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RESEARCH ARTICLE

Phospho-specific plasma p-tau181 assay detects clinical as well as asymptomatic Alzheimer's disease

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Abstract

Objective: Plasma phosphorylated-tau-181 (p-tau181) reliably detects clinical Alzheimer's disease (AD) as well as asymptomatic amyloid-β (Aβ) pathology, but is consistently quantified with assays using antibody AT270, which cross-reacts with p-tau175. This study investigates two novel phospho-specific assays for plasma p-tau181 and p-tau231 in clinical and asymptomatic AD. **Methods**: Plasma p-tau species were quantified with Simoa in 44 AD patients, 40 spouse controls and an independent cohort of 151 cognitively unimpaired (CU) elderly who underwent Aβ-PET. Simoa plasma Aβ42 measurements were available in a CU subset ($N = 69$). Receiver operating characteristics and Aβ-PET associations were used to evaluate biomarker validity. **Results**: The novel plasma p-tau181 and p-tau231 assays did not show cross-reactivity. Plasma p-tau181 accurately detected clinical AD (area under the curve (AUC) = 0.98, 95% CI 0.95–1.00) as well as asymptomatic Aβ pathology (AUC = 0.84, 95% CI 0.76–0.92), while plasma p-tau231 did not (AUC = 0.74, 95% CI 0.63–0.85 and 0.61, 95% CI 0.52–0.71, respectively). Plasma p-tau181, but not p-tau231, detected asymptomatic Aβ pathology more accurately than age, sex and *APOE* combined (AUC = 0.64). In asymptomatic elderly, correlations between plasma p-tau181 and Aβ pathology were observed throughout the cerebral cortex ($\rho = 0.40$, $p < 0.0001$), with focal associations within AD-vulnerable regions, particularly the precuneus. The plasma Aβ42/p-tau181 ratio did not reflect asymptomatic Aβ pathology better than p-tau181 alone. **Interpretation**: The novel plasma p-tau181 assay is an accurate tool to detect clinical as well as asymptomatic AD and provides a phospho-specific alternative to currently employed immunoassays.

Introduction

Alzheimer's disease (AD) is pathologically characterised by aggregation of misfolded amyloid-β (Aβ) and phosphorylated-tau (p-tau) into respectively plaques and tangles.[1] Although these pathological processes ultimately result in cognitive dysfunction, the latter typically emerges years later.[2] Hence, AD has a long preclinical phase in which irreversible brain damage could possibly be averted and therapeutic intervention would thus be most advantageous. Biomarkers enable in vivo detection and monitoring of AD pathology within this asymptomatic phase. Aβ and tau aggregates and consequent brain damage can be visualised and quantified by positron emission

tomography (PET) and are reflected by alterations in the core cerebrospinal fluid (CSF) biomarkers Aβ42, p-tau and total tau.[2] However, the high cost, invasive nature and/or limited availability of these techniques prevent their implementation in contexts requiring large-scale testing such as clinical trial recruitment, or primary care diagnostics. Fortunately, recent technological advancements have opened the opportunity to extend the use of fluid biomarkers to peripheral blood.[3] So far, p-tau has shown most promise as an early and highly specific blood-based biomarker for AD.[4–6] Most p-tau assays target tau phosphorylated at T181 (p-tau181), yet tau hyperphosphorylation in AD involves several phosphorylation sites and recently two additional p-tau species, p-tau217 and p-tau231, have gained increased attention.[7] While some studies reported comparable performances,[8–10] others suggested earlier elevations of p-tau217 and p-tau231 as well as slightly better performances to detect asymptomatic Aβ pathology relative to p-tau181.[6,10–13] However, a limitation of these studies is the use of the antibody AT270 for plasma p-tau181 quantification, which cross-reacts with p-tau175, thus limiting assay specificity.[14] A novel prototype plasma p-tau181 assay that uses the antibody ADx252, which does not cross-react with p-tau175, has been shown to detect clinical AD with higher accuracy than the commercial AT270-based plasma p-tau181 assay (Quanterix v.2 Cat#103714). A prototype p-tau231 assay with identical analytical features showed only moderate performance to detect clinical AD.[15]

In this study, we aim to extend previous findings to the asymptomatic AD phase by investigating the performances of the phospho-specific prototype p-tau181 and p-tau231 assays to detect not only clinical AD, but also cerebral Aβ pathology in cognitively unimpaired (CU) elderly. Second, we assessed the correlation between p-tau species and Aβ deposition in CU elderly. Since the performance of CSF p-tau to detect both clinical and asymptomatic AD has been shown to improve when assessed in conjunction with Aβ levels in a ratio,[16–19] we also explored the additional value of Aβ42/p-tau ratios in plasma.

Methods

Study population

The memory clinic cohort consisted of 44 CSF biomarker-proven AD patients presenting with mild to severe dementia (median Mini-Mental State Examination (MMSE): 18, range: 5–29) and 40 spouse controls, prospectively recruited through the memory clinic of University Hospitals Leuven. Plasma was collected from 2018 to 2019.

An independent cohort of 151 CU elderly was selected from the Flemish Prevent AD Cohort KU Leuven (F-PACK) based on sample availability. F-PACK is a community-recruited prospective cohort enriched for the risk of developing AD through a genetic stratification scheme.[20] Recruitment took place between 2009 and 2019 and inclusion criteria consisted of age between 50 and 80 years, a MMSE score $\geq$ 27/30, a Clinical Dementia Rating = 0 and test scores on neuropsychological examinations within published norms.[20] Aβ-PET imaging and plasma sampling were performed between 2009 and 2021 with a median time interval of 4 months (IQR: 5 months). Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki and all study procedures were approved by the Ethics Committee of University Hospitals Leuven (reference numbers S65105 and S51125).

Amyloid-β-PET imaging

Within the F-PACK cohort, Aβ-PET scans were acquired on a 16-slice Biograph PET/CT scanner (Siemens, Erlangen, Germany) using [^{11}C]Pittsburgh compound B (PiB) (n = 81, mean activity = 270 MBq, SD: 39, range: 172–356) or [^{18}F]flutemetamol (n = 70, mean activity = 149 MBq, SD: 5, range: 127–162) at University Hospitals Leuven. PET processing was performed using Statistical Parametric Mapping 12 (SPM12, Wellcome Trust Centre for Neuroimaging, London, UK, http://www.fil.ion.ucl.ac.uk/spm) implemented in Matlab R2014b (Mathworks, Natick, USA).[21] In short, standardised uptake value ratio (SUVR) images were reconstructed from the 40–60 minute postinjection window for [^{11}C]PiB-PET and the 90–110 minute postinjection window for [^{18}F]flutemetamol-PET using a participant-specific cerebellar grey matter reference region. For both tracers, a composite SUVR was calculated in a volume of interest involving five bilateral cortical areas (frontal, parietal, anterior cingulate, precuneus-posterior cingulate and lateral temporal) derived from the Automated Anatomical Labelling atlas and intersected with participant-specific grey matter thresholded at 0.3.[21] Aβ-PET measurements were harmonised across tracers through conversion of SUVR values to Centiloids using our previously validated analysis pipeline.[22–24] Individuals were considered to be Aβ-positive when Centiloids > 23.5, thus identifying Thal phase 3-to-5.[25] For voxelwise analyses, Centiloid maps were constructed by applying the conversion formula to each voxel of the SUVR image followed by smoothing with an isotropic 3D Gaussian kernel at 8 mm full width at half maximum. For the memory clinic cohort, no Aβ-PET imaging data were available.

Plasma collection and phosphorylated-tau measurements

Blood was collected in K2EDTA-coated polyethylene terephthalate tubes (BD Diagnostics, BD367864). In the memory clinic cohort, the collected blood sample was centrifuged at 400 *g* for 5 min at room temperature followed by transfer of the supernatant to polypropylene cryovials (Thermo Fisher Scientific, 363401) and subsequent storage at −80°C. In the F-PACK cohort, blood was centrifuged at 1200 *g* for 10 min at 4°C and plasma was transferred to polypropylene cryovials (Thermo Fisher Scientific, 363401) and stored at −20°C for 24 h prior to long-term storage at −80°C. Prior to analysis, plasma samples were thawed, vortexed and centrifuged at 1000 *g* and 4°C for 8 min to remove debris.

Plasma p-tau181 and p-tau231 levels were quantified by means of prototype Simoa-based immunoassays developed by ADx NeuroSciences (Ghent, Belgium)[15] (Supplement 1). All samples were measured in duplicate and randomised over four runs using the R Package *WPM*.[26] Two reruns were performed to reanalyse samples with intra-assay coefficients of variation (CV%) above 20% or single/missing values. Average enzyme per bead (AEB) signals below the lower limit of quantification (LLOQ) were imputed to 0.1 pg/mL since physiological plasma p-tau concentrations were assumed to never equal 0. The LLOQ was determined through recalculation of calibrator concentrations by interpolation from a mean standard curve of the six runs and was equal to the concentration of the lowest calibrator for which the inter-assay CV% was below 20%. Intra-assay precision was derived from duplicate measurements from all 235 study subjects. Since both quality control (QC) samples demonstrated p-tau181 concentrations within the lower range of the calibration curve, inter-assay precision was calculated based on the recalculated calibrator concentrations for a more accurate representation of assay precision across the calibration range. The specificities of the prototype ADx and commercial Quanterix plasma p-tau181 assays were verified through measurement of phospho-specific variants of the calibrator peptide with both assays. Plasma Aβ42 levels were quantified in an F-PACK subset ($N = 69$, of which 17 were Aβ-positive) using the Amyblood Simoa assay as described previously.[22,27]

Statistical analyses

Standard statistical analyses were performed using R, version 4.1.2 (The R Foundation for Statistical Computing, https://www.r-project.org/). Voxelwise analyses were performed with SPM12. Normality was assessed with D'Agostino-Pearson test. Group-wise comparisons of continuous variables were conducted using unpaired *t*-tests or Mann–Whitney *U* tests, depending on normality. X^2 tests were used for analysis of contingency tables. Effect sizes of differences in plasma p-tau levels between groups were indicated by robust *d* values using the R package *WRS2*.[28] Bonferroni correction was applied to adjust for the comparison of different biomarkers ($\alpha = 0.05$ corresponding to an uncorrected $p = \alpha/k$ compared biomarkers).

As a primary outcome analysis, the performances of the prototype assays with respect to detecting clinical AD or asymptomatic Aβ-PET positivity were assessed by receiver operating characteristic (ROC) analyses using the R package *pROC*.[29] Areas under the curve (AUCs) with 95% CI were reported as measures of performance. In addition to biomarker-only models, logistic regression models including multiple predictors were constructed. These encompass i) demographic models with age, sex and *APOE*, but no biomarker, as predictors, and ii) covariate-adjusted biomarker models including age, sex, *APOE* as well as a biomarker as predictors. Since the *APOE* genotype was only known for a subgroup (75/84) of the memory clinic cohort, all logistic regression models were constructed and compared to the biomarker-only model within this subgroup. Pairwise AUC comparisons were performed with the DeLong method.[30]

As a secondary outcome analysis, the associations between plasma p-tau species and CLs were assessed by means of Spearman rank correlations and univariate linear regressions. In addition, multivariate linear regression models were constructed in which age, sex and *APOE* were used as covariates to assess whether they modify the relationship between plasma p-tau and Centiloids. Predictors for which p-tau-independent contributions to Centiloids were found, were included as covariates in voxelwise multivariate regression analyses between plasma p-tau and Centiloid maps. The significance threshold was set at a cluster-level whole-brain family-wise error (FWE) threshold of $P_{FWE} < 0.05$ with voxel-level set at $P_{uncorrected} < 0.001$.[31]

All analyses were repeated in an F-PACK subgroup ($N = 69$) for which plasma Aβ42 levels had been determined[22] in order to assess the additional value of the plasma Aβ42/p-tau ratio.

Results

Analytical performance of the prototype assays

While the prototype ADx plasma p-tau181 assay only targeted p-tau181, the commercial Quanterix p-tau181 assay showed cross-reactivity with p-tau175 (Fig. 1). Across the study population, ADx plasma p-tau181 and p-tau231 assays, respectively, had mean intra-assay CVs% of 10.2%

and 11.4% and inter-assay CVs% of 12.1% and 6.22%. The absolute between-run differences in low QC concentrations were on average 1.43 pg/mL for p-tau181 and 0.84 pg/mL for p-tau231 (Supplement 2). The functional LLOQ was equal to 0.8 pg/mL for the p-tau181 assay and 0.3 pg/mL for the p-tau231 assay. No plasma samples had p-tau181 values below this functional LLOQ, yet 16 (6%) subjects, of which one Aβ-positive CU and one AD patient, had p-tau231 concentrations below the functional LLOQ.

Cohort demographics and biomarker data

Cohort characteristics are summarised in Table 1. Within the memory clinic cohort, the AD patients did not differ in age or sex from spouse controls, but had a higher proportion of *APOE-ε4* carriers. Plasma p-tau181 ($d = 0.96$) and plasma p-tau231 ($d = 0.58$) levels were respectively 4.6- and 1.9-fold higher in AD patients than controls (Fig. 2A). Within the F-PACK cohort, Aβ-positive CU subjects were slightly older than Aβ-negative CU subjects and had a higher proportion of *APOE-ε4* carriers, but an equal sex distribution. Aβ-positive and Aβ-negative CU elderly had equal Auditory Verbal Learning Test total learning and MMSE scores, yet Aβ-positive CU elderly scored lower on the Buschke Selective Reminding Test total retention. Aβ-positive CU elderly had 2.3-fold higher plasma p-tau181 levels ($d = 0.77$) than Aβ-negative CU elderly, while for plasma p-tau231 this was only 1.1-fold and did not remain significant after Bonferroni correction (Fig. 2B).

Accuracy of plasma p-tau181 and p-tau231 to discriminate AD from controls

Within the memory clinic cohort, both plasma p-tau species reliably distinguished AD dementia patients from controls, yet plasma p-tau181 showed a higher AUC (0.98) than p-tau231 with a difference (ΔAUC) of 0.24 ($p = 0.0002$). Combining both p-tau species in a biomarker model did not improve performance relative to plasma p-tau181 alone (Fig. 2C). AUCs of biomarker-only models observed in the memory clinic subgroup with known *APOE* genotype were identical to those observed in the total memory clinic cohort. Within this subgroup, plasma p-tau181 was a better predictor of clinical AD than not only plasma p-tau231, but also a demographic model of age, sex and *APOE* (ΔAUC = 0.14, $p = 0.008$). When adjusting biomarker models for age, sex and *APOE*, plasma p-tau181 detected clinical AD with a perfect AUC of 1.00 (95% CI 1.00–1.00) thus also outperforming the demographic model (ΔAUC = 0.16, $p = 0.0006$). Adding plasma p-tau231 as a predictor to the demographic model did not increase performance (Fig. 2E).

Within the F-PACK cohort, plasma p-tau181 species accurately reflected Aβ-PET status (AUC = 0.84), thereby outperforming p-tau231 (ΔAUC = 0.23, $p = 0.0001$) as well as the demographic model (ΔAUC = 0.20, $p = 0.001$). Incorporation of both plasma p-tau181 and plasma p-tau231 in a combined biomarker model did not increase performance with respect to distinguishing Aβ-positive from Aβ-negative CU elderly (AUC = 0.83) relative to p-tau181 alone (Fig. 2D). At maximised Youden index, plasma p-tau181 detected Aβ status in CU elderly with much higher specificity (0.82 vs. 0.48) and positive predictive value (0.57 vs. 0.33) than p-tau231, yet no differences in sensitivities or negative predictive values were observed (Supplement 3). Correction for age, sex and *APOE* did not change the performance of either plasma p-tau species to detect asymptomatic Aβ pathology. Addition of plasma p-tau181 to the demographic model increased its performance to predict Aβ-PET status (ΔAUC = 0.20, $p = 0.0003$), which was not the case for p-tau231 (Fig. 2F).

Correlation of plasma p-tau species with asymptomatic cerebral Aβ burden

Plasma p-tau181 was moderately correlated with Aβ-PET CLs in CU elderly ($\rho = 0.40$, Fig. 3A) of the F-PACK cohort. For every 1 pg/mL increase in plasma p-tau181, Aβ-PET tracer uptake increased with 2.17 CLs (Supplement 4A). Partialling out the variance shared between plasma p-tau181, age, sex and *APOE-ε4* carrier status did not remove this association (Supplement 4B). In addition to plasma p-tau181, *APOE-ε4* carrier status, unlike age and sex, independently contributed to CLs. Strongest associations between plasma p-tau181 and CLs followed the typical distribution of Aβ load with high values in the precuneus, inferior parietal gyrus and middle frontal gyrus (Fig. 3B, Supplement 5). Plasma p-tau231 did not show any associations with global nor regional cerebral Aβ burden, regardless of correction for age, sex and *APOE-ε4* carrier status (Fig. 3C, Supplement 4C–D).

The added value of plasma Aβ42/p-tau ratios in asymptomatic AD

The plasma Aβ42/p-tau181 ratio was 2.5-fold lower in Aβ-positive than Aβ-negative CU subjects (Fig. 4A) with an effect size ($d = 0.81$) similar to that of plasma p-tau181 alone ($d = 0.82$, Fig. 4B). The plasma Aβ42/p-tau181 ratio and plasma p-tau181 alone identified asymptomatic Aβ-PET positivity with comparable accuracies (AUCs = 0.87 and 0.86, respectively, Fig. 4C) and

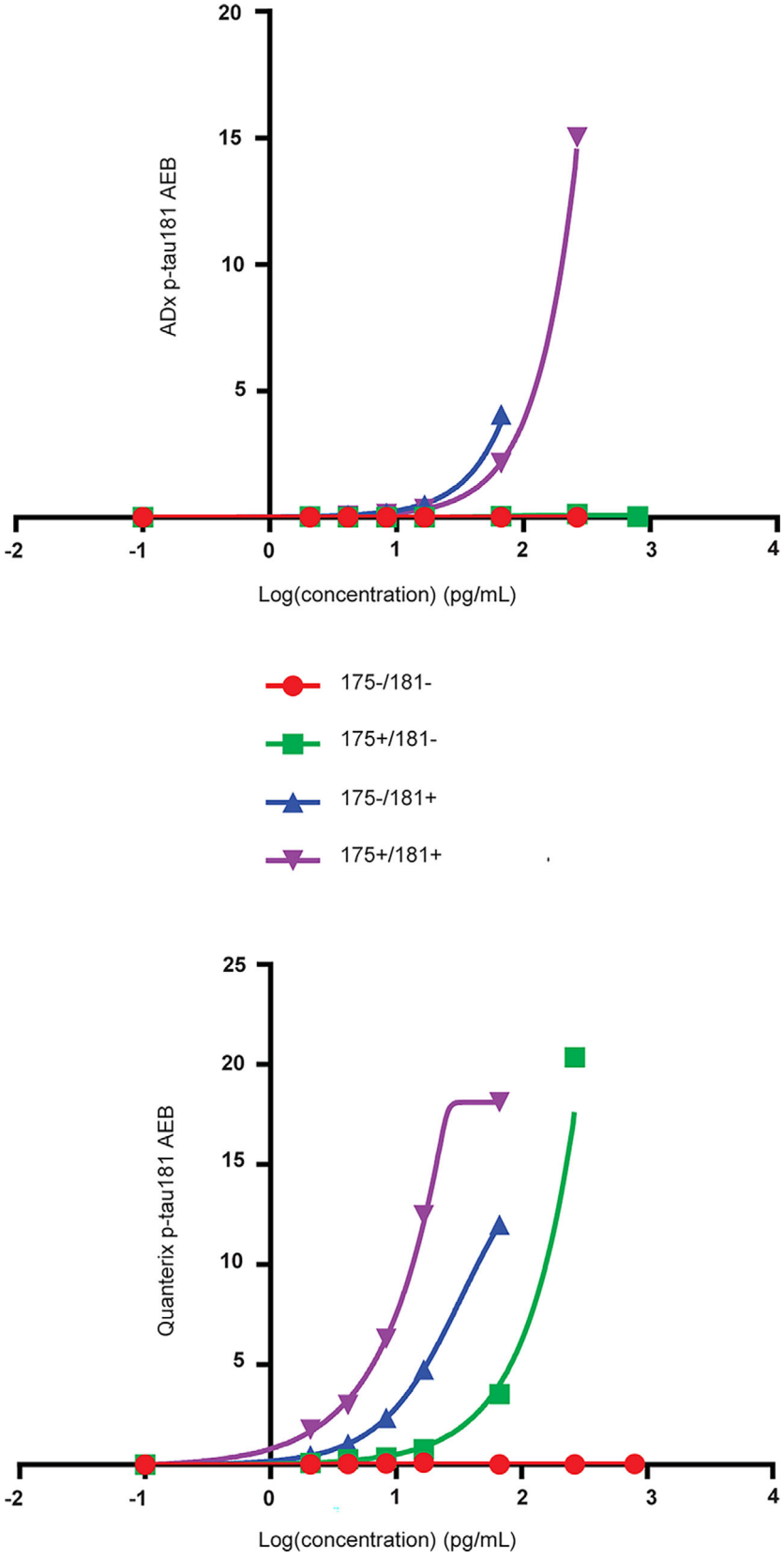


Figure 1. Specificity of the novel versus commercial p-tau181 Simoa assay. Synthetic peptides representing different phosphorylation statuses of T175 and T181 of the tau protein were tested in the ADx prototype assay (top panel) and in the commercial Quanterix test kit (Simoa® p-tau181 v.2 Advantage Kit, bottom panel), respectively. Peptide concentrations ranged between 2 pg/mL and 800 pg/mL (X axis). The assay readouts (average enzyme per beads signal) are shown on the Y axis. Peptide concentrations below the detection limit are not shown.

Table 1. Characteristics of the study cohorts.

	Memory clinic		F-PACK		
	Controls	AD	All	Aβ-negative	Aβ-positive
n (%)	40	44	151	114 (75)	37 (25)
Age, years	65 ± 7	64 ± 7	70 ± 6	69 ± 7	72 ± 5*
Female, *n* (%)	18 (45)	19 (43)	79 (52)	59 (52)	20 (54)
APOE-ε4 carriers, *n* (%)	3 (9)	27 (66)‡	70 (47)	42 (37)	28 (76)‡
Aβ-PET, CL	NA	NA	7.7 [21.8]	4.1 ± 7.7	56.1 ± 27.1‡
MMSE, /30	NA	18 [12]	29 [1]	29 [1]	29 [1]
AVLT TL, /75	NA	NA	47 ± 10	48 ± 10	45 ± 10
Mean BSRT TR, /12	NA	NA	8.0 ± 1.6	8.1 ± 1.6	7.5 ± 1.5*
Plasma p-tau181, pg/mL	6.9 [4.9]	31.8 [15.7]‡	8.9 [7.5]	7.4 [5.1]	17.1 [10.5]‡
Plasma p-tau231, pg/mL	3.8 [5.7]	7.1 [4.1]†	3.4 [2.5]	3.1 [2.6]	3.5 [1.7]*

Continuous data are expressed as mean ± SD when normally distributed and median [IQR] when not. Categorical data are expressed as number (%). Comparisons between cohort subgroups were made using either an unpaired *t*-test (normal data), a Mann–Whitney *U* test (non-normal data) or a χ^2 test (categorical data). *$p < 0.05$, †$p < 0.001$, ‡$p < 0.0001$ for subgroup comparisons.

Abbreviations: Aβ, amyloid-β; APOE, Apolipoprotein E; AVLT TL, Auditory Verbal Learning Test total learning; BSRT TR, Buschke Selective Reminding Test total retention; CL, Centiloid; F-PACK, Flemish Prevent AD Cohort KU Leuven; IQR, interquartile range; MMSE, Mini-Mental State Examination; p-tau, phosphorylated-tau; SD, standard deviation.

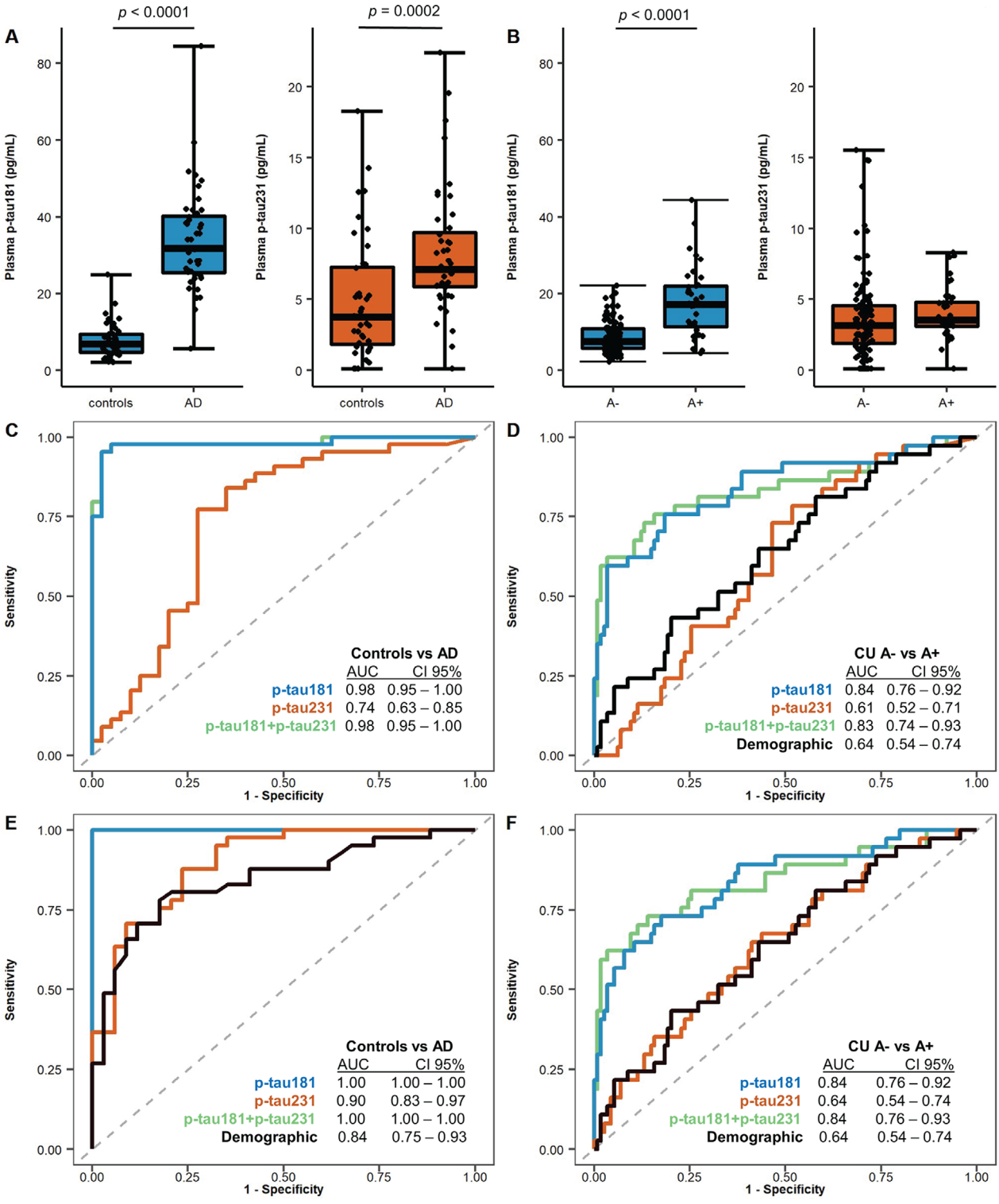
correlated equally strong—yet inversely—with cerebral Aβ load ($\rho = -0.50$ and 0.44 respectively, Fig. 5A and B). However, in univariate linear regression models, plasma p-tau181 explained 16% more of the Centiloid variance observed among F-PACK subjects than the plasma Aβ42/p-tau181 ratio (adjusted $R^2 = 0.37$ for p-tau181 and 0.21 for Aβ42/p-tau181, Supplement 6A and B). No differences in the plasma Aβ42/p-tau231 ratio nor plasma p-tau231 nor Aβ42 alone were detected between Aβ-positive and Aβ-negative CU subjects and neither of these biomarkers predicted Aβ-PET status better than the demographic model (Fig. 4C), nor associated with CLs (Supplement 6C–E).

Discussion

The novel highly phospho-specific plasma p-tau181 Simoa assay is a reliable tool to detect not only clinical, but also asymptomatic AD, since it accurately discriminates Aβ-positive from Aβ-negative CU elderly. Moreover, plasma p-tau181 levels measured by this novel assay correlated with cerebral Aβ load within CU elderly. These correlations were strongest in brain regions typically affected in early AD, such as the precuneus.[32,33] The prototype plasma p-tau231 assay showed lower performance to detect both clinical and asymptomatic AD and was not a better predictor of clinical AD or Aβ-PET status than the combination of age, sex and *APOE*. Combining both plasma p-tau species in a biomarker model did not increase performance to detect clinical or asymptomatic AD relative to a biomarker model based on plasma p-tau181 alone. No global nor regional correlations between p-tau231 and Aβ-PET burden were observed in CU elderly. The ratio of plasma Aβ42 with either plasma p-tau181 or p-tau231 did not increase performance to detect asymptomatic Aβ pathology. Both assays showed good intra- and inter-assay precision, which were situated well within the FDA acceptance criteria (<15%) for non-zero calibrators.[34]

CSF-based p-tau species have been established as early and reliable markers of underlying AD pathology.[35–38] In recent years, a growing body of evidence has supported

Figure 2. Alterations in plasma p-tau181 and p-tau231 levels in clinical and asymptomatic AD. Box- and whisker plots show differences in plasma phosphorylated-tau (p-tau)181 (blue, left) and p-tau231 (orange, right) levels between Alzheimer's disease (AD) dementia patients and spouse controls of the memory clinic cohort (A) and between Aβ-positive (A+) and Aβ-negative (A-) CU elderly of the F-PACK cohort (B). The middle line of the box represents the median while the lower and upper line denote the 25th and 75th percentiles. Whiskers represent the range. Individual datapoints are presented on top of the plot. *P* values were obtained using Mann–Whitney *U* tests and are shown if significant after multiple comparison correction (significance level $\alpha = 0.05/2 = 0.03$). ROC curves of plasma p-tau181 and p-tau231 are shown with AD diagnosis (C, E) or PET-based evidence of Aβ pathology (Centiloid > 23.5) (D,F) as the standard-of-truth, either unadjusted (C,D) or adjusted (E,F) for age, sex and *APOE*. The demographic model (black) includes age, sex and *APOE* genotype as predictors without inclusion of any p-tau biomarker. Areas under the curve (AUCs) with 95% confidence intervals (CIs) are superimposed on the plot.



their translatability to blood. Plasma p-tau181, as defined by AT270, is by far the most investigated form and several immunoassays targeting this epitope have been developed.[8,11,12,39,40] However, we showed that an assay using AT270, does not only target p-tau181, but also p-tau175.[15] Hence, performances reported in previous

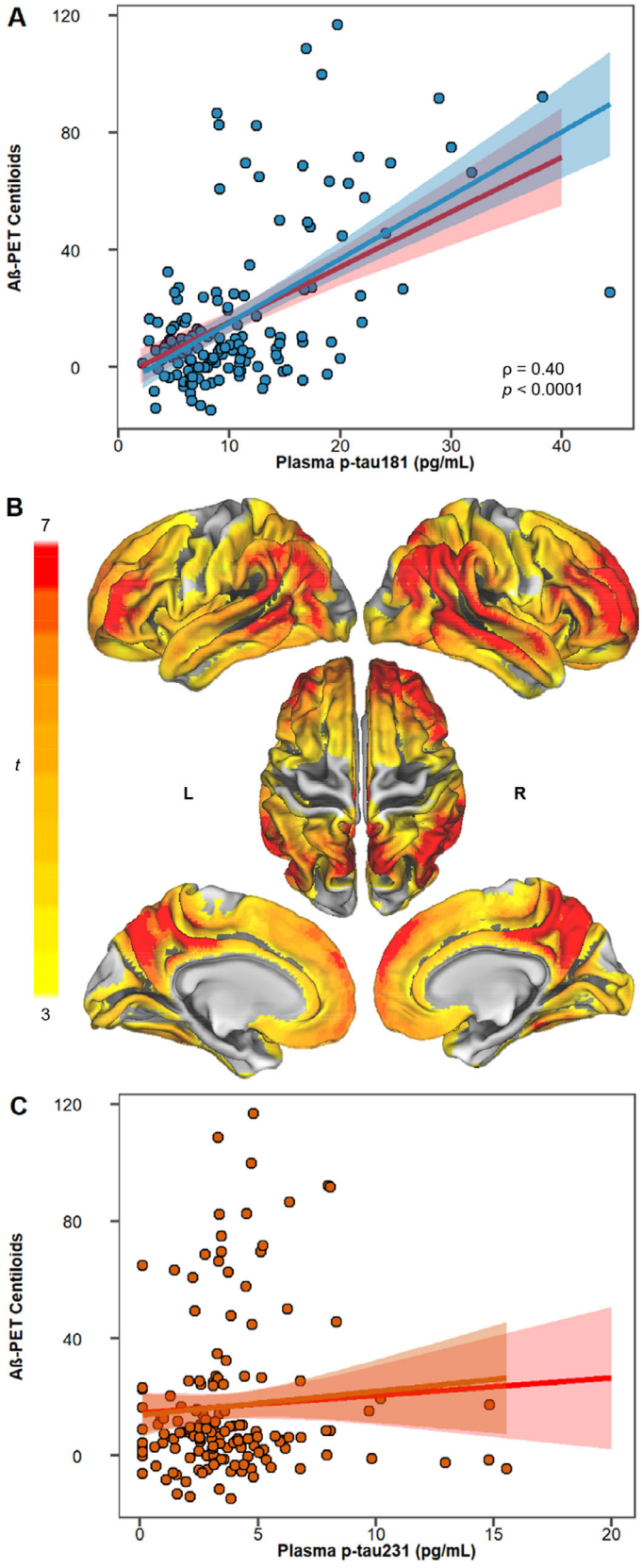


Figure 3. Relationship between novel plasma p-tau biomarkers and cerebral Aβ burden in asymptomatic elderly. (A) Plasma phosphorylated-tau (p-tau)181 levels (blue) were plotted against amyloid-β (Aβ)-PET Centiloids and Spearman rank correlations were calculated in the total F-PACK cohort (N = 151). Linear fits are shown on top of the plot, either unadjusted (blue) or adjusted for age, sex and *APOE-ε4* carrier status (red). (B) Parametric maps of the *APOE*-adjusted regional relationships between plasma p-tau181 levels and Centiloids in the total F-PACK cohort calculated through voxelwise multivariate linear regression models. The significance threshold was set at a cluster-level whole-brain family-wise error (FWE) threshold of $P_{FWE} < 0.05$ with voxel-level set at $P_{uncorrected} < 0.001$. Thresholded maps were superimposed on the left and right hemisphere of the PALS cortical surface (PALS-B12) using CARET v5.65 (Van Essen Lab, http://brainvis.wustl.edu). (C) Scatterplot of plasma p-tau231 and Aβ-PET CLs with calculated Spearman rank correlations in the total F-PACK cohort with linear fits shown on top, either unadjusted (orange) or adjusted for age, sex and *APOE*.

studies are, strictly speaking, not p-tau181 phospho-specific.[12,15,40] The current study employed a highly phospho-specific prototype p-tau181 Simoa assay incorporating ADx252 as a capture antibody. A recent study showed very high performance of this novel p-tau181 assay to detect clinical AD in blood, which was equal to that of plasma p-tau217 (Eli Lily) and plasma p-tau231 (Gothenburg) Simoa assays, and higher than that of two AT270-based plasma p-tau181 Simoa assays (commercial Quanterix and prototype Eli Lily assays).[15] Although both the p-tau231 and p-tau217 Eli Lily assays employed in that study were originally developed on the Mesoscale

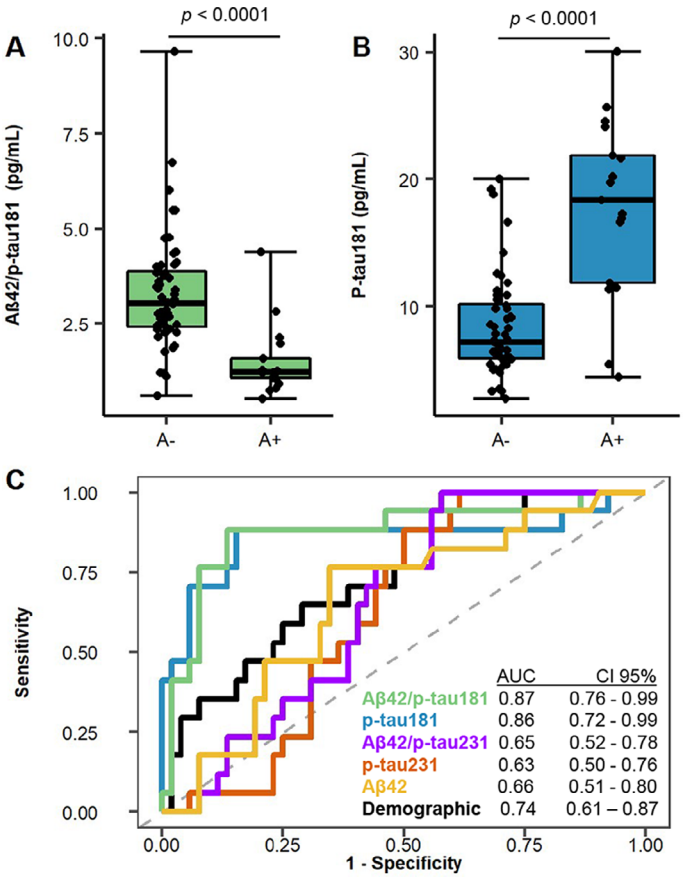


Figure 4. Alterations in plasma Aβ42, p-tau181, p-tau231 and their ratios in asymptomatic AD. Box- and whisker plots of the plasma amyloid-β (Aβ42)/phosphorylated-tau (p-tau)181 ratio (A, green) and p-tau181 (B, blue) in the F-PACK cohort stratified by Aβ-PET status are shown (N = 69). F-PACK subjects were Aβ-positive (A+) when Centiloid >23.5. The middle line of the box represents the median while the lower and upper line denote the 25th and 75th percentiles. Whiskers represent the range. Individual data points are presented on top of the plot. *P* values were obtained using Mann–Whitney *U* tests (significance level $\alpha = 0.05$ corresponding to an uncorrected $p = 0.05/5$ biomarkers = 0.01). (B) Receiver operating characteristic (ROC) curves of plasma p-tau181, p-tau231, Aβ42 and their ratios are shown with Aβ-positivity as the standard-of-truth. Areas under the curve (AUCs) with 95% confidence intervals (CIs) are superimposed on the plot.

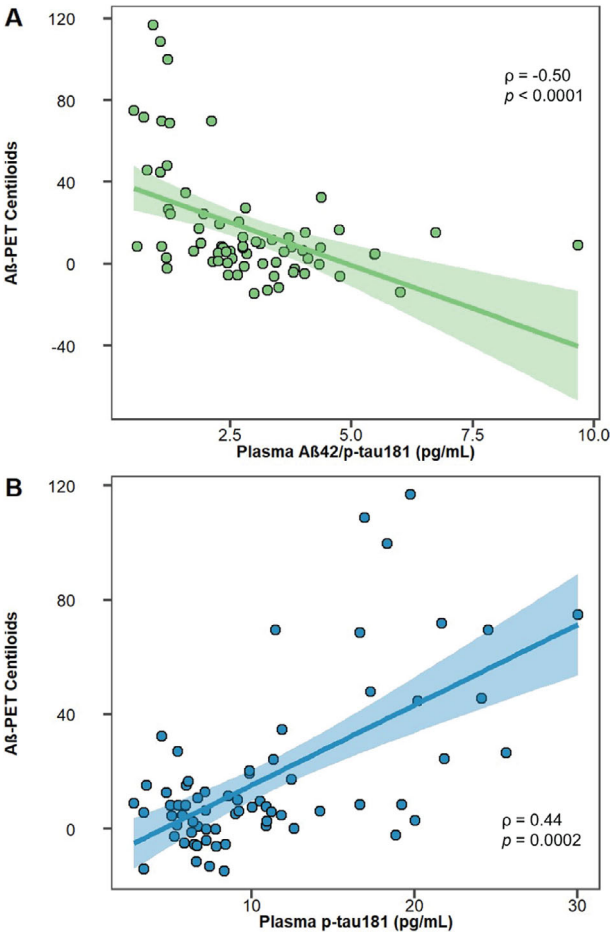


Figure 5. Relationship of the plasma Aβ42/p-tau181 ratio and p-tau181 alone with global cerebral Aβ burden. Plasma amyloid-β (Aβ) 42/phosphorylated-tau (p-tau)181 ratio (A, green) and p-tau181 alone (B, blue) were plotted against Aβ-PET Centiloids and Spearman rank correlations were calculated in the F-PACK subset with available Aβ42 measurements (N = 69). Linear fits are shown on top of the plot.

Discovery (MSD) platform, they were translated to the Simoa platform for comparison purposes.

The current study replicates these results and additionally demonstrates the accuracy of the novel p-tau181 assay for detecting cerebral amyloidosis in CU elderly. Plasma p-tau181 levels in Aβ-positive CU elderly were over double (2.3X) those observed in Aβ-negative CU elderly, while previous studies using AT270-based p-tau181 assays reported differences of only 1.2 to 1.5 fold.[9,10,12,39,41] Moreover, we reported AUCs for distinguishing Aβ-positive from Aβ-negative CU elderly that are numerically higher than those previously reported for AT270-based plasma p-tau181 assays in unadjusted models (AUC = 0.84 vs. 0.70–0.81[5,9,10,39,41]) and approximate those of models adjusted for age, sex and/or *APOE* (AUC = 0.84 vs. 0.81–0.86[6,9,12,42]). In addition, we showed a moderate but widespread correlation of plasma p-tau181 with cerebral Aβ load. Although few studies to date have examined the relationship between plasma p-tau and cerebral Aβ burden within CU elderly, similar findings have been reported in two previous studies using Simoa and/or MSD AT270-based plasma p-tau181 assays.[9,39] Contrarily, two other studies reported only weak correlations which were restricted to small regions within the precuneus, temporal and superior-frontal areas.[13,43] In addition to plasma p-tau181, plasma p-tau217 and p-tau231 have also shown promise as blood-based markers of asymptomatic AD.[5,6,9–11,44,45] In line with observations of the recent pilot study,[15] the ADx prototype p-tau231 assay could not distinguish AD patients from controls as accurately as the p-tau181 assay. We now also demonstrated that it performs worse than p-tau181 in the asymptomatic phase of AD. Yet, the sensitivity of the p-tau231 assay reported here matched that of the p-tau181 assay, suggesting that, although the p-tau181 assay is preferred, both assays might be useful in contexts requiring high sensitivity, such as population screening for early diagnosis. Conversely, only the p-tau181 assay is useful for clinical trial recruitment where high specificity is warranted. The lower performance of the novel plasma p-tau231 assay compared to the p-tau181 assay to detect early AD contrasts previous reports on the Gothenburg p-tau231 assay.[9–11,13] This discrepancy suggests that assay conditions (e.g. incubation time, diluents or temperature) applied in this previous plasma p-tau231 assay are more favourable for plasma p-tau231 quantification. A recent study even demonstrated higher performance of plasma p-tau231, as measured by the Gothenburg assay, than plasma p-tau181, as measured by an AT270-based Simoa assay, to detect asymptomatic Aβ pathology. Of note, the AUC reported as the performance parameter for p-tau231 in that study was not higher (and even numerically lower) than the AUC of the novel ADx252-based p-tau181 assay reported here. In fact, in this previous study, plasma p-tau181 demonstrated rather poor (AUC = 0.70) performance to detect Aβ pathology as well as weaker and more restricted correlations with cerebral Aβ than presented for the novel p-tau181 assay in the current study. Taken together with the better performance of the novel p-tau181 assay compared to AT270-based p-tau181 assays shown previously,[15] this suggests that the increased performance of plasma p-tau231 versus p-tau181 might be a consequence of the aspecificity of the employed p-tau181 assay shown here, rather than superiority of plasma p-tau231 over p-tau181 species for detection of early AD.

Lastly, no additional value of including plasma Aβ42 in an Aβ42/p-tau ratio was found over p-tau alone. This supports the hypothesis that blood-based Aβ42 and p-tau

reflect closely related pathological processes in AD. Initially, the ATN framework postulated that while Aβ42 is a biomarker of Aβ plaques, p-tau levels reflect fibrillar tau that is passively released in the extracellular space after neuronal damage or death.[46] However, blood-based p-tau species are predominantly N-terminal fragments missing the aggregation-prone microtubule binding regions found in cerebral tau tangles.[47,48] Moreover, plasma p-tau correlates more strongly with Aβ-PET than tau-PET,[4,9,39,41] acts as a mediator between the two[44,49] and is not increased in other tauopathies.[6] This, together with our findings, supports the role of plasma p-tau as a direct marker of Aβ pathology that is released into the blood through Aβ-mediated active secretion from neurons.

A limitation of the present study is the lack of direct comparison between ADx prototype p-tau assays and those used in previous studies, thus preventing formal conclusions on their relative validities. Furthermore, the cross-sectional nature of our findings warrants confirmation with longitudinal data, particularly how plasma p-tau measurements of these prototype assays relate to underlying changes in brain pathology to assess their monitoring potential.

In conclusion, the present study shows high performance of the novel prototype plasma p-tau181 assay to detect not only clinical AD, but also cerebral amyloidosis in asymptomatic AD. Although the plasma p-tau231 assay demonstrated similar sensitivity, its overall performance was poor and did not seem to adequately reflect cerebral Aβ pathology. While the novel plasma p-tau181 assay was a better predictor of Aβ-PET status than age, sex and *APOE* combined, the equivalent p-tau231 assay was not. Hence, the prototype p-tau181 assay proves to be better suited for clinical trial recruitment in clinical or asymptomatic AD.

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Author Contributions

RV contributed to the study concept and design. SDM analysed the data and performed statistical analyses. JV, EV, ES and SDM acquired plasma biomarker data. JMS, MR, ESL and KVL contributed to image acquisition and/or blood processing. SDM, MR and PD contributed to image processing. SDM, RV and KP drafted the manuscript. All authors revised and approved the final manuscript.

Conflict of Interest

SDM, JMS, MR, ESL, PD and KP report no disclosures. JV, ES and EV are full-time paid employees and EV is co-founder of ADx Neurosciences, the company that developed the novel plasma p-tau181 and p-tau231 assays investigated here. RV's institution has had a clinical trial agreement for phase 1 and 2 studies with GE Healthcare, which provided [^{18}F]flutemetamol for this study. KVL has performed contract research through UZ/KU Leuven as principal investigator for GE Healthcare and received speaker fees from GE Healthcare.

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Supporting Information

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

Data S1.
Supplement 1. Assay characteristics
Supplement 2. Inter-assay CV%
Supplement 3. Performance parameters of unadjusted biomarker models with 95% CI
Supplement 4. Linear regression models of plasma p-tau biomarkers for asymptomatic cerebral Aβ burden in the F-PACK cohort
Supplement 5. Brain regions in which amyloid load is associated with plasma p-tau181
Supplement 6. Linear regression models of plasma p-tau, Aβ42 and their ratios for asymptomatic cerebral Aβ burden.

