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Head-to-head comparison of plasma p-tau181, p-tau231 and glial fibrillary acidic protein in clinically unimpaired elderly with three levels of *APOE4*-related risk for Alzheimer's disease

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ABSTRACT

Plasma phosphorylated tau (p-tau) and glial fibrillary acidic protein (GFAP) both reflect early changes in Alzheimer's disease (AD) pathology. Here, we compared the biomarker levels and their association with regional β-amyloid (Aβ) pathology and cognitive performance head-to-head in clinically unimpaired elderly ($n = 88$) at three levels of *APOE4*-related genetic risk for sporadic AD (*APOE4/4* $n = 19$, *APOE3/4* $n = 32$ or non-carriers $n = 37$). Concentrations of plasma p-tau181, p-tau231 and GFAP were measured using Single molecule array (Simoa), regional Aβ deposition with ^{11}C-PiB positron emission tomography (PET), and cognitive performance with a preclinical composite. Significant differences in plasma p-tau181 and p-tau231, but not plasma GFAP concentrations were present between the *APOE4* gene doses, explained solely by brain Aβ load. All plasma biomarkers correlated positively with Aβ PET in the total study population. This correlation was driven by *APOE3/3* carriers for plasma p-tau markers and *APOE4/4* carriers for plasma GFAP. Voxel-wise associations with amyloid-PET revealed different spatial patterns for plasma p-tau markers and plasma GFAP. Only higher plasma GFAP correlated with lower cognitive scores. Our observations suggest that plasma p-tau and plasma GFAP are both early AD markers reflecting different Aβ-related processes.

1. Introduction

The underlying pathological changes in Alzheimer's disease (AD), namely accumulation of β-amyloid (Aβ) peptides and hyperphosphorylation of intracellular tau protein, are known to start decades before the manifestation of cognitive symptoms (Jack et al., 2018).

Abbreviations: AD, Alzheimer's disease; APCC, Alzheimer's Prevention Initiative Preclinical Cognitive Composite; *APOE*, apolipoprotein E gene; Aβ, beta-amyloid; CNS, central nervous system; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; MMSE, Mini-Mental State Examination; PET, positron emission tomography; p-tau, phosphorylated tau; Simoa, Single Molecule Array; SUVR, standardized uptake value ratio; VOI, volume of interest.

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Thus, characterizing the pathological profile of healthy individuals at increased risk of AD, such as homozygous or heterozygous carriers of apolipoprotein E ε4 (*APOE4*) allele, is important for future screening, early and accurate diagnosis, and initiation of treatment, especially as novel disease-modifying therapies for AD finally begin to emerge (Cummings et al., 2021; van Dyck et al., 2022). At present, the ongoing AD pathological process can be depicted using various biofluid or imaging-based markers: cerebrospinal fluid (CSF) markers are available for measurements of $A\beta_{42/40}$, total tau (t-tau) and phosphorylated tau (p-tau) and positron emission tomography (PET) for monitoring fibrillar Aβ and tau deposition in the brain (Dubois et al., 2021). However, these measures are either invasive, expensive, not suitable for serial measurements, or measuring alterations that occur only at late stages of the AD continuum. Thus, the recent development of highly sensitive blood-based biomarkers for AD pathological processes have been recognized as an asset for *e.g.*, pre-screening of suitable at-risk volunteers for future disease-modifying drug trials and even as measures of pharmacodynamic effects of novel drugs (Karikari et al., 2022; Pontecorvo et al., 2022).

In recent years, an extensive number of studies have shown different forms of p-tau to be specific and sensitive blood biomarkers for AD (Karikari et al., 2020; Mattsson-Carlgren et al., 2020; Palmqvist et al., 2020; Ashton et al., 2021; Mielke et al., 2018; Janelidze et al., 2022; Lantero Rodriguez et al., 2020; Triana-Baltzer et al., 2021). Plasma p-tau181, p-tau217 and p-tau231 have all been shown to gradually increase during the long AD continuum (Karikari et al., 2020; Palmqvist et al., 2020; Ashton et al., 2021); to discriminate AD from other neurodegenerative disorders (Palmqvist et al., 2020; Lantero Rodriguez et al., 2020); and to correlate both with Aβ and tau pathology (Karikari et al., 2020; Ashton et al., 2021; Karikari et al., 2021; Meyer et al., 2022). Interestingly, plasma p-tau concentrations begin to increase already in cognitively unimpaired individuals with subtle changes in Aβ and without neurofibrillary tangle pathology measurable by tau PET (Milà-Alomà et al., 2022; Ashton et al., 2022), likely caused by a change in the metabolism of tau associated with the initiation of Aβ pathology (Sato et al., 2018; Maia et al., 2013; Kanmert et al., 2015). In a recent comparison of several available blood p-tau markers, p-tau181, p-tau231 and p-tau217 levels correlated strongly (Bayoumy et al., 2021; Ashton et al., 2023). However, plasma p-tau231 and plasma p-tau217 have been shown to be the best measures of the earliest Aβ pathology present in preclinical AD (Milà-Alomà et al., 2022), which is also shown in CSF studies (Ashton et al., 2022c).

Another interesting biomarker measurable from blood is glial fibrillary acidic protein (GFAP), an intermediate filament of brain astrocytes. Increased levels of GFAP are generally seen to reflect reactive astrogliosis related to neuronal injury or ongoing pathology (Abdelhak et al., 2022). In addition to p-tau species, plasma GFAP has been shown to increase in AD dementia and mild cognitive impairment (MCI) (Oeckl et al., 2019; Benedet et al., 2021; Cicognola et al., 2021) as well as in cognitively unimpaired Aβ-positive individuals (Benedet et al., 2021; Chatterjee et al., 2021; Pereira et al., 2021), and predict conversion to AD dementia in MCI-AD participants (Cicognola et al., 2021). Similar to p-tau markers, plasma GFAP levels are associated with Aβ, and less with tau pathology (Pereira et al., 2021). Plasma p-tau181, p-tau231 and GFAP all have been shown to detect Aβ-pathology measured by PET in preclinical AD (Milà-Alomà et al., 2022).

APOE4 is the strongest risk factor for sporadic AD, known to be associated with both higher Aβ and tau PET already in older individuals without dementia (Salvadó et al., 2021). In this study, we wanted to compare plasma p-tau181, plasma p-tau231 and plasma GFAP concentrations and their association with regional Aβ pathology and cognitive performance head-to-head in clinically unimpaired elderly. Our primarily objectives were to investigate i) whether the number of *APOE4* alleles (one, two or no copies of *APOE4*, referred throughout this paper as *APOE* genotype) affects plasma p-tau181, plasma p-tau231 or plasma GFAP concentrations in cognitively normal elderly, and ii) whether these differences are independent of brain Aβ load measured by amyloid-PET. In addition, we investigated association between the plasma biomarkers and cognitive performance in the whole elderly population. We also performed a secondary analysis stratifying the cohort based on Aβ-positivity, with the secondary objective to replicate previous findings concerning the ability of the plasma biomarkers to differentiate individuals based on Aβ-status already prior to cognitive impairment. Based on previous research we hypothesized that higher plasma p-tau181, plasma p-tau231 and plasma GFAP levels would be detected already in cognitively unimpaired *APOE4* carriers in comparison to non-carriers in a gene dose-related matter (*APOE4/4* > *APOE3/4* > Non-carriers), and that all plasma biomarkers would be associated with Aβ PET in the whole study population.

2. Materials and methods

2.1. Study participants

This cross-sectional study includes individuals from two independent research cohorts recruited at the Turku PET Centre during years 2018–2022 including clinically unimpaired "at-risk" individuals (Cohort 1: ASIC-E4 ($n = 61$ (Snellman et al., 2022);; Cohort 2: CIRI-5Y ($n = 37$ (Ekblad et al., 2018);). Briefly, the ASIC-E4 project aims to study cognitively unimpaired individuals with varying *APOE4* gene dose, and thus varying risk for sporadic AD, whereas CIRI-5Y is a 5-year follow-up of a study that examined the associations between midlife insulin resistance, *APOE* genotype and late-life AD-related cerebral changes and cognition (Ekblad et al., 2018; Toppala et al., 2021; Toppala et al., 2019). The recruitment and screening process for ASIC-E4 study and the CIRI baseline study have been described previously (Snellman et al., 2022; Ekblad et al., 2018). In the CIRI-5Y study all the 60 individuals who participated in the baseline study in 2014–2016 were contacted and invited to the CIRI-5y follow-up. Altogether 47 (78%) of the study volunteers participated in some parts of the follow-up study; 43 underwent a ^{11}C-PiB-PET scan; 37 had plasma sampling for biomarkers of AD. For this head-to-head comparison, we included 88 clinically unimpaired participants from both studies who had **i)** *APOE2/3*, *APOE3/3*, *APOE3/4* or *APOE4/4* genotype; **ii)** MMSE ≥24, and **iii)** plasma p-tau181, plasma p-tau231, plasma GFAP measurements and ^{11}C-PiB PET available (Fig. 1). The participants who were included had no neurological or psychiatric diseases and they did not report subjective cognitive impairment.

2.2. Ethical considerations

For both cohorts, protocols have been approved by the Ethics Committee of the Hospital District of Southwest Finland. All participants signed a written informed consent at enrollment to the studies in accordance with the declaration of Helsinki.

2.3. APOE genotyping

For cohort 1, *APOE* genotyping was performed during the screening stage by Auria biobank at Turku University Hospital, Clinical Microbiology and Immunology Laboratory with a Taqman SNP genotyping assay (Applied Biosystems, ThermoFisher). For cohort 2, *APOE* genotyping was performed as previously reported (Ekblad et al., 2018).

2.4. Blood draw and plasma biomarker measurements

Venous blood samples were drawn during a study visit after a 10–12 h fasting period and plasma creatinine levels were measured in a local testing laboratory (TYKSLAB) as previously described (Snellman et al., 2022). Additional EDTA-plasma samples (Vacuette EDTA-K2 tube no. 454411) were collected for plasma biomarker measurements. Samples were frozen and stored at −80 prior to analysis. All biomarker

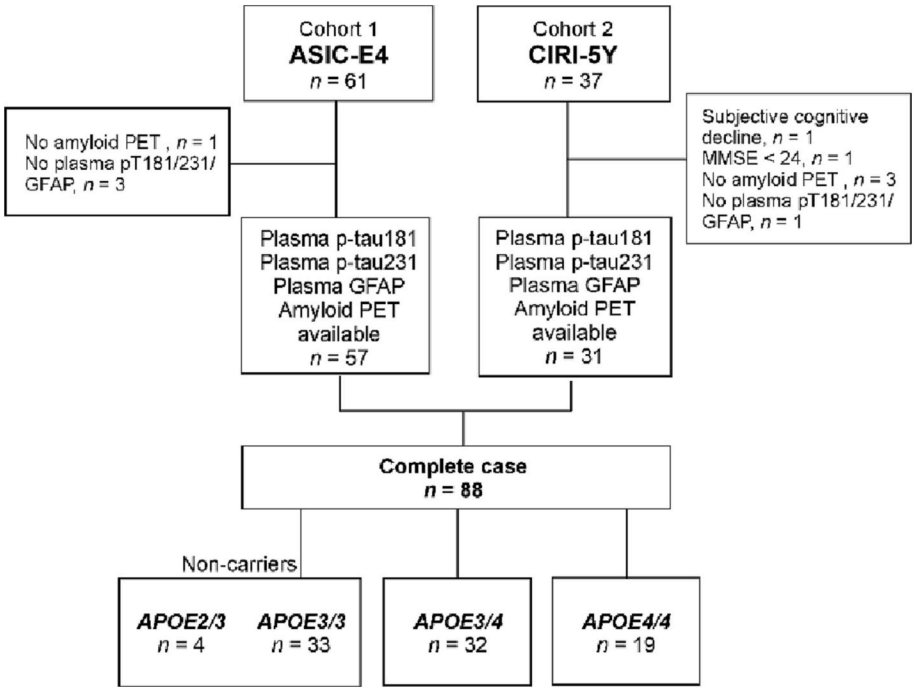

Fig. 1. Flowchart for the study sample selection.

measurements were performed at the Clinical Neurochemistry Laboratory, University of Gothenburg (Mölndal, Sweden). Plasma p-tau181 and plasma p-tau231 were measured using in-house assays (Karikari et al., 2020; Ashton et al., 2021). Briefly, the plasma p-tau181 assay used a mouse monoclonal antibody targeted for phosphorylated T181 for capture (AT270; MN1050, Invitrogen, Waltham, MA) and biotinylated Tau12 mouse monoclonal antibody targeting N-terminal epitope 6–18 for detection (Tau12, Biolegend, San Diego, DA). For plasma p-tau231 assay, a mouse monoclonal ADx253 antibody targeting phosphorylated tau T231 was used for capture and Tau12 for detection. Recombinant full-length tau441 protein phosphorylated *in vitro* (TO8-50FN, Signal-Chem) was used as an assay calibrator for both p-tau assays. Prior to analysis, all plasma samples were thawed, vortexed (2000 rpm, 30s) and centrifuged (4000 ˣ g, 10 min at room temperature) before plating and diluting 1:2 with an assay diluent (Tau 2.0, Quanterix). Samples were randomized and analysed using the same batch of p-tau181 and p-tau231 reagents on two analytical runs on Simoa HD-X instrument. Quality control plasma samples with a mean concentration of 19 pg/ml and 41 pg/ml for p-tau181 and 7.1 pg/ml and 14.6 pg/ml for p-tau231 were included in the beginning and end of each plate to control inter- and intraplate variation. Calibrators and controls were analysed as dublicates and samples as singlicates. The within and between run variations were < 6% for p-tau181 and < 10% for p-tau231.

Plasma GFAP was measured at the the Clinical Neurochemistry Laboratory of University of Gothenburg, using a commercial GFAP discovery kit (#102336, Quanterix). Prior to analysis, all plasma samples were thawed, vortexed (2000 rpm, 30s) and centrifuged (4000 xg, 10 min at room temperature) before plating. Calibrators and controls were analysed as dublicates, samples as singlicates. Samples were analysed in two analytical runs using the same kit lot and Simoa HD-1 instrument. QC samples with mean concentration of 100 pg/ml and 608 pg/ml were included in the beginning and end of each plate, and the within and between run variations were < 15%. Plasma GFAP findings for cohort 1 have been published before (Snellman et al., 2023), and were combined with readings from cohort 2 and to enable comparison to the novel plasma p-tau markers.

2.5. Neuroimaging

A 3D T1-weighed MRI scan was performed for each individual with either Philips Ingenuity 3.0 T TF PET-MR at Turku PET Centre (Philips Healthcare, Amsterdam, the Netherlands) or Philips Ingenia 3.0 T systems at Turku University Hospital (Philips Healthcare, Amsterdam, the Netherlands).

All participants underwent ^{11}C-Pittsburgh compound B (PiB) PET scans at Turku PET Centre with High Resolution Research Tomograph (HRRT; Siements Medical Solutions, Knoxville, TN, USA) during 2018–2021. 500 MBq of ^{11}C-PiB was injected intravenously and emission data was collected 40–90 min post injection. Cortical ^{11}C-PIB uptake was quantified as standardized uptake value ratios (SUVRs) calculated for 60–90 min time frame, with cerebellar cortex as a reference region. A composite volume-of-interest (VOI) using volume weighted means of pre-frontal cortex, parietal cortex, anterior cingulum, posterior cingulum, precuneus and lateral temporal cortex was created and used as a proxy of cortical Aβ load. Participants with a composite VOI SUVR <1.5 were classified as Aβ-negative and ≥ 1.5 as Aβ-positive.

2.6. Cognitive assessment

All participants underwent cognitive testing during one of the study visits. The performed tests included the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS (Randolph et al., 1998)) test battery and Raven's Matrices that together with Mini-Mental State Examination (MMSE) orientation to time and place were used to calculate the Alzheimer's Prevention Initiative Preclinical Cognitive Composite (APCC (Langbaum et al., 2020)). Briefly, the APCC battery is a cognitive composite that has been developed and tested for decline in preclinical AD and previously used in the API Generation Program clinical trials (Langbaum et al., 2020).

2.7. Statistics

All statistical analyses were performed with JMP Pro 16.0.0 (SAS Institute Inc., Cary, North Carolina), and data visualizations with GraphPad Prism (version 9.4.1). Normality assumption for the data was

inspected both visually from the histograms and by the Shapiro-Wilk test. Demographic data are presented as mean (standard deviation) for normally distributed and median (interquartile range) for skewed data. For continuous demographic variables, differences between *APOE4* genotypes were analysed using 1-way ANOVA followed by *post hoc* Tukey's honest significance test or Kruskal-Wallis test followed by *post hoc* Steel-Dwass test for all pairs. Fisher's exact test was used for categorical variables.

All plasma biomarker data were skewed, therefore $\log_{10}$ transformation was performed before group comparisons. For all analysis including *APOE4* genotype, *APOE2/3* and *APOE3/3* were combined into one group (Non-carriers, $n = 37$) due to the low number of *APOE2/3* carriers ($n = 4$). Two individuals (both *APOE3/4*) were excluded based on outlier values for plasma GFAP (1006 pg/ml) or plasma p-tau181 (62.7 pg/ml), resulting to final complete case analysis with 86 subjects.

Differences in $\log_{10}$-transformed biomarker levels between *APOE4* genotypes were tested using linear models, adjusting for age that differed significantly between the groups. If a significant effect was found, all pairs were compared using *post hoc* Tukey's honest significance test for multiple comparisons. Subsequently, to investigate if the found *APOE4* effect was independent of Aβ-deposition, we added brain Aβ-load (estimated by $\log_{10}$-transformed composite ^{11}C-PiB-PET SUVR) and its interaction with *APOE* genotype (*APOE* genotype × Aβ-PET) to the model. If significant interaction term was found, similar models were constructed separately for each *APOE* genotype.

Correlations of raw blood biomarker concentrations with each other, with brain Aβ load (estimated with ^{11}C-PiB SUVR in the composite VOI) and with cognitive MMSE/APCC scores were first tested with Spearman's correlation. If a significant correlation was found, independent associations and interactions were further examined using linear regression analysis including age as a covariate. Normality assumption for the models was always inspected from the residuals.

Secondary analysis testing differences between Aβ-negative and Aβ-positive individuals were performed using linear models, adjusting for age that differed significantly between the groups. Secondary analysis for the capability of blood biomarkers to distinguish Aβ-positive individuals (PiB PET ≥1.5) from Aβ-negative individuals in our cohort was tested using logistic regression and receiver operating characteristic (ROC) analysis.

Voxel-wise association between ^{11}C-PiB binding and ($\log_{10}$) plasma biomarker concentrations were tested with linear regression analysis using Statistical Parametric Mapping (SPM12). Parametric ^{11}C-PiB SUVR images normalized to Montreal Neurological Institute (MNI) space were used for voxel-wise analysis. Significant results were thresholded using combined $p_{\text{uncorrected}} = 0.001$ at the voxel level, and a false discovery rate (FDR) corrected $p_{\text{FDR}} < 0.05$ at the cluster level.

3. Results

3.1. Cohort demographics and correlations

Demographics of the whole study population ($n = 88$) are presented in Table 1. Median age of the total study population was 71 years (range 60–84 years), and 59.1% (52 out of 88) of the participants were females. Groups were matched for sex, education, plasma creatinine levels and number of participants using hypertensive medication, but homozygous *APOE4/4* carriers were younger than the two other groups. Thus, all further analyses for differences between groups were adjusted for age.

For a secondary analysis, we also stratified the population based on Aβ-positivity (using a cutoff of composite cortical SUVR >1.5) resulting in Aβ-positive ($n = 62$, presenting AD pathological change or preclinical AD (Jack et al., 2018)) and Aβ-negative groups ($n = 26$) (Supplemental Table 1). Groups were matched for sex and education, but Aβ-positive subjects were older and had lower cognitive scores compared to Aβ-negative group. Due to difference between groups, age was added as a covariate in following analysis.

In the whole study population, there was no correlation between age and plasma p-tau levels (Rho <0.099 $p > 0.36$ for both), whereas higher plasma GFAP and older age had a weak and borderline significant correlation (Rho = 0.21, $p = 0.055$). Plasma p-tau181 and plasma p-tau231 had a strong positive correlation with each other in the whole cohort (Rho = 0.72, $p < 0.0001$), and within all *APOE* genotypes. Plasma GFAP did not correlate with plasma p-tau markers in any of the *APOE4* genotypes (Rho <0.31, $p > 0.081$ for all). None of the plasma biomarkers correlated with levels of plasma creatinine (estimating kidney function) in our sample (plasma p-tau181: Rho = 0.12, $p = 0.26$; plasma p-tau231: Rho = 0.095, $p = 0.39$; plasma GFAP: Rho = 0.035, $p = 0.75$).

3.2. Group comparisons

3.2.1. Aβ load across the APOE genotypes

Statistically significant differences in brain Aβ load measured by ^{11}C-PiB were detected between the *APOE4* genotypes ($p = 0.0002$, Kruskall-Wallis test, **Supplementary Fig. 1**). Gradual increase was present from non-carriers (Median SUVR 1.59 (IQR 1.43–1.71)) to *APOE3/4* (SUVR 1.71 (1.48–2.30), $p = 0.16$ compared to non-carriers), and further to

Table 1
Demographics.

	Total sample	Non-carriers	*APOE4/3*	*APOE4/4*	*p*
n	88	37	32	19	
Age (y), median (IQR)	71 (66–73)	72 (69–75)	72 (65–73)	69 (63–72)	0.068
Sex (M/F), *n* (%)	36/52 (41/59)	17/20 (46/54)	11/21 (34/66)	8/11 (42/58)	0.64
Education, n (%)					0.85
Primary school	28 (32)	11 (30)	10 (31)	7 (37)	
Middle or comprehensive school	21 (24)	10 (27)	6 (19)	5 (26)	
High school	21 (24)	9 (24)	7 (22)	5 (26)	
College or university	18 (20)	7 (19)	9 (28)	2 (11)	
Diabetes, n (%)	3 (3.4)	3 (8.11)	0 (0)	0 (0)	0.23
Hypertensive medication, n (%)	42 (47.73)	22 (59.46)	11 (34.38)	9 (47.37)	0.12
Plasma creatinine (μmol/L), median (IQR)	78 (68–88)	80 (69–93)	75.5 (68–86)	79 (67–85)	0.74
APCC score, mean (SD)	70.0 (8.55)	71.5 (6.88)	69.5 (9.70)	66.1 (8.68)	0.086
MMSE score, median (IQR)	29 (27–30)	29 (28–30)	29 (28–30)[c]	28 (26–29)[b]	**0.015**
[a]Plasma p-tau181 (pg/ml), median (IQR)	13.5 (11.1–16.9)	12.1 (11.1–15.9)	12.5 (10.6–16.7)[b]	15.9 (14.4–18.5)[b]	**0.014**
[a]Plasma p-tau231 (pg/ml), median (IQR)	5.35 (3.93–7.01)	5.00 (3.70–6.41)	5.48 (3.68–7.35)	5.80 (4.67–9.32)[b]	**0.041**
[a]Plasma GFAP (pg/ml), median (IQR)	150 (110–197)	137 (112–192)	150 (107–181)	187 (137–269)	0.098
^{11}C-PiB SUVR, median (IQR)	1.67 (1.47–2.23)	1.59 (1.43–1.71)	1.71 (1.48–2.30)	2.53 (1.84–2.88)[b]	**< 0.0001**

[a] Plasma biomarkers are Log10 transformed, *p*-values adjusted for age.
[b] $p < 0.05$ compared to non-carriers.
[c] $p < 0.05$ compared to *APOE4/4*.

APOE4/4 (SUVR 2.53 (1.83–2.88), $p = 0.027$ compared to *APOE3/4,* and $p = 0.0002$ compared to non-carriers).

3.2.2. Plasma p-tau181, plasma p-tau231 and plasma GFAP levels across the APOE genotypes

Median concentrations of plasma p-tau181, plasma p-tau231 and plasma GFAP for the *APOE4*-related risk groups are presented in Table 1 and scatterplots presented in Fig. 2. In our cognitively unimpaired population, both plasma p-tau181 and plasma p-tau231 showed significant differences between the *APOE4* genotypes ($p = 0.014$ for p-tau181; $p = 0.041$ for p-tau231, adjusted for age); Both plasma p-tau181 ($p = 0.014$) and plasma p-tau231 ($p = 0.031$) were significantly higher in *APOE4/4* compared to non-carriers, whereas differences between homozygous *APOE4/4* and heterozygous *APOE3/4* were present only with plasma p-tau181 ($p = 0.040$). For plasma GFAP, differences between *APOE4* gene doses did not reach statistical significance ($p = 0.098$), although an increasing trend was visible in *APOE4/4* homozygotes compared to non-carriers.

When the models were further adjusted for brain Aβ-load (estimated by composite ^{11}C-PiB SUVR), significant effect of *APOE* genotype was lost in both plasma p-tau181 ($p = 0.30$), plasma p-tau231 ($p = 0.71$) and plasma GFAP ($p = 0.50$).

3.3. Associations between plasma biomarkers, Aβ-PET and cognitive variables

3.3.1. ROI-level association between plasma biomarkers and composite Aβ-PET

Spearman correlations between the plasma biomarkers and brain Aβ load for the whole cohort and stratified by *APOE* genotype are presented in Table 2. As expected, all plasma biomarkers had a moderate positive correlation with composite PiB SUVR (p-tau181: Rho = 0.31, $p = 0.0032$; p-tau231: Rho = 0.34, $p = 0.0015$; plasma GFAP: Rho = 0.25, $p = 0.018$). The correlation was driven by the *APOE3/3* non-carriers for both p-tau181 (Rho = 0.31, $p = 0.065$) and plasma p-tau231 (Rho = 0.35, $p = 0.033$). On the contrary, a strong positive correlation between plasma GFAP and brain Aβ load was present only in the *APOE4/4* homozygote group (Rho = 0.65, $p = 0.0028$) (Fig. 4A).

We then further investigated the associations between plasma biomarkers and Aβ PET, and their interaction with *APOE* genotype using multivariate linear regression, where plasma biomarkers were set as outcome, Aβ-PET (composite ^{11}C-PiB SUVR), and an interaction (*APOE4* gene dose × Aβ PET) term as predictors, and age always as a covariate (Table 3). In line with previously found correlations, there was a significant association between brain Aβ-PET and plasma p-tau181 (β = 0.33 (95% CI 0.053–0.60), $\beta_{Std} = 0.30$, $p = 0.020$), plasma p-tau231 (β = 0.49 (95% CI 0.13–0.86), $\beta_{Std} = 0.33$, $p = 0.0090$) and plasma GFAP (β = 0.48 (95% CI 0.056–0.90), $\beta_{Std} = 0.28$, $p = 0.027$, Table 3). Significant *APOE* genotype × Aβ-PET interaction effect was found for plasma GFAP ($p = 0.032$), and when the analysis was repeated stratified for *APOE* genotype, a significant association between plasma GFAP and Aβ-PET was present only for *APOE4/4* homozygotes (β = 1.22 (95% CI 0.019–2.42), $\beta_{Std} = 0.58$, $p = 0.047$).

3.3.2. Voxel-wise associations between plasma biomarkers and Aβ PET

In the full study sample, voxel-wise results supported the ROI-level data, showing significant associations with Aβ PET for all plasma biomarkers. For plasma p-tau231, associations were present in the regions well known for early Aβ deposition, such as the frontal cortex, cingulum and precuneus (Grothe et al., 2017). Similar regional distribution for associations were present for plasma p-tau181, with some additional cortical areas affected (**Fig. 3B**). Interestingly, plasma GFAP showed less significant associations in the early Aβ regions, as most significant associations with Aβ PET were present in parietal regions known to accumulate amyloid later, such as the motor cortex (Fig. 3B).

3.3.3. Associations between plasma biomarkers and cognitive measurements

In the whole population, lower scores on both MMSE and APCC score correlated with higher plasma GFAP concentrations (MMSE: Rho = −0.27, $p = 0.011$; APCC: Rho = −0.24, $p = 0.029$), but not with plasma p-tau181 (MMSE: Rho = 0.021, $p = 0.85$; APCC: Rho = −0.011, $p = 0.92$) or plasma p-tau231 (MMSE: Rho = 0.087, $p = 0.43$; APCC: Rho = −0.031, $p = 0.78$) (Table 2).

Based on the found correlation between plasma GFAP and cognitive variables, we further tested this association with linear regression models where cognitive variables were the outcomes, plasma GFAP predictor, and age a covariant. Again, higher plasma GFAP concentrations were associated with both lower MMSE (β = −2.61 (95% CI -4.28 - -0.95), $\beta_{Std} = -0.33$, $p = 0.0024$) and lower APCC score (β = −8.74

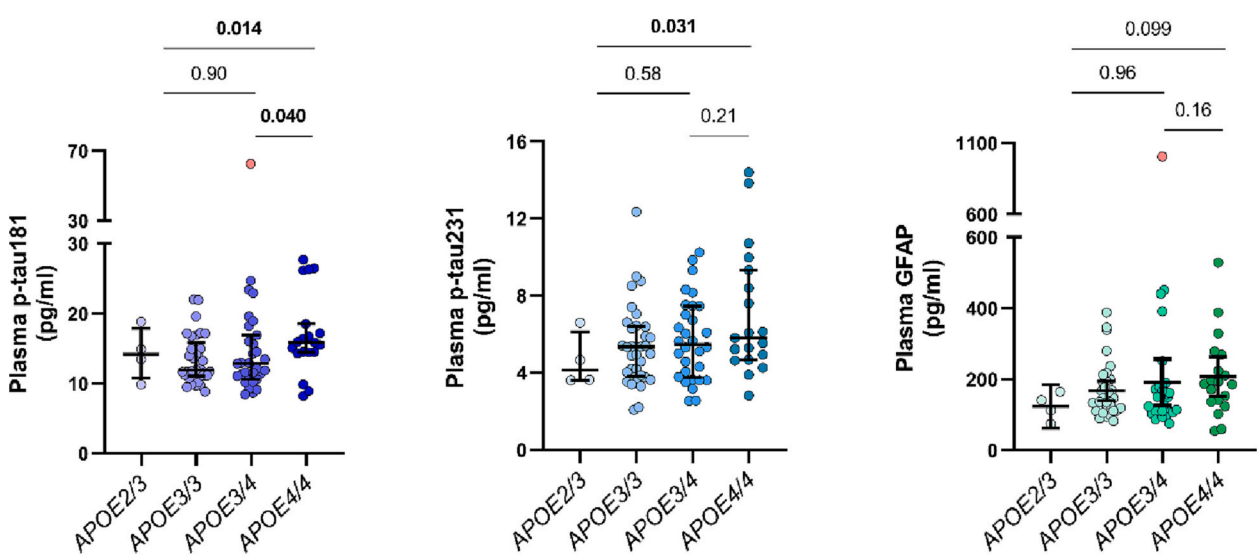

Fig. 2. Comparison of plasma p-tau181, plasma p-tau231 and plasma GFAP concentrations in clinically unimpaired elderly stratified by *APOE4* genotype. *APOE2/3* are presented separately for visualization but combined with *APOE3/3* for statistical analysis. Raw data is presented in the figure, and statistical testing between groups was done using $\log_{10}$-transformed values adjusted for age. Outliers excluded from the analysis are included for visualization as red symbols. GFAP, glial fibrillary acidic protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
APOE4 genotype stratified Spearman's correlations between plasma biomarkers, Aβ PET and cognitive measurements.

	Plasma p-tau181						Plasma p-tau231						Plasma GFAP					
	Aβ PET		MMSE		APCC		Aβ PET		MMSE		APCC		Aβ PET		MMSE		APCC	
	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*	Rho	*p*
Total sample ($n = 86$)	**0.32**	**0.003**	0.021	0.85	−0.011	0.92	**0.34**	**0.002**	0.087	0.43	−0.031	0.78	**0.26**	**0.018**	**−0.27**	**0.011**	−0.24	**0.029**
Non-carriers ($n = 37$)	**0.31**	**0.065**	0.17	0.31	0.28	0.10	**0.35**	**0.033**	0.11	0.54	0.21	0.21	0.23	0.17	−0.059	0.73	−0.079	0.65
APOE3/4 ($n = 32$)	0.25	0.18	0.18	0.33	−0.10	0.59	0.23	0.21	0.24	0.20	−0.055	0.77	−0.081	0.67	−0.14	0.46	−0.19	0.33
APOE4/4 ($n = 19$)	0.13	0.61	−0.16	0.51	−0.20	0.42	0.12	0.64	0.089	0.72	−0.24	0.31	**0.65**	**0.003**	**−0.57**	**0.011**	**−0.48**	**0.037**

(95% CI -17.2 - -0.22), $\beta_{Std} = -0.21$, $p = 0.044$). Association with MMSE survived also further adjustment for education level ($\beta = -2.35$ (95% CI -4.01 - -0.69), $\beta_{Std} = -0.30$, $p = 0.0061$), whereas association with APCC was no longer statistically significant ($\beta = -6.74$ (95% CI -14.7–1.26), $\beta_{Std} = -0.16$, $p = 0.097$).

3.4. Plasma p-tau181, plasma p-tau231 and plasma GFAP concentrations between Aβ-positive and Aβ-negative

Finally, for secondary analysis, we stratified our cohort according to Aβ-positive and Aβ-negative to be able to see how well the blood-based biomarkers could distinguish these groups in clinically unimpaired elderly (Fig. 4; Supplementary table 1). Here, plasma p-tau231 showed significantly higher concentrations in Aβ-positive participants in comparison with Aβ-negative (+18.4%; $p = 0.016$, adjusted for age), whereas plasma p-tau181 (+19.3%; $p = 0.082$) and plasma GFAP (+18.9%; $p = 0.099$) showed increasing trends in the Aβ-positive group. However, in our clinically unimpaired population, the overlap between groups was still high, and univariate ROC analysis showed similar performance for all the biomarkers for discriminating Aβ-positive and Aβ-negative individuals ($AUROC_{p\text{-}181} = 0.62$; 95% CI: 0.49–0.74; $AUROC_{p\text{-}231} = 0.65$; 95% CI: 0.52–0.78; $AUROC_{GFAP} = 0.63$; 95% CI: 0.50–0.75).

4. Discussion

In this study, we performed a cross sectional comparison of plasma p-tau181, plasma p-tau231 and plasma GFAP levels and their association with Aβ PET in healthy elderly volunteers with three levels of *APOE4* related genetic risk for sporadic AD. Plasma p-tau181 and plasma p-tau231 concentrations were increased already in cognitively unimpaired at-risk individuals. Notably, both markers were associated with Aβ deposition in the regions known for early Aβ pathology, and *APOE4* gene dose effect was no longer present when accounted for brain Aβ load. For plasma GFAP, differences in concentrations between the *APOE* genotype groups did not reach statistical significance, but *APOE* genotype was found to modulate the association between plasma GFAP levels and brain Aβ load, suggesting that plasma GFAP concentration increases in parallel with brain Aβ deposition only in *APOE4/4* homozygotes. In addition, only plasma GFAP correlated with lower cognitive performance in our cognitively unimpaired cohort enriched by *APOE4* carriers.

Over the last few years, concentrations of plasma p-tau181 and plasma p-tau231 have been consistently shown to be increased in AD, and thus hold great promise as easily accessible biomarkers of early AD pathology (reviewed recently in (Karikari et al., 2022)). Our findings are in line with the existing literature: plasma p-tau181 and p-tau231 concentrations were seen to increase already in cognitively unimpaired homozygous *APOE4/4* carriers with increased risk for sporadic AD, compared to non-carriers. With p-tau181, but not p-tau231, significantly higher levels were also present in *APOE4/4* homozygotes compared to *APOE3/4* heterozygotes, suggesting that plasma p-tau231 levels start to increase earlier, already in the heterozygotes with more modest Aβ load measured by PET, in line with previous studies comparing these two markers (Ashton et al., 2021; Milà-Alomà et al., 2022). However, this significance was lost if the outlying value (an *APOE3/4* carrier with p-tau181 concentration = 62.7 pg/ml) was included, and overall differences between plasma p-tau181 and p-tau231 in our cohort were modest.

Even though phosphorylated tau measured from CSF has traditionally been classified as a marker for tau pathology (T), plasma p-tau181 and plasma p-tau231 (in addition to plasma p-tau217 not included to this study) are known to increase even before measurable increase in tangle pathology evaluated by PET, and associate strongly with ongoing Aβ pathology (Milà-Alomà et al., 2022). Also here, we detected *APOE4* gene dose effect on both plasma p-tau biomarker concentrations already in cognitively unimpaired elderly individuals. However, when brain Aβ

Table 3
Parameter estimates from the linear regression models testing the effects of Aβ-PET, *APOE* genotype (0, 1 or 2 copies of the *APOE4* allele) and their interaction on plasma p-tau181, plasma p-tau231 and plasma GFAP concentrations.

	Plasma p-tau181 ($n = 86$)				Plasma p-tau231 ($n = 86$)				Plasma GFAP ($n = 86$)			
Predictors	R^2_{Adj} (%)	β	95% CI	β_{Std}	R^2_{Adj} (%)	β	95% CI	β_{Std}	R^2_{Adj} (%)	β	95% CI	β_{Std}
Model 1	**12.3**				**12.9**				**9.8**			
Aβ-PET		**0.40**	**0.18 to 0.63*** **	**0.37**		**0.56**	**0.26 to 0.86*** **	**0.380**		**0.450**	**0.092 to 0.80***	**0.26**
Age		0.0011	−0.0037 to 0.0059	0.047		0.0008	−0.0057 to 0.0072	0.024		0.007	−0.00064 to 0.015	0.19
Model 2	**7.5**				**4.8**				**7.2**			
APOE genotype [0]		**−0.04**	**−0.076** to **−0.0017***	**0.24**		**−0.06**	**−0.11** −to **−0.0054***	**-0.260**		−0.044	−0.1 to0.015	−0.17
Age		0.0041	−0.00092 to 0.0092	0.180		0.0049	−0.0020 to 0.012	0.160		**0.010**	**0.0024 to 0.018***	**0.28**
Model 3	**12.2**				**13.4**				**14.6**			
Aβ-PET		**0.33**	**0.053 to 0.60***	**0.30**		**0.49**	**0.13 to 0.86****	**0.33**		**0.48**	**0.056 to 0.90***	**0.28**
APOE genotype [1]		−0.032	−0.073 to 0.085	−0.19		−0.032	−0.086 to 0.023	-−0.14		−0.0058	−0.069 to 0.057	−0.022
Aβ-PET x *APOE* genotype [1]		−0.036	−0.37 to 0.30	−0.027		−0.14	−0.59 to 0.31	−0.078		**−0.64**	**−1.15** to **−0.11***	**−0.30**
Age		0.0027	−0.0027 to 0.0081	0.12		0.0035	−0.0037 to 0.011	0.11		0.0078	−0.0004 to 0.016	0.23

The data are presented as estimates (β), 95% confidence intervals (CI) and standardized estimates (β_{std}). R^2_{Adj}, Adjusted R square for the whole model.
Aβ-PET (Model 1) and *APOE* genotype (Model 2) were first analysed in separate linear regression models adjusted for age.
Subsequently both were added to the same model (Model 3) also including the interaction term and age.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

PET was added to the multivariate regression models, these differences were no longer significant, implying that the effect is Aβ dependent. Interestingly, in our cohort enriched with *APOE4* carriers, correlations between p-tau markers and Aβ PET SUVRs were driven by the *APOE3/3* noncarriers, with lowest level of Aβ pathology. When we further stratified our cohort by Aβ PET positivity (^{11}C-PiB composite SUVR higher than 1.5), median concentrations of all plasma biomarkers were 18–19% higher in Aβ positive individuals but differences reached statistical significance only for plasma p-tau231. Here, the performance for differentiating between Aβ positive from Aβ negative cognitively unimpaired participants was modest for both plasma p-tau markers ($AUROC_{181}$ = 0.62; $AUROC_{231}$ = 0.65), compared to previous studies (*e.g.* 0.71 (Keshavan et al., 2021), 0.77 (Ashton et al., 2021) and 0.70 (Mielke et al., 2018) for plasma p-tau181, and 0.83 (Ashton et al., 2021) for plasma p-tau231. This discrepancy could be explained by *e.g.,* lower number of participants or differences in the cut-off/reference region used for defining Aβ positivity; however, we cannot exclude possible cofounding effect of much higher number of *APOE4* carriers included in our population. Overall, it should be noted that when investigating fluid biomarkers, especially plasma p-tau concentrations known to increase early before Aβ PET positivity, binary classification of study populations into Aβ-positive and Aβ-negative based on PET is not optimal. Together, our findings support the existing view that CSF and plasma p-tau231 (together with p-tau217) start to increase in parallel with the earliest Aβ related changes in preclinical AD, and possibly reach a plateau with high Aβ levels (Palmqvist et al., 2020; Ashton et al., 2021; Milà-Alomà et al., 2022; Suárez-Calvet et al., 2020).

GFAP is a cytoskeletal protein highly expressed in astrocytes, and thus not a marker of a specific AD related pathological process (Abdelhak et al., 2022). Similar to plasma p-tau, increases in plasma GFAP concentrations related to Aβ pathology have been repeatedly reported in AD, suggested to be explained by reactive astrocytosis around the forming Aβ plaques (Escartin et al., 2021). In our cognitively unimpaired cohort enriched with *APOE4* carriers, we did not observe significant differences in plasma GFAP concentration between *APOE4* gene doses or in Aβ-positive compared to Aβ-negative individuals, even though an increasing trend in the homozygous *APOE4/4* carriers compared to non-carriers was present. Previously, changes in plasma GFAP concentrations were reported early within the AD continuum (Benedet et al., 2021), already in Aβ-positive compared to Aβ-negative cognitively unimpaired individuals (Chatterjee et al., 2021; Prins et al., 2022). This discrepancy between the previous studies and ours could be explained for example by the much larger percentage of *APOE4* carriers in the present study (51/86, 51.3%) compared to previous studies (19/96, 19.7% (Chatterjee et al., 2021) and 60/200, 30.0% (Shir et al., 2022)).

Previously, higher GFAP levels have been reported to associate with higher Aβ load in older functionally intact adults, whereas a nonlinear, inverted U-shape relationship was present at later AD stages (Asken et al., 2020). Similarly, we found a moderate association between plasma GFAP levels and brain Aβ load, and interestingly, this association seemed to be modulated by *APOE4* gene dose. Here, a strong correlation between brain Aβ load and plasma GFAP levels was seen only in *APOE4/4* homozygotes, not in *APOE3/4* heterozygotes or non-carriers. In addition, in our study plasma GFAP levels seem to start increasing rather late, in individuals with ^{11}C-PiB SUVR ~2.5 or higher. This suggests that in our cohort enriched with *APOE4* carriers, plasma GFAP could be associated with the "advanced" stage of preclinical brain Aβ accumulation where Aβ levels already start to plateau. Possibly, the association between plasma GFAP and Aβ at these stages could reflect astrocytic involvement in - or reaction to Aβ accumulation. Since GFAP is an astrocytic protein, and astrocytes are known to be the main source of apolipoprotein E, the protein coded by *APOE* in the CNS (Chernick et al., 2019), it is not surprising that such an interaction exists. Since *APOE4/4* homozygotes are rare, most previous studies have not been investigating *APOE4* gene doses separately, even though *APOE4* carriership is commonly used as a covariate in group comparisons.

As an astrocytic marker, plasma GFAP is less specific to AD-related pathology and known to be increased also in other neurodegenerative dementias (Baiardi et al., 2022), after a stroke (Katsanos et al., 2017), as well as with age (Chatterjee et al., 2022). Higher levels of GFAP in frontotemporal dementia patients were associated with disease severity and cognitive decline (Zhu et al., 2021). As *APOE4* is also known as a

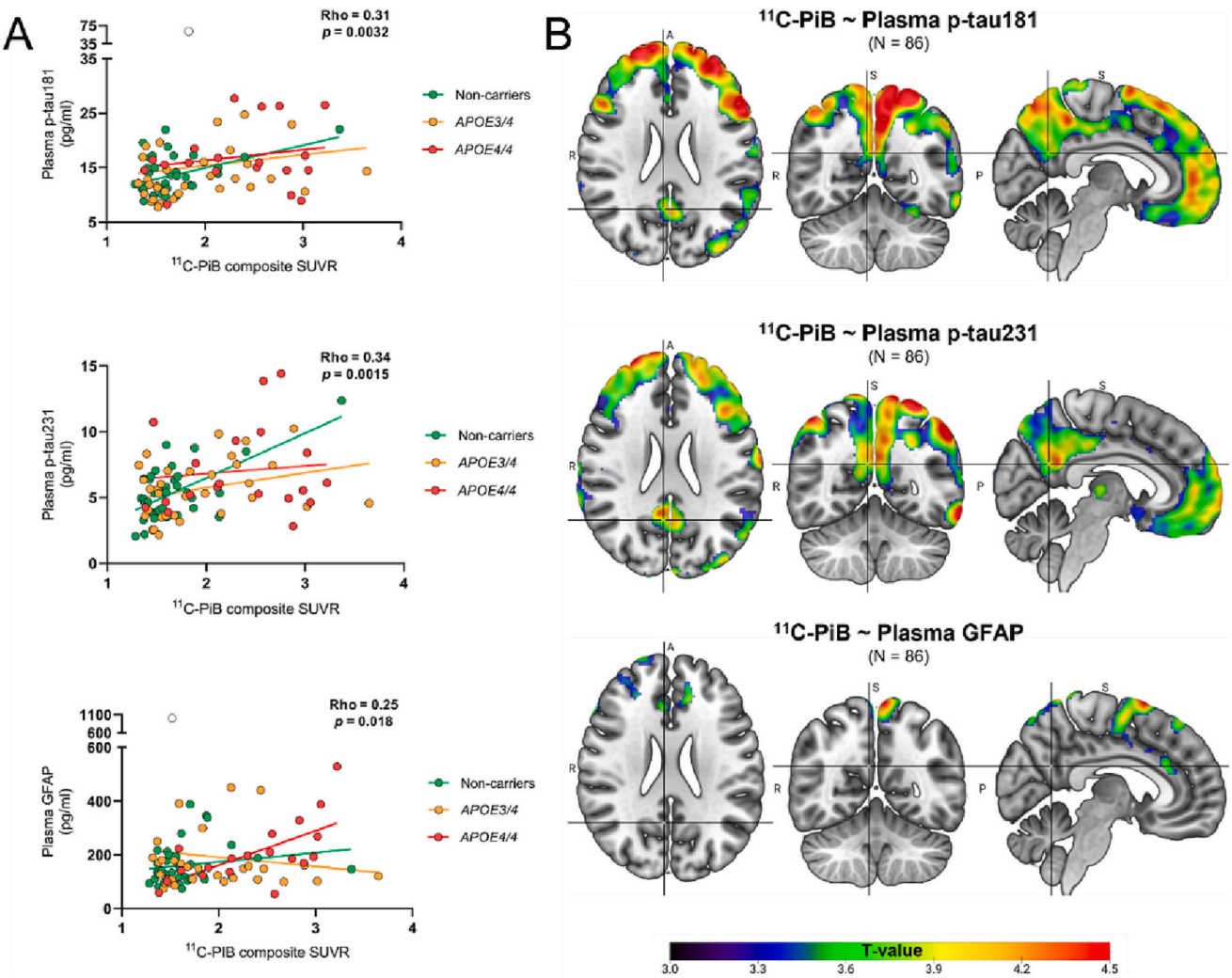

Fig. 3. Region-of-interest and voxel-wise association between plasma p-tau181, plasma p-tau231, plasma GFAP and brain Aβ load measured by ^{11}C-PiB-PET. Positive correlation was seen between cortical composite ^{11}C-PiB SUVR and all plasma biomarkers, driven by non-carriers in p-tau181 and p-tau231 and *APOE4/4* homozygotes in plasma GFAP. Voxel-wise analysis showed regional differences between the plasma biomarkers. Voxel-level analysis was performed with Statistical Parametric Mapping (SPM), and level for statistical significance was set combining voxel-level $p < 0.001$ with cluster level false discovery rate (FDR) corrected $p < 0.05$ to account for multiple comparisons.

risk factor for vascular pathologies, this could in part explain the findings with plasma GFAP in *APOE4/4* homozygotes, even though other neurological and psychiatric diseases as well as previous strokes were exclusion criteria during recruitment for both cohorts included in this study (Snellman et al., 2022; Ekblad et al., 2018). Plasma GFAP levels have been shown to associate with higher white matter hyperintensities (Shir et al., 2022; Elahi et al., 2020) and with a risk of cerebral microbleeds (Shir et al., 2022), that could increase parallel with Aβ pathology in *APOE4/4* homozygotes. In addition, age was a significant covariate in our regression models explaining plasma GFAP, similar as what has been reported in previous studies in preclinical AD populations (Prins et al., 2022; Chatterjee et al., 2022).

Finally, only higher plasma GFAP, not plasma p-tau181 or plasma p-tau231, was seen to be associated with lower cognitive performance, evaluated both with clinically used MMSE score and a preclinical composite score (APCC) developed for research purposes (Langbaum et al., 2020). This is verified by a recent longitudinal study comparing multiple plasma biomarkers (Ashton et al., 2022). Again, using both measures, the association was driven by the *APOE4/4* homozygotes for plasma GFAP. We have previously reported this association for cohort 1 (Snellman et al., 2023) and here, we present a similar finding in a larger cognitively unimpaired sample. Presumably, the *APOE4/4* homozygotes, despite being classified as cognitively unimpaired, already present subtle cognitive impairment that associates with the early pathological change of AD. Plasma GFAP has been seen to be associated with cognitive impairment (Oeckl et al., 2019; Chatterjee et al., 2022) and conversion to AD dementia (Stocker et al., 2022) also in other studies, while in others including participants without dementia this association was not found (Shir et al., 2022). Higher plasma p-tau levels have also been associated with prospective cognitive decline (Meyer et al., 2022; Chatterjee et al., 2022; Moscoso et al., 2021), and (weak) correlations between cognitive measures and plasma p-tau181 and p-tau231 have been reported also cross-sectionally (Karikari et al., 2020; Chatterjee et al., 2022). However, here, in our cross-sectional study including only cognitively unimpaired individuals, such association between MMSE score, or the APCC score, and plasma p-tau markers were not found.

The strength of our study is a well-defined cohort of cognitively unimpaired individuals, including a relatively large group of rare *APOE4/4* carriers. However, this study has also several limitations. One limitation of our study is the lack of CSF or PET tau biomarkers, prohibiting us to stratify our Aβ-positive individuals between AD pathological change (A + T-) and preclinical AD (A + T+). In addition, the size of our cohort is relatively small. However, due to identical data

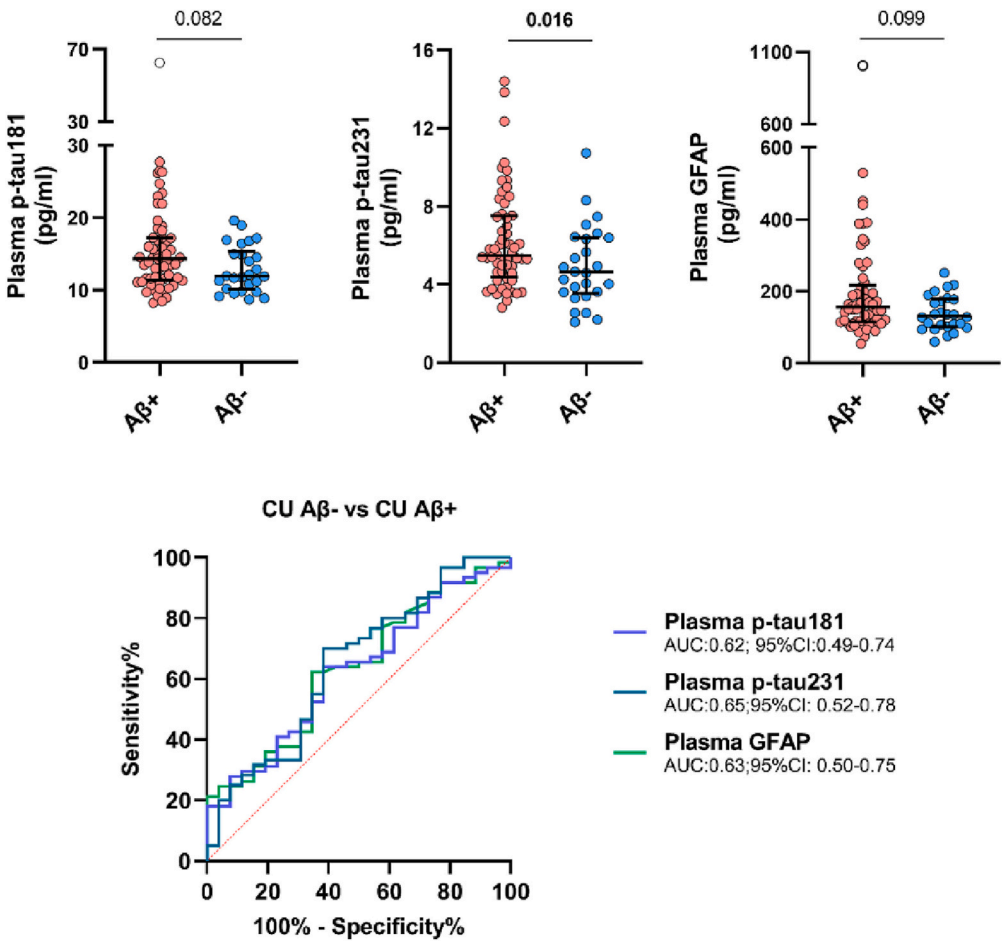

Fig. 4. Concentrations of plasma p-tau181, plasma p-tau231 and plasma GFAP in clinically unimpaired elderly stratified by Aβ-positivity. Threshold for Aβ-positivity was composite PiB SUVR >1.5. Statistical analysis was done using $\log_{10}$-transformed biomarker concentrations, adjusting for age. Outliers excluded from the analysis are included for visualization as clear symbols.

collecting procedures we were able to combine data from two ongoing studies at the Turku PET Centre investigating individuals at risk of sporadic AD. Cross-sectional study design is limiting our analysis to one time-point, however, future follow-up of the population will be able extend these findings with longitudinal data.

5. Conclusion

Our cross-sectional observative head-to-head comparison study supports the view that both plasma p-tau181 and plasma p-tau231 are early markers of Aβ pathology, increasing already in cognitively unimpaired *APOE4* carriers. However, the differences between genotypes were fully explained by differences in brain Aβ load. On the contrary, plasma GFAP did not yet show significant differences between *APOE4* genotypes, but higher concentrations were associated with both lower cognitive performance. These associations were driven strongly by the *APOE4/4* homozygotes who had higher levels of Aβ in their cerebral cortex than *APOE3/4* or non-carriers of *APOE*. Our observations suggest that plasma p-tau and plasma GFAP are both early AD markers but reflect different Aβ-related processes.

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CRediT authorship contribution statement

Anniina Snellman: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Project administration, Funding acquisition. **Laura L. Ekblad:** Resources, Investigation, Formal analysis, Writing – review & editing. **Nicholas J. Ashton:** Conceptualization, Writing – review & editing. **Thomas K. Karikari:** Writing – review & editing, Supervision. **Juan Lantero-Rodriguez:** Investigation, Writing – review & editing. **Elina Pietilä:** Investigation, Writing – review & editing. **Mikko Koivumäki:** Investigation, Writing – review & editing. **Semi Helin:** Investigation, Writing – review & editing. **Mira Karrasch:** Conceptualization, Writing – review & editing. **Henrik Zetterberg:** Writing – review & editing, Resources, Supervision. **Kaj Blennow:** Writing – review & editing, Resources, Supervision. **Juha O. Rinne:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, BioArctic, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, Pharmatrophix, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. Other authors do not report any conflicts of interest.

Data availability

De-identified data will be made available to qualified researchers on reasonable request.

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Appendix A. Supplementary data

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