Diagnostic performance and clinical applicability of blood-based biomarkers in a prospective memory clinic cohort

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Number of characters in title: 113

Abstract Word count: 332

Word count of main text: 4481

References: 35

Figures: 5

Tables: 2

Supplemental: - Reporting guidelines - Supplementary material (eFigure 1 and eFigure 2 legends and eTable 1) - eFigure 1 - eFigure 2 - Revised manuscript with tracked changes

Search Terms: [26] Alzheimer's disease, [28] Dementia with Lewy bodies, [29] Frontotemporal dementia, [38] Assessment of cognitive disorders/dementia, [111] Diagnostic test assessment

Study Funding: The authors report no targeted funding

Disclosures: The authors report no disclosures relevant to the manuscript.

Abstract

Background and Objectives: Blood-based biomarkers have emerged as minimally-invasive options for evaluating cognitive impairment. Most studies to date have assessed them in research cohorts, limiting their generalization to everyday clinical practice. We evaluated their diagnostic performance and clinical applicability in a prospective, real-world, memory clinic cohort.

Methods: All patients referred with suspected cognitive impairment between July 2019 and June 2021, were prospectively invited to participate. Five plasma biomarkers (p-tau181, GFAP, NfL, t-tau, UCH-L1) were determined with SiMoA. Performance was assessed in comparison to clinical diagnosis (blinded to plasma results) and amyloid status (CSF/PET). A group of cognitively unimpaired (CU) controls was also included.

Results: Three hundred forty-nine participants (mean age 68, SD 8.3 years) and 36 CU controls (mean age 61.7, SD 8.2 years) were included. In the sub-cohort with available AD biomarkers (n=268), plasma p-tau181 and GFAP had a high diagnostic accuracy to differentiate AD from non-neurodegenerative causes (AUC 0.94 and 0.92, respectively), with p-tau181 systematically outperforming GFAP. Plasma p-tau181 levels predicted amyloid status (85% sensitivity and specificity) with accurate individual prediction in approximately 60% of the subjects. Plasma NfL differentiated frontotemporal dementia syndromes (FTD) from CU (0.90) and non-neurodegenerative causes (0.93), while the discriminative capacity with AD and between all neurodegenerative and non-neurodegenerative causes was less accurate. A combination of p-tau181 and NfL identified FTD with 82% sensitivity and 85% specificity and had a negative predictive value for neurodegenerative diagnosis of 86%, ruling out half of the non-neurodegenerative diagnoses. In the sub-cohort without AD biomarkers similar results were obtained. T-tau and UCH-L1 did not offer added diagnostic value.

Discussion: Plasma p-tau181 predicted amyloid status with high accuracy and could have potentially avoided CSF/amyloid PET testing in approximately 60% of subjects in a memory-clinic setting. NfL was useful for identifying FTD from non-neurodegenerative causes but behaved worse than p-tau181 in all other comparisons. Combining p-tau181 and NfL improved diagnostic performance for FTD and non-neurodegenerative diagnoses. However, the 14% false-negative results suggest that further improvement is needed before implementation outside memory clinics.

Classification of Evidence: This study provides Class I evidence that plasma p-tau181 correlates with the presence or absence of AD and a combination of plasma p-tau181 and NfL correlate moderately well with a diagnosis of FTD.

Introduction

Cognitive impairment is one of the biggest challenges of today's society, with a prediction of 150 million cases worldwide by 2050.[1] A prompt and accurate diagnosis is essential for initiating treatment, care-planning and avoiding complications.[2] Current diagnostic criteria for Alzheimer's disease (AD) and other neurodegenerative dementias include the use of biomarkers (biochemical in cerebrospinal fluid [CSF] and neuroimaging) for increasing diagnostic certainty.[3–12] However, widespread use of positron emission tomography (PET) and CSF biomarkers is limited due to their economic cost, invasiveness and limitations of access. There is a stringent need for validation and implementation in clinical practice of accessible and scalable biomarkers. The best candidates for this role are blood-based biomarkers.

The development of highly-sensitive techniques, like the Single-Molecule Array (SiMoA), where analytes are captured by antibody-coated magnetic beads and trapped in femtoliter sized microcavities with a digital ELISA readout,[13] have allowed an accurate measurement of distinct brain-derived proteins in blood.

Blood amyloid-β (Aβ) and total-tau (t-tau) biomarkers have shown conflicting results depending on the technique and cohort analyzed.[14–16] Ubiquitin C-terminal hydrolase-L1 (UCH-L1), an enzyme that regulates protein degradation in the proteasome, is increased in CSF and blood following brain injury and has been proposed as a non-specific marker of neurodegeneration.[17] Glial fibrillary acidic protein (GFAP) is a marker of astrocytic activation and proliferation. Plasma GFAP increases throughout the AD continuum and in Lewy body dementia (LBD), predicts Aβ positivity even in preclinical stages and is associated with cognitive decline in AD.[18,19] Plasma neurofilament light chain (NfL), a structural component of the neural cytoskeleton, has shown high correlation with CSF levels, disease severity, longitudinal decline in cognitive and behavioral deficits and atrophy and plasma NfL levels are higher in neurodegenerative disorders compared to non-neurodegenerative diseases, especially in frontotemporal dementia (FTD).[16,20–23] Plasma levels of tau phosphorylated at threonine 181 (p-tau181), 217 (p-tau217) and 231 (p-tau231) have shown good diagnostic discrimination between the AD continuum and other diagnosis such as cognitively unimpaired (CU) controls, non-neurodegenerative causes of cognitive impairment, LBD and FTD; Moreover, p-tau181 predicts greater cognitive decline, MRI atrophy, FDG-PET hypometabolism and amyloid and tau-PET positivity.[19,22–33] Although advancing in knowledge, most of prior studies focused on highly-selected research cohorts. Therefore, data from routine clinical settings is needed for pushing forward the implementation of blood-based biomarkers as diagnosis tools in clinical practice.[32,33]

In this study, our primary research goals were to assess the discriminative capacities of five blood-based biomarkers (p-tau181, t-tau, NfL, GFAP and UCH-L1) and to explore their potential utility in real clinical practice scenarios. To do so, we measured these five blood-based biomarkers in a real clinical practice cohort of subjects referred for suspected cognitive impairment. We hypothesized that p-tau181 and GFAP would be differentially increased in the AD group and could discriminate between AD and the other diagnostic categories. In contrast, the other biomarkers would differentiate between neurodegenerative and non-neurodegenerative diagnoses.

Methods

Study population

All subjects who were referred for diagnosis with suspected cognitive impairment to the Alzheimer's Disease and other Cognitive disorders Unit (ADCU), Hospital Clínic Barcelona, between July 1st, 2019 and June 30th, 2021, were prospectively invited to participate in the study. Patients with severe dementia at the first evaluation, subjects referred for a second opinion or for genetic counseling were excluded. Additionally, CU control participants, aged 50 years or older, were retrospectively included.

Clinical assessment

Subjects underwent the same clinical diagnostic protocol irrespective of whether they were included in the study or not. This included a neurological assessment, neuropsychological testing and structural neuroimaging. AD biomarkers were systematically performed, according to current guidelines, to subjects with mild cognitive impairment (MCI) below 75 years of age, early-onset dementia and non-amnestic phenotypes. At our site, CSF is the first line AD biomarker used; amyloid PET is performed when lumbar puncture is contraindicated or technically difficult. When deemed appropriate to reach a final diagnosis, other diagnostic procedures, such as fluorodeoxyglucose (FDG) PET or dopamine transporter scan were also performed.

Diagnosis was established according to current diagnostic criteria by the treating neurologist and blinded to plasma biomarkers. In the whole cohort, syndromic diagnostic categories included subjective cognitive decline (SCD), MCI (irrespective of suspected etiology, both AD and non-AD), probable AD dementia, probable Lewy Body Dementia (LBD), semantic variant primary progressive aphasia (svPPA), nonfluent variant PPA (nfPPA), behavioural variant FTD (bvFTD), progressive supranuclear palsy (PSP), corticobasal syndrome (CBS) and Creutzfeldt-Jakob disease (CJD). SvPPA, nfPPA, bvFTD, PSP and CBS were unified under the umbrella "FTD" term.

In the sub-cohort with AD biomarkers performed (CSF/amyloid PET), participants were classified depending on suspected etiology of cognitive impairment: suspected non-neurodegenerative cognitive impairment (SND), AD (MCI due to AD + probable AD dementia with positive AD biomarkers), probable LBD and FTD. SND was defined as stable cognitive impairment that was not suggestive of any neurodegenerative disease and with negative AD biomarkers (grouping subjects from SCD and MCI initial syndromic diagnostic categories). All controls had no cognitive complaints, normal neuropsychological testing and CSF analysis (A-T-N-).

Procedures and measurements

A blood sample was obtained from all participants at the time of inclusion, centrifuged to obtain plasma, aliquoted and stored at -80ºC. Plasma biomarkers concentrations were measured using the Quanterix Simoa Neurology 4-Plex A (including t-tau, GFAP, NfL and UCH-L1) and the Quanterix Simoa p-tau181 Advantage V2 assays following the manufacturer's protocol (Quanterix, USA). Blood sample measurements were performed after finalizing recruitment, during November and December 2021. Lab technicians running the SiMoA analysis had no access to clinical or demographic information. *APOE* genotype was determined through analysis of two single nucleotide polymorphisms (rs429358 and rs7412) by Sanger sequencing.

Lumbar puncture was performed according to current clinical guidelines. CSF amyloid β_{1-42}, p-tau181 and t-tau were measured with Lumipulse following manufacturer's instructions (Fujirebio, Belgium) and using local cut-offs. Amyloid positive (Aβ+) status was defined as CSF amyloid β_{1-42} below 600 pg/mL. In cases with borderline results of amyloid β_{1-42} in CSF, a ratio $A\beta_{1-42}/A\beta_{1-40}$ <0.07 was used to define the Aβ+ status. CSF NfL was measured with the ELISA kit of Uman Diagnostics (IBL International, Germany).

Amyloid PET images were acquired in a Biograph molecular computed tomography PET (Siemens) using florbetaben or flutemetamol ligands and its status (Aβ+ or Aβ-) was evaluated by a nuclear medicine specialist in accordance with current guidelines.

Statistical analyses

We assessed normality with Kolmogorov-Smirnov or Shapiro Wilk tests. Continuous variables were compared depending on the normality tests using t-test/analysis of variance or Mann-Whitney U/Kruskal-Wallis test with Dunn's multiple comparison test. Fisher's exact test and χ^2 were used for categorical variables. Since plasma biomarker levels were not normally distributed, they were log10 transformed for normality, which allowed linear modeling and parametric testing. Plasma biomarker concentrations were compared using an analysis of covariance to test for the effect of diagnostic group, with age and sex as covariates and the adjusted η^2 as a measure of effect size, with Bonferroni correction for pairwise comparisons. Correlations between plasma and CSF results and demographics were calculated using Spearman rank correlation test. To test for the potential effect of sample storage time on plasma biomarkers results, we used linear regression analyses with each individual plasma biomarker as the dependent variable and time from collection to analysis (in days) as the independent variable, for each separate diagnostic group, both with and without adjustment for demographic variables. To analyze the diagnostic and CSF/amyloid PET status prediction performance of blood biomarkers, the area under the receiver operating characteristic (ROC) curves (AUC) was assessed. To study the performance of plasma biomarkers in distinct clinical scenarios, models were calculated using binary logistic regression, including a parsimonious model (the minimum number of variables that maximized predictive power) with the stepwise forward selection method based on the probability of a likelihood-ratio statistic based on conditional parameter estimates. The predicted probability of each model was used to calculate the resulting AUCs. Cut-offs were first selected when they maximized sensitivity and specificity per Youden index. Also, new cut-off values were chosen for optimized sensitivity or specificity in order to evaluate the biomarkers in distinct clinical practice scenarios. SPSS (version 25) and GraphPad Prism (version 9) were used for statistical analyses and significance was set at $p<0.05$.

Standard Protocol Approvals, Registrations, and Patient Consents

All participants gave written informed consent for participation in the study. The study protocol was approved by the Hospital Clínic de Barcelona Research Ethics Committee (HCB/2019/0600).

Data Availability

Anonymized data will be shared upon reasonable request from any qualified investigator if approved by the local Research Ethics Committee.

Results

Whole cohort

Demographics

Three hundred forty-nine participants (39 SCD, 183 MCI, 54 AD dementia, 28 LBD, 41 FTD [26 bvFTD, 8 svPPA, 5 nfPPA, 1 CBS and 1 PSP] and 4 CJD) were prospectively included, plus an additional 36 CU controls, accounting for a total of 385 subjects. One patient with cognitive impairment related to multiple sclerosis, 3 with recent brain hemorrhage and 1 diagnosed with end-stage pancreatic cancer shortly after inclusion, were initially recruited but were excluded from analysis.

Demographic, cognitive, APOE and biomarker characteristics are summarized in Table 1. Symptomatic subjects (n=349) had a mean age (standard deviation [SD]) of 68 (8.3) years and were older than CU participants (61.7 [8.2]) years ($p<0.001$). The control group had more women compared to symptomatic participants (78% vs 53%, $p=0.004$) and less APOE ε4 allele carriers (22% vs 43%, $p=0.015$).

Plasma biomarkers

Plasma p-tau181, GFAP, NfL and t-tau biomarker concentrations are plotted for each diagnostic group in Figure 1 with a color code depending on amyloid status: not performed, Aβ+ or Aβ-.

In the whole cohort, all 5 biomarker concentrations were different among the diagnostic groups. P-tau181 levels were higher in AD dementia compared to the other diagnoses, except for CJD; MCI also had higher concentrations than CU participants. A pattern of higher p-tau181 levels for Aβ+ compared with Aβ- subjects was observed for each diagnostic category. GFAP behaved similar to p-tau181, with higher concentrations in AD and for Aβ+ patients in each diagnostic category. NfL was higher in FTD compared to the other groups and in AD compared to CU, SCD and MCI. In CJD, GFAP, NfL, t-tau and UCH-L1 levels were increased the most, and p-tau181 levels were similar to AD dementia. T-tau and UCH-L1 levels were only differentially elevated in the CJD group compared to the other diagnostic categories.

Sub-cohort with AD biomarkers (CSF/amyloid PET) performed

The sub-cohort with available AD biomarkers (235 subjects with cognitive impairment and 36 CU) was used to evaluate the diagnostic performance and establish cut-offs. CJD subjects were excluded from further analysis due to low sample size. Therefore, a total of 268 subjects were analyzed.

In the sub-cohort with AD biomarkers available, etiological diagnostic categories included CU and SND (Aβ- SCD and MCI), AD (including MCI-Aβ+ and AD dementia Aβ+) and clinical diagnosis of LBD and FTD (most subjects Aβ-, with some Aβ+ cases interpreted as co-pathology). Demographic, cognitive, APOE and biomarker characteristics are summarized in Table 2.

Diagnostic performance of individual plasma biomarkers

P-tau181, GFAP and NfL concentrations of participants with AD biomarkers available are plotted for each diagnostic group in the left part of Figure 2 (p-tau181 and NfL) and eFigure 1 (GFAP), while the corresponding AUCs are represented on heatmaps on the right part of the figures.

P-tau181

P-tau181 levels were different between diagnostic categories (adjusted η^2=0.426, p<0.001) and were higher in AD. There were no differences between AD and Aβ+ LBD or FTD. P-tau181 levels discriminated AD from CU and SND participants with high accuracy (AUC of 0.96 and 0.94, respectively) and with moderate accuracy from LBD and FTD (0.79 and 0.80, respectively). There was only low to moderate accuracy to differentiate between the other diagnostic groups.

GFAP

GFAP concentrations were different between groups (adjusted η^2=0.308, p<0.001) and were higher in AD compared to CU, SND and LBD. GFAP levels were similar in AD and Aβ+ LBD or FTD. GFAP discriminated AD from CU and SND with high accuracy (AUC 0.85 and 0.92, respectively) but with low-moderate accuracy between AD and LBD (0.76) and FTD (0.65) and between the rest of the diagnoses. Diagnostic performance was globally inferior compared to p-tau181.

NfL

NfL had different concentrations between diagnoses (adjusted η^2=0.364, p<0.001) and was increased the most in patients with FTD. NfL was also increased in AD compared to CU and SND participants. NfL levels were similar between LBD, CU and SND. NfL had an excellent diagnostic performance when differentiating FTD from CU and SND (AUC 0.90 and 0.93, respectively) and a moderate performance to discriminate FTD from AD (0.79) and LBD (0.82). NfL also had moderate performance for differentiating AD from CU and SND (AUC 0.75 and 0.81, respectively) with a diagnostic accuracy inferior to p-tau181 and GFAP.

T-tau

T-tau levels were different between diagnoses (adjusted η^2=0.054, p=0.038). They were only statistically higher in AD compared to the SND diagnostic category, with a low diagnostic accuracy (AUC of 0.65).

UCH-L1

UCH-L1 concentrations were not statistically different between diagnostic groups (adjusted η^2=0.019, p=0.309).

Correlations between plasma biomarkers, demographic data, cognition, time from blood collection to measurement and CSF biomarkers

In the AD group, only a low correlation of age with plasma p-tau181 was found (ρ=-0.31). In the non-neurodegenerative group (CU+SND), age correlated with NfL (ρ=0.53) and GFAP (ρ=0.3) but not with p-tau181. Plasma p-tau181 and NfL were inversely correlated with MMSE in AD (ρ=-0.3 and -0.28, respectively). No correlation between cognition, sex or education with plasma biomarkers was found in CU and SND groups. Time from blood sample collection to

measurement did not significantly influence the concentration of any plasma biomarker (linear regressions) nor was this variable significantly correlated with blood biomarker levels.

Moderate correlations (eFigure 2) were observed between plasma p-tau181 and CSF $A\beta_{1-42}$, p-tau and t-tau (ρ=0.52-0.57), between plasma GFAP and CSF biomarkers (ρ=0.43-0.56) and between plasma NfL and CSF t-tau (ρ=0.36) and NfL (ρ=0.63). Partial correlations adjusting for age and sex yielded minimal differences in correlation coefficients.

Plasma biomarkers in distinct practical clinical scenarios

To study the practical clinical utility of plasma biomarkers, we calculated distinct predictive models and cut-offs to address different clinical questions (Fig. 3).

Clinical scenario #1: Amyloid status prediction

A combination of the 5 plasma biomarkers, age, sex, MMSE and APOE (complete model) predicted Aβ status with and AUC of 0.96 (Fig. 3A). The parsimonious model (PM) included p-tau181, GFAP and APOE (AUC 0.94). P-tau181 had a greater explanatory power than GFAP, which in turn was better than APOE (AUCs 0.91, 0.82 and 0.72, respectively). A model with no biomarkers, using only sex, age, MMSE and APOE had an AUC of 0.83, similar to GFAP but worse than p-tau181 alone.

Since plasma p-tau181 was the biomarker with a higher explanatory power to predict Aβ status, our next goal was to establish useful cut-offs in clinical practice in individual subjects. A balanced cut-off of 1.37 pg/mL had 85% sensitivity and specificity to predict Aβ status. We established two other cut-off values, one inferior with optimized sensitivity and NPV (cut-off 0.89, sensitivity 97% and NPV 95%) and one superior with optimized specificity and PPV (cut-off 1.92, specificity 96% and PPV 94%). Values below 0.89 pg/mL were deemed to have a high probability of being Aβ- and those above 1.92 pg/mL probably would be Aβ+. The algorithm is represented on Figure 4 and both thresholds are plotted in Fig 1A and 2A.

Twenty-six percent of all individuals with AD biomarkers available had p-tau181 levels below the threshold of 0.89 pg/mL, with 4 (5%) Aβ+ subjects misclassified (3 MCI due to AD and 1 Aβ+ LBD). Conversely, 34% of all participants with available AD biomarkers had p-tau181 above the cut-off of 1.92 pg/mL, with 5 Aβ- subjects (6%) misclassified (3 Aβ- FTD, 1 Aβ- LBD, 1 SND). In sum, using an inferior and a superior cut-off for plasma p-tau181, in 60% of all subjects Aβ status could have been predicted with reasonable high accuracy and more advanced biomarkers testing (CSF/amyloid PET) could have been potentially avoided. The remaining 40% of subjects fell in the "grey-zone", with no added value of p-tau181 in the decision of performing or not CSF/PET biomarkers.

P-tau181 had a better accuracy in young (age <65 years, 37% of subjects, AUC of 0.98) than in older (age ≥65 years, AUC of 0.85) participants. The optimized p-tau181 cut-off for predicting Aβ status for <65 years was 1.58 pg/mL and had a 97% sensitivity and 96% specificity.

Clinical scenario #2: FTD diagnosis

The complete predictive model could discriminate FTD subjects from all other participants (AUC 0.89, Fig. 3B). The PM (AUC 0.84) included plasma NfL (AUC 0.83) and plasma p-tau181

(AUC 0.63). NfL was able to differentiate FTD from subjects with SND (AUC 0.93, balanced cut-off of 11.3 pg/mL, 84% sensitivity and specificity). Diagnostic performance was lower when differentiating FTD from AD (AUC 0.79, balanced cut-off 16.6, 75% sensitivity and 74% specificity. Combining both biomarkers improved diagnostic performance, with an NfL/p-tau181 ratio differentiating AD from FTD (AUC 0.93, cut-off 10.3, 88% sensitivity, 89% specificity).

Based on these results, in our cohort, participants with low p-tau181 (<1.37 pg/mL) would be suggestive of FTD if NfL is elevated (≥11.3 pg/mL). In cases with p-tau181 above the cut-off ≥1.37 pg/mL, the NfL/p-tau181 ratio would be used, with a ratio ≥10.3 being suggestive of FTD. The proposed algorithm identified FTD diagnosis with a sensitivity of 82%, a specificity of 85% and an NPV of 97%. The distribution of p-tau181 and NfL concentrations between clinical diagnoses and Aβ status, as well as proposed cut-offs, are represented in Fig. 3D; the algorithm is schematized on Figure 5.

Clinical scenario #3: Screening for neurodegenerative causes of cognitive impairment

We studied the diagnostic performance of distinct plasma biomarkers to differentiate between neurodegenerative (AD, LBD and FTD) and non-neurodegenerative (SND) etiologies. The complete model (Fig. 3C) had an AUC of 0.94, while the PM (AUC 0.92) included p-tau181, NfL and APOE (individual AUCs of 0.87, 0.82 and 0.68, respectively). The model with no biomarkers had an AUC of 0.83, similar to NfL but inferior to p-tau181 alone.

A sensitivity-optimized p-tau181 cut-off had 78% NPV for identifying non-neurodegenerative causes of cognitive impairment, while NPV was only 67% for NfL. Since these individual values were not deemed good enough to be effectively used for screening, we evaluated a combined algorithm of both p-tau181 and NfL. Fifteen percent of subjects (including half of SND diagnoses), had a "negative" result (i.e., p-tau181 and NfL levels below their respective cut-offs), with a sensitivity of 97% and a NPV of 86%. However, 5 (14% of all negative results) participants with a neurodegenerative disease (2 AD, 3 LBD), would have been misclassified as non-neurodegenerative.

In subjects <65 years of age, NfL performed better than p-tau181 (AUC 0.88 and 0.84, respectively), with specific cut-offs for this younger subgroup having a NPV of 82% for NfL and 73% for p-tau181 and, combining both biomarkers in a similar algorithm, 28% would have tested "negative", resulting in a 91% NPV.

Sub-cohort without AD biomarkers

Prospectively included patients without AD biomarkers (n=113) were older than those with AD biomarkers (mean age 70.6 vs 66.7, respectively, p<0.001). The rest of demographic, cognitive and plasma biomarkers data were distributed similarly between both groups (Fig. 1 and eTable 1).

We then applied the cut-offs and algorithms calculated previously to the sub-cohort without AD biomarkers performed (Fig. 4 and 5). Applying algorithm #1, based on individual p-tau181 results, 18% subjects could be classified as probably Aβ- (32% of SCD diagnoses, 14% of MCI, 7% AD dementia, 13% LBD and 13% FTD) and 35% could be classified as probably Aβ+ (9% of

SCD, 44% of MCI, 60% AD dementia, 38% LBD and 13% FTD). In total, in 53% of participants Aβ status could have been predicted with a probably high accuracy based on plasma p-tau181 results (Fig. 1A).

Algorithm #2, for FTD diagnosis, was suggestive of FTD in all cases of FTD (100% sensitivity and NPV), while specificity was 77%. The third algorithm, for identifying neurodegenerative causes of cognitive impairment, was suggestive of a non-neurodegenerative etiology in only 11% of patients (69% SCD and 31% MCI participants).

Classification of Evidence

This study provides Class I evidence that plasma p-tau181 correlates with the presence or absence of AD and a combination of plasma p-tau181 and NfL correlate moderately well with a diagnosis of FTD.

Discussion

In this prospective, everyday clinical practice cohort in a memory clinic, we have evaluated the diagnostic performance of several plasma biomarkers and have proposed specific algorithms for implementation in daily clinical routine.

Plasma p-tau181 has been established as an AD pathophysiological process biomarker, even in presymptomatic individuals.[24,27,34] In our cohort, p-tau181 levels could accurately identify symptomatic patients in the AD continuum and differentiate them from subjects with a non-neurodegenerative cause of cognitive impairment, with AUCs above 0.90, in line with previous studies.[26,28,30] Diagnostic performance was inferior when trying to differentiate AD from LBD and FTD, probably due to the presence of concomitant AD pathology.

Plasma GFAP also had a good diagnostic performance when differentiating patients with a non-neurodegenerative cognitive impairment from AD. We found no elevated GFAP levels in LBD, as opposed to a recent work,[19] which could be due to our lower number of LBD participants. Overall, GFAP diagnostic performance was inferior to p-tau181 in all comparisons. Previous studies also found a higher performance of p-tau181 in subjects with cognitive decline,[19,26] in contrast with studies with cognitively unimpaired participants, where plasma GFAP had a similar or better performance than p-tau181,[18] suggesting that p-tau181 might be better in symptomatic AD while GFAP could be more valuable in preclinical disease.

Plasma NfL discriminated with high accuracy FTD from subjects with a non-neurodegenerative cause but performed worse for the contrast FTD vs AD, as NfL were also mildly elevated in most AD subjects. The proposed NfL/p-tau181 ratio clearly improved the diagnostic accuracy between AD and FTD, in line with the body of evidence available from CSF biomarkers where combined biomarkers improve the diagnostic accuracy of individual results.

Finally, plasma UCH-L1 and t-tau levels showed poor diagnostic performance. A recent study using assays targeting other t-tau epitopes found better accuracy for identifying the AD pathophysiological process, which suggests that other plasma t-tau assays could be useful.[35] All plasma biomarkers were higher in CJD, in line with the rapid and massive neuronal death characteristic to this condition.

Plasma markers could not accurately discriminate LBD from AD or other diagnoses, stressing out the need for validation of new biochemical biomarkers for LBD.

Diagnostic performance evaluated with the AUCs values show the biomarker performance at group level. For a successful implementation in clinical practice, the added value for individual subjects in different clinical scenarios should be tested.

In our cohort, plasma p-tau181 could predict Aβ status with high sensitivity and specificity. Based on individually predicted results, we do not propose to substitute CSF AD biomarkers or amyloid PET by plasma p-tau181 but to reduce the number of subjects requiring CSF/PET biomarkers based on p-tau181 levels. Patients below the lower cut-off would probably be Aβ- (in our cohort 97% sensitivity, 95% NPV) while those above the upper cut-off would probably be Aβ+ (in our cohort 96% specificity, 94% PPV). Both groups represent 60% of the subjects in our cohort, and in these patients, plasma p-tau181 levels would potentially make unnecessary CSF AD biomarkers and/or amyloid PET for diagnostic purposes.

As in other high prevalence diseases such as diabetes, it is imperative to develop biochemical screening tools that allow an effective triaging of subjects with a higher probability of underlying neurodegeneration to be referred to specialized settings.[32,33] Previous studies have suggested that plasma NfL might identify neurodegenerative causes.[21] Although we confirmed the good discriminative capacity of NfL levels, our results suggest that NfL is not ready yet to be systematically used as a stand-alone screening tool for this purpose previous to clinical assessment in specialized memory clinics. Plasma NfL showed an AUC value of 0.83, similar to the model using only demographic data and APOE, for discriminating neurodegenerative (AD, LBD and FTD) from non-neurodegenerative etiologies. This can be due to several factors. First, LBD group had relatively low NfL levels, as previous studies with LBD/Parkinson's disease dementia and second, as seen in the study of Ashton et al., plasma NfL accuracy was better in younger (<65 years) participants if specific cut-offs for this age range were calculated.[21] In our cohort, plasma p-tau181 had greater diagnostic value than NfL for this aim (i.e. neurodegenerative vs non-neurodegenerative diagnosis), probably biased by the high number of participants with AD. Adding plasma p-tau181 to NfL and optimizing cut-offs improved diagnostic performance, screening-out half of subjects with a non-neurodegenerative cause but at the cost of 1 in 7 (14%) false negative results in subjects with a neurodegenerative diagnosis. Specific cut-offs for individuals <65 years old slightly improved the diagnostic performance of the algorithm to approximately 1 in 10 false negatives (9%). In this sense, we do not believe that NfL are ready to be used at this point to screen-out patients in the primary care for referral to a memory clinic due to the potential high number of false negative results.

The main strength of our study is its prospective design and the routine clinical practice methodology used. There is a clear need for real-world data for a successful implementation of plasma biomarkers in daily clinical practice. As for limitations, we do not have post-mortem pathological confirmation of any participant or AD biomarkers performed in the whole cohort and Aβ positivity was evaluated with a mix of CSF and PET results (inherent to the routine clinical practice methodology used). Second, the mean age of patients referred to our center is relatively young, and our data may not necessarily be valid in older populations (i.e above 80 years). Third, subjects with other neurological disorders and cognitive complaints (e.g., epilepsy, multiple sclerosis, large-vessel stroke) are not usually referred to our clinic and thus were not represented in our cohort. Fourth, the cut-offs proposed here are based on the results of one laboratory, and as other biomarkers cut-offs, should be individualized in each center. Fifth, some diagnostic categories were relatively underpowered and we used the "FTD"

umbrella term to unify several clinical entities. Finally, having measured all plasma biomarkers following completion of study recruitment diverges from usual clinical laboratory practice and potentially could have decreased the variability of results as compared with repeated measurements.

In conclusion, in a prospective real-world memory clinic cohort, we have shown that plasma p-tau181 is an excellent biomarker for identifying amyloid positivity, outperforming the other plasma biomarkers studied. Using an algorithm with two p-tau181 cut-offs, 60% participants could potentially have avoided undergoing further specific AD biomarker (CSF/amyloid PET) testing. NfL differentiated FTD from non-neurodegenerative causes and a combination of plasma p-tau181 and NfL improved differential diagnosis of FTD from AD. Our results do not support implementation of plasma biomarkers for routine screening for neurodegenerative disease previous to specialized clinical evaluation. Further studies are needed with real-world data to validate these results, especially in older patients.

Study funding

This work was supported by Spanish Ministry of Science and Innovation - Instituto de Salud Carlos III and Fondo Europeo de Desarrollo Regional (FEDER), Unión Europea, "Una manera de hacer Europa" (PI20/00448 to RSV, PI19/00449 to AL, and PI19/00198 to MB). JS received funding from a PFIS grant (FI21/00015). NG received funding from a PFIS grant (FI20/00076). ORC received funding from a PFIS grant (FI18/00121). NF received funding from a Rio Hortega grant (CM21/00024), Alzheimer's Association grant (ALZASSOC_CSF_21_01) and Global Brain Health Institute grant (GBHI ALZ UK-21-723831). SBE is a recipient of the Joan Rodés-Josep Baselga grant from the FBBVA.

Disclosures

The authors report no disclosures relevant to the manuscript.

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Table 1. Demographic, cognitive, APOE and plasma biomarkers characteristics of the whole cohort according to the clinical syndromic diagnosis (n=385).

	CU (n=36)	SCD (n=39)	MCI (n=183)	AD dementia (n=54)	LBD (n=28)	FTD (n=41)	CJD (n=4)	p-value
Age (years)	61.7 (8.2)	64.9 (8.7)	69.2 (7.6)	66.9 (7.9)	69.9 (6.9)	65.6 (10.4)	65.2 (11.2)	p<0.001*
Disease duration (years)	NA	2.8 (2.7)	2.5 (2.1)	2.7 (1.9)	3 (2.4)	2.8 (2)	0 (0)	NS**
Sex (female)	78%	54%	55%	63%	32%	44%	75%	p=0.005***
Education (years)	10.3 (4.8)	11.7 (3.8)	10.6 (4.9)	10 (4.1)	10.5 (4.7)	11.1 (4.6)	10 (3.5)	NS
MMSE	28.8 (1.1)	28.2 (2.9)	25 (3.4)	20.4 (4.6)	24.1 (3.9)	23.6 (5.3)	16 (2.8)	p<0.001^
APOE ε4 carriers	22%	35%	46%	56%	29%	26%	50%	p=0.009^^
AD biomarkers available (CSF/PET)	100%	21%	68%	72%	71%	80%	75%	NA
Plasma p-tau181 (pg/mL)	0.8 (0.3)	1.2 (0.5)	1.8 (1)	2.6 (1.2)	1.4 (0.7)	1.4 (0.8)	2.1 (1.1)	p<0.001
Plasma t-tau (pg/mL)	3.8 (1.6)	3.3 (1.3)	3.7 (1.5)	4.1 (1.5)	3.8 (1.6)	4 (1.5)	30.5 (45.2)	p<0.001^^^
Plasma NfL (pg/mL)	9.2 (3.9)	8.5 (3.4)	12.1 (5.7)	15.6 (5.7)	14 (7.4)	33.1 (22.4)	91.8 (68.5)	p<0.001
Plasma GFAP (pg/mL)	98 (58.2)	97.8 (47.3)	156.1 (89.6)	222.3 (114.5)	133.5 (81.1)	157.1 (90.5)	1019.9 (763.5)	p<0.001
Plasma UCH-L1 (pg/mL)	15.6 (18.6)	16 (13)	18.1 (13.1)	16.4 (9.6)	17.6 (10.4)	20.4 (14.3)	48 (16.3)	p=0.012^^^

Data are shown as mean (standard deviation) or percent. *MCI and LBD subjects were older than CU participants. **CJD patients were excluded from this analysis. ***There were more women in the CU group and more men with LBD. ^MMSE was higher in the CU and SCD groups, while the AD group had the lowest MMSE scores. ^^There were more APOE ε4 allele carriers in the MCI and the AD dementia group. ^^^Plasma t-tau and UCH-L1 concentrations were only significantly different in CJD compared to the other diagnostic groups. **Abbreviations:** CU, cognitively unimpaired; SCD, subjective cognitive decline; MCI, mild cognitive impairment; AD, Alzheimer's disease; LBD, dementia with Lewy bodies; FTD, frontotemporal dementia; CJD, Creutzfeldt-Jakob disease; MMSE, mini-mental state examination; p-tau181, tau phosphorylated at threonine 181; t-tau, total tau; NfL, neurofilament light chain; GFAP, glial fibrillary acidic protein; UCH-L1, Ubiquitin C-terminal hydrolase-L1; NS, not significant; NA, not applicable.

Figure 1. Plasma concentrations of p-tau181, GFAP, NfL and t-tau in the whole cohort per diagnostic syndromic group.

Box-and-whisker plots with the central horizontal box line showing the median plasma concentration in each diagnostic group and lower and upper box boundaries showing the 25^{th} and 75^{th} percentile, respectively. Plasma biomarker concentrations between diagnostic groups were log10 transformed and compared using an analysis of covariance, adjusting for age and sex, and pairwise comparisons were assessed with the Bonferroni correction method. For visualization purposes, raw (not log10-transformed) biomarker concentrations were plotted in the graph and the scale of the upper segment of the Y axis was adjusted, as marked by a discontinuous line. Participants were represented by a different color depending on amyloid status (not performed/negative/positive). Two additional horizontal discontinuous lines were represented in A, corresponding to a plasma p-tau181 concentration of 0.89 and 1.92, which were the ones with an optimized negative and positive predictive value for amyloid beta status discrimination, respectively (see results section).

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. Brackets indicate the diagnostic groups compared and a left-pointing arrow marks that all diagnostic categories to the left have the same statistical significance. **Abbreviations:** CU, cognitively unimpaired; SCD, subjective cognitive decline; MCI, mild cognitive impairment; AD, Alzheimer's disease; LBD, dementia with Lewy bodies; FTD, frontotemporal dementia; CJD, Creutzfeldt-Jakob disease; p-tau181, tau phosphorylated at threonine 181; t-tau, total tau; NfL, neurofilament light chain; GFAP, glial fibrillary acidic protein; Aβ-/+, amyloid beta negative/positive.

Table 2. Demographic, cognitive, APOE and plasma biomarkers characteristics of the sub-cohort with AD biomarkers available (CSF or amyloid PET) by diagnostic etiological category (n=268).

	CU (n=36)	SND (n=60)	AD (n=119)	LBD-Aβ- (n=13)	LBD-Aβ+ (n=7)	FTD-Aβ- (n=28)	FTD-Aβ+ (n=5)	p-value
Age (years)	61.7 (8.2)	63.4 (8)	68.2 (6.1)	69.9 (6.8)	65.9 (6.1)	65.2 (9.8)	71.6 (4.6)	p<0.001*
Disease duration (years)	NA	2.5 (1.9)	2.7 (2.1)	3 (2.8)	3.6 (2.1)	2.8 (2.1)	3.2 (1.6)	p=0.699
Sex (female)	78%	47%	61%	39%	15%	46%	40%	p=0.001**
Education (years)	10.3 (4.8)	10.3 (4.8)	10.8 (4.5)	11.1 (5.5)	11.4 (4.4)	11.4 (4.6)	9.6 (7)	p=0.945
MMSE	28.7 (1.1)	26.8 (3.2)	22.7 (4.4)	25.4 (4.1)	23.3 (4.2)	23.6 (5.6)	21.8 (5.7)	p<0.001^
APOE ε4 carriers	22%	19%	63%	18%	80%	19%	67%	p<0.001^^
Plasma p-tau181 (pg/mL)	0.8 (0.3)	0.9 (0.4)	2.4 (1)	1.1 (0.5)	1.9 (0.9)	1.4 (0.8)	1.7 (0.9)	p<0.001
Plasma t-tau (pg/mL)	3.8 (1.6)	3.2 (1.3)	4 (1.7)	3.6 (1.1)	4.4 (2.4)	3.9 (1.1)	5.2 (1.9)	p=0.038
Plasma NfL (pg/mL)	9.2 (4)	8.6 (4.2)	14 (5.6)	14.2 (7.8)	11 (3.6)	29.3 (17.2)	42.6 (30.1)	p<0.001
Plasma GFAP (pg/mL)	98 (58)	81.9 (35.6)	203.8 (101.7)	137.2 (85.1)	95.6 (21.2)	144.4 (83.3)	210.1 (81.6)	p<0.001
Plasma UCH-L1 (pg/mL)	15.5 (18.5)	18.9 (13.9)	17.9 (14.2)	18.7 (10.6)	19.3 (11.6)	21.4 (14.9)	18.7 (9.3)	p=0.309

Please note that in the sub-cohort with AD biomarkers available, diagnostic categories are grouped depending on the etiology of cognitive impairment, in contrast to the syndromic category used for the whole cohort. Data are shown as mean (standard deviation) or percent. *CU individuals were younger than AD and LBD-Aβ- participants and the AD group was older than the SND one. **There were significantly more women in the CU and AD diagnostic categories. ^MMSE was higher in the CU and SND group compared to the other groups. ^^There were more APOE ε4 allele carriers in the AD, LBD-Aβ+ and FTD-Aβ+ groups. **Abbreviations:** CU, cognitively unimpaired; SND, suspected nondegenerative cognitive impairment; MCI, mild cognitive impairment AD, Alzheimer's disease; LBD, dementia with Lewy bodies; FTD, frontotemporal dementia; MMSE, mini-mental state examination; p-tau181, tau phosphorylated at threonine 181; t-tau, total tau; NfL, neurofilament light chain; GFAP, glial fibrillary acidic protein; UCH-L1, Ubiquitin C-terminal hydrolase-L1; Aβ-/+, amyloid beta negative/positive; NA, not applicable.

Figure 2. Plasma p-tau181 and NfL concentrations in the sub-cohort with AD biomarkers performed per diagnostic group (left) and area under the ROC curve for discrimination between diagnostic etiological categories (right).

A and C. Box-and-whisker plots with the central horizontal box line showing the median plasma concentration in each diagnostic group and lower and upper box boundaries showing the 25th and 75th percentile, respectively. Plasma biomarker concentrations between diagnostic groups were log10 transformed and compared using an analysis of covariance, adjusting for age and sex, and pairwise comparisons were assessed with the Bonferroni correction method. For visualization purposes, raw (not log10-transformed) biomarker concentrations were plotted in the graph and the scale of the upper segment of the Y axis was adjusted for plasma NfL (C), as marked by a discontinuous line. Participants were represented by a different color depending on amyloid beta positivity or negativity, which was defined using our center definitions (see methods section). Two additional horizontal discontinuous lines were represented in A, corresponding to a plasma p-tau181 concentration of 0.89 and 1.92, which were the ones with an optimized negative and positive predictive value for amyloid beta status discrimination, respectively (see results section). B and D. Diagnostic accuracy of plasma biomarkers to differentiate between each pair of diagnoses is represented by a heatmap of each AUC, with a value of 0.5 meaning no discrimination and a value of 1 a perfect discrimination.

*: p<0.05, **: p<0.01, ***: p<0.001. Brackets indicate the diagnostic groups compared and a left-pointing arrow marks that all diagnostic categories to the left have the same statistical significance. **Abbreviations:** CU, cognitively unimpaired; SND, suspected nondegenerative cognitive impairment; AD, Alzheimer's disease; LBD, dementia with Lewy bodies; FTD, frontotemporal dementia; p-tau181, tau phosphorylated at threonine 181; NfL, neurofilament light chain; ; Aβ-/+, amyloid beta negative/positive; ROC, receiver operating curve; AUC, area under the ROC curve.

Figure 3. Receiver operating characteristic plots of predictive models and individual plasma biomarkers for distinct clinical scenarios and scatter plot for clinical and amyloid beta status classification using plasma NfL and p-tau181.

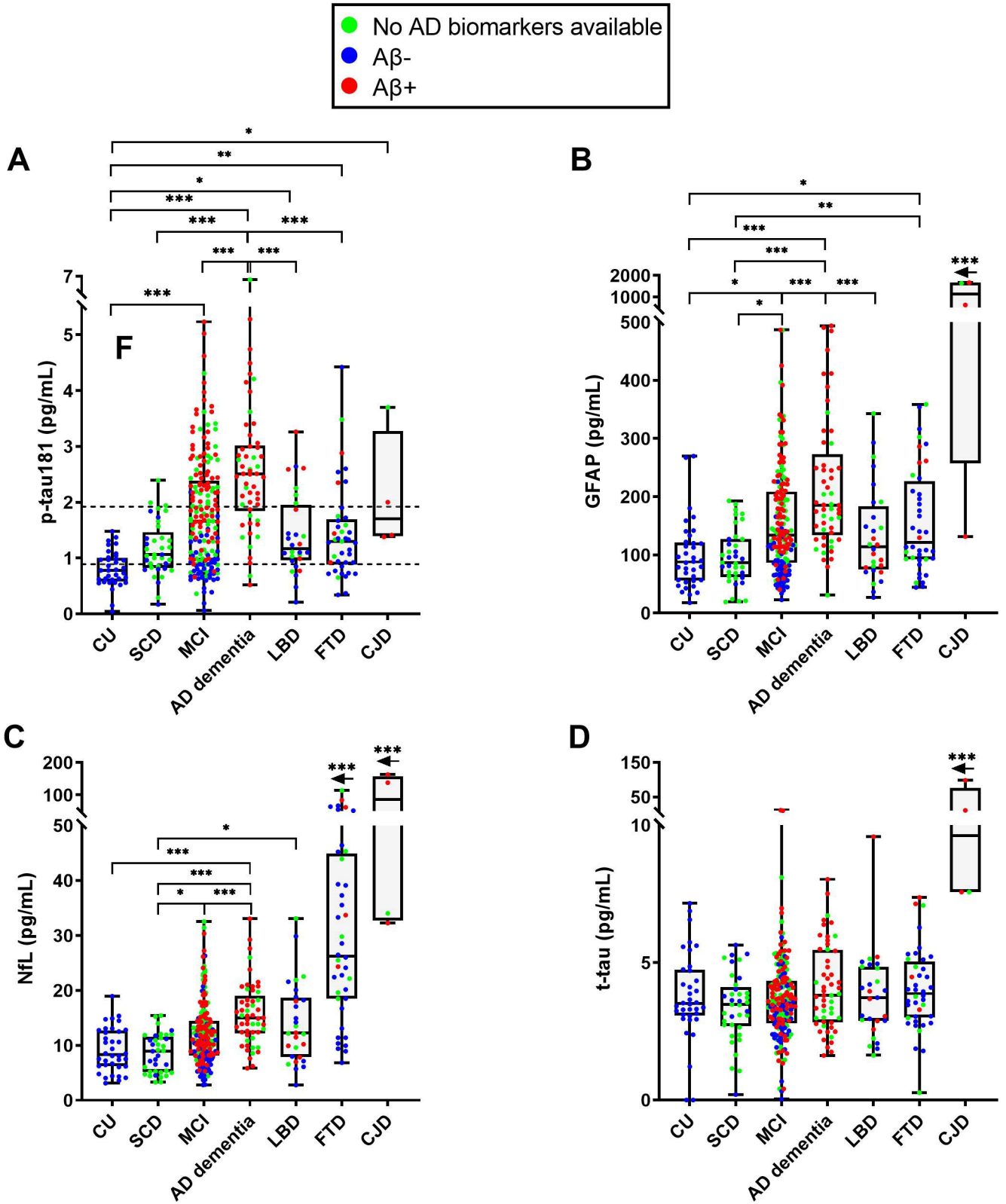
A, B and C. Receiver operating characteristic plots showing the area under the curve of the predicted probability of distinct logistic regression models and individual plasma biomarkers in the proposed practical scenarios, with 95% confidence interval in brackets. The complete model included age, sex, APOE, MMSE and the 5 plasma biomarkers, while the model without biomarkers included only age, sex, APOE and MMSE. D. Scatter plot showing the distribution of diagnoses (represented by shape) and amyloid beta status (represented by color) by plasma p-tau181 and NfL concentrations. Three cut-offs are represented in the scatter plot by dotted lines: plasma p-tau181 1.37 pg/mL (balanced cut-off for amyloid beta status discrimination), plasma NfL 11.3 pg/mL (balanced cut-off for FTD diagnosis when p-tau181 is below 1.37 pg/mL, for optimal differentiation of FTD from SND) and plasma NfL/p-tau181 ratio of 10.3 (balanced cut-off for FTD diagnosis when p-tau181 is equal or higher than 1.37 pg/mL, for optimal differentiation of FTD from AD). For visualization purposes, the scale of the upper segment of the Y axis was adjusted, as marked by a discontinuous line. **Abbreviations:** PM, parsimonious model; CU, cognitively unimpaired; SND, suspected nondegenerative cognitive impairment; AD, Alzheimer's disease; LBD, dementia with Lewy bodies; FTD, frontotemporal dementia; p-tau181, tau phosphorylated at threonine 181; NfL, neurofilament light chain; GFAP, glial fibrillary acidic protein; Aβ-/+, amyloid beta negative/positive; MMSE, mini-mental state examination.

Figure 4. Proposed algorithm for amyloid status prediction using plasma p-tau181.

Abbreviations: P-tau181, tau phosphorylated at threonine 181; Aβ-/+, amyloid beta negative/positive.

Figure 5. Proposed algorithm for FTD diagnosis using plasma p-tau181 and NfL.

Abbreviations: SND, suspected nondegenerative cognitive impairment; AD, Alzheimer's disease; LBD, dementia with Lewy bodies; FTD, frontotemporal dementia; p-tau181, tau phosphorylated at threonine 181; NfL, neurofilament light chain.


No AD biomarkers available
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Aβ+
A
F
p-tau181 (pg/mL)
B
GFAP (pg/mL)
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MCI
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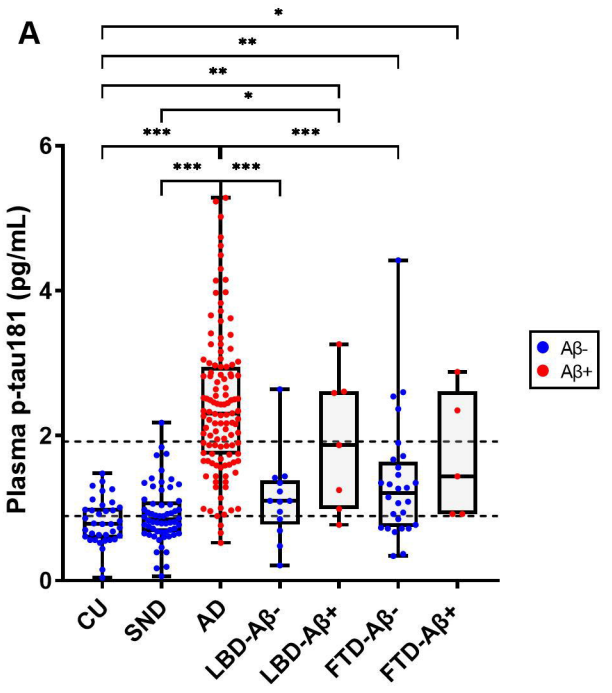
LBD
FTD
CJD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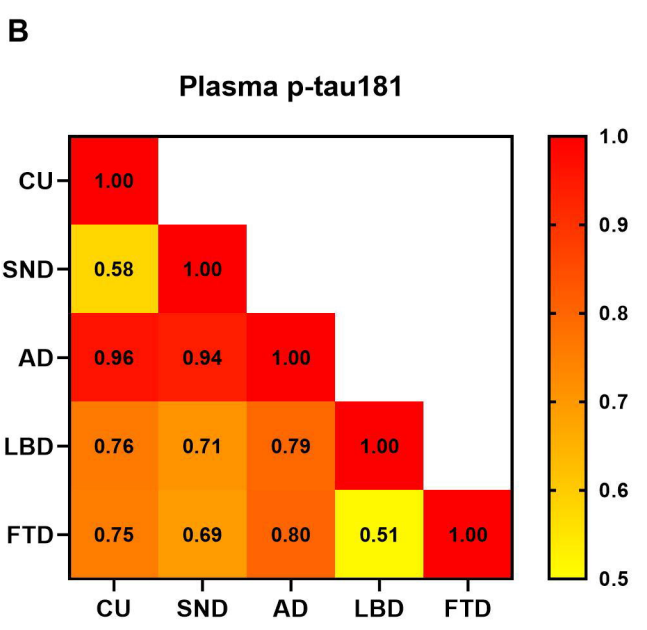
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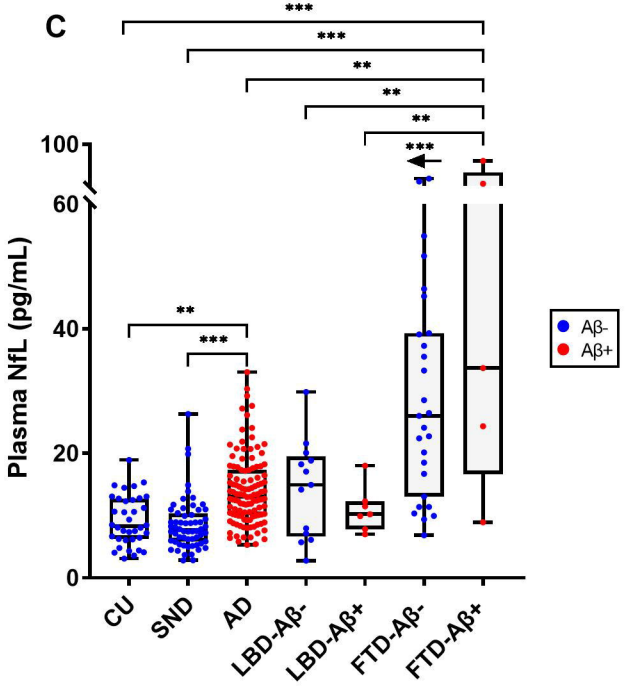
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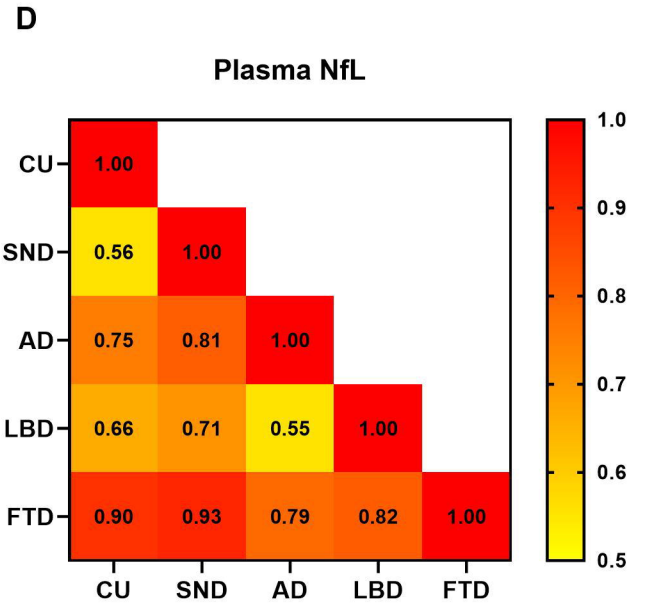
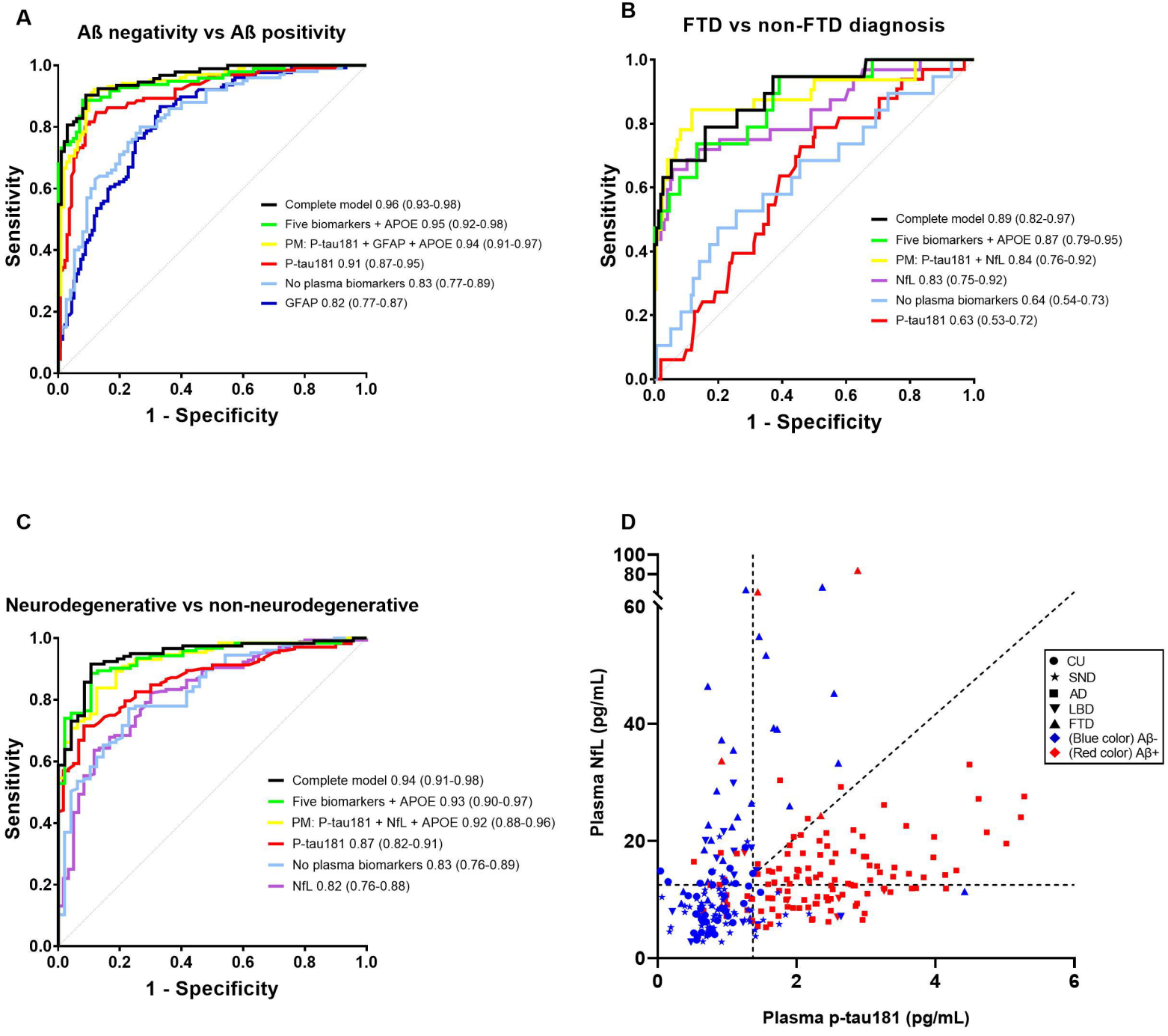
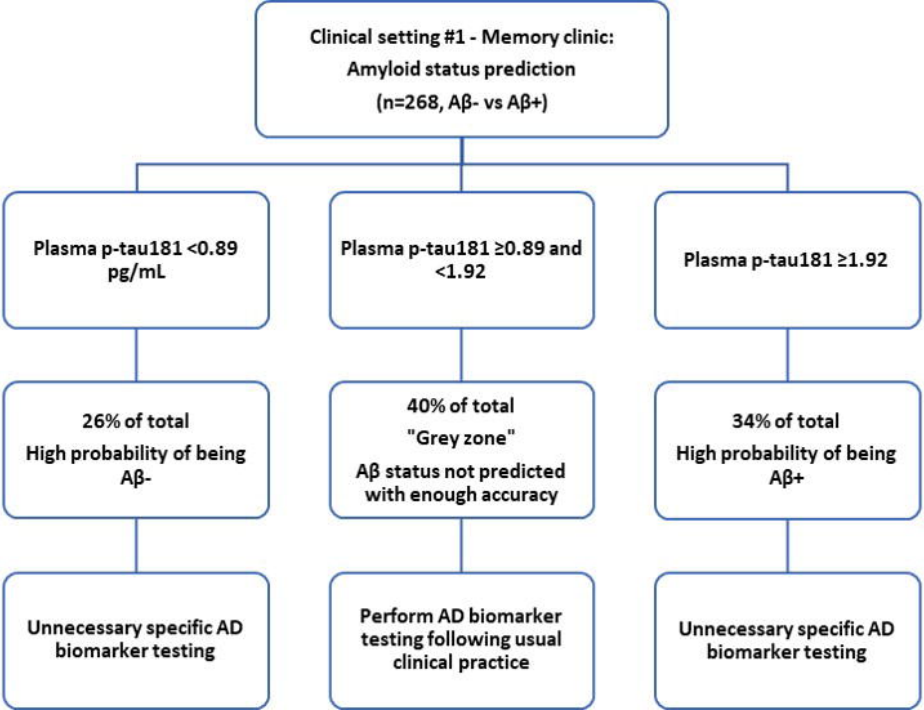
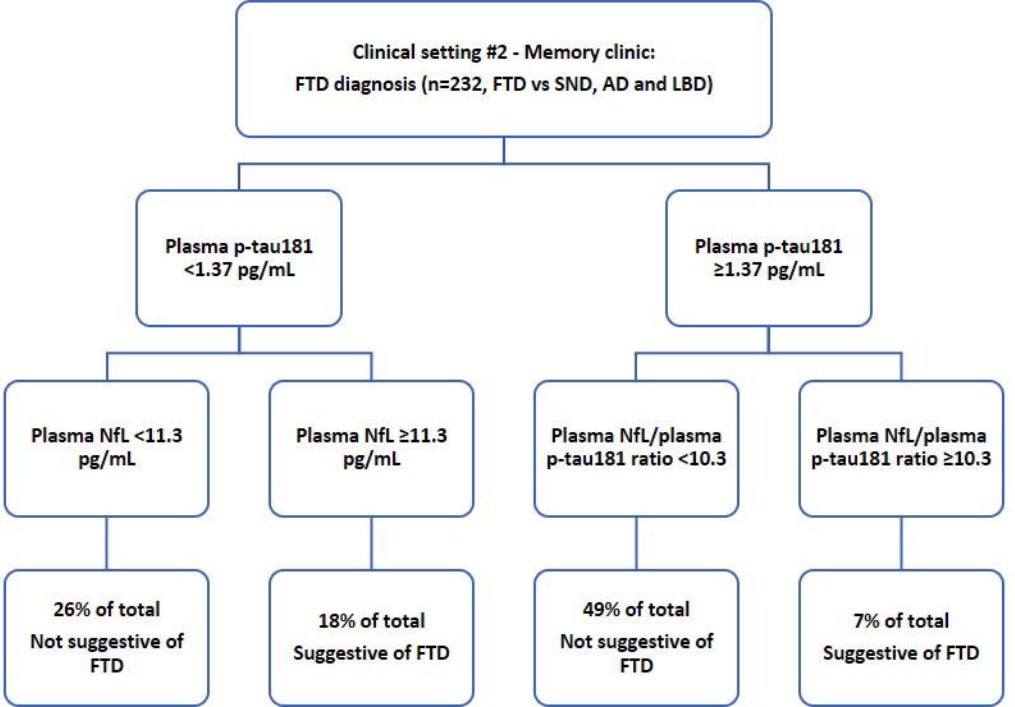
FTD-Aβ-
FTD-Aβ+


D
Plasma NfL
CU
SND
AD
LBD
FTD
1.00
0.56 1.00
0.75 0.81 1.00
0.66 0.71 0.55 1.00
0.90 0.93 0.79 0.82 1.00


A
Aß negativity vs Aß positivity
Sensitivity
1 - Specificity
Complete model 0.96 (0.93-0.98)
Five biomarkers + APOE 0.95 (0.92-0.98)
PM: P-tau181 + GFAP + APOE 0.94 (0.91-0.97)
P-tau181 0.91 (0.87-0.95)
No plasma biomarkers 0.83 (0.77-0.89)
GFAP 0.82 (0.77-0.87)
B
FTD vs non-FTD diagnosis
Sensitivity
1 - Specificity
Complete model 0.89 (0.82-0.97)
Five biomarkers + APOE 0.87 (0.79-0.95)
PM: P-tau181 + NfL 0.84 (0.76-0.92)
NfL 0.83 (0.75-0.92)
No plasma biomarkers 0.64 (0.54-0.73)
P-tau181 0.63 (0.53-0.72)
C
Neurodegenerative vs non-neurodegenerative
Sensitivity
1 - Specificity
Complete model 0.94 (0.91-0.98)
Five biomarkers + APOE 0.93 (0.90-0.97)
PM: P-tau181 + NfL + APOE 0.92 (0.88-0.96)
P-tau181 0.87 (0.82-0.91)
No plasma biomarkers 0.83 (0.76-0.89)
NfL 0.82 (0.76-0.88)
D
Plasma NfL (pg/mL)
Plasma p-tau181 (pg/mL)
CU
SND
AD
LBD
FTD
(Blue color) Aβ-
(Red color) Aβ+


Clinical setting #1 - Memory clinic:
Amyloid status prediction
(n=268, Aβ- vs Aβ+)
Plasma p-tau181 <0.89 pg/mL
Plasma p-tau181 ≥0.89 and <1.92
Plasma p-tau181 ≥1.92
26% of total
High probability of being Aβ-
40% of total
"Grey zone"
Aβ status not predicted with enough accuracy
34% of total
High probability of being Aβ+
Unnecessary specific AD biomarker testing
Perform AD biomarker testing following usual clinical practice
Unnecessary specific AD biomarker testing


Clinical setting #2 - Memory clinic:
FTD diagnosis (n=232, FTD vs SND, AD and LBD)
Plasma p-tau181 <1.37 pg/mL
Plasma p-tau181 ≥1.37 pg/mL
Plasma NfL <11.3 pg/mL
Plasma NfL ≥11.3 pg/mL
Plasma NfL/plasma p-tau181 ratio <10.3
Plasma NfL/plasma p-tau181 ratio ≥10.3
26% of total
Not suggestive of FTD
18% of total
Suggestive of FTD
49% of total
Not suggestive of FTD
7% of total
Suggestive of FTD