Diagnostic and prognostic plasma biomarkers for preclinical Alzheimer's disease

Pratishtha Chatterjee, Ph.D. [a,b], Steve Pedrini, M.Sc. [b], Nicholas J. Ashton, Ph.D. [c,d, e], Michelle Tegg, M.Sc. [b], Kathryn Goozee, MCN [a,b,f,g,h], Abhay K. Singh, Ph.D. [i], Thomas K. Karikari, Ph.D. [c], Joel Simrén, Ph.D. [c,o], Eugeen Vanmechelen, Ph.D. [t], Nicola J. Armstrong, Ph.D. [s], Eugene Hone, Ph.D [b], Prita R. Asih, Ph.D. [a,u], Kevin Taddei, B.Sc. [b,j], Vincent Doré, Ph.D. [k,l], Victor L. Villemagne, M.D. [l,m], Hamid R. Sohrabi, Ph.D. [a,b,f,j,n], Henrik Zetterberg, Ph.D. [c,o,p,q], Colin L. Masters, Ph.D. [r], Kaj Blennow, Ph.D. [c,o], Ralph N. Martins, Ph.D.[a,b,f,g,h,j,*]

a: Department of Biomedical Sciences, Macquarie University, North Ryde, NSW, Australia
b: School of Medical and Health Sciences, Edith Cowan University, Joondalup, WA, Australia
c: Department of Psychiatry and Neurochemistry, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden
d: Department of Old Age Psychiatry, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK
e: Wallenberg Centre for Molecular and Translational Medicine, University of Gothenburg, Gothenburg, Sweden
f: School of Psychiatry and Clinical Neurosciences, University of Western Australia, Crawley, WA, Australia
g: The Cooperative Research Centre for Mental Health, Carlton South, Australia
h: KaRa Institute of Neurological Disease, Sydney, Macquarie Park, Australia
i: Macquarie Business School, Macquarie University, North Ryde, NSW, Australia
j: Australian Alzheimer's Research Foundation, Nedlands, WA, Australia
k: eHealth, CSIRO Health and Biosecurity, Herston, Queensland, Australia
l: Department of Nuclear Medicine and Centre for PET, Austin Health, Heidelberg, Victoria, Australia
m: Department of Psychiatry, University of Pittsburgh, Pennsylvania, USA
n: Centre for Healthy Ageing, Health Future Institute, Murdoch University, Murdoch, WA, Australia
o: Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden
p: Department of Neurodegenerative Disease, UCL Queen Square Institute of Neurology, London, UK
q: UK Dementia Research Institute at UCL, London, UK
r: The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, VA, Australia
s: Department of Mathematics & Statistics, Curtin University, Bentley, WA, Australia
t: ADx NeuroSciences, Gent, Belgium
u: College of Medicine and Public Health, Flinders University, Adelaide, SA, Australia

Contact information of corresponding author: Professor Ralph N. Martins, School of Medical and Health Sciences, Edith Cowan University, Ralph & Patricia Sarich Neuroscience Research Institute, 8 Verdun Street, Nedlands, WA 6009, Australia, email: r.martins@ecu.edu.au. Telephone: (61 8) 6304 5456; Facsimile: (61 8) 6304 5851.

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Abstract

INTRODUCTION: This study involved a parallel comparison of the diagnostic and longitudinal monitoring potential of plasma glial fibrillary acidic protein (GFAP), total-tau (t-tau), phosphorylated-tau (p-tau181 and p-tau231) and neurofilament light (NFL) in preclinical Alzheimer's disease (AD).

METHODS: Plasma proteins were measured using Simoa assays in cognitively unimpaired older adults (CU), with either absence (Aβ-) or presence (Aβ+) of brain amyloidosis.

RESULTS: Plasma GFAP, t-tau, p-tau181 and p-tau231 concentrations were higher in Aβ+ CU compared with Aβ- CU cross-sectionally. GFAP had the highest effect size and AUC in differentiating between Aβ+ and Aβ- CU, however, no statistically significant differences were observed between AUCs of GFAP, p-tau181 and p-tau231, but all were significantly higher than the AUC of NFL, and the AUC of GFAP was higher than the AUC of t-tau. The combination of a base model (BM), comprising the AD risk factors, age, sex and *APOE* ε4 status with GFAP was observed to have a higher AUC (>90%) compared with the combination of BM with any of the other proteins investigated in the current study. Longitudinal analyses showed increased GFAP and p-tau181 in Aβ+ CU and increased NFL in Aβ- CU, over a 12-month duration. GFAP, p-tau181, p-tau231 and NFL showed significant correlations with cognition, while no significant correlations were observed with hippocampal volume.

DISCUSSION: These findings highlight the diagnostic and longitudinal monitoring potential of GFAP and p-tau for preclinical AD.

Keywords: Alzheimer's disease, preclinical Alzheimer's disease, blood biomarkers, diagnosis, longitudinal monitoring, glial fibrillary acidic protein, amyloid-beta, tau, p-tau181, p-tau231, neurofilament light, brain amyloid-β, single molecule array

1. INTRODUCTION

Alzheimer's disease (AD), a progressive neurodegenerative disease that causes cognitive deterioration and ultimately death, is the most common form of dementia and accounts for nearly 60-70% of its cases. In 2020, approximately 50 million people were living with dementia globally, and there are close to 10 million new cases every year [1]. Given that only symptomatic drugs are available, but yet no cure or disease-modifying treatment for AD, the identification of diagnostic and longitudinal monitoring biomarkers for at-risk populations is paramount to aid in assessing the efficacy of clinical trials.

The existence of a long preclinical phase, i.e., prior to the manifestation of clinical symptoms, during which the hallmark proteinopathies (amyloid plaques and neurofibrillary tangles) develop, has provided the opportunity for the investigation of biomarkers that can assist diagnosis and prognosis for such at-risk populations. For instance, positron emission tomography (PET) and cerebrospinal fluid (CSF) analysis are able to reveal abnormal levels of brain amyloid-β (Aβ) and hyperphosphorylated tau (p-tau), pathologies that begin to accumulate approximately 20 years prior to symptom onset [2, 3]. However, routine application of these markers in the clinical setting may be hampered by their limited availability, high costs and invasiveness, and therefore more accessible diagnostic approaches such as blood-based biomarkers are being investigated intensively.

Several recent studies have reported that plasma glial fibrillary acidic protein (GFAP), total- and phosphorylated-tau 181 and 231 (t-tau, p-tau181, p-tau 231) and neurofilament light (NFL) levels are higher in AD and have suggested that they could serve as potential blood-biomarkers for AD, given that they likely reflect AD-related neuropathological processes such as astrogliosis and the disruption of the axonal cytoskeletal structure [4-9]. Within the current

study, we conducted a parallel investigation of plasma GFAP, tau (including t-tau, p-tau181 and p-tau231) and NFL in preclinical AD, by comparing the circulating levels of these proteins between cognitively unimpaired older adults with absence of brain amyloidosis (Aβ-) and cognitively unimpaired older adults who were classified as being within the preclinical stage of AD, characterised by presence of brain amyloidosis (Aβ+). Plasma GFAP, tau (including t-tau, p-tau181 and p-tau231) and NFL levels were measured using an ultra-sensitive, single-molecule array (Simoa) platform, and analysed cross-sectionally at baseline and at a 12-month follow up timepoint, to determine if they could differentiate between these groups. We hypothesized that these plasma biomarkers would be higher in the Aβ+ group compared with the Aβ- group at both, baseline and the 12-month follow-up timepoint. Validating our cross-sectional observations 12 months apart, would provide insight into the reliability of these biomarkers in preclinical AD and assess if they have value to assist with the identification of Aβ+ cognitively unimpaired older adults for recruitment into clinical trials. We observed higher plasma GFAP, p-tau181 and p-tau231 concentrations in the Aβ+ group compared with the Aβ- group, and GFAP had the highest effect size. No statistically significant differences were observed between GFAP, p-tau181 and p-tau231 in distinguishing between Aβ+ and Aβ- groups, however, GFAP had the highest discriminative accuracy when added to a model comprising the AD risk factors, age, sex and *APOE* ε4 status compared with the other proteins added to a model comprising the AD risk factors.

Additionally, we assessed longitudinal changes in plasma GFAP, t-tau, p-tau181, p-tau231, and NFL in the Aβ- and Aβ+ groups over a 12-month period, given that understanding longitudinal changes in blood biomarkers over time would provide valuable insight into determining whether the use of these biomarkers as outcome measures may have value for improving the efficacy of designing and interpreting disease modifying clinical therapeutic

trials. We posited that these plasma measures will increase over a 12-month duration in the Aβ+ group. We observed increased GFAP and p-tau181 in the Aβ+ group and increased NFL in the Aβ- group, over this 12-month duration.

We also evaluated the correlations of the plasma markers with cognition and hippocampal volume and observed that GFAP, p-tau181, p-tau231 and NFL showed significant correlations with cognition, while no significant correlations were observed with hippocampal volume.

2. METHODS

2.1 Cohort

The Kerr Anglican Retirement Village Initiative in Ageing Health (KARVIAH) cohort volunteers (N=206) were required to meet a set of screening inclusion and exclusion criteria to be eligible for the cohort. Briefly, the inclusion criteria comprised an age range of 65-90 years, good general health, no known significant cerebrovascular disease, fluent in English, adequate/corrected vision and hearing to enable testing, no objective cognitive impairment as screened by a Montreal Cognitive Assessment (MoCA) score ≥26. MoCA scores lying between 18-25 were assessed on a case by case basis by the study neuropsychologist following stratification of scores according to age and education [10]. The exclusion criteria comprised, the diagnosis of dementia based on the revised criteria from the National Institute on Aging - Alzheimer's Association [11], presence of acute functional psychiatric disorder (including lifetime history of schizophrenia or bipolar disorder), history of stroke, severe or extremely severe depression (based on the Depression, Anxiety, Stress Scales; DASS) and uncontrolled hypertension (systolic BP > 170 mm Hg or diastolic BP > 100 mm Hg). One-hundred and thirty-four volunteers met the inclusion/exclusion criteria. These 134 participants underwent a 12-month placebo-curcumin intervention (UTN: U1111-1144-1011). One hundred and five out of these 134 participants underwent neuropsychometric evaluation, blood collection and

cerebral Aβ imaging. Within these 105 participants, 100 participants (comprising 50 placebo and 50 curcumin intervention) were considered to have normal global cognition based on their Mini-Mental State Examination score (MMSE ≥26) [12] at baseline and were included in the current study.

At baseline, plasma GFAP, t-tau, and NFL concentrations are reported in all 100 (n(Aβ-)=67, n(Aβ+)=33) participants included in the current study while, p-tau181 and p-tau231 are reported in 97 (n(Aβ-)=67, n(Aβ+)=30) and 96 (n(Aβ-)=67, n(Aβ+)=29) participants, respectively, due to sample availability. At the 12-month follow-up timepoint, plasma GFAP, t-tau, and NFL concentrations are reported in 95 (n(Aβ-)=64, n(Aβ+)=31) participants while, p-tau181 and p-tau231 are reported in 95 (n(Aβ-)=64, n(Aβ+)=31) and 93 (n(Aβ-)=63, n(Aβ+)=30) participants, respectively. Additionally, participants with a Memory Assessment Clinic - Questionnaire (MAC-Q) score ≥ 25 were considered as subjective memory complainers (SMC, n=76; a specific form of subjective cognitive decline, defined by self-reported memory complaints). At baseline, plasma GFAP, t-tau, and NFL concentrations are reported in all 76 (n(Aβ-)=52, n(Aβ+)=24) SMC participants included in the current study while, p-tau181 and p-tau231 are reported in 75 (n(Aβ-)=52, n(Aβ+)=23) and 73 (n(Aβ-)=52, n(Aβ+)=21) SMC participants, and at the 12-month follow-up timepoint, plasma GFAP, t-tau, and NFL concentrations are reported in 74 (n(Aβ-)=49, n(Aβ+)=25) SMC participants while, p-tau181 and p-tau231 are reported in 74 (n(Aβ-)=49, n(Aβ+)=25) and 72 (n(Aβ-)=48, n(Aβ+)=24) SMC participants, respectively. Details of the participants analysed within the current study have been reported in Supplementary Figure 1. All participants were based in Sydney, Australia. All volunteers provided written informed consent prior to participation, and the Bellberry and Macquarie University Human Research Ethics Committees provided approval for the study.

2.2 Neuroimaging

Neuroimaging was conducted within three months of blood collection at Macquarie Medical Imaging in Sydney. PET studies were conducted over as a 20 min static scan (4x5min frames) that was acquired fifty minutes after an intravenous bolus of ^{18}F-florbetaben (FBB). Neocortical Aβ load was calculated as the mean standard uptake value ratio (SUVR) of the frontal, superior parietal, lateral temporal, lateral occipital, and anterior and posterior cingulate regions using image processing software, CapAIBL (v2.0) [13, 14]. Participants with an FBB PET SUVR ≥1.35 were considered Aβ+, while those with an FBB PET SUVR<1.35 were considered Aβ-. Available Aβ-PET data for participants at baseline and at the 12-month follow-up timepoints have been illustrated in Supplementary Figure 1.

Additionally, participants passing all standard MRI inclusion/exclusion criteria underwent MRI as described previously using a General Electric (GE) 3 Tesla scanner (Model 750W) [15]. Hippocampal volume calculated from the images acquired was normalized with the total intracranial volume comprising the cerebrospinal fluid, grey matter and white matter. Data for 94 participants at baseline and 81 participants at the 12-month follow-up timepoint were available.

2.3 Blood collection, APOE genotyping, measurement of plasma GFAP, t-tau, p-tau181, p-tau231 and NFL

A minimum of 10 hours overnight fasted blood was collected from participants using standard processing methods [16]. Apolipoprotein E (*APOE)* genotype was determined from purified genomic DNA extracted from 0.5 mL whole blood as previously described [16].

EDTA plasma protein concentrations were measured employing the ultra-sensitive single-molecule array (Simoa) platform. GFAP, t-tau and NFL were measured using the Neurology 4-Plex A kit (QTX-102153, Quanterix, Billericam MA), which also includes UCH-L1, but this biomarker failed our quality control criteria because of high (> 20%) coefficients of variation (CVs). P-tau181 and p-tau231 were measured using the in-house assays developed at the University of Gothenburg, Sweden [5, 8]. Additionally, p-tau181 was also measured using the P-Tau 181 V2 Simoa Advantage Assay (QTX-103714, Quanterix, Billerica, MA) in nineteen samples, each at baseline and at the 12-month follow-up timepoint and Supplementary Figure 2 shows the correlation between the two assays. Calibrators and samples were run in duplicates for all assays. Quality control (QC) was achieved by assessing in duplicates the levels of two controls included in the Simoa kits at the beginning of each plate. The analytical lowest limit of quantification was 0.467 pg/ml for GFAP, 0.053 pg/ml for t-tau, 1 pg/ml for p-tau181, 1 pg/ml for p-tau231 and 0.241 pg/ml for NFL. The average %CV was 2.72% for the GFAP assay, 7.48% for the t-tau assay, 8% for the p-tau181 assay, 12% for the p-tau231 assay and 3.65% for the NFL assay, respectively.

2.4 Neuropsychological tests

Study participants underwent a comprehensive battery of neuropsychological testing at baseline and 12 months as described previously and composite scores were generated for verbal and visual episodic memory and working memory and executive function. Additionally, a global composite z-score was constructed using the verbal and visual episodic memory z-scores, working memory and executive function z-scores and MMSE z-scores as described previously [17].

2.5 Statistical analyses

Descriptive statistics including means and standard deviations were calculated for Aβ- and Aβ+ groups with comparisons employing Student's t-tests or Chi-square tests as appropriate. Linear models were employed to compare continuous variables between Aβ- and Aβ+ groups corrected for covariates age, sex and *APOE* ε4 carrier status both cross-sectionally and longitudinally (repeated measures). Dependent variables were natural log transformed to better approximate normality and variance homogeneity as required. Spearman's correlation coefficient (r_s) was employed to investigate correlations between continuous variables. Logistic regression with Aβ-/+ as response was used to evaluate predictive models and receiver operating characteristic (ROC) curves constructed from the logistic scores. The AUCs for different plasma proteins were compared using DeLong test. To determine the accuracy of each protein in distinguishing between Aβ- and Aβ+ groups, the R package cutpointr was used. All analyses and data visualization were carried out using IBM® SPSS® (v27), GraphPad Prism (v8) or R (v4.0.3).

3. RESULTS

3.1 Cohort characteristics

No significant differences were observed between Aβ- and Aβ+ group characteristics in age, sex, body mass index (BMI), subjective memory complaint (SMC) status, MMSE scores and hippocampal volumes, however, a significantly higher frequency of *APOE* ε4 allele carriers was observed in the Aβ+ group compared with the Aβ- group ($p<.0001$), as expected (Table 1).

3.2 Associations of AD related risk factors, age, sex and *APOE* ε4 allele status, with plasma GFAP, t-tau, p-tau181, p-tau231 and NFL measures

Plasma GFAP, p-tau181, p-tau231 and NFL measures were observed to have significant positive correlations with age, at both baseline and the 12-month timepoint ($p<.05$; Supplementary Table 1A). Plasma t-tau was observed to be significantly higher in females compared with males before and after adjusting for covariates, age and *APOE* ε4 allele status, at both baseline and the 12-month timepoint (Supplementary Table 1B). Plasma p-tau231was observed to be significantly higher in *APOE* ε4 allele carriers compared with non-carriers before and after adjusting for covariates, age and sex, at both baseline and the 12-month timepoint (Supplementary Table 1C). No associations were observed for age, sex or *APOE* ε4 allele status with protein measures not listed above.

3.3 Cross-sectional comparisons of plasma GFAP, t-tau, p-tau181, p-tau231 and NFL between Aβ- and Aβ+ groups

Plasma GFAP, p-tau181 and p-tau231 were significantly higher in the Aβ+ group compared with the Aβ- group, at both baseline and the 12-month timepoint, before and after adjusting for covariates age, sex and *APOE* ε4 allele carrier status ($p<.05$). Plasma t-tau was higher in the Aβ+ group compared with the Aβ- group with a trend towards significance at baseline and appeared significant at the 12-month timepoint, both before and after adjusting for covariates. Plasma NFL, however, was not significantly different between Aβ- and Aβ+ groups, at baseline and the 12-month timepoint, before and after adjusting for covariates (Table 2A, Supplementary Table 2A, Figure 1). Additionally, in these analyses, a large effect size was observed for GFAP, medium to large for p-tau181 and p-tau231, medium for t-tau and small for NFL [18]. Further, within the SMC subset, similar observations were found for plasma GFAP, t-tau, p-tau181, p-tau231 and NFL between the Aβ+ and Aβ- group, at both baseline and the 12-month timepoint (Table 2B, Supplementary Table 2B).

3.4 Evaluation of plasma GFAP, t-tau, p-tau181, p-tau231 and NFL as predictors of brain Aβ status

At baseline, the diagnostic accuracies between Aβ- and Aβ+ groups are illustrated using ROC curves in Figure 2. Plasma GFAP was observed to have the highest AUC (79%, CI: 69%-89%) in differentiating between Aβ- and Aβ+ groups when compared with all other proteins considered independently. However, while there was a significant difference in the AUC for GFAP versus t-tau ($p<.05$) and GFAP versus NFL ($p<.005$), there was no statistically significant difference in the AUC for GFAP versus p-tau181 ($p>.05$) or p-tau231 ($p>.05$). Additionally, the AUCs of p-tau181 and p-tau231 were also observed to be significantly higher than the AUC of NFL ($p<.05$) (Supplementary Table 3A). Similar observations were also noted at the 12-month timepoint (Supplementary Table 3A).

Additionally, at baseline, we generated a base model (BM) incorporating the AD risk factors age, sex and *APOE* ε4 allele status, and observed that this base model was significantly outperformed when the plasma protein GFAP ($p=.001$) was added to it, while a trend towards significance was observed when p-tau181 ($p=.054$) or p-tau231 ($p=.077$) were added to the BM. However, the addition of t-tau or NFL to the BM did not have a significant additional contribution to the BM AUC in distinguishing Aβ+ from Aβ- (Figure 2, Supplementary Table 3B). Similar observations were noted at the 12-month timepoint (Figure 2, Supplementary Table 3B).

Further at baseline, the AUC for BM+GFAP was observed to be significantly higher than the AUCs observed for BM+t-tau ($p=.002$), BM+p-tau181 ($p=.002$), BM+p-tau231 ($p=.014$) and BM+NFL ($p=.001$). At the 12-month timepoint, the AUC for BM+GFAP was observed to be significantly higher than the AUCs observed for BM+t-tau ($p=.049$) and BM+NFL ($p=.01$),

however, became non-significant when compared with BM+p-tau181 (p=.10) and BM+p-tau231 (p=.15) (Supplementary Table 3B).

On comparing AUCs from the ROC comprising the combination of the three plasma proteins demonstrating the highest AUCs individually, namely GFAP, p-tau181 and p-tau231, between the Aβ- and Aβ+ groups at baseline and at the 12-month timepoint gave an AUC= 85% (CI: 76%-93%) (Figure 2) and 83% (CI: 74%-91%) (Figure 2), respectively. At baseline, the AUC of the combination of these three proteins was significantly higher than the AUCs of GFAP, t-tau, p-tau181 and NFL individually, and a trend towards significance was observed for p-tau231. At the 12-month timepoint, the AUC of the combination of these three proteins was only observed to be significantly higher than the AUCs of t-tau, p-tau231 and NFL.

Additionally, when these three proteins were combined with the BM, the AUC was further improved to 94% (CI: 89%-98%) (Figure 2) and 91% (CI: 84%-98%) (Figure 2), at baseline and at the 12-month timepoint, respectively (Supplementary Table 3B). At baseline and the 12-month timepoint, the AUC of the combination of these three proteins and the BM was significantly higher than the AUCs of t-tau+BM, p-tau181+BM, p-tau231+BM and NFL+BM individually, however, no significant difference was observed with GFAP+BM.

Further, at 80% sensitivity, p-tau181 alone was observed to have the highest diagnostic accuracy to detect preclinical AD (BL: accuracy=68%, specificity=63%, NPV=88%, PPV=47%; 12m: accuracy=69%, specificity=63%, NPV=87%, PPV=50%) when compared with the other proteins independently, while the accuracy for GFAP+BM was observed to be the highest (BL: accuracy=86%, specificity=88%, NPV=91%, PPV=74%; 12m: accuracy=85%, specificity=87%, NPV=90%, PPV=74%) compared with all proteins

considered individually or in combinations, as shown in Supplementary Table 4A. Sensitivity, specificity, accuracy, Youden's cut point, NPV and PPV at Youden's index are provided in Supplementary Table 4B.

3.5 Longitudinal changes in plasma GFAP, t-tau, p-tau181, p-tau231 and NFL over a 12-month duration in Aβ- and Aβ+ groups

There was a significant interaction effect of time*Aβ status on GFAP levels in all participants; GFAP increased more over 12-months in the Aβ+ group versus the Aβ- group before adjusting for covariates. However, the interaction term became non-significant after adjusting for covariates age, sex and *APOE* status. No interaction effects between time and Aβ status were observed for t-tau, p-tau181, p-tau231 and NFL changes over 12-months between the Aβ- group and Aβ+ group before and after adjusting for covariates (Table 3A, Supplementary Table 5A).

The main effect of time was significant for plasma GFAP and p-tau181 in all participants and approached significance for plasma NFL, where these protein measures were observed to increase over a 12-month duration, before and after adjusting for covariates. No significant main effect of time was observed for t-tau and p-tau231.

Further investigation of pairwise comparisons for plasma GFAP, p-tau181 and NFL within each Aβ status group in all participants, showed the significant effect of time on GFAP was restricted to the Aβ+ group only, before and after adjusting for covariates. In addition, a significant effect of time on p-tau181 was similarly observed only in the Aβ+ group, but only reached statistical significance after adjusting for covariates. Interestingly, a significant effect of time on NFL was observed in the Aβ- group only, before and after adjusting for covariates (Table 3A, Supplementary Table 5A, Figure 3). However, the absolute differences in NFL levels cross-sectionally and in change over time were similar in Aβ- and Aβ+ in the overall

cohort, with considerable overlap between groups, hence NFL may have limited value as a preclinical AD biomarker. The estimates of effect size are presented in Supplementary Table 6, wherein small to medium effect sizes were observed for GFAP and p-tau181 in the Aβ+ and for NFL in the Aβ- groups [18].

The longitudinal analyses also confirmed our cross-sectional observations with a significant main effect of Aβ-/+ status on plasma GFAP, t-tau, p-tau181 and p-tau231 levels, wherein these proteins were observed to be higher in the Aβ+ group at both timepoints (Table 3A, Supplementary Table 5A). GFAP and p-tau isoforms were significantly different between the Aβ+ and Aβ- group, supporting a potential role for these plasma proteins as diagnostic and longitudinal monitoring biomarkers in preclinical AD. Similar observations were also noted within the SMC subset (Table 3B, Supplementary Table 5B, Supplementary Figure 3).

3.6 Association of plasma GFAP, t-tau, p-tau181, p-tau231 and NFL with cognition and hippocampal volume

At baseline, plasma GFAP was observed to be inversely correlated with the working memory and executive function composite score (r_s= -.257, p=.010) and the global composite score (r_s= -.200, p=.047). P-tau181 inversely correlated with the global composite score (r_s= -.209, p=.040) which was also seen for p-tau231 (r_s= -.278, p=.006), and p-tau231 additionally correlated inversely with the verbal, visual and episodic memory composite score (r_s= -.254, p=.013). As expected, plasma NFL inversely correlated with the verbal, visual and episodic memory composite score (r_s= -.335, p=.001), the working memory and executive function composite score (r_s= -.347, p<.0001) and the global composite score (r_s= -.438, p<.0001) [17].

At the 12-month follow-up timepoint, plasma GFAP remained to be inversely correlated with the working memory and executive function composite score (r_s= -.234, p=.021) and the global

composite score (r_s= -.273, p=.007), while a trend towards a significant inverse correlation was observed between p-tau181 and the global composite score (r_s= -.174, p=.086). As expected, plasma NFL continued to inversely correlate with the verbal, visual and episodic memory composite score (r_s= -.342, p=.001), working memory and executive function composite score (r_s= -.371, p<.001) and the global composite score (r_s= -.456, p<.001).

No significant correlation was observed between the plasma proteins and hippocampal volume at baseline, except for a trend towards significance for p-tau231 (r_s= -.213, p=.078), however this trend was not observed at the 12-month timepoint.

4. Discussion

In the current study, for the first time to the best of our knowledge, we evaluated plasma GFAP, t-tau, p-tau181, p-tau231 and NFL in parallel between a cognitively unimpaired Aβ+ older adult group (preclinical AD) and a cognitively unimpaired Aβ- older adult group. We found higher plasma GFAP, p-tau181 and p-tau231 in the cognitively unimpaired Aβ+ group. We further validated our cross-sectional findings observed at baseline, in a 12-month follow-up timepoint to re-examine the differences in plasma protein levels, and continued to find higher plasma GFAP, p-tau181 and p-tau231 in the preclinical AD group, suggesting that the plasma protein differences observed between these two groups are consistent and potentially reliable candidate markers for diagnosis of preclinical AD. Our results held in the subjective memory complainer subset, further supporting the potential utility of GFAP and p-tau isoforms as preclinical AD biomarkers.

We found no significant difference between the AUCs for GFAP, p-tau181 and p-tau231 in differentiating between cognitively unimpaired Aβ+ older adults and cognitively unimpaired Aβ- older adults, although GFAP showed the highest AUC among these three proteins.

However, when GFAP was added to the AD risk factors, age, sex and *APOE* ε4 status, the AUC was significantly higher compared with the AUCs of p-tau181 or p-tau231 added to the AD risk factors, at baseline. Interestingly, although the AUC of the combination of the AD risk factors with the three highest performing proteins (i.e., BM+GFAP+p-tau181+p-tau231) was significantly higher than the AUC of t-tau, p-tau181, p-tau231 or NFL combined with the AD risk factors (i.e., t-tau+BM/p-tau181+BM/p-tau231+BM/NFL+BM), no significant difference was observed with GFAP+BM at baseline and the 12-month timepoint. Corroborating this observation, GFAP+BM was also observed to have the highest accuracy. While further studies are required to validate these observations, it may be suggested that plasma GFAP levels may reflect pathological mechanisms additional to those associated with the well-known risk factors for AD within the preclinical stage.

Most interestingly, we also show for the first time that GFAP and p-tau181 increased with time in cognitively unimpaired Aβ+ older adults and NFL increased with time in cognitively unimpaired Aβ- older adults, over a 12-month duration. Similar observations were also noted in the subjective memory complainer subset. Together, these observations suggest that GFAP and p-tau181 may have potential in serving as longitudinal monitoring markers and outcome measures for relatively shorter clinical trials conducted in preclinical AD populations, while the longitudinal increase in NFL observed with time in the Aβ- group could possibly indicate that NFL reflects other ongoing neurodegenerative processes that are not Aβ associated.

Among the five proteins investigated in the current study, GFAP, p-tau181 and p-tau231 showed the highest estimates of effect size for the cross-sectional analyses between the Aβ- and Aβ+ groups in all participants (GFAP>p-tau231>p-tau181). Given that these estimates of effect sizes mostly met the ‘large’ cut-off, may be indicative of their clinical utility value.

Longitudinally, GFAP was observed to have the highest estimates of effect size, followed by p-tau181 in the Aβ+ group while NFL was observed to have the highest estimates of effect size in the Aβ- group. However, these effect sizes mostly fell within the small to moderate range but may still have utility in assessing the efficacy of clinical trials.

In line with our observations of higher GFAP levels observed in the Aβ+ group in this study, GFAP, a marker of astrogliosis [19], has been reported to be higher in preclinical AD and is associated with brain amyloidosis [4, 7, 20-22]. Higher GFAP levels have also been reported in AD patients compared with controls [4, 23]. Increased GFAP has also been observed around Aβ plaques in the brains of individuals with mild cognitive impairment (MCI) due to AD [24] and its expression has been observed to correlate with Aβ plaque density in AD [25]. Higher plasma GFAP levels observed in the Aβ+ group within the current study could thus be due to GFAP upregulation associated with astrogliosis in Aβ+ individuals. Astrogliosis has been reported to occur within the early stages of AD pathogenesis and cultured astrocytes exposed to amyloid isolated from human AD brains have been observed to trigger astrogliosis [26, 27]. Additionally, studies employing ^{11}C-deuterium-L-deprenyl PET, further support that reactive astrocytosis is a prodromal feature in the early stages of AD development [24]. Further, similar to our longitudinal findings, Cicognola and colleagues have shown that plasma GFAP increases at a faster rate in Aβ+ MCI compared with Aβ- MCI [21]. Additionally, Oeckl and colleagues report that GFAP distinguished between AD and behaviour variant frontal temporal dementia with 89% sensitivity and 79% specificity [23]. However, further studies comparing plasma GFAP levels in AD versus other neuropathologically defined non-AD neurodegenerative diseases are required to confirm the specificity of plasma GFAP alterations for AD, given the existence of mixed pathologies.

Further, higher plasma p-tau181 levels have been reported in individuals with MCI and AD compared with cognitively unimpaired older adult groups and individuals with other neurodegenerative diseases [5, 28-31]. These studies have also showed that p-tau181 levels are higher in cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ- older adults and our findings from the current study are in line with these observations. It has been suggested that the early dysregulation in neuronal tau metabolism, is likely to be associated with early Aβ pathology, attributing to the release of soluble p-tau181 in blood [28, 30]. Similarly, p-tau231, has relatively recently been reported to be elevated in the blood in individuals with MCI and AD compared with cognitively unimpaired older adult groups and individuals with other neurodegenerative diseases [8]. This study [8] has also reported that p-tau231 levels are higher in cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ- older adults and our findings at baseline and the 12-month timepoint are in line with these observations. Interestingly, p-tau231 has been reported to identify the clinical stages of AD and neuropathology as strongly as p-tau181, however, increases relatively earlier when compared with p-tau181, with subtle Aβ deposition [8]. It is important to note that plasma p-tau217, like p-tau181 and p-tau231, has also been reported to be higher in cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ- older adults [32], but has been observed to have a higher discriminative accuracy between AD and non-AD neurodegenerative diseases compared with plasma p-tau181 [33]. Interestingly, plasma p-tau217 has been reported to correlate with brain amyloidosis in early disease stages [34]. Further, plasma p-tau217 has been observed to increase in *PSEN1* E280A autosomal dominant AD (ADAD) mutation carriers approximately 20 years prior to symptom onset [33].

Plasma t-tau is known as a marker of neuronal injury and shows a very marked increase in disorders with acute neuronal injury, such as cardiac arrest [35]. In contrast, while CSF t-tau

shows a marked increase in AD [36], plasma t-tau levels only shows a discrete change in AD, and there is no correlation between plasma and CSF t-tau levels in AD-control cohorts [37]. The reason for this discrepancy is not known, but a possible explanation may be that, in contrast to p-tau, non-phosphorylated tau is also produced in peripheral nerves or tissue [38], and it is estimated that only around 20% of plasma t-tau comes from the CNS [39], and thus peripherally produced tau will blur possible differences in brain-derived tau in plasma in AD. Although hypothetical, this could explain why the difference in t-tau levels were non-significant between cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ- older adults at baseline, however became significant after 12-months, which may be attributed to a possible increase in preclinical AD pathogenesis severity in 12-months.

Plasma NFL, reflecting neuronal injury, was not significantly higher in cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ- older adults at baseline or at the 12-month timepoint, suggesting that NFL may not have value as a preclinical AD marker for identifying cognitively unimpaired older adults at risk for AD. These observations are in line with those reported by Mattsson and colleagues, wherein no significant differences in plasma NFL were observed between Aβ- and Aβ+ controls [40]. In contrast, in a *PSEN1* E280A ADAD Colombian kindred, higher plasma NFL levels and a higher annual rate of plasma NFL change have been observed 22 years prior to the estimated age at symptom onset in the mutation carriers compared with non-carriers [41]. Similarly, a higher annual rate of change of serum NFL has been observed 6.8 years prior to the estimated age at symptom onset in mutation carriers compared with non-carriers from the Dominantly Inherited Alzheimer Network cohort [42]. Elevated blood NFL levels (or a higher annual rate of change of blood NFL levels), reflecting neurodegeneration, observed so early in the ADAD pathogenesis trajectory, prior to

symptom onset, could be attributed to the aggressive nature of ADAD mutations when compared with sporadic AD.

In the current study, we also noted inverse correlations of plasma GFAP, p-tau181, p-tau231 and NFL with cognitive performance, however the strength of these associations was at best small to moderate in this cognitively unimpaired cohort. No significant associations were observed for GFAP, p-tau181, p-tau231 and NFL with hippocampal volume. These observations could be attributed to the very early stage within the AD pathogenesis trajectory, the study Aβ+ participants in this study may lie in. However, it is interesting to note that such prominent changes appear in the cognitively unimpaired Aβ+ blood prior to any apparent hippocampal atrophy.

Findings from the current study highlight potential blood biomarkers for the diagnosis and longitudinal monitoring of cognitively unimpaired individuals within the preclinical AD stage. Further studies in larger research cohorts for example, the Australian Imaging, Biomarker and Lifestyle (AIBL) Study of Aging cohort, are required to validate the current findings. Additionally, future studies also need to establish clinical cut-off points for implementation in clinical settings, employing standardised blood collection, processing and storage protocols. The establishment of clinical cut-off scores will also be assay-dependent, for example, the difference in absolute levels observed between the two p-tau181 assays (i.e., the in-house assay developed at the University of Gothenburg versus the Quanterix assay) are visible in Supplementary Figure 2, even though a near perfect correlation was observed between the two assays. Additionally, a majority of the studies conducted on the afore-discussed proteins are primarily in Caucasian cohorts and therefore further data from multiple races and ethnic

backgrounds need to be investigated. Studies will also need to validate established cut-off points in individuals with other co-morbidities in the future.

It is acknowledged that the current study had limitations with regard to its modest sample size. However, the cross-sectional comparisons investigated were consistent, 12-months apart, which is a strength of the study. While plasma biomarker data available for the maximum number of participants was used in the current study, cross-sectional differences for a direct comparison using the same participants for all biomarkers showing similar observations are presented in Supplementary Table 7. Another limitation to be considered within the current study is that the longitudinal change in plasma GFAP, t-tau, p-tau181, p-tau231 and NFL was investigated over a 12-month duration, which may not have been long enough to observe changes in the other proteins i.e., t-tau and p-tau231. However, considering budget constraints for small scale clinical trials, outcome measures that show changes within a 12-month duration may also be considered suitable. Further, this cohort underwent a 12-month duration placebo-curcumin intervention, although all statistical analyses were conducted with and without adjusting for this intervention. Further, longitudinal comparison of plasma protein measures between Aβ- and Aβ+ participants within the placebo group only, also had similar observations (Supplementary Table 8). Another limitation within the current study is that Aβ42/Aβ40 ratios and p-tau217 were not included. However, in a previous study we noted that Aβ42/Aβ40 ratios had an AUC<70% in differentiating between cognitively unimpaired Aβ- older adults and cognitively unimpaired Aβ+ older adults, using the same platform in the same cohort [20] and therefore, more sensitive assays such as immunoprecipitation followed by mass-spectrometry approaches for e.g., that employed by Nakamura and colleagues may be required [43]. As we did not have access to the p-tau217 assay, this was not included in the current study.

To conclude, in the current study we observed higher plasma GFAP, t-tau, p-tau181 and p-tau231 in preclinical AD. Further, plasma GFAP and p-tau181 increased with time in preclinical AD. These observations strongly highlight the diagnostic and longitudinal monitoring potential of plasma GFAP and p-tau isoforms in preclinical AD.

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