was assessed by pooled plasma samples from the NILVAD study from 12 donors selected to represent the extremes of age, education, body mass index, with an equal distribution of males and females. This pool was included in each assay plate in order to calculate CVs. The intra-assay CV was calculated from duplicate pooled samples on the same plate and the inter-assay CV from pooled samples across the four plates. Analytes with both inter and intra-assay CVs > 20% were deemed not to pass quality control and excluded from further analysis.

Statistical Analysis

Descriptive statistics included means with standard deviations, medians with interquartile ranges (IQR) and frequencies with percentages as appropriate. Plasma samples with low sequencing reads were excluded from further analysis, in line with manufacturer's recommendations and those with available NULISA-Seq™ data included. NPQ data expressed on a log-2 scale were subsequently z-scored prior to analyses to facilitate comparison of effect estimates across proteins.

For the primary analysis, linear regression was initially used to assess associations between each protein (independent variable) and baseline CDR-Sb/ADAS-Cog/DAD score (dependent variable). For all analyses, CDR-Sb was considered the primary clinical outcome of interest with ADAS-Cog and DAD considered co-secondary outcomes. The Benjamini-Hochberg (BH) method was used to control for multiple testing. Proteins significant following BH correction were subsequently examined for pairwise correlation using Spearman correlation coefficients. To reduce redundancy prior to multivariable modelling, highly correlated proteins (Spearman $\rho \geq 0.70$) were reviewed using a structured data-reduction approach incorporating the strength and consistency of associations across clinical outcomes and biological plausibility. Representative proteins retained following this process were carried forward to multivariable models. Multivariable models first included all other retained proteins carried forward and variance-inflation-factors (VIFs) and residual-vs-fit/Q-Q plots examined for multicollinearity (VIFs < 2.0 were considered low evidence of multicollinearity). Subsequently, multivariable models were also adjusted for age, sex, BMI, education and medical comorbidity. Results are reported as regression coefficients (β), 95% Confidence Intervals (95% CI) and p-values.

Longitudinal associations between baseline plasma proteins and subsequent clinical progression were assessed using mixed-effects linear regression, with a participant as a random effect. Change from baseline in CDR-Sb/ADAS-Cog/DAD was modelled as the dependent variable, with *biomarker*time(in years)* interaction term as the independent variable representing the association between baseline biomarker concentration and subsequent rate of change in clinical severity. The BH method was again used to adjust for multiple testing. Proteins remaining significant again underwent correlation-based data reduction as above and retained biomarkers carried forward for multivariable analysis. Mixed-effects multivariable analysis first examined all retained proteins, with models examined for multicollinearity as above, with subsequent adjustment for important clinical covariates age, sex, education, BMI, comorbidity, baseline cognitive severity score (on CDR-Sb/ADAS-Cog/DAD as appropriate) and study group (Nilvadipine vs placebo). Results for the *biomarker*time(in years)* (independent variable) are reported as β, 95% CIs and p-values as appropriate.

As a complementary secondary analysis to prioritise proteins of interest for further evaluation in the presence of substantial correlation between groups of similar proteins, penalised elastic-net regression with bootstrap stability was used. Separate Gaussian elastic-net models were fitted for each measure of disease severity separately (CDR-Sb, ADAS-Cog, DAD). An alpha level of $\alpha = 0.5$ was used. For baseline models, clinical covariates (age, sex, BMI, education, comorbidity) were included as unpenalized

variables whilst plasma proteins were penalised. The optimal penalty parameter (λ_{min}) was selected by five-fold cross-validation. Stability of protein selection was assessed across 300 resamples, with selection frequency defined as the proportion of bootstrap samples in which a protein retained a non-zero coefficient at λ_{min}. Proteins selected at ≥ 80% of bootstrap iterations were considered bootstrap-stable. Median elastic-net coefficients across bootstrap iterations were used to describe the direction/relative magnitude of associations. As a secondary model-reduction step, backward Bayesian Information Criterion (BIC)-based model selection was subsequently applied to proteins meeting this ≥ 80% stability threshold for each clinical outcome. BIC was used to identify a more parsimonious subset of the bootstrap-stable proteins while penalising increasing model complexity.

For longitudinal associations, a corresponding elastic-net analysis was conducted to identify baseline proteins associated with subsequent clinical progression. Baseline *protein*time* interactions were penalised terms whilst age, sex, BMI, education, comorbidity, baseline severity score and study group were included as unpenalized terms. Five-fold cross validation was performed at participant level. Stability selection was based on 300 participant-level bootstrap resamples. An alpha level of $\alpha = 0.5$ was used and λ_{min} selected as above, with ≥ 80% selection frequency considered bootstrap-stable selection. Again, BIC-based model selection was used as a secondary model reduction step to produce a smaller subset of bootstrap-stable proteins.

Following the primary conventional regression and complementary elastic-net analyses, six proteins were prioritised for focused evaluation based on established biomarker status and convergence across conventional regression, bootstrap stability selection and BIC-based model refinement. These comprised the established AD biomarkers BD-pTau217, GFAP and NfL, together with NPTXR, NPTX2 and FABP3 for baseline analyses, and NPTXR, FABP3 and β-synuclein (SNCB) for longitudinal analyses. Biomarkers were first examined unadjusted, followed by adjustment for the other five selected biomarkers (Model 2) and followed by adjustment for the other five biomarkers and additional clinical variables as described above (Model 3). Associations persisting in fully-adjusted models were subsequently examined and visualised in tertiles to aid clinical interpretation of effects.

To examine the incremental explanatory value of prioritised candidate biomarkers, their performance was examined using sequential nested models. For baseline outcomes, a clinical model including age, sex, BMI, education and comorbidity was first fitted. BD-pTau217, GFAP and NfL were each individually added to the clinical model to examine their incremental contribution before all three were fitted to an established biomarker model. Each prioritised biomarker (NPTXR, NPTX2 and FABP3) was then added individually to the established-biomarker model, followed by a final model containing all prioritised candidate biomarkers. Incremental R^2 was calculated and nested models compared using partial F-tests. For longitudinal outcomes, the corresponding approach used linear mixed-effects models, with clinical models additionally including treatment group and baseline disease severity. Again, BD-pTau217, GFAP and NfL were first examined individually and subsequently together as an established biomarker model. Each prioritised candidate (NPTXR, SNCB, FABP3) was then added individually to the established

biomarker, followed by a final model containing all prioritised candidate biomarkers. Marginal R^2 was calculated using the Nakagawa method, and nested models compared using likelihood-ratio tests evaluating the joint contribution of biomarker main effect and *biomarker*time* interaction. The corresponding *biomarker*time* interaction coefficient was used to determine the direction and magnitude of associations with subsequent clinical progression.

As NILVAD recruitment was based on clinical criteria alone (NINCDS-ADRA) and AD diagnosis has evolved to a clinical-biological diagnosis (46), we conducted sensitivity analysis based on plasma levels of p-tau217 consistent with a very high-likelihood of AD. For these analyses, we used a validated in-house cut-off of p-Tau217 > 6.01 pg/mL on the Meso Scale Discovery S-PLEX® assay for plasma p-Tau217 consistent with a very high likelihood (97.5% specificity) of amyloid pathology – as previously described (12, 47, 48). Analyses for biomarkers significantly associated with baseline disease severity and longitudinal decline were thus repeated only in individuals with very high likelihood of AD pathology.

All tests were two-sided and $p < 0.05$ considered statistically significant. Analyses were performed in RStudio 2024.04.2 + 764 and STATA v17.0/BE. Ethical approval was granted from appropriate National Competent Authorities, Independent Ethics Committees and Institutional Review Boards for all 23 sites. A full list of Independent Ethics Committees and Institutional Review Boards are provided in Supplementary Material (Table S1).

Results

Participant Characteristics

In total, 327 participants with mild-to-moderate dementia due to AD from the NILVAD sub-study had baseline plasma samples available for analysis. After exclusion of 20 samples with low sequencing reads, 307 were included. Included participants had a mean age of 72.4 (± 8.3) years and 62.9% (n = 193/307) were female. Median AD diagnosis duration was just under one year (0.92; IQR: 0.4–2.1 years). Baseline dementia severity was 5.2 (± 2.7) on the CDR-Sb, 34.5 (± 10.7) on the ADAS-Cog and 29.7 (± 7.8) on the DAD (Supplementary Table S2). Of 307 participants, 278 had dementia severity assessed at 12 months and 275 were assessed at 18 months. Nilvadipine treatment was not associated with longitudinal change in CDR-Sb (β: 0.05; -0.51, 0.61, p = 0.86), ADAS-Cog (β: 0.11; -1.78, 2.01, p = 0.91) or DAD (β: 0.50; -0.90, 1.91, p = 0.48) (Figure S1) and participants receiving Nilvadipine and placebo were pooled for biomarker analysis. Across both groups, participants demonstrated clinically-significant progression on the CDR-Sb (mean change: +3.7 ± 3.11), ADAS-Cog (mean change: +9.5 ± 9.4) and DAD (mean change: -8.3 ± 8.5).

Analysis of Baseline Plasma Samples Using NULISA-Seq™

Baseline plasma samples were analysed using the NULISA-Seq™ CNS 120 + Panel. Following manufacturer's quality control recommendations, 20 plasma samples were excluded due to low read

counts, distributed equally across assay plates. Excluded samples did not differ by age, sex, BMI or disease severity. Analytical precision was generally very high for nearly all 131 analytes (Table S3). Two analytes, VGF and SFRP1, had both intra and inter-assay CVs > 20% and were excluded from analysis, leaving 129 proteins (Figure S2).

Plasma Synaptic Biomarkers are Associated with Baseline AD Severity

Cross-sectional associations between 129 plasma proteins and baseline disease severity were first examined using univariate linear regression. Following correction for multiple testing, 35 proteins were associated with AD severity on the CDR-Sb, 28 on the ADAS-Cog and 18 on the DAD - reflecting 44 unique proteins (Fig. 1A). Associations spanned multiple biological domains including protein aggregation, inflammation, energy homeostasis, proteostasis and DNA/RNA dysfunction (Fig. 1B). As many candidate proteins were highly correlated (Fig. 1C), a structured data reduction strategy prioritised proteins for further analysis according to the strength and consistency of their association across clinical outcomes together with biological plausibility before multivariable modelling. This prioritised reproducible biological signals whilst reducing redundancy from highly correlated analytes. The rationale for retaining/excluding individual proteins for further analysis is provided in detail in Table S4 (Supplementary Material).

<Insert Fig. 1 Here>

Of the 44 unique proteins associated with one or more measure of baseline disease severity, 18 biologically representative proteins spanning neurodegeneration, synaptic biology, inflammation, energy homeostasis and stress-response pathways were retained for multivariable analysis (Fig. 1D). In multivariable analysis, BD-pTau217 remained the strongest independent association with ADAS-Cog severity after adjustment for the other representative biomarkers and clinical covariates (Fig. 1E). Of the novel biomarkers, lower NPTXR and greater NPTX2 demonstrated consistent independent associations with greater baseline disease severity across all three measures (CDR-Sb, ADAS-Cog, DAD), which remained significant following clinical covariate adjustment (Fig. 1E). Additional outcome-specific associations are visualised in Fig. 1.

Next, as a complementary secondary analysis approach, we used elastic net regression as a feature selection approach to identify proteins independently associated with baseline disease severity whilst accounting for collinearity between markers (Fig. 2A). Mirroring the primary analysis, NPTXR emerged as the most consistently selected novel biomarker across the three clinical outcomes (Fig. 2B). Additional synaptic and neuronal network proteins, including CNTN2, ACHE and PDLIM5 were also selected with high frequency. Among established AD biomarkers, BD-pTau217 demonstrated particularly consistent selection for ADAS-Cog, whilst GFAP and NfL were also frequently retained in models of disease severity.

<Insert Fig. 2 Here>

The effect size and direction of the median elastic-net coefficients further highlighted the importance of synaptic and neural network biomarkers, and in particular neuronal pentraxins (Fig. 2C), with NPTXR consistently retained across baseline CDR-Sb, ADAS-Cog and DAD models, with lower NPTXR levels associated with greater disease severity. When proteins selected in 80% or more of bootstrap iterations were grouped according to biological category, synaptic and neuronal network proteins were the most consistently represented category for the CDR-Sb and ADAS-Cog, whilst proteins in the inflammatory category predominated in the DAD models (Table S5). Proteins selected in 80% or more of bootstrap iterations were subjected to backward BIC-based selection (Table S6). NPTXR and NPTX2 were the only proteins retained across all three measures of baseline disease severity, supporting the consistent neuronal pentraxin signal identified using conventional regression and elastic-net analysis (Table S6).

Plasma Synaptic Biomarkers and Subsequent Clinical Progression

Next, longitudinal associations between baseline plasma proteins and subsequent clinical progression were examined. Following correction for multiple testing, baseline concentrations of 18 proteins were associated with CDR-Sb progression, 19 with ADAS-Cog progression and 15 with DAD progression - representing 22 unique proteins (Fig. 3A). These proteins spanned pathological protein aggregation, synaptic and neuronal network dysfunction, inflammation, energy homeostasis and vascular/extracellular matrix biology (Fig. 3B). A structured data reduction strategy was again used, given that several proteins (particularly phosphorylated tau isoforms) were highly correlated (Fig. 3C). Of the 22 proteins, 16 were carried forward for multivariable analysis (Fig. 3D). The rationale for retaining/excluding individual proteins is provided in Table S7 (Supplementary Material).

<Insert Fig. 3 Here>

Among the 16 retained proteins, the strongest longitudinal associations were observed for the established biomarkers BD-pTau217 and NfL which were associated with decline across multiple outcome measures (Fig. 3). Amongst the novel biomarkers, NPTXR again demonstrated consistent independent associations with progression on both CDR-Sb and DAD. β-synuclein (SNCB) was also independently associated with accelerated decline on both CDR-Sb and DAD (Fig. 3). The synaptic biomarker Glial Cell Line-Derived Neurotrophic Factor (GDNF) and the protein aggregation marker phosphorylated α-synuclein (pS129-α-synuclein) were also associated with longitudinal progression. Outside of synaptic/neuronal network and protein aggregation markers, additional associations were observed for proteins from inflammatory (CRP, GFAP, CCL3), metabolic (FABP3) and proteostasis (MME) categories with clinical severity measures (Fig. 3).

As a complementary secondary approach, elastic net regression with bootstrap stability selection was also used to identify combinations of baseline proteins associated with subsequent clinical progression (Fig. 4A). NfL demonstrated the highest selection frequency for CDR-Sb and DAD progression, whereas FOLR1 predominated for progression on the ADAS-Cog (Fig. 4B). Among novel biomarkers, NPTXR was highly stable for CDR-Sb and DAD progression and was also selected in ADAS-Cog models. SNCB was

frequently selected across both CDR-Sb and DAD progression, mirroring results from the primary analysis. Additional synaptic proteins including GDNF, CNTN2, TREM2, IGF1R, and AGRN were reproducibly retained across bootstrap resamples (Fig. 4B).

<Insert Fig. 4 Here>

When proteins selected in 80% or more of bootstrap iterations were grouped by biological function, synaptic and neuronal network proteins represented the predominant category across all three clinical outcomes, and in particular for CDR-Sb progression (Fig. 4D). Full results are provided in Supplementary Table S8. Using backward BIC-based selection, NfL and FABP3 were retained across all three longitudinal outcomes, whilst NPTXR was retained for CDR-Sb and DAD progression and BD-pTau217 for ADAS-Cog and DAD (Table S9), reflective of the primary analysis.

Clinically-Interpretable Analysis of Candidate Protein Associations

To facilitate clinical interpretation of above results, six representative proteins were selected for further analysis based on the primary and secondary complementary analyses above. These included three established biomarkers of AD pathology (BD-pTau217), neuro-axonal injury (NfL) and neuro-inflammation (GFAP) for both baseline and longitudinal analysis. The three strongest/most robust candidate proteins for baseline (NPTXR, NPTX2 and FABP3) and longitudinal (NPTXR, SNCB and FABP3) analyses were also taken forward for further analysis.

At baseline, six associations remained significant after adjustment for the other five selected biomarkers and important clinical covariates. These comprised greater BD-pTau217 and greater ADAS-Cog severity, lower levels of NPTXR and greater disease severity across all three severity measures; and greater NPTX2 with greater disease severity on the CDR-Sb and DAD remained significant (Supplementary Table S10). For these six associations, biomarker tertiles were then used to aid interpretation. Under fully-adjusted models including clinical covariate adjustment, the highest (vs lowest) tertile of BD-pTau217 was associated with significantly greater baseline severity on the ADAS-Cog (β: 6.75; 95% CI: 3.36, 10.15; $p < 0.001$). The highest (vs lowest) tertile of NPTXR was associated with lesser disease severity on the CDR-Sb (β: -1.83; 95% CI: -2.70, -0.96; $p < 0.001$), ADAS-Cog (β: -6.98; 95% CI: -10.27, -3.69; $p < 0.001$) and DAD (β: 5.16; 95% CI: 2.67, 7.65; $p < 0.001$). The highest tertile of NPTX2 was associated with greater disease severity on the CDR-Sb (β: 0.93; 95% CI: 0.07, 1.79; $p = 0.03$) and DAD (β: -3.71; 95% CI: -6.18, -1.23; $p < 0.001$). Results are visualised in Fig. 5 and included in full in Table S11 (Supplementary Material).

<Insert Fig. 5 Here>

To evaluate whether the biomarkers NPTX2, NPTXR and FABP3 provided additional explanatory value in addition to established biomarkers, each candidate was separately added to a reference model containing clinical covariates, BD-pTau217, GFAP and NfL. NPTXR was the only candidate biomarker to

significantly improve model fit across all three clinical outcomes, increasing explained variance by 7.0%, 8.1% and 4.5% on the CDR-Sb, ADAS-Cog and DAD respectively (all nested models $p < 0.001$; Table 1).

Table 1
Incremental Explanatory Value of Plasma Biomarkers for Baseline Clinical Severity

Outcome	Model Covariates	Variable Added	R^2	ΔR^2	P
CDR-Sb	Clinical covariates	—	0.03	—	—
	Clinical covariates + BD-pTau217	BD-pTau217	0.04	0.01	0.101
	Clinical covariates + GFAP	GFAP	0.03	0.00	0.237
	Clinical covariates + NfL	NfL	0.03	0.00	0.411
	Clinical covariates + BD-pTau217 + GFAP + NfL	BD-pTau217 + GFAP + NfL	0.04	0.01	0.420
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTXR	**NPTXR**	**0.11**	**0.07**	**< 0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTX2	NPTX2	0.04	0.01	0.212
	Clinical covariates + BD-pTau217 + GFAP + NfL + FABP3	FABP3	0.04	0.00	0.410
ADAS-Cog	Clinical covariates	—	0.05	—	—
	Clinical covariates + BD-pTau217	**BD-pTau217**	**0.14**	**0.09**	**< 0.001**
	Clinical covariates + GFAP	**GFAP**	**0.09**	**0.04**	**< 0.001**
	Clinical covariates + NfL	**NfL**	**0.07**	**0.02**	**0.017**
	Clinical covariates + BD-pTau217 + GFAP + NfL	**BD-pTau217 + GFAP + NfL**	**0.14**	**0.09**	**< 0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTXR	**NPTXR**	**0.22**	**0.08**	**< 0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTX2	NPTX2	0.14	0.00	0.952
	Clinical covariates + BD-pTau217 + GFAP + NfL + FABP3	**FABP3**	**0.17**	**0.03**	**0.002**
DAD	Clinical covariates	—	0.04	—	—
	Clinical covariates + BD-pTau217	BD-pTau217	0.04	0.00	0.283

Outcome	Model Covariates	Variable Added	R^2	ΔR^2	P
	Clinical covariates + GFAP	**GFAP**	**0.06**	**0.02**	**0.016**
	Clinical covariates + NfL	NfL	0.05	0.01	0.055
	Clinical covariates + BD-pTau217 + GFAP + NfL	BD-pTau217 + GFAP + NfL	0.06	0.02	0.088
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTXR	**NPTXR**	**0.11**	**0.04**	**< 0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTX2	NPTX2	0.07	0.01	0.136
	Clinical covariates + BD-pTau217 + GFAP + NfL + FABP3	FABP3	0.07	0.01	0.056

Values are derived from nested linear regression models. All models included the clinical covariates age, sex, body mass index, education and comorbidity. BD-pTau217, GFAP and NfL were first evaluated individually by adding each biomarker separately to the clinical covariate model. Their combined contribution was evaluated by adding all three established biomarkers as a block to the clinical model. Each candidate biomarker (NPTXR, NPTX2 and FABP3) was then evaluated separately against the same reference model containing the clinical covariates and all three established biomarkers. Biomarker concentrations were standardised as z-scores before analysis. R^2 denotes the proportion of outcome variance explained by each model. ΔR^2 denotes the increase in R^2 relative to the relevant reference model: the clinical model for individual established biomarkers and the established-biomarker block, and the clinical plus established-biomarker model for individual candidate biomarkers and the candidate-biomarker. P-values were derived from partial F-tests comparing each expanded model with its specified nested reference model. Significant results are highlighted in bold. CDR-Sb, Clinical Dementia Rating–Sum of Boxes; ADAS-Cog, Alzheimer's Disease Assessment Scale–Cognitive Subscale; DAD, Disability Assessment for Dementia; BD-pTau217, brain-derived phosphorylated tau 217; GFAP, glial fibrillary acidic protein; NfL, neurofilament light; NPTXR, neuronal pentraxin receptor; NPTX2, neuronal pentraxin 2; FABP3, fatty acid-binding protein 3.

<Insert Table 1 Here>

For longitudinal analyses, 11 associations remained significant after adjustment for five selected biomarkers and clinical covariates. BD-pTau217 was associated with significant progression across all three outcomes, whilst NfL and GFAP were independently associated with CDR-Sb progression (Supplementary Table S12). Of the novel biomarkers, greater NPTXR was associated with significantly slower disease progression on the CDR-Sb and DAD and greater SNCB with greater disease progression on both the CDR-Sb and DAD, which persisted after adjustment for other biomarkers and important clinical covariates (Supplementary Table S11). Finally, greater FABP3 was significantly associated with slower CDR-Sb and DAD decline (Supplementary Materials; Table S11).

Biomarker tertiles were again examined to aid clinical interpretation. Under fully-adjusted models (for the other five biomarkers and clinical covariates), the highest (vs lowest) tertile of BD-pTau217 was associated with greater yearly decline on the CDR-Sb (β: 0.93; 95% CI 0.47, 1.40; $p < 0.001$), ADAS-Cog (β:

6.36; 95% CI 2.55, 10.15; p = 0.001) and DAD (β: -3.02; 95% CI -4.31, -1.74; p < 0.001). The highest (vs lowest) tertile of NfL and GFAP were independently associated with yearly decline on the CDR-Sb (β: 0.63; 95% CI 0.16, 1.10; p = 0.01 for NfL; β: 0.83; 95% CI 0.36, 1.30; p < 0.001 for GFAP). The highest (vs lowest) tertile of NPTXR was associated with significantly slower yearly progression on the CDR-Sb (β: -0.51; 95% CI -0.99, -0.03; p = 0.04) and DAD (β: 1.71; 95% CI: 0.39, 3.02; p = 0.01). The highest (vs lowest) tertile of SNCB was associated with significantly greater yearly decline on the CDR-Sb (β: 0.65; 95% CI: 0.18, 1.11; p = 0.01) and DAD (β: -1.34; 95% CI: -2.62, -0.05; p = 0.04). Finally, the highest tertile of FABP3 was significantly associated with slower yearly progression on the CDR-Sb (β: -0.72; 95% CI: -1.19, -0.25; p = 0.003) and DAD (β: 2.44; 95% CI: 1.15, 3.73; p < 0.001). Results of all models are provided in Supplementary Material (Table S13) and Fig. 6.

<Insert Fig. 6 Here>

For longitudinal analyses, each candidate biomarker was separately added to a mixed-effects reference model containing clinical covariates, BD-pTau217, GFAP and NfL. NPTXR increased marginal R^2 by 6.0%, 1.0% and 6.0% for CDR-Sb, ADAS-Cog and DAD, respectively, while FABP3 produced corresponding increases of 6.0%, 3.0% and 4.0% (all p < 0.001). SNCB demonstrated more limited incremental value, increasing marginal R^2 only for CDR-Sb progression (2.0%, p = 0.03; Table 2)

Table 2
Incremental explanatory value of plasma biomarkers for longitudinal clinical progression.

Outcome	Model Covariates	Variable Added	Marginal R^2	Δ Marginal R^2	P
CDR-Sb	Clinical covariates	—	0.10	—	—
	Clinical covariates + BD-pTau217	**BD-pTau217**	**0.14**	**0.03**	**0.001**
	Clinical covariates + GFAP	**GFAP**	**0.14**	**0.04**	**0.001**
	Clinical covariates + NfL	**NfL**	**0.15**	**0.05**	**< 0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL	**BD-pTau217 + GFAP + NfL**	**0.17**	**0.06**	**< 0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTXR	**NPTXR**	**0.22**	**0.06**	**< 0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL + SNCB	**SNCB**	**0.18**	**0.02**	**0.026**
	Clinical covariates + BD-pTau217 + GFAP + NfL + FABP3	**FABP3**	**0.23**	**0.06**	**< 0.001**
ADAS-Cog	Clinical covariates	—	0.09	—	—
	Clinical covariates + BD-pTau217	**BD-pTau217**	**0.11**	**0.02**	**< 0.001**
	Clinical covariates + GFAP	GFAP	0.09	0.01	0.218
	Clinical covariates + NfL	NfL	0.10	0.01	0.174
	Clinical covariates + BD-pTau217 + GFAP + NfL	**BD-pTau217 + GFAP + NfL**	**0.12**	**0.03**	**0.005**
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTXR	**NPTXR**	**0.13**	**0.01**	**0.018**
	Clinical covariates + BD-pTau217 + GFAP + NfL + SNCB	SNCB	0.12	0.00	0.533
	Clinical covariates + BD-pTau217 + GFAP + NfL + FABP3	**FABP3**	**0.15**	**0.03**	**0.002**

Outcome	Model Covariates	Variable Added	Marginal R^2	Δ Marginal R^2	P
DAD	Clinical covariates	—	0.09	—	—
	Clinical covariates + BD-pTau217	**BD-pTau217**	**0.12**	**0.04**	**< 0.001**
	Clinical covariates + GFAP	**GFAP**	**0.10**	**0.01**	**0.019**
	Clinical covariates + NfL	**NfL**	**0.11**	**0.02**	**0.029**
	Clinical covariates + BD-pTau217 + GFAP + NfL	**BD-pTau217 + GFAP + NfL**	**0.14**	**0.05**	**0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL + NPTXR	**NPTXR**	**0.19**	**0.06**	**< 0.001**
	Clinical covariates + BD-pTau217 + GFAP + NfL + SNCB	SNCB	0.14	0.01	0.382
	Clinical covariates + BD-pTau217 + GFAP + NfL + FABP3	**FABP3**	**0.18**	**0.04**	**< 0.001**

Values are derived from maximum-likelihood linear mixed-effects models with a participant-specific random intercept. The clinical model included continuous time, age, sex, body mass index, education, comorbidity score, study group and the corresponding baseline severity score (CDR-Sb, ADAS-Cog or DAD). Each established plasma biomarker (BD-pTau217, GFAP and NfL) was first evaluated separately by adding its main effect and biomarker × time interaction to the clinical model. Their joint contribution was evaluated by adding all three established biomarkers and their respective time interactions as a block. Each candidate biomarker identified through the discovery and machine-learning analyses (NPTXR, SNCB and FABP3) was then evaluated separately against the same reference model containing the clinical covariates and all three established biomarkers. Marginal R^2 represents the proportion of variance explained by the fixed effects. Δ marginal R^2 represents the increase relative to the relevant reference model: the clinical model for individual established biomarkers and the established-biomarker block, and the clinical plus established-biomarker model for individual candidate biomarkers. P values were obtained from likelihood-ratio tests and evaluate the joint incremental contribution of each added biomarker main effect and biomarker × time interaction, or all corresponding terms in block comparisons. CDR-Sb, Clinical Dementia Rating–Sum of Boxes; ADAS-Cog, Alzheimer's Disease Assessment Scale–Cognitive Subscale; DAD, Disability Assessment for Dementia; BD-pTau217, brain-derived phosphorylated tau 217; GFAP, glial fibrillary acidic protein; NfL, neurofilament light; NPTXR, neuronal pentraxin receptor; SNCB, β-synuclein; FABP3, fatty acid-binding protein 3.

<Insert Table 2 Here>

Sensitivity Analysis of Findings by AD Pathology

Given that recruitment to the NILVAD study was based on clinical criteria for AD, we next repeated analysis of key findings in only those individuals with plasma p-tau217 levels consistent with a very-high

likelihood of AD pathology (Specificity > 97.5%). At this threshold, 90.9% (279/307) of participants had plasma p-tau217 levels consistent with AD pathology. On repeating analysis only in those with very high likelihood of AD pathology, BD-pTau217 retained its associations with baseline disease severity and subsequent clinical progression, whilst NPTXR continued to demonstrate robust independent associations with baseline disease severity and clinical progression. Similarly, NPTX2 was consistently associated with baseline disease severity and FABP3 retained its association with longitudinal progression. Effect estimates were similar or slightly larger in those with very high likelihood of AD. Longitudinal associations for SNCB were somewhat attenuated. Tables S14-S17 (Supplementary Material) provide results of the sensitivity analysis.

Discussion

We used high-sensitivity multiplex plasma proteomics to comprehensively examine biomarkers associated with disease severity and clinical progression in mild-to-moderate AD. Complementary conventional regression, elastic-net modelling with bootstrap stability selection and BIC-based model refinement consistently highlighted associations between biomarkers of synaptic and neuronal network dysfunction and both baseline clinical severity and clinical progression over 18 months. NPTXR emerged as the most consistent novel biomarker, demonstrating independent associations with baseline disease severity and subsequent clinical progression. Importantly, NPTXR consistently added additional explanatory information beyond clinical characteristics and more well-studied biomarkers such as BD-pTau217, NfL and GFAP. FABP3 was also independently associated with longitudinal AD progression. Several additional proteins also identified through this approach demonstrated outcome-specific associations. Together, these findings highlight the potential importance of synaptic and neuronal network dysfunction in the clinical expression and progression of established AD.

The most consistent novel finding was observed for NPTXR, a member of the neuronal pentraxin family of proteins with important roles in synaptic organisation and plasticity.(49, 50) Neuronal pentraxins are involved in synapse formation and remodelling during activity-dependent plasticity, and NPTXR is enriched at excitatory glutamatergic synapses, where it contributes to synaptic organisation.(51) Several early proteomic studies identified NPTXR as reduced in the CSF in both clinically and biologically-defined AD compared to healthy controls (52–55) and may be associated with disease stage and clinical progression. (56) (55, 57) More recent data from over 600 individuals in two independent cohorts demonstrated associations between lower baseline CSF NPTXR, accelerated brain atrophy and progression from MCI to dementia.

Comparatively fewer studies have demonstrated associations between plasma NPTXR and AD, potentially reflecting limitations in analytical sensitivity in plasma. (37) However, lower NPTXR was recently demonstrated in individuals with AD in a large NULISA-Seq™ study of over 3,000 samples spanning healthy controls and multiple neurodegenerative disorders. (33) NPTXR was also amongst the most consistently selected protein in a NULISA-Seq™ multi-protein biomarker signature identifying neocortical tau tangle pathology with high accuracy against tau-PET (36). Additionally, a recent study

demonstrated that plasma NPTXR provided prognostic information beyond p-tau217 in predicting progression from cognitively unimpaired to symptomatic AD.(38) Our findings extend this literature by demonstrating that lower NPTXR is associated with not only greater baseline clinical severity, but also faster subsequent progression in established symptomatic AD, independent of BD-pTau217, GFAP and NfL. Moreover, NPTXR provided additional explanatory information beyond these established biomarkers across multiple clinical outcomes. Together, these findings support the further evaluation of NPTXR as a complementary marker of synaptic and neuronal network dysfunction associated with the clinical expression and progression of AD.

The broader neuronal pentraxin signal was also notable in the current study. Greater plasma NPTX2 was independently associated with greater baseline disease severity across multiple clinical measures, contrasting with the direction of associations observed for NPTXR. Although these findings require replication, they may indicate that individual neuronal pentraxins reflect distinct aspects or stages of synaptic response in established AD. More broadly, the repeated prioritisation of neuronal pentraxins across complementary analytical approaches further supports the potential relevance of this protein family to heterogeneity in the clinical expression of AD.

FABP3 was also consistently associated with subsequent clinical progression. FABP3 is a neuronal fatty-acid transport protein involved in lipid homeostasis and has previously been investigated as a marker of neuronal membrane injury in AD. (58) Previous studies have found an increase in CSF FABP3 in AD – particularly in mild disease – and levels are associated with more advanced neurodegeneration (59, 60). Evidence for plasma FABP3 remains considerably more limited however. In the current study, greater plasma FABP3 was unexpectedly associated with slower subsequent clinical progression, which persisted in participants with a very high likelihood of AD pathology. Notably, FABP3 was also one of the bootstrap-stable proteins with a negative elastic net coefficient in a recent NULISA-Seq™ analysis of neocortical tau burden, consistent with the direction of our findings.(36). Further studies are required to fully elucidate the dynamics of plasma FABP3 across the AD continuum and further evaluate its prognostic utility in established AD.

Additional findings provide further support for a relationship between biomarkers of synaptic dysfunction and the clinical expression/progression of AD.

Plasma β-synuclein, a pre-synaptic protein involved in vesicle trafficking and neurotransmitter release, was independently associated with greater progression on the CDR-Sb and DAD.(18) Previous studies have demonstrated increased β-synuclein in AD and associations with cognitive impairment, neuroimaging abnormalities and subsequent clinical progression (61–63),(64–66) In the current analysis, its incremental explanatory contribution was more modest than NPTXR and longitudinal associations attenuated in analyses restricted to those with very high likelihood of AD pathology. β-synuclein should therefore be considered a less consistent finding than NPTXR, but provides additional evidence implicating synaptic dysfunction in the clinical expression of established AD.

These novel associations were observed independent of, and in addition to strong associations between more established biomarkers and clinical severity/cognitive decline. Plasma BD-pTau217, the leading BBM for AD pathology, was the biomarker most significantly associated with baseline cognitive severity (ADAS-Cog) and subsequent clinical progression on all three measures. This is congruent with existing literature supporting the prognostic utility of pTau217 in AD (12, 67). Additionally, both GFAP and NfL reflecting astroglia activation and neurodegeneration, were also associated with subsequent clinical progression on the CDR-Sb – the primary dementia progression measure in the current study, although associations were not as consistent as those seen for BD-pTau217. The novel biomarkers identified here should therefore be considered complementary rather than competitive with established AD biomarkers, potentially capturing additional aspects of downstream disease biology not fully reflected by biomarkers of core AD pathology.

These findings may have implications for biological stratification in established AD. Biomarkers such as p-tau217 increasingly provide accurate information regarding the presence of core AD pathology, but individuals with established AD demonstrate substantial heterogeneity in subsequent clinical progression. Synaptic and neuronal network biomarkers may provide complementary information regarding downstream disease expression and clinical trajectory. If independently replicated, biomarkers such as NPTXR could therefore contribute to prognostic enrichment and clinical-trial stratification, including identification of individuals with differing trajectories despite similar evidence of core AD pathology. Such applications will require external validation and prospective assessment of predictive performance before clinical utility can be established.

Our study has several strengths. The NILVAD cohort was deeply phenotyped, with repeated assessment of global disease severity, cognition and functional ability over 18 months, enabling associations with progression to be examined across three complementary clinical outcomes. The high sensitivity and multiplexing capability of NULISA-Seq™ enabled simultaneous measurement of 129 plasma proteins spanning multiple aspects of AD biology. Further, complementary analytical approaches incorporating correction for multiple testing, multivariable regression, elastic-net modelling with bootstrap stability selection and BIC-based model refinement enabled assessment of convergence across methods with different underlying assumptions. Prioritised biomarkers were then examined using clinically interpretable models and for incremental explanatory information beyond established BBMs.

Limitations

Several limitations should be acknowledged. Most importantly, whilst NILVAD was a multi-centre European study, we did not have external validation which is crucial, particularly for the novel biomarker associations. Whilst the sample was comparatively large and deeply-phenotyped, the sample size remains modest for high-dimensional proteomic analysis. We attempted to mitigate this through correction for multiple testing, correlation-based data reduction and complementary bootstrap-stability selection. At the time NILVAD was conducted, diagnosis was based on clinical criteria alone, and since then the standard for AD care has evolved into a clinical-biological diagnostic paradigm. We attempted

to mitigate this with sensitivity analysis restricted only to those with very high likelihood of AD pathology based on a previously validated plasma p-tau217 cut-off – importantly this did not materially alter our findings. As this was a single cohort, the incremental explanatory analyses should not be interpreted as demonstrating improved prediction or clinical utility – independent replication and formal evaluation of multi-biomarker panels will be required before their clinical utility can be established.

Samples had been stored for up to eight years before the current analysis. Although samples were stored in a monitored biobank at -80^{o}C and aliquots used had not undergone prior freeze-thaw cycles, we cannot exclude the influence of sample degradation over time. However, despite this, our data demonstrate clear associations between biomarkers of synaptic integrity and neuronal network function beyond more established AD biomarkers. As plasma biomarkers were only measured at baseline and not on multiple occasions, temporal relationships between biomarker changes over time and the clinical expression of AD cannot be inferred from the current data. However, our findings support further evaluation of synaptic and neuronal network proteins as prognostic biomarkers in established AD.

Conclusions

In conclusion, plasma biomarkers of synaptic and neuronal network biology were associated with both baseline disease severity and longitudinal clinical progression in established AD. NPTXR emerged as the most consistent novel marker and provided explanatory information beyond established plasma biomarkers. These findings support further evaluation of synaptic and neuronal network biomarkers as complementary markers of downstream disease expression and progression in AD.

Abbreviations

ACHE
Acetylcholinesterase
AD
Alzheimer disease
ADAS-Cog
Alzheimer Disease Assessment Scale–Cognitive Subscale
AGRN
Agrin
ARSA
Arylsulfatase A
BBM
Blood-based biomarker
BD-pTau217
Brain-derived tau phosphorylated at threonine 217
BH
Benjamini–Hochberg

BIC
Bayesian information criterion
BMI
Body mass index
CCL3
C-C motif chemokine ligand 3
CCL17
C-C motif chemokine ligand 17
CCL26
C-C motif chemokine ligand 26
CD40LG
CD40 ligand
CDR-SB
Clinical Dementia Rating–Sum of Boxes
CI
Confidence interval
CNS
Central nervous system
CNTN2
Contactin-2
CRH
Corticotropin-releasing hormone
CRP
C-reactive protein
CSF
Cerebrospinal fluid
CV
Coefficient of variation
CXCL8
C-X-C motif chemokine ligand 8
DAD
Disability Assessment for Dementia
EDTA
Ethylenediaminetetraacetic acid
eMTBR-tau243
Endogenously cleaved microtubule-binding region of tau containing residue 243
FABP3
Fatty acid-binding protein 3
FGF2
Fibroblast growth factor 2

FOLR1
Folate receptor alpha
GDNF
Glial cell line-derived neurotrophic factor
GFAP
Glial fibrillary acidic protein
GOT1
Glutamic-oxaloacetic transaminase 1 (cytoplasmic aspartate aminotransferase)
HBA1/HBA2
Haemoglobin subunit alpha 1/2
ICH GCP
International Conference on Harmonisation–Good Clinical Practice
IGF1R
Insulin-like growth factor 1 receptor
IL-6
Interleukin-6
IL-18
Interleukin-18
IQR
Interquartile range
MCI
Mild cognitive impairment
MME
Membrane metalloendopeptidase (neprilysin)
MSLN
Mesothelin
MTA
Materials transfer agreement
NEFL
Neurofilament light chain
NfL
Neurofilament light
NINCDS-ADRDA
National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association
NPQ
NULISA protein quantification
NPTX2
Neuronal pentraxin 2
NPTXR

Neuronal pentraxin receptor
NULISA
Nucleic acid linked immuno-sandwich assay
PDLIM5
PDZ and LIM domain protein 5
PET
Positron emission tomography
pS129-α-syn
Alpha-synuclein phosphorylated at serine 129
pTau217
Tau phosphorylated at threonine 217
pTDP43-409
TAR DNA-binding protein 43 phosphorylated at serine 409
Q–Q
Quantile–quantile
RMSE
Root mean squared error
S100B
S100 calcium-binding protein B
SFRP1
Secreted frizzled-related protein 1
SNCB
Beta-synuclein
STROBE
Strengthening the Reporting of Observational Studies in Epidemiology
TREM2
Triggering receptor expressed on myeloid cells 2
VGF
Neurosecretory protein VGF
VIF
Variance inflation factor

Declarations

Ethics Approval and Consent to Participate

Ethical approval was granted from appropriate National Competent Authorities, Independent Ethics Committees and Institutional Review Boards for all 23 sites. Written informed consent was obtained from all participants and the trial adhered to the Declaration of Helsinki and to International Conference on Harmonisation of Good Clinical Practice (ICH GCP) guidelines at the time of study conduct. A full list

of Independent Ethics Committees and Institutional Review Boards are provided in Supplementary Material (Table S1).

Consent for Publication

Not Applicable

Competing Interests

The authors have no competing interests to declare

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GR and AHD directly accessed and verified the underlying individual participant data. The NILVAD Study Group assisted with participant recruitment, assessment, and completion of the clinical trial in accordance with the trial protocol. All authors read and approved the final manuscript.

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Availability of Data and Materials

Due to the NILVAD study protocols, consortium agreement, Irish/European data protection law and risk of patient identification, the data presented in the current manuscript is not publicly available. All requests for anonymized data will be promptly reviewed by the corresponding author (AHD) and anonymised data provided on reasonable request, providing it is in agreement with EU legislation and the NILVAD ethical approval, and with a suitable Materials Transfer Agreement (MTA) in place. Requests can be addressed to the corresponding author (dyera@tcd.ie).

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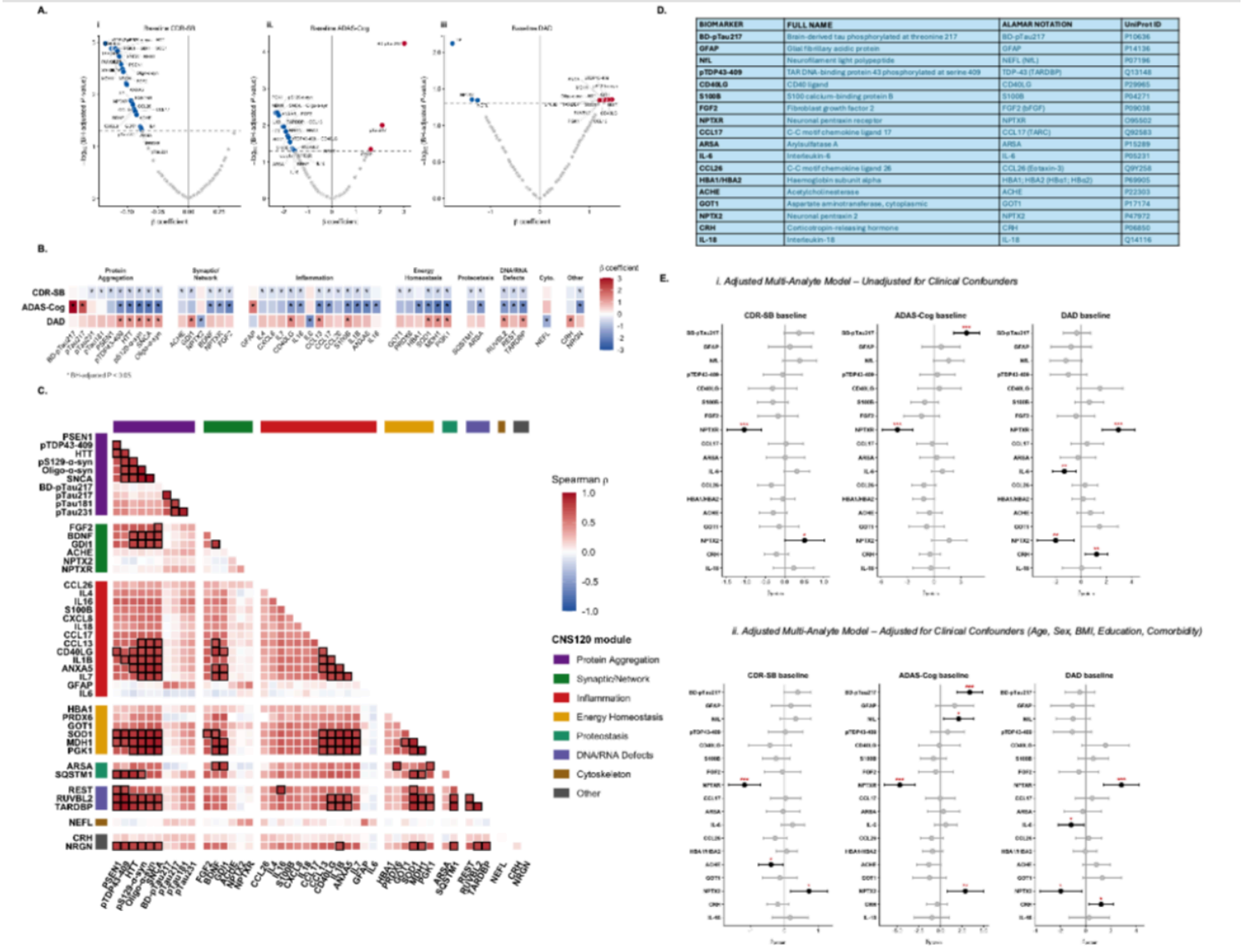
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Figures



BIOMARKER	FULL NAME	ALAMAR NOTATION	UniProt ID
BD-pTau217	Brain-derived tau phosphorylated at threonine 217	BD-pTau217	P10636
GFAP	Glial fibrillary acidic protein	GFAP	P14136
NfL	Neurofilament light polypeptide	NEFL (NfL)	P07196
pTDP43-409	TAR DNA-binding protein 43 phosphorylated at serine 409	TDP-43 (TARDBP)	Q13148
CD40LG	CD40 ligand	CD40LG	P29965
S100B	S100 calcium-binding protein B	S100B	P04271
FGF2	Fibroblast growth factor 2	FGF2 (bFGF)	P09038
NPTXR	Neuronal pentraxin receptor	NPTXR	O95502
CCL17	C-C motif chemokine ligand 17	CCL17 (TARC)	Q92583
ARSA	Arylsulfatase A	ARSA	P15289
IL-6	Interleukin-6	IL-6	P05231
CCL26	C-C motif chemokine ligand 26	CCL26 (Eotaxin-3)	Q9Y258
HBA1/HBA2	Haemoglobin subunit alpha	HBA1; HBA2 (HBa1; HBa2)	P69905
ACHE	Acetylcholinesterase	ACHE	P22303
GOT1	Aspartate aminotransferase, cytoplasmic	GOT1	P17174
NPTX2	Neuronal pentraxin 2	NPTX2	P47972
CRH	Corticotropin-releasing hormone	CRH	P06850
IL-18	Interleukin-18	IL-18	Q14116

Figure 1

Plasma Biomarkers Associations with Baseline Disease Severity in Mild-to-moderate Alzheimer Disease. **(A)**Volcano plots of univariate associations between plasma proteins and baseline disease severity measures using linear regression. Dashed horizontal lines indicate the Benjamini-Hochberg significance threshold to account for multiple testing. Significant proteins are coloured in blue (negative

coefficients) and red (positive coefficients). **(B)** Distribution of BH-significant proteins according to manufacturer-defined CNS120 biological categories. Colour intensity reflects effect size (beta coefficient) of the association (univariate linear regression), with asterisks indicating significance <0.05. **(C)** Spearman correlation matrix of BH-significant proteins, grouped according to manufacturer-defined categories/modules. Black boxes denote highly correlated protein clusters (absolute Spearman correlation of 0.70 or greater), which informed correlation-based data reduction before multi-analyte modelling. **(D)** Proteins retained for multivariable analysis following sequential correlation-based reduction/biological review. **(E)** Multivariable linear regression models demonstrating independent associations between retained plasma biomarkers and baseline disease severity. **i.** Models adjusted for all other retained biomarkers and **ii.** models adjusted for age, sex, body mass index, education and comorbidity. Regression coefficients are shown with 95% confidence intervals, with significant associations in black and asterisks to indicate statistical significance. *p<0.05, **p<0.01, ***p<0.001. CDR-Sb: Clinical Dementia Rating Scale – Sum of Boxes; ADAS-Cog: Alzheimer Disease Assessment Scale – Cognitive Subscale; DAD: Disability Assessment for Dementia. BMI: Body Mass Index.

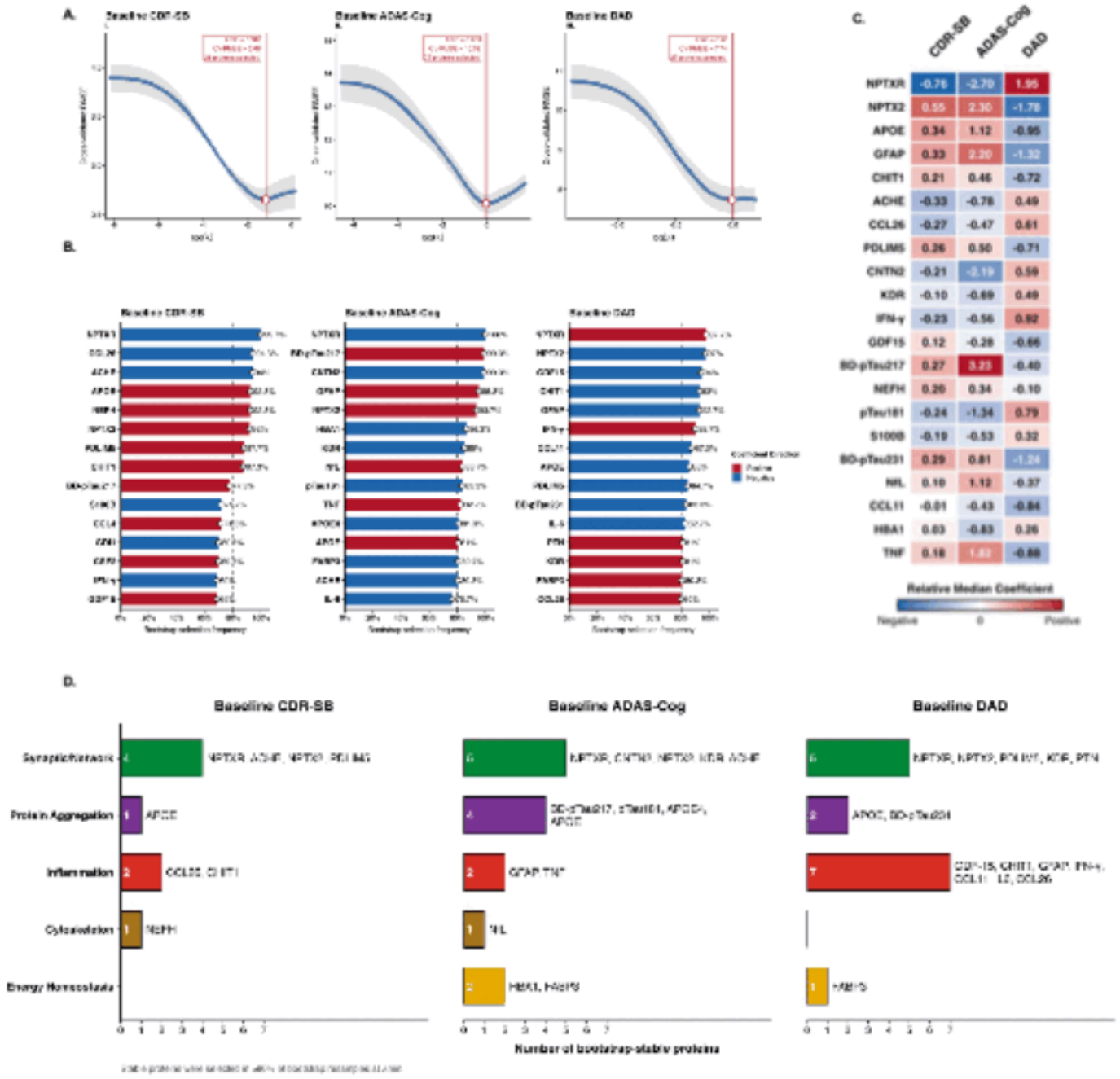


Figure 2

Elastic Net Regression/Bootstrap Stability Selection Prioritise Synaptic Biomarkers Associated with Baseline Disease Severity. (A) Cross-validation curves from elastic net regression for baseline CDR-SB, ADAS-Cog and DAD models. The mean cross-validation error (root mean squared error [RMSE]) is shown across values of the penalty parameter (λ), with shaded areas representing ±1 standard error. Vertical lines indicate the optimal penalty parameter (λ_{min}) selected by cross-validation. **(B)** Bootstrap stability selection results from 300 bootstrap resamples showing the frequency with which each protein was retained in elastic net models at λ_{min}. Colours of the bars indicate direction of association (red: positive coefficients; blue: negative coefficients). Selection frequencies are expressed as the percentage of

bootstrap resamples in which each protein was retained. **(C)** Heatmap of median elastic net regression coefficients across bootstrap resamples for proteins retained in at least one bootstrap model. Colour intensity reflects the magnitude and direction of the median coefficient for each clinical outcome. **(D)** Distribution of bootstrap-stable proteins (selection frequency ≥80%) according to manufacturer-defined categories. Numbers indicate the count of bootstrap-stable proteins within each category, with protein names shown adjacent to each bar. CDR-SB, Clinical Dementia Rating Scale–Sum of Boxes; ADAS-Cog, Alzheimer's Disease Assessment Scale–Cognitive Subscale; DAD, Disability Assessment for Dementia; RMSE, root mean squared error.

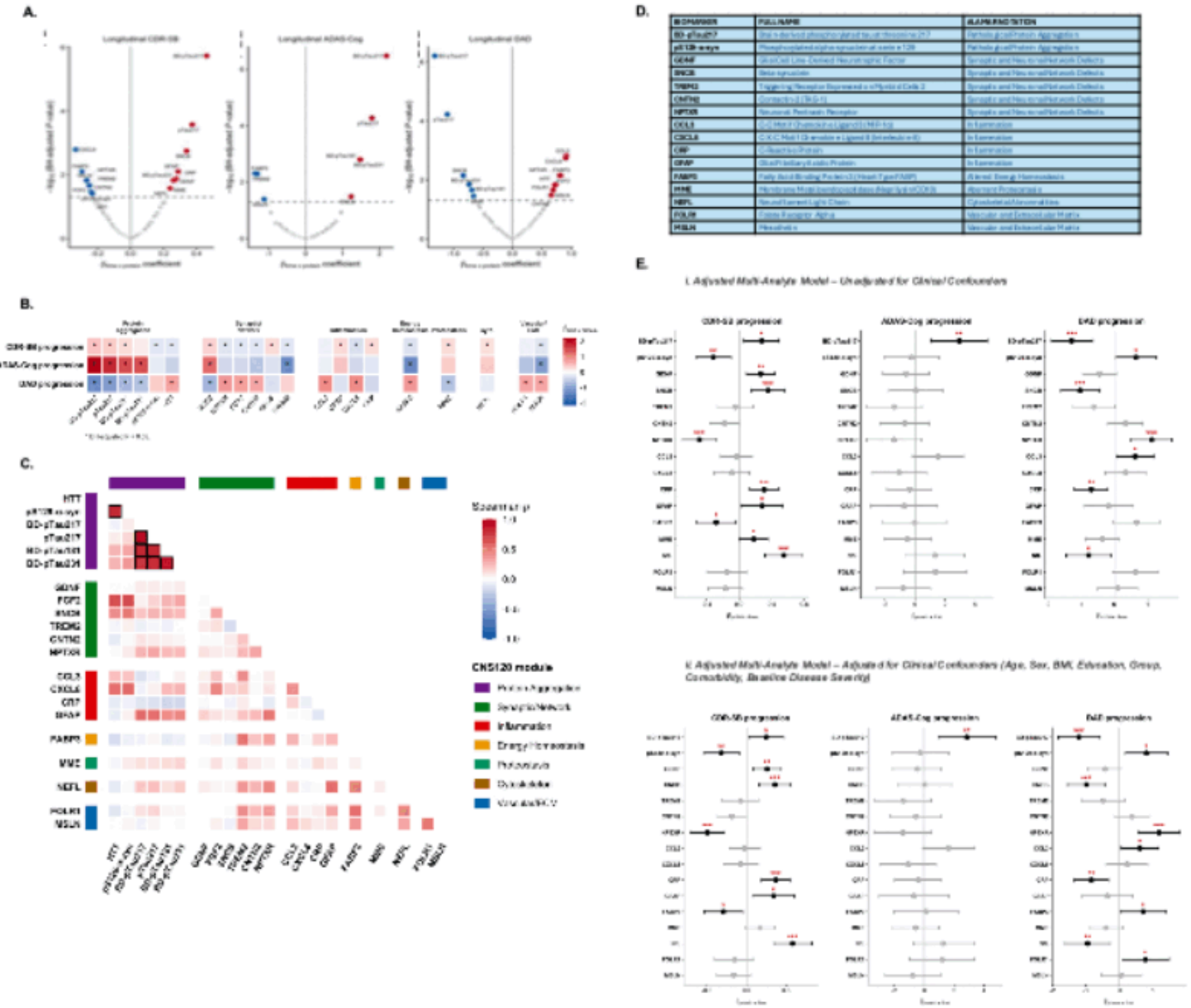


Figure 3

Plasma Biomarkers Associated with Clinical Progression Over 18 Months in Mild-to-moderate Alzheimer Disease. (A) Volcano plots of univariate associations between baseline plasma proteins and

subsequent clinical progression on the CDR-SB, ADAS-Cog and DAD using linear mixed-effects models. Dashed horizontal lines indicate the Benjamini–Hochberg significance threshold to account for multiple testing. Significant proteins are coloured in blue (negative *biomarker*time* interaction coefficients) and red (positive *biomarker*time* interaction coefficients). **(B)** Distribution of BH-significant proteins according to manufacturer-defined CNS120 biological categories. Colour intensity reflects the *biomarker*time* interaction coefficient from univariate linear mixed-effects models, with asterisks indicating statistical significance following BH correction ($p<0.05$). **(C)** Spearman correlation matrix of BH-significant proteins, grouped according to manufacturer-defined categories/modules. Correlations were calculated using baseline plasma protein concentrations. Black boxes denote highly correlated protein clusters (absolute Spearman correlation of 0.70 or greater), which informed correlation-based data reduction before multi-analyte modelling. **(D)** Proteins retained for multivariable analysis following sequential correlation-based reduction and biological review. **(E)** Multivariable linear mixed-effects models demonstrating independent associations between retained baseline plasma biomarkers and subsequent clinical progression. **i.** Models adjusted for all other retained biomarkers and **ii.** models additionally adjusted for age, sex, body mass index, education, comorbidity, baseline disease severity and study group (Nilvadipine vs placebo). Regression coefficients represent *biomarker*time* terms (annual rate of change) and are shown with 95% confidence intervals. Significant associations are shown in black with asterisks indicating statistical significance. *$p<0.05$, **$p<0.01$, ***$p<0.001$. CDR-SB: Clinical Dementia Rating Scale–Sum of Boxes; ADAS-Cog: Alzheimer's Disease Assessment Scale–Cognitive Subscale; DAD: Disability Assessment for Dementia; BMI: Body Mass Index.

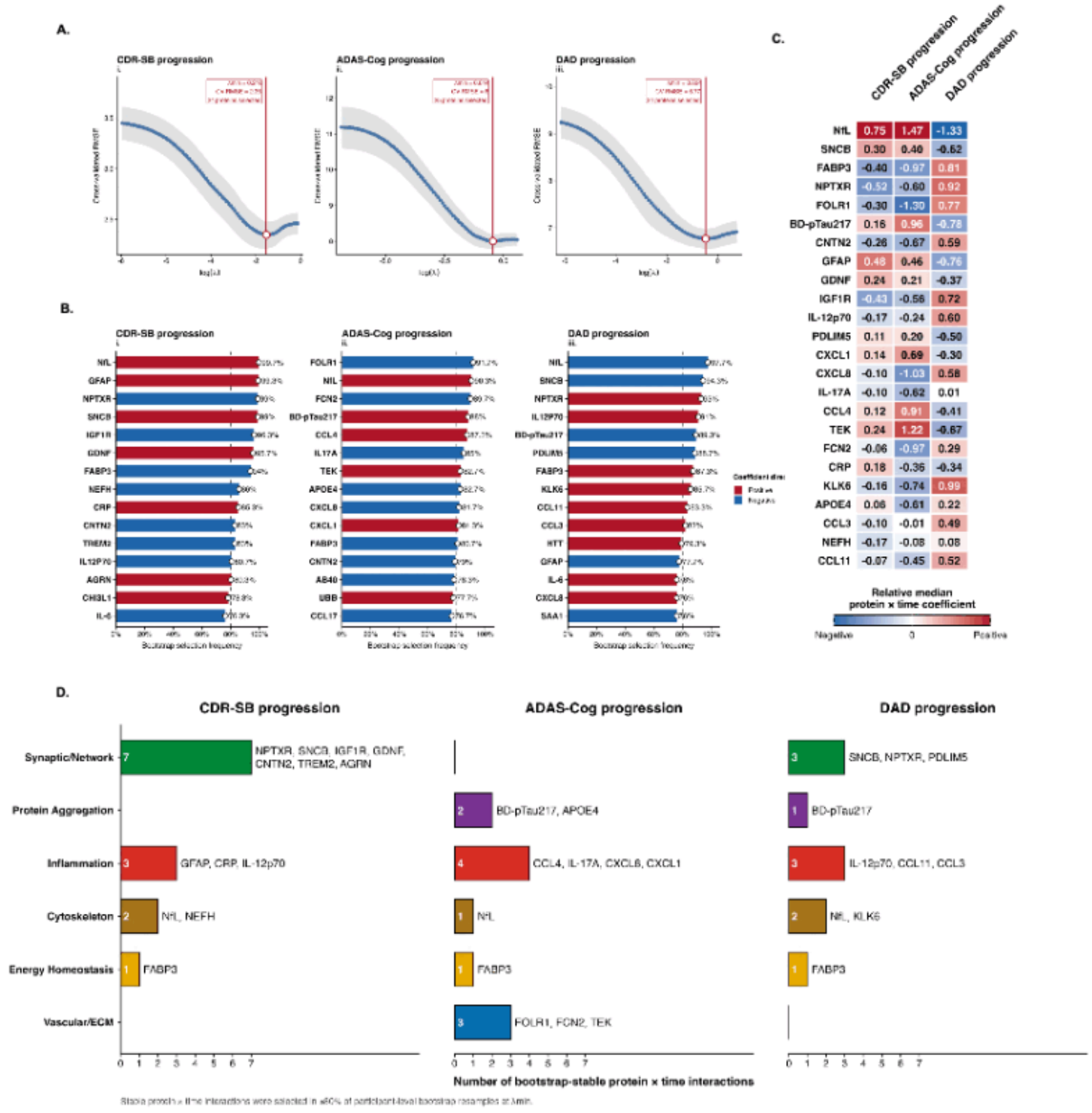


Figure 4

Elastic Net Regression and Bootstrap Stability Selection Prioritise Synaptic Biomarkers Associated with Clinical Progression. (A) Cross-validation curves from elastic net regression for CDR-SB, ADAS-Cog and DAD progression models. The mean cross-validation error (root mean squared error [RMSE]) is shown across values of the penalty parameter (λ), with shaded areas representing ±1 standard error. Vertical lines indicate the optimal penalty parameter (λ_{min}) selected by cross-validation. **(B)** Bootstrap stability selection results from 300 participant-level bootstrap resamples showing the frequency with

which each baseline plasma *protein*time* interaction was retained in elastic net models at λ_{min}. Bar colours indicate the direction of the *protein*time* interaction coefficient (red: positive interaction; blue: negative interaction). Selection frequencies are expressed as the percentage of bootstrap resamples in which each protein was retained. **(C)** Heatmap of median *protein*time* interaction coefficients across bootstrap resamples for proteins retained in at least one bootstrap model. Colour intensity reflects the magnitude and direction of the median interaction coefficient for each clinical outcome. **(D)** Distribution of bootstrap-stable *protein*time* interactions (selection frequency ≥80%) according to manufacturer-defined biological categories. Numbers indicate the count of bootstrap-stable proteins within each category, with protein names shown adjacent to each bar. CDR-SB, Clinical Dementia Rating Scale–Sum of Boxes; ADAS-Cog, Alzheimer's Disease Assessment Scale–Cognitive Subscale; DAD, Disability Assessment for Dementia; RMSE, root mean squared error.

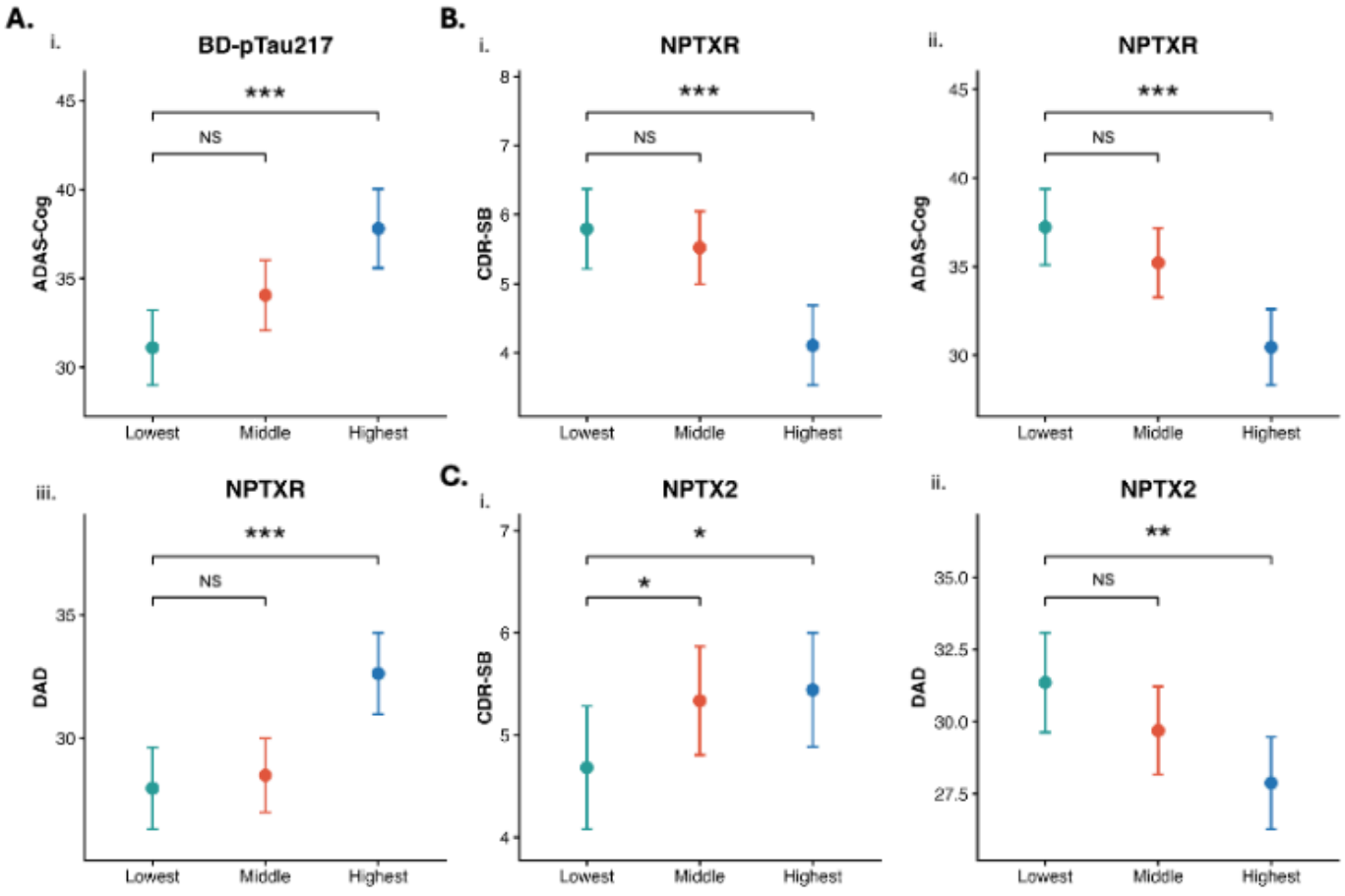


Figure 5

Associations Between Baseline Biomarker Tertile and Baseline Disease Severity. Mean disease severity (with 95% Confidence Intervals) are given according to baseline biomarker tertiles. Associations between baseline biomarker tertile and disease severity were analysed for those associations significant under all three models in continuous analysis above to appreciate differences, with lowest tertile as the reference group. **(A)**The highest (vs lowest) tertile of BD-pTau217 was associated with greater cognitive severity on the ADAS-Cog. **(B)**The highest (vs lowest) NPTXR tertile was consistently associated with

lesser disease severity on the CDR-SB, ADAS-Cog and DAD. **(C)** The highest (vs lowest) NPTX2 tertile was associated with greater dementia severity on the CDR-SB and DAD. Models are adjusted for the other five plasma biomarkers, age, sex, body mass index, education and comorbidity. *p<0.05, **p<0.01, ***p<0.001; NS, not significant. CDR-Sb: Clinical Dementia Rating, Sum of Boxes; ADAS-Cog: Alzheimer Disease Assessment Scale, Cognitive Subscale; DAD: Disability Assessment for Dementia.

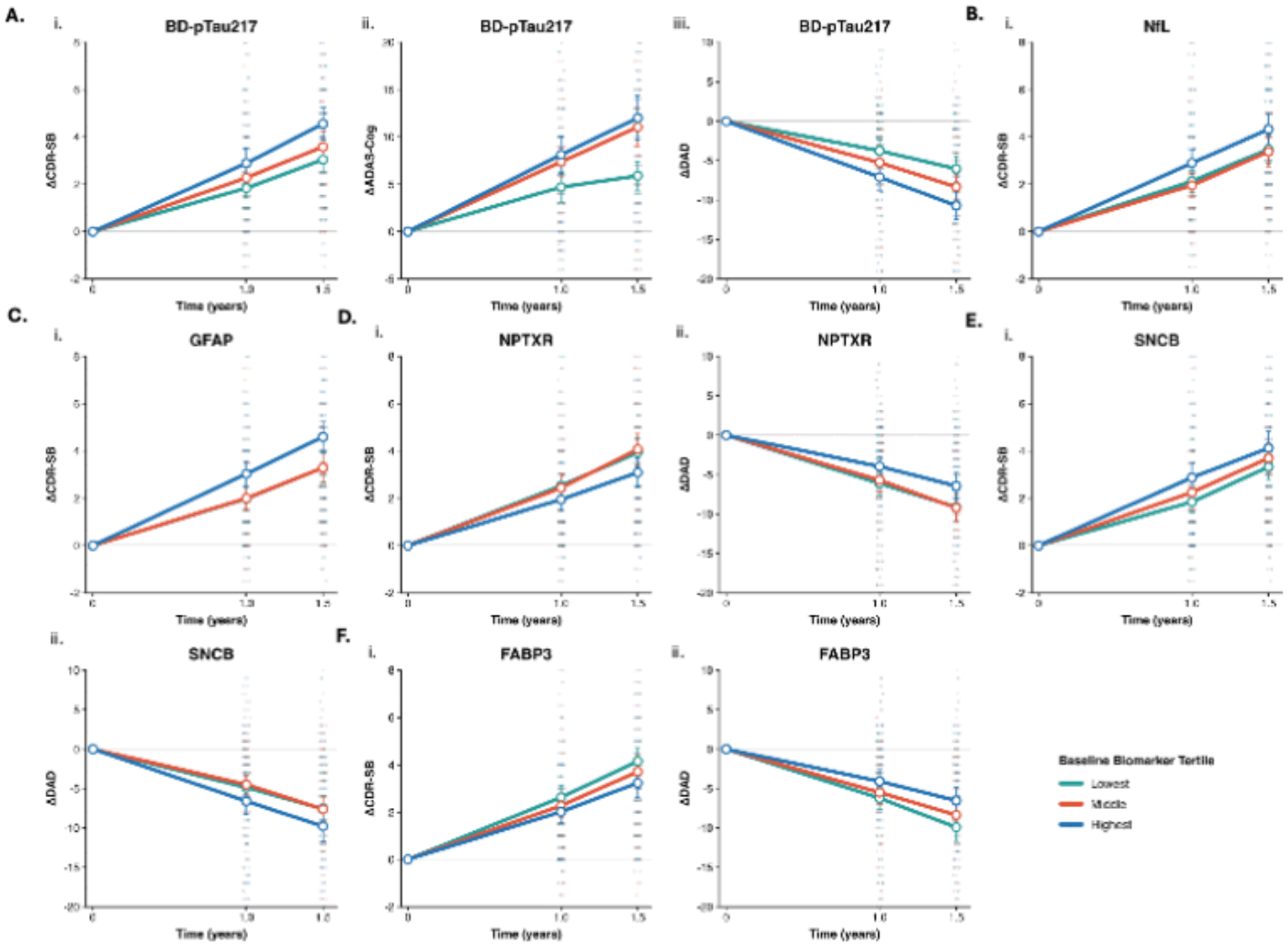


Figure 6

Associations Between Baseline Biomarker Tertile and Clinical Progression Over 18 Months. Change in clinical outcomes over 18 months according to baseline plasma biomarker tertile for biomarker-outcome associations that remained significant following full multivariable adjustment. Models were adjusted for the other five selected biomarkers, age, sex, body mass index, education, comorbidity, baseline disease severity and study group (Nilvadipine vs placebo). Higher CDR-Sb and ADAS-Cog scores indicate greater disease severity, whereas lower DAD scores indicate greater functional impairment. Biomarker tertiles are shown as lowest (green), middle (red), and highest (blue). CDR-Sb, Clinical Dementia Rating–Sum of Boxes; ADAS-Cog, Alzheimer Disease Assessment Scale–Cognitive Subscale; DAD, Disability Assessment for Dementia.

Supplementary Files

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