Title: Plasma p-tau231, p-tau181, PET biomarkers and cognitive change in older adults

Running head: Plasma p-tau biomarkers in pre-symptomatic AD

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Current knowledge on this topic: Plasma p-tau epitopes have demonstrated excellent ability to detect Aβ pathology and to distinguish AD dementia from controls and other neurodegenerative diseases. However, there remains a need for a strong plasma biomarker in pre-symptomatic AD.

What question did this study address: Can plasma p-tau231 and p-tau181 detect AD pathology and predict cognitive decline in cognitively unimpaired older adults at risk for AD dementia.

What does this study add to our knowledge? This study shows that plasma p-tau231 may be a more robust indicator of Aβ and tau pathologies in pre-clinical disease than p-tau181. Combining plasma Aβ and tau biomarkers may improve our ability to detect cognitively unimpaired older adults at increased risk of cognitive decline.

How might this potentially impact on the practice of neurology? Further validation efforts are required before plasma biomarkers can be readily implemented in clinic. However, the availability of these tools may facilitate early diagnosis of AD and patient recruitment in clinical trials for disease-modifying drugs.

Abstract

Objective: To evaluate novel plasma p-tau231, p-tau181 as well as $A\beta_{40}$ and $A\beta_{42}$ assays as indicators of tau and Aβ pathologies measured with positron emission tomography (PET), and their association with cognitive change, in cognitively unimpaired older adults.

Methods: In a cohort of 244 older adults at risk of AD owing to a family history of AD dementia, we measured single molecule array (Simoa)-based plasma tau biomarkers (p-tau231, p-tau181), $A\beta_{40}$ and $A\beta_{42}$ with immunoprecipitation mass spectrometry, and Simoa NfL. A subset of 129 participants underwent amyloid-β (^{18}F-NAV4694) and tau (^{18}F-flortaucipir) PET assessments. We investigated plasma biomarker associations with Aβ and tau PET at the global and voxel level and tested plasma biomarker combinations for improved detection of Aβ-PET positivity. We also investigated associations with 8-year cognitive change.

Results: Plasma p-tau biomarkers correlated with flortaucipir binding in medial temporal, parietal and inferior temporal regions. P-tau231 showed further associations in lateral parietal and occipital cortices. Plasma $A\beta_{42/40}$ explained more variance in global Aβ-PET binding than $A\beta_{42}$ alone. P-tau231 also showed strong and widespread associations with cortical Aβ-PET binding. Combining $A\beta_{42/40}$ with p-tau231 or p-tau181 allowed for good distinction between Aβ-negative and -positive participants (AUC range 0.81-0.86). Individuals with low plasma $A\beta_{42/40}$ and high p-tau experienced faster cognitive decline.

Interpretation: Plasma p-tau231 showed more robust associations with PET biomarkers than p-tau181 in pre-symptomatic individuals. The combination of p-tau and $A\beta_{42/40}$ biomarkers detected early AD pathology and cognitive decline. Such markers could be used as pre-screening tools to reduce the cost of prevention trials.

Introduction

Alzheimer's disease (AD) pathogenesis spans several decades before onset of cognitive impairment. [1-3] When symptoms appear, brain damage may therefore be too widespread and advanced for treatments to modify the disease course. [4] Thus, research has focused considerably on developing meaningful biomarkers of incipient disease. To date, the most promising candidates have been markers of amyloid-β (Aβ) plaques and *tau* tangles, the pathological hallmarks of the disease. These proteins are measured by cerebrospinal fluid (CSF) assays and positron emission tomography (PET). [5-8] Although promising for prediction of subsequent AD dementia, [9, 10] these are either too invasive or costly to permit widespread use. Thus, there is an urgent need for biomarkers with potential for routine use in clinic. Because venipuncture is minimally invasive and can be performed routinely, plasma biomarkers would be ideal. However, development of such markers has been difficult because AD-related plasma Aβ and tau are confounded by peripheral expression or exist in low concentrations, respectively.[11] Recent availability of high sensitivity immunoassay technologies and immunoprecipitation coupled with mass spectrometry (IP-MS) assays has allowed practical assessment of brain Aβ pathology from plasma.[12-16] Blood-based biomarkers of brain tau pathology have also been developed. Specifically, novel p-tau181[17-22] and p-tau217[23-25] assays appear to: efficiently distinguish AD, controls, and non-AD dementias; improve detection of cerebral Aβ plaque deposition; and correlate with cerebral tau pathology in cohorts including cognitively impaired patients. These biomarkers also appear to be altered prior to cognitive decline.[21, 22, 26] More recently, levels of a novel plasma p-tau231 marker were also found to exhibit similar traits as p-tau181 and p-tau217 but were elevated even among individuals below a typical Aβ-PET positivity threshold.[27] In a cohort of cognitively unimpaired (CU) relatively young elderly at increased risk of AD dementia, we evaluated p-tau231 and p-tau181 as indicators of early tau deposition and neuronal response to Aβ

pathology. We also assessed IP-MS and Simoa measures of plasma Aβ as indicators of brain Aβ deposition and plasma neurofilament light (NfL) as a marker of non-specific neurodegeneration.

Methods

Participants. Participants were part of the PREVENT-AD cohort of healthy older adults with increased risk of AD dementia owing to a family history of the disease.[28] Starting in 2011, PREVENT-AD enrolled 386 CU participants aged 60 years or older having a parent or at least two siblings diagnosed with AD dementia (aged 55+ years if within 15 years of their youngest-affected relative's symptom onset). At study enrolment, all participants underwent brief cognitive screening using the Clinical Dementia Rating (CDR)[29] and Montreal Cognitive Assessment (MoCA)[30] to confirm normal cognition.[31] In a few cases of ambiguous CDR (0.5) or MoCA (≤26), individuals underwent a complete neuropsychological evaluation by a certified neuropsychologist; three such participants were included in our analyses. These participants were screened in 2015 and, as of their 2021 visits, were still cognitively normal. Removing these participants from cognitive endpoint analyses did not affect study results. At annual follow-up visits, the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS)[32] and AD8 questionnaire[33] were administered and the scores reviewed by certified neuropsychologists to detect change in cognitive status. Suspicion of MCI following neuropsychological evaluation resulted in referral to a memory clinic. Participants also underwent annual structural magnetic resonance imaging (MRI) and medical assessments. A sample of 244 older adults donated blood samples which were analyzed for plasma p-tau and Aβ biomarkers. Of these plasma donors, 129 persons volunteered for PET assessment of Aβ and *tau* pathologies *in vivo* (PET sub-sample). All participants were CU at the time of blood

sample collection and PET scanning. Participants provided written informed consent, and all research procedures complied with the ethical principles of the Declaration of Helsinki and were approved by the Institutional Review Board at McGill University.

Plasma measurements. Blood specimens were collected at an annual study visit either in the fasting or non-fasting state. Samples of 20mL of blood were collected in two EDTA(K2) tubes and centrifuged for 10 minutes at 3000rpm (2000 × *g*) at 4°C. Plasma was separated from the buffy coat and red blood cells with a polypropylene transfer pipet, further aliquoted into 500µL polypropylene tubes and stored at -80°C. Plasma $A\beta_{40}$ and $A\beta_{42}$ concentrations were measured using ultrasensitive Simoa immunoassay (Quanterix, Billerica, MA), as previously described. Plasma $A\beta_{40}$ and $A\beta_{42}$ levels were also measured using immunoprecipitation mass spectrometry (IP-MS).[15] In brief, calibrators were prepared using recombinant $A\beta_{1–40}$ and $A\beta_{1–42}$ (rPeptide) added to 8% bovine serum albumin in phosphate-buffered saline. Recombinant "heavy" peptides (^{15}N-uniformly labelled $A\beta_{1–40}$ and $A\beta_{1–42}$; rPeptide) were added to samples and calibrators prior to sample preparation and used as internal standards. Pooled plasma samples from the University of Gothenburg were used to track assay performance over different days and showed inter-assay coefficients of variation <5%. After a single thaw, Aβ peptides were extracted from 0.25 mL of each sample using immunoprecipitation with anti-Aβ antibodies 4G8 (epitope 17-27 in the Aβ sequence) and 6E10 (epitope 1-16, both antibodies from BioLegend) coupled to Dynabeads™ M-280 Sheep Anti-Mouse IgG magnetic beads (Thermo Fisher Scientific). Immunoprecipitation was performed using a KingFisher™ Flex Purification System (Thermo Fisher Scientific). After immunoprecipitation, eluates in 0.1 mL of 0.5% formic acid were vacuum centrifuged and stored at -80°C. Prior to analysis by liquid chromatography-tandem mass spectrometry (LC–MS/MS), the dried eluates were re-suspended in 20% acetonitrile and 4% concentrated

ammonia in water and injected into the LC–MS system (a Dionex Ultimate LC-system and a Thermo Scientific Q Exactive quadrupole-Orbitrap hybrid mass spectrometer). Chromatographic separation was achieved using basic mobile phases and a reversed-phase monolith column at a flow rate of 0.3 mL/min. The mass spectrometer, operating in parallel reaction monitoring (PRM) mode, was set to isolate the 4+ charge state precursors of the Aβ peptides. Product ions (14–15 depending on the peptide) specific for each precursor were selected and summed to calculate the chromatographic areas for each peptide and its corresponding internal standard. The area ratio of the analyte to the internal standard was used for quantification in samples and calibrators.

Plasma p-tau231 and p-tau181 concentrations were measured using the Simoa platform as described previously.[17, 20, 27] Plasma p-tau181 and p-tau231 concentrations were measured using ultrasensitive in-house single molecule array (Simoa) assays developed at the Clinical Neurochemistry Laboratory, Department of Psychiatry & Neurochemistry, University of Gothenburg, Sweden, on the HD-X platform (Quanterix, Billerica, MA, USA). The plasma p-tau181 Simoa assay is comprised of paramagnetic beads coupled with a mouse monoclonal capture antibody specifically targeting phosphorylated threonine 181 (AT270, Invitrogen) and biotinylated mouse monoclonal detector antibody directed against the N-terminal region of tau (Tau12, BioLegend). Full-length recombinant tau-441 phosphorylated in vitro by glycogen synthase kinase 3β (#TO8-50FN, SignalChem) was used as the calibrator. Immediately before analysis, plasma samples were thawed, vortexed (2000 rpm) and centrifuged (10 min at 4000 × *g* at RT) and then diluted two-fold with Tau2.0 buffer (Quanterix, Billerica, MA, USA). Plasma samples were randomized and analysed using identical batches of reagents. Plasma p-tau181 data was collected over three analytical runs, and all samples measured above the lower limit of quantification set for the assay (1.0 pg/mL). As a measure of assay precision, two quality control (QC) plasma samples were

analysed in duplicate at the start and end of each run. The within- and between-run coefficients of variation for both QC samples were <10%. For the novel plasma p-tau231 Simoa assay, monoclonal mouse antibodies were generated using a synthetic peptide (K_{224}KVAVVR(pT)PPKSPSSAK_{240}C) as a KLH-coupled antigen, numbered according to full-length tau-441 phosphorylated on threonine 231. Candidate hybridomas were selected on brain extracts of AD and control brain tissue. The final cloned and purified monoclonal antibody, ADx253, was characterized on synthetic peptides spanning amino acids threonine 217 till serine 241 of full-length tau, This was done to assess its affinity, its phospho-specificity using both phosphorylated and non-phosphorylated peptides and its preferred selectivity in which position 232 was replaced by a Pip. A biotin-conjugated N-terminal anti-tau mouse monoclonal antibody was used for detection. Full-length recombinant tau41 phosphorylated in vitro by glycogen synthase kinase 3β was used as the calibrator. The within- and between-run coefficients of variation for both QC samples were <8%. Further details about the assay and validation performance have been previously described.[27] Plasma NfL concentration was measured at the Clinical Neurochemistry Laboratory in Gothenburg using the commercially available Simoa kit (Quanterix; 103186). The intra-assay and inter-assay coefficients of variation were <6% and <10% respectively for all internal control samples. The limit of detection (LOD) was 0.038 pg/mL and the lower limit of quantification (LLOQ) was 0.176 pg/mL when compensated for a 4-fold sample dilution. Note that the use of fasting or non-fasting blood only resulted in higher measures for Simoa $A\beta_{40}$ and $A\beta_{42}$ in fasting blood (Figure 1).

Image acquisition and processing. Cortical Aβ and tau pathologies were measured using ^{18}F-NAV4694 (Navidea Biopharmaceuticals, Dublin, Ohio) and ^{18}F-AV1451(flortaucipir; Eli Lilly & Company, Indianapolis, Indiana). PET was performed at the McConnell Brain

Imaging Centre at the Montreal Neurological Institute (Montreal, Quebec) between February 16, 2017 and May 10, 2019. Scans were typically performed on two consecutive days and were never separated in time by more than eight months (median 1 day, range 1-246 days). NAV4694 scans and flortaucipir scans were acquired 40-70 minutes and 80-100 minutes post-injection, respectively.

A T1-weighted structural MRI scan was obtained separately on a 3T Siemens Trio scanner at the Douglas Mental Health University Institute (Montreal), as described previously.[34] These MRIs were processed using FreeSurfer 5.3 and parcellated following the Desikan-Killiany atlas.[35]

The preprocessing pipeline for PET images has been described[34] and is available at https://github.com/villeneuvelab/vlpp. Briefly, the 4D PET images were averaged and co-registered to the T1-weighted MRI from the closest available visit. Registered PET images were then masked to exclude CSF binding and smoothed using a Gaussian kernel of 6 mm^3. Standardized uptake value ratios (SUVR) were computed dividing the signal in regions of interest (ROI) by that in the reference region (cerebellar grey matter for NAV4694,[35] and inferior cerebellar grey matter for flortaucipir).[36] To assess Aβ deposition, we calculated NAV4694 SUVR values in a global cortical ROI.[35] Participants with SUVR ≥1.37 were considered Aβ-positive.[34] AD-related tau deposition was assessed by flortaucipir SUVR in the entorhinal and inferior temporal cortices, and in a temporal metaROI.[37, 38] For whole-brain voxelwise analyses, we normalized the preprocessed T1-coregistered PET images to the Montreal Neurological Institute (MNI)-ICBM152 stereotaxic symmetric template, using the transformation parameters obtained from non-linear normalization of the individual T1-

weighted images to the MNI-ICBM152 template in SPM12 (Segment routine; Wellcome Department of Imaging Neuroscience, London, England).

APOE genotyping. *APOE* genotype was determined using RT-PCR amplified DNA and the PyroMark Q96 pyrosequencer (Qiagen, Toronto, Canada), as described previously.[39]

Cognitive testing. Participants' cognitive performance was measured annually for up to eight years using the RBANS,[32] which gives index scores for the five cognitive domains evaluated (Immediate Memory, Delayed Memory, Attention, Language and Visuospatial) as well as a Total Scale score. The index scores are standardized by the participant's age such that a score of 100 represents the expected cognitive performance for a given age range. Four equivalent versions of the RBANS were administered in a randomized manner across the years in French or English depending on the participants' preferred language. Cognitive follow-up ranged from 0 to 8 years (median 4 years).

Statistical analyses. We compared demographic characteristics of the plasma donor sample and the PET sub-sample using Fisher exact or Kruskal-Wallis tests where appropriate. We then tested for associations between the various plasma biomarkers. We also evaluated the association of plasma p-tau biomarkers with flortaucipir tracer retention. We then examined associations of plasma biomarkers ($A\beta_{42}$, $A\beta_{42/40}$, p-tau231 and p-tau181) with NAV4694 SUVR. Because the distribution of cortical Aβ-PET did not meet assumptions for linear modelling, the data were normalized using Boxcox transformation (λ=-4.0363) and standardized using a z-score. Associations of plasma and PET biomarkers were assessed using robust regression to avoid extreme values driving associations. Results are presented unadjusted but were similar when adjusted for time difference between the plasma collection

and PET assessments, whether fasting blood samples were analyzed and whether or not participants volunteered for a PET scan. Finally, we investigated voxelwise associations of plasma and PET biomarkers in the statistical parametric mapping toolbox (SPM12, Wellcome Department of Imaging Neuroscience). Note that all presented associations were comparable when PET was corrected for partial volume effects.[36, 40-42]

We also sought to identify whether a combination of plasma biomarkers could improve detection of cerebral Aβ deposition, as measured with PET. Aβ-PET positivity status was assessed using logistic regression models to estimate coefficients for these predictors, Aβ-PET positivity probability and area under the receiver operating characteristic curve (AUC). Thresholds for individual biomarkers or their combinations were selected if they corresponded to the maximum Youden Index (Sensitivity + Specificity - 1).

Finally, the identified thresholds were used to classify all plasma donors as having abnormal plasma $A\beta_{42/40}$, p-tau231 or p-tau181 (i.e., four combinations of four groups). Because p-tau181 and particularly p-tau231 have been shown to associate with Aβ deposition[17, 18, 21, 41] and because PREVENT-AD participants for the most part only have evidence of nascent tau deposition, we chose to define p-tau abnormality as the thresholds that best discriminated between Aβ-PET positive and negative persons. General linear models tested for group differences in RBANS performance, and linear mixed-effects (LME) models assessed group differences in longitudinal cognitive change. For LME models, time was centered on the visit corresponding to donation of the assayed plasma sample. All cognitive outcome models were adjusted for age at the time of plasma sampling, *APOE* ε4 status, sex and education years. Analyses used Matlab R2019a (Mathworks Inc.; Natick, Massachusetts) and SPSS (IBM Corp.).

Voxelwise analyses considered two-sided P-values ≤0.001 using a cluster size >200 voxels to be statistically significant. Family-wise error (FWE) corrected maps are also presented. All other analyses considered P-value ≤0.05 as significant.

Results

Sample characteristics

PREVENT-AD participants had a mean age at plasma assessment of 65.33 [s.d. 5.31] years, and 71% were female. Of the 244 studied here, 20 (8%) developed mild cognitive impairment over the course of the study. Importantly, all participants were cognitively normal when they donated plasma and when they underwent PET scans. Additional demographic data are reported in Table 1. The PET sub-sample was comparable to the rest of the PREVENT-AD cohort with regard to *APOE* ε4 carrier status frequency, sex distribution, education years, plasma p-tau231 and p-tau181 levels, but was older, performed better on the RBANS total scale and had lower plasma NfL levels.

Associations between plasma biomarkers

Among 244 older adults, Simoa and IP-MS assays of plasma $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{42/40}$ levels were correlated ($A\beta_{40}$ β=0.79, 95% confidence interval [CI] 0.67—0.92, R^2=0.40; $A\beta_{42}$ β=0.24, 95% CI 0.19—0.28, R^2=0.33; and $A\beta_{42/40}$ β=0.20, 95% CI 0.14—0.25, R^2=0.18; all P<0.001). P-tau181 was correlated with p-tau231 (β=0.48, 95% CI=0.37—0.59, R^2=0.25, P<0.001). Plasma NfL levels did not correlate with either Simoa or IP-MS measures of $A\beta_{42/40}$ but were correlated with plasma p-tau181 (β=0.12, 95% CI=0.02—0.23, R^2=0.02, P=0.02) and p-tau231 levels (β=0.18, 95% CI=0.07—0.28, R^2=0.04, P<0.001). However, when adjusting plasma NfL models for age at the time of plasma assessment, only the association of

plasma NfL and plasma p-tau231 levels in the entire plasma donor cohort remained apparent. We observed similar associations when considering only the PET sub-sample.

Plasma p-tau231 and p-tau181 associate with early tau deposition

Among 129 PET volunteers, p-tau231 was associated with flortaucipir tracer retention in the entorhinal (β=7.88, 95% CI 3.22—12.54, R^2=0.09, P<0.002) and inferior temporal (β=15.86, 95% CI 11.07—20.66, R^2=0.26, P<0.001) cortices. Similarly, plasma p-tau181 was associated with flortaucipir binding in the entorhinal (β=6.54, 95% CI 2.16—10.90, R^2=0.07, P<0.005) and inferior temporal (β=10.88, 95% CI 6.47—15.30, R^2=0.16, P<0.001) cortices. Both markers also associated with flortaucipir retention in the temporal metaROI (Fig. 2A and 2C). These analyses were performed using robust regression to avoid extreme values driving associations. In voxelwise analyses, we observed associations of flortaucipir with p-tau231 in medial and lateral temporal and parietal regions that further extended to the lateral occipital and frontal cortices (Fig. 2B). Associations of p-tau181 with flortaucipir appeared restricted to medial and inferior temporal lobes (Fig. 2D). Only associations of p-tau231 with flortaucipir survived FWE correction. Plasma NfL levels correlated with flortaucipir binding in the temporal metaROI (R^2=0.06, P < 0.05) and this association was robust to adjustment for participant age. However, in voxelwise analyses, we did not find any significant clusters of associations of NfL with flortaucipir binding.

Plasma biomarkers associate with PET Aβ biomarkers.

Plasma $A\beta_{42}$ levels measured using Simoa and IP-MS assays were associated with global cortical Aβ-PET load (Simoa β=-0.69, 95% CI -1.03—-0.36, R^2=0.12; and IP-MS β=-1.23, 95% CI -2.07—-0.39, R^2=0.06; both P≤0.005). Specification of the $A\beta_{42/40}$ ratio resulted in some improvement in the association of Simoa measures with cortical Aβ-PET binding

(β=2.68x10^{-3}, 95% CI -3.66x10^{-3}—-1.71x10^{-3}, R^2=0.19, P<0.001; Fig. 3A). IP-MS-derived $A\beta_{42/40}$ measures also explained more variance in cortical Aβ-PET binding than $A\beta_{42}$ alone (β=-5.40x10^{-3}, 95% CI -7.55x10^{-3}—-3.25x10^{-3}, R^2=0.16, P<0.001; Fig. 3C). At the voxel level, we observed widespread association of Aβ-PET with plasma $A\beta_{42/40}$ measures. These associations were strong in precuneal and medial prefrontal regions, which are known to harbor early accumulation of plaques. Associations of IP-MS measures of plasma $A\beta_{42/40}$ with Aβ-PET tracer retention were more widespread than were Simoa measures (Fig. 3B and 3D). Plasma NfL levels did not correlate with Aβ-PET tracer retention either at the global or voxelwise level.

As expected, plasma p-tau231 (β=1.38, 95% CI 0.80—1.96, R^2=0.17, P<0.001; Fig. 3E) and p-tau181 (β=0.66, 95% CI 0.63—1.26, R^2=0.04, P<0.05; Fig. 3G) were also associated with global neocortical Aβ-PET binding. At the voxel level, p-tau231 associations with Aβ-PET survived FWE correction suggesting more robust associations than for any other plasma biomarker, including $A\beta_{42/40}$ (Fig. 3F and 3H).

Plasma biomarkers for the detection of cerebral Aβ deposition

The results from the logistic regression models for associations with Aβ-PET positivity are summarized in Fig. 4. IP-MS and Simoa measures of plasma $A\beta_{42/40}$ had similar accuracy in predicting Aβ-PET positivity (Simoa AUC=0.756, 95% CI 0.655—0.857; IP-MS AUC=0.808, 95% CI 0.725—0.891; all P<0.001). The threshold for the highest Youden Index for IP-MS $A\beta_{42/40}$ corresponded to a value of 0.089 (sensitivity=77%, specificity=78%) and for Simoa $A\beta_{42/40}$ corresponded to a value of 0.034 (sensitivity=60%, specificity=85%). Plasma NfL was not a good predictor of Aβ-PET positivity (AUC=0.588, 95% CI 0.465—0.712). P-tau181 alone had poor ability to predict Aβ-PET positivity (AUC=0.695, 95% CI

0.572—0.819, P=0.003) with an optimal threshold of 10.59 pg/mL (sensitivity=46%, specificity=91%). P-tau231 had the best ability to predict Aβ-PET positivity (AUC=0.811, 95% CI 0.71—0.91, P<0.001), with an optimal threshold of 12.72 pg/mL (sensitivity=60%, specificity=91%). Combining p-tau231 with IP-MS plasma $A\beta_{42/40}$ improved the predictive ability of the p-tau231 model to determine Aβ-PET positivity (AUC =0.869, 95% CI 0.80—0.94, P<0.001), with the optimal threshold of 15.54 showing improved sensitivity (83%) but reduced specificity (76%).

Association of plasma $A\beta_{42/40}$/p-tau231 categorization with longitudinal cognitive change in unimpaired elderly

When using the aforementioned thresholds of IP-MS $A\beta_{42/40}$ and p-tau231 to categorize the 244 plasma donors as having normal or abnormal plasma $A\beta_{42/40}$ and p-tau231 levels, 142 were $A\beta_{42/40}$-/p-tau231-, 53 were $A\beta_{42/40}$+/p-tau231-, 24 were $A\beta_{42/40}$+/p-tau231+, 14 $A\beta_{42/40}$-/p-tau231+ and 11 were not categorizable. No group performed differently on any of the RBANS scales at the time of plasma collection. In linear mixed-effects models, $A\beta_{42/40}$+/p-tau231+ participants experienced faster cognitive decline than $A\beta_{42/40}$-/p-tau231- participants on the RBANS total scale (time-by-group interaction β=-0.08 points/month, 95% CI=-0.14 – -0.03, P=0.004), immediate memory (time-by-group interaction β=-0.08 points/month, 95% CI=-0.16 – -0.01, P=0.03) and delayed memory (time-by-group interaction β=-0.08 points/month, 95% CI=-0.14 – -0.02, P=0.007) subscales (Fig. 5). When compared with participants who were either $A\beta_{42/40}$+/p-tau231- or $A\beta_{42/40}$-/p-tau231+, participants who were $A\beta_{42/40}$+/p-tau231+ experienced faster 8-year decline in total RBANS score (time-by-group interaction *vs.* $A\beta_{42/40}$+/p-tau231- β=-0.08 points/month, 95% CI=-0.14 – -0.03, P=0.003 ; time-by-group interaction *vs.* $A\beta_{42/40}$-/p-tau231+ β=-0.08 points/month, 95% CI=-0.15 – -

0.01, P=0.03), immediate memory score (time-by-group interaction *vs.* $A\beta_{42/40}$+/p-tau231- β=-0.08 points/month, 95% CI=-0.16 – -0.01, P=0.03; time-by-group interaction *vs.* $A\beta_{42/40}$-/p-tau231+ β=-0.11 points/month, 95% CI=-0.20 – -0.01, P=0.02) and delayed memory score (time-by-group interaction *vs.* $A\beta_{42/40}$+/p-tau231- β=-0.08 points/month, 95% CI=-0.14 – -0.02, P=0.007; time-by-group interaction *vs.* $A\beta_{42/40}$-/p-tau231+ β=-0.09 points/month, 95% CI=-0.17 – -0.02, P=0.01). When adjusting further for cognitive performance at time of plasma collection and baseline NfL levels, all associations were attenuated but remained statistically significant. In these models, plasma NfL was not a significant predictor of cognitive decline. $A\beta_{42/40+}$/p-tau231- and $A\beta_{42/40}$-/p-tau231$_+$ groups did not differ from the $A\beta_{42/40}$-/p-tau231- group. Results were similar when combining p-tau231 with Simoa-based measures of $A\beta_{42/40}$ or when replacing p-tau231 by p-tau181.

When creating similar fully adjusted linear mixed-effects models where plasma biomarker abnormality was defined using only a single biomarker (p-tau231, p-tau181, Simoa $A\beta_{42/40}$, or IP-MS $A\beta_{42/40}$), none of the biomarker-abnormal groups differed in their rate of cognitive change from the biomarker-normal group.

Discussion

In a relatively young group of aging CU persons at elevated risk for AD, we investigated associations of plasma and PET biomarkers of Aβ and tau, the disease's core pathologies. We also studied whether plasma biomarkers of AD pathology could predict longitudinal cognitive change. Compared with p-tau181, the novel p-tau231 assay displayed more robust and widespread associations with flortaucipir binding. Furthermore, among all plasma markers tested, p-tau231 displayed the most robust association with Aβ-PET. Over several years,

$A\beta_{42/40}$+/p-tau231+ participants experienced faster cognitive decline, particularly in immediate and delayed memory.

The availability of plasma biomarkers able to detect cerebral tau and Aβ pathologies has the potential to transform the AD field. Neurofibrillary tangles associate with dementia symptoms more than Aβ plaques.[43] Flortaucipir PET tracks tau deposition in the brain and accurately predicts atrophy patterns.[44] A non-invasive, inexpensive tool that tracks cerebral tau deposition could facilitate identification of individuals with notable AD pathology likely to experience cognitive decline. Plasma p-tau231 may be such a tool. While plasma p-tau181 correlates with tau-PET binding, detects cerebral Aβ deposition and discriminates AD vs. non-AD dementias,[17-19, 21, 45] it appears to perform less well in CU persons. In the PREVENT-AD cohort, plasma p-tau231 proved superior to p-tau181 for detection of flortaucipir binding. Importantly, combining plasma IP-MS $A\beta_{42/40}$ with p-tau231 or p-tau181 measures can identify individuals likely to experience cognitive decline.

A potential advantage of these fluid biomarkers may be their indication of earlier AD pathogenesis,[38, 46, 47] and some may provide more specific implications for specific brain regions.[48-50] Specifically, early changes in plasma p-tau231 may reflect earlier increments in Aβ-PET signal,[27] therefore performing better than p-tau181 when screening for early pre-clinical AD pathogenesis.

Because Aβ deposition is believed to be the earliest detectable manifestation of AD, it has been the usual target for potentially disease-modifying preventive interventions. Unfortunately, even Aβ-targeting interventions that achieve robust removal of plaques have failed to slow cognitive decline.[51] As is true in many chronic diseases, early treatment may improve chances of success,[4] thus indicating the potential value of tools for detection and

measurement of AD pathogenesis. Although randomized trials using CSF and PET measures of Aβ deposition are now in progress, both techniques are unlikely to achieve widespread clinical use. Some 25-30% of CU older adults have evident Aβ pathology,[52, 53] but these results come from studies of older cohorts. Prevention trials are better conducted in younger samples. Presumably because of its elevated age-specific risk, even the relatively young PREVENT-AD PET-subset yielded 26 Aβ-PET-positive persons out of 129 (20%). Plasma p-tau231 assessment of the PET-subset using a threshold of 12.72 pg/mL identified 24 persons who screened positive, 15 of whom were Aβ-PET-positive using a conservative threshold. Scaling up, a typical AD prevention trial might require 1000 Aβ-positive CU individuals. To identify these using Aβ-PET without pre-screening, the A4 study required ~3400 scans ($10.2 million at an estimated $3000/scan).[54] By contrast, plasma screening of 8600 persons would yield 1600 who screen positive for $2.15 million, assuming a commercial cost of $250 per plasma screen. To identify 1000 persons who meet the "gold standard" of being Aβ-PET-positive, one could perform Aβ-PET on these 1600 at a cost of $4.80 million, for an overall savings in screening costs of $3.25 million (with the added advantages of yielding a sample of "doubly-positive" persons and requiring recruitment of 1600 rather than 3400 persons to undergo PET). However, further validation of p-tau231 as a stand-alone marker of cerebral Aβ burden is required to precisely estimate the cost-savings associated with its use. The cost-effectiveness of pre-screening using plasma biomarkers may be reduced if a panel of multiple markers is required.

Strengths and Limitations

This study's principal strength is its reliance on a large cohort of well characterized CU older adults having plasma, and PET measures of Aβ and tau pathologies as well as up to 8 years of

cognitive evaluations. Plasma biomarkers correlated readily with PET measures of tau and Aβ, adding credence that they reflect AD pathological changes. Furthermore, p-tau181 and p-tau231 were measured using the same platform, reducing methodological influences. However, the availability of plasma p-tau217 would be desirable to evaluate whether p-tau231 outperforms this epitope for detection of AD pathological changes in pre-symptomatic AD. Another weakness is reliance on a single cohort of predominantly well-educated, Caucasian females, potentially limiting generalizability of our findings to other populations. The relatively small number of Aβ-PET-positive individuals found among this relatively young cohort would appear also to limit the robustness of the plasma biomarker thresholds identified here.

Conclusions

Plasma Aβ and p-tau measures associate with PET measures of Aβ and tau pathologies in CU older adults at risk of AD dementia. Plasma p-tau231 appears to be a superior indicator of early tau deposition. Combining several plasma biomarkers may achieve improved detection of cerebral Aβ deposition and identification of persons at greatest risk of cognitive decline.

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Author Contributions

P.F.M, N.J.A, T.K.K, J.Po., M.S., J.C.S.B, H.Z., K.B., and S.V. contributed to study concept and design. P.F.M, N.J.A, T.K.K, T.K., J.G., A.P.B., H.O., Y.Y., C.S.B, J.S., J.Pa., J.L.R., A.L., M.S., S.L.B and E.V. contributed to data acquisition and analysis. P.F.M., N.J.A, Y.Y., C.S.B., and S.V. drafted the manuscript and figures.

Potential Conflicts of Interest

P.F.M, N.J.A, T.K.K., C.S.B., T.K., J.G., A.P.B, H.O., Y.Y. , J.S., J.Pa., J.L.R., A.L., S.L.B., M.S. ,J.Po, J.C.S.B, H.Z., K.B. and S.V. report no relevant conflicts of interest. E.V. is co-founder of ADx NeuroSciences, a company which works on the scientific development and commercialization of biomarker assays.

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Figure Legends

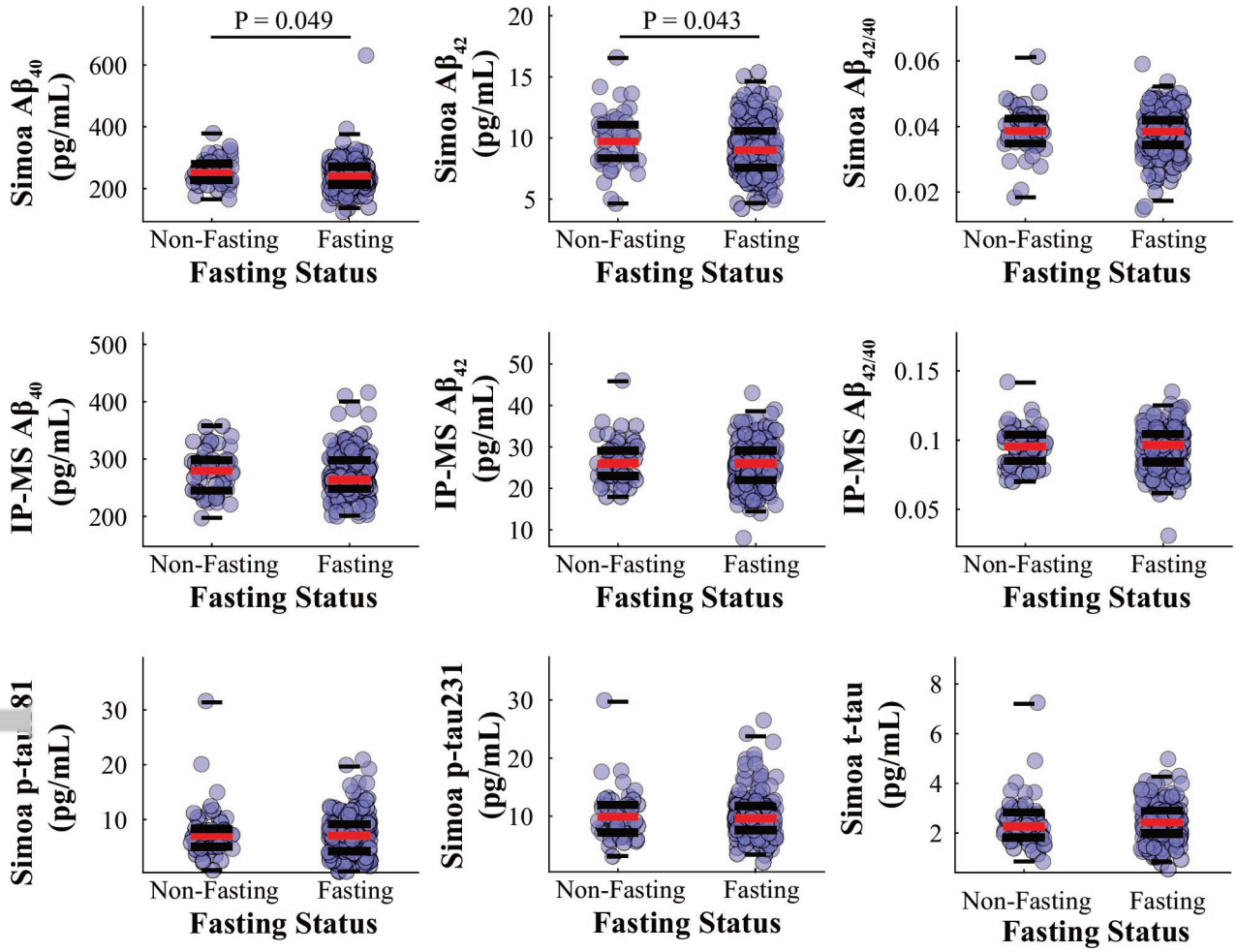
Figure 1. Plasma biomarker levels in fasting *vs.* non-fasting blood.
Among 244 participants, 192 (mean age 64.77 ± 5.33 years, 69% female) donated fasting blood and 52 (mean age 67.24 ± 4.82 years, 81% female) donated non-fasting blood. Mann-Whitney U tests were used to compare plasma biomarker levels in fasting and non-fasting blood donors. Simoa $A\beta_{40}$ and $A\beta_{42}$ levels were higher in non-fasting compared to fasting blood (both $p < 0.05$). However, this was no longer apparent when considering Simoa $A\beta_{42}/_{40}$. Other plasma biomarkers were apparently unaffected by fasting or non-fasting blood donation. Red bar is the group median, thick black bars depict 25th and 75th percentile, small black lines are 1st and 99th percentile.

Figure 2. Association of plasma p-tau181 and p-tau231 with flortaucipir PET binding
Among 129 PET volunteers, flortaucipir binding correlated with plasma p-tau231 in the temporal metaROI ***(A).*** At the voxel level, association of p-tau231 with flortaucipir binding was mainly observable in the precuneus, medial and lateral temporal, and parietal regions (***B***). P-tau181 was also associated with flortaucipir PET binding in the temporal metaROI ***(C)*** with no associations surviving FWE correction ***(D)***.

Figure 3. Association of plasma Aβ and p-tau biomarkers with Aβ-PET binding
Global cortical Aβ-PET tracer retention was associated with Simoa $A\beta_{42/40}$ ***(A)***. At the voxel level, associations of Aβ-PET with Simoa $A\beta_{42/40}$ were located focally in the precuneus and medial prefrontal cortex ***(B)***. Aβ-PET tracer retention was also associated with IP-MS $A\beta_{42/40}$ ***(C)***. At the voxel level, associations of Aβ-PET and IP-MS $A\beta_{42/40}$ were also located mainly in in the precuneus and medial prefrontal cortex ***(D).*** P-tau231 was associated with global cortical Aβ-PET binding (***E***). At the voxel level, most associations of Aβ-PET with p-tau231 survived FWE correction ***(F)***. P-tau181 was associated with Aβ-PET tracer retention ***(G)*** with no associations surviving FWE correction ***(H).***

Figure 4. Plasma biomarkers distinguish Aβ-PET positive and negative individuals
Optimized ROC curves and corresponding areas under the curve (AUC) for individual plasma biomarkers ***(A)*** or their combination ***(B)***.

Figure 5. Plasma p-tau231 biomarker status and cognitive change using IP-MS $A\beta_{42/40}$
From linear mixed-effects models, we obtained group-level cognitive slopes for participants who were either IP-MS plasma $A\beta_{42/40}$-/p-tau231-, $A\beta_{42/40}$+/p-tau231-, $A\beta_{42/40}$+/p-tau231+ or $A\beta_{42/40}$-/p-tau231+. These are shown for RBANS ***(A)*** total score, ***(B)*** immediate memory, ***(C)*** delayed memory, ***(D)*** visuospatial/constructional ability, ***(E)*** language and ***(F)*** attention subscales. Over 8 years of follow-up, $A\beta_{42/40}$+/p-tau231+ participants showed stronger decline than $A\beta_{42/40}$-/p-tau231- in total, immediate memory and delayed memory scores. Small lines represent individual cognitive trajectories. Follow-up time was centered on the visit at which the plasma sample was collected.



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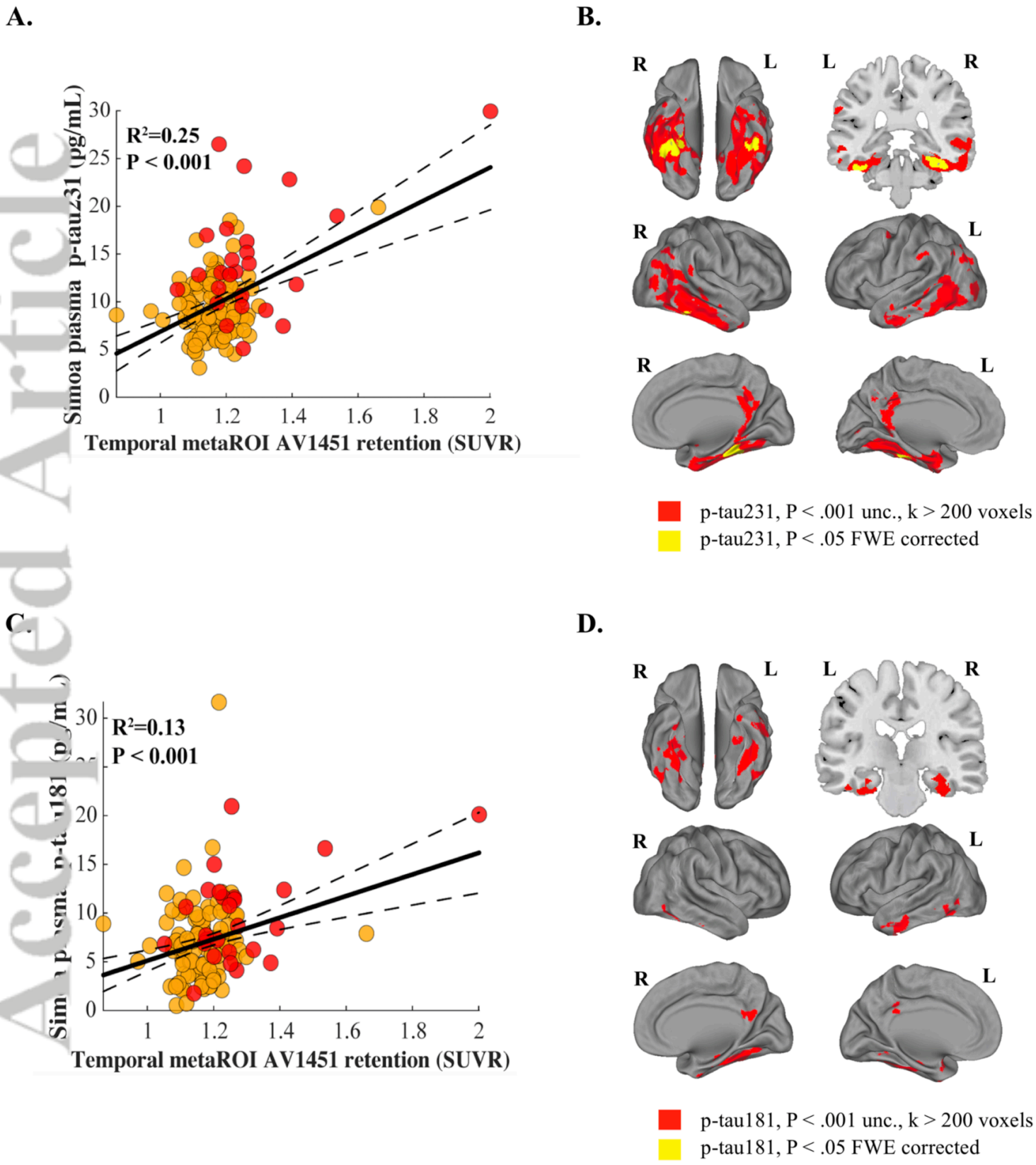

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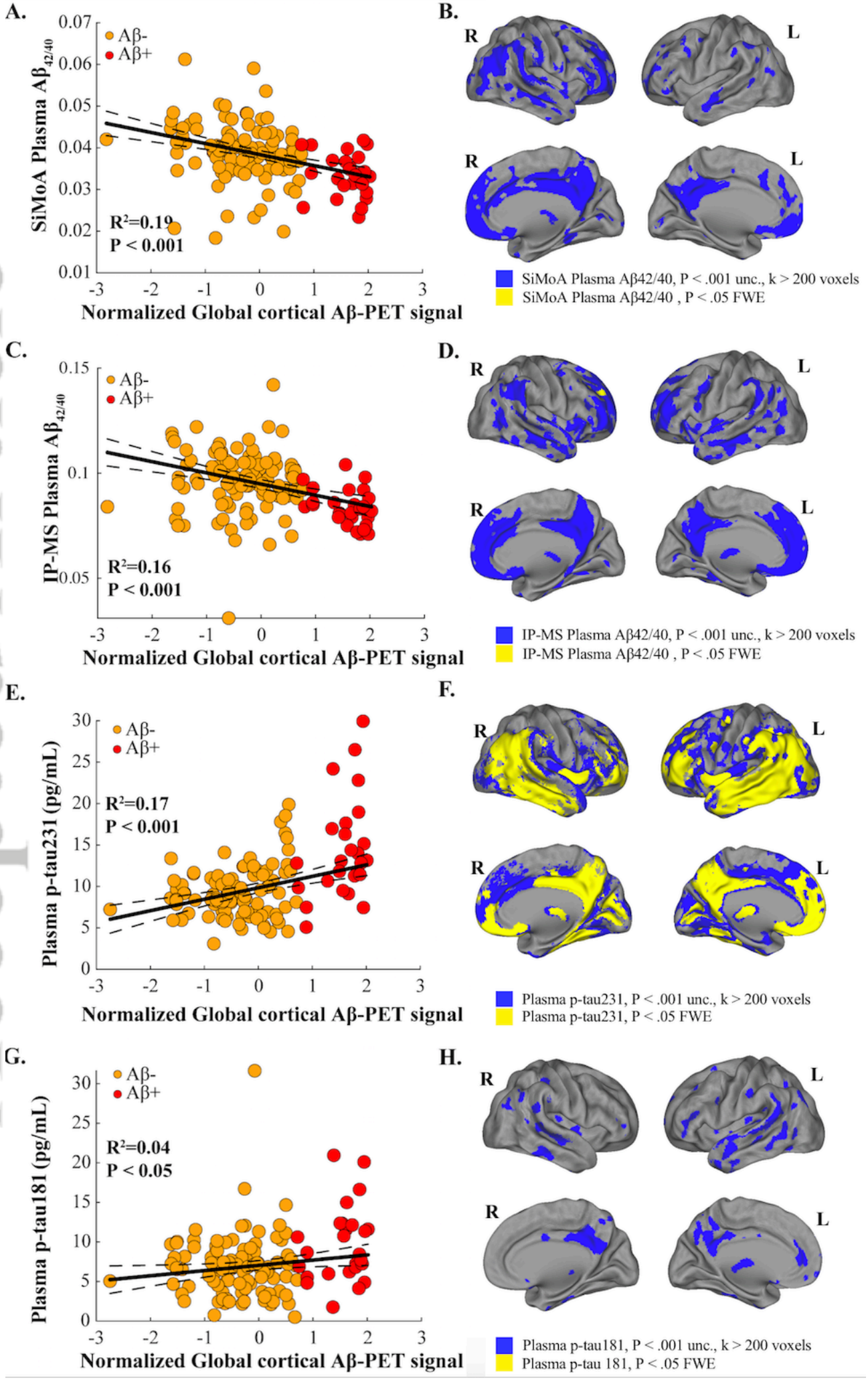

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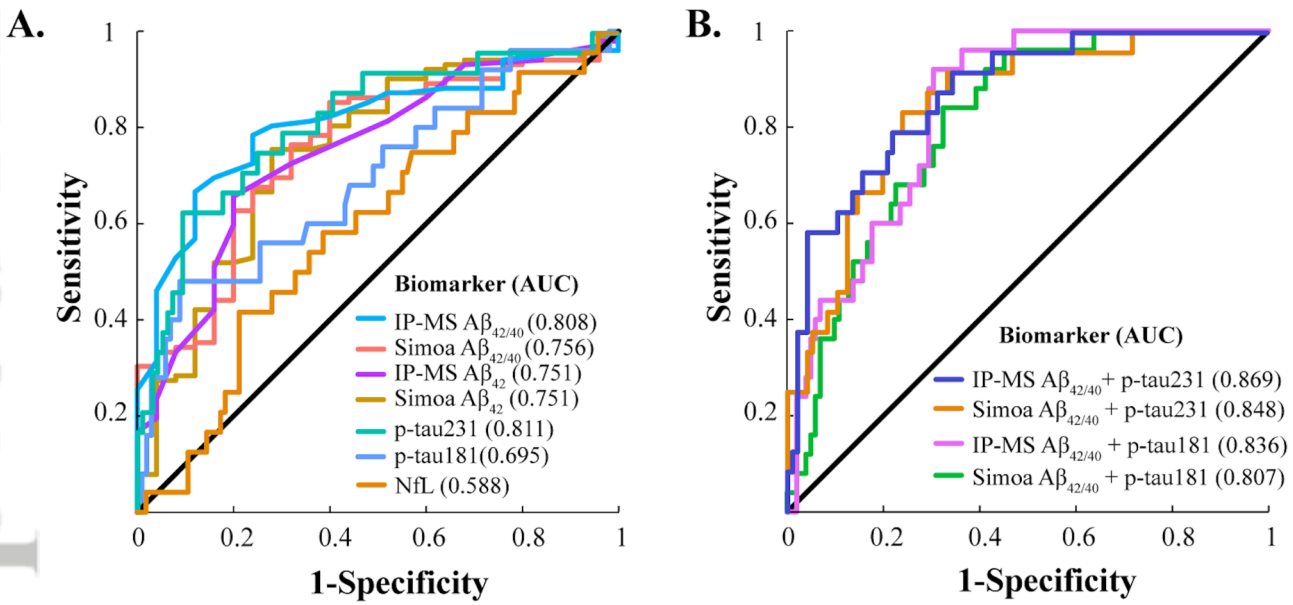

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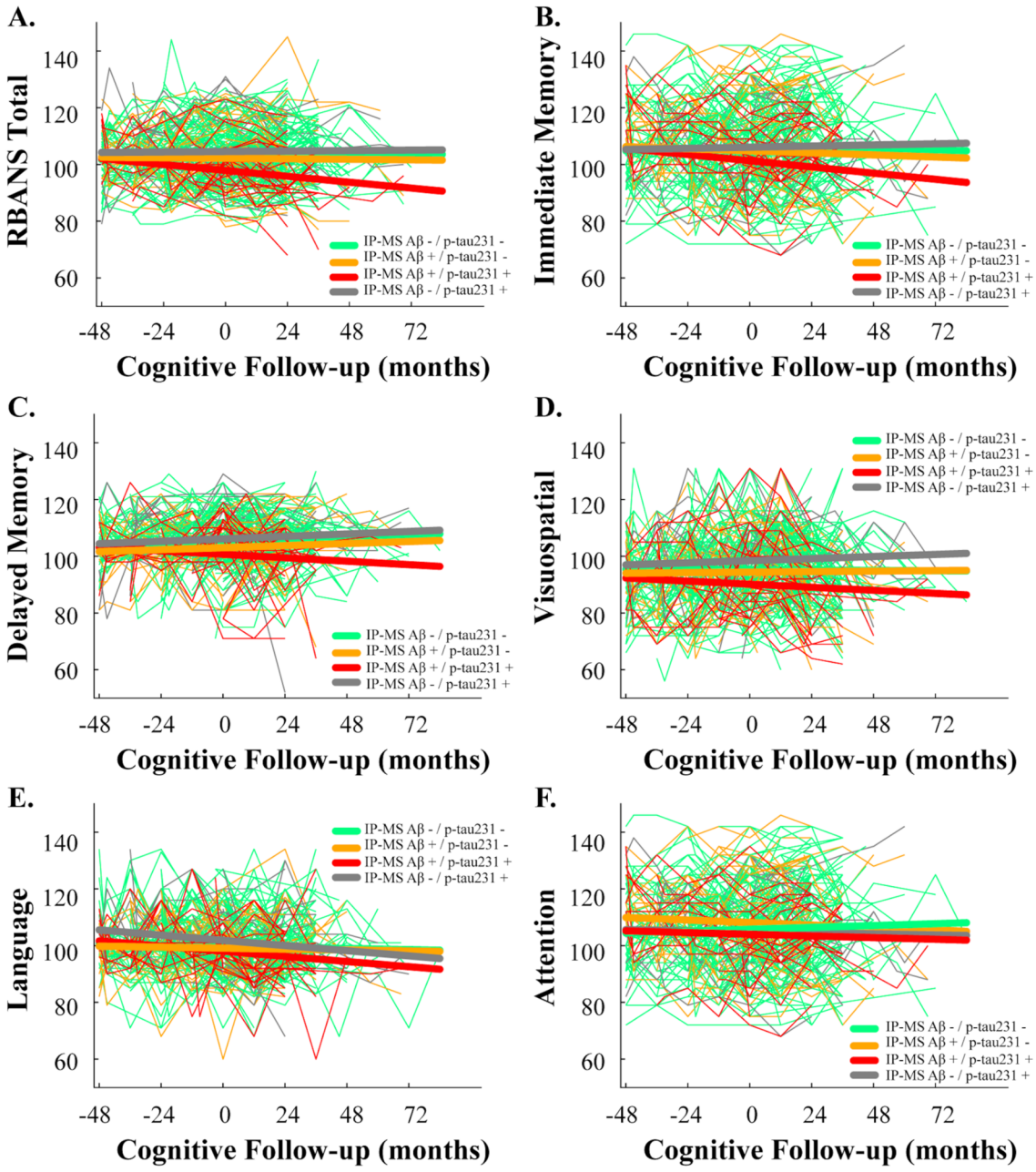

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Table 1. Sample Demographics

	All Sample	PET Sub-sample	P-value
Sample Size	244	129	
Age	65.33 (5.31)	66.58 (4.90)	< 0.001
Sex %F (N)	71.31 (174)	74.4 (96)	0.89
***APOE* ε4 carriers % (N)**	36.89 (90)	41.09 (53)	0.79
Education years	15.28 (3.25)	15.15 (3.28)	0.59
Total RBANS score	102.81 (10.46)	104.85 (10.85)	0.01
Plasma p-tau181 (pg/mL)	7.23 (3.91)	7.47 (4.21)	0.43
Plasma p-tau231 (pg/mL)**	10.10 (3.97)	10.35 (4.42)	0.82
Plasma Nfl***	13.73 (4.84)	13.40 (4.49)	0.03
Global Aβ SUVR	*NA*	1.34 (0.34)	*NA*
metaROI Flortaucipir SUVR	*NA*	1.18 (0.12)	*NA*
Plasma-PET Delay (days)	*NA*	277.69 [6-1613]	*NA*

APOE apolipoprotein-E genotype; F Female; SUVR Standard Uptake Value Ratio; RBANS: Repeatable Battery for the Assessment of Neuropsychological status

** 11 participants (4 with unknown Aβ-PET status, 6 Aβ-PET negative and 1 Aβ-PET positive) had p-tau231 values below the lower limit of detection of the assay.

*** 3 participants did not have NfL values available.