Published in final edited form as:

J Alzheimers Dis. 2020 ; 78(1): 265–276. doi:10.3233/JAD-200755.

Plasma Glial Fibrillary Acidic Protein Levels Differ Along the Spectra of Amyloid Burden and Clinical Disease Stage

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Abstract

Background: Measuring plasma glial fibrillary acidic protein (GFAP) alongside cortical amyloid- β (A β) may shed light on astrocytic changes in aging and Alzheimer's disease (AD).

Objective: To examine associations between plasma GFAP and cortical A β deposition in older adults across the typical aging-to-AD dementia spectrum.

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Authors' disclosures available online (<https://www.j-alz.com/manuscript-disclosures/20-0755r1>).

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <https://dx.doi.org/10.3233/JAD-200755>.

Methods: We studied two independent samples from UCSF (Cohort 1, N = 50; Cohort 2, N = 37) covering the spectra of clinical severity (CDR Sum of Boxes; CDR-SB) and A β -PET burden. A β -PET was completed with either florbetapir or Pittsburgh Compound B and standardized uptake value ratios were converted to the Centiloid (CL) scale for analyses. All participants with CDR-SB > 0 were A β -PET positive, while clinically normal participants (CDR-SB = 0) were a mix of A β -PET positive and negative. Regression analyses evaluated main effect and interaction associations between plasma GFAP, A β -PET, and clinical severity.

Results: In both cohorts, plasma GFAP *increased* linearly with A β -PET CLs in clinically normal older adults. In Cohort 2, which included participants with more severe clinical dysfunction and A β -PET burden, the association between A β and GFAP became curvilinear (inverted U-shape; quadratic model R² change = 0.165, p = 0.009), and A β -PET interacted with CDR-SB (R² change = 0.164, p = 0.007): older adults with intermediate functional impairment (CDR-SB = 0.5–4.0) showed a weak (negative) association between A β -PET CLs and plasma GFAP, while older adults with dementia (CDR-SB > 4.0) showed a strong, *negative* association of higher A β -PET CLs with *lower* plasma GFAP.

Conclusion: The relationship between astrocytic integrity and cortical A β may be highly dynamic, with linear, positive associations early in disease that diverge in more severe disease stages.

Keywords

Alzheimer's disease; amyloid; astrocyte; biomarker; glial fibrillary acidic protein; plasma

INTRODUCTION

The pathophysiology of Alzheimer's disease (AD) development and progression is a highly dynamic process with multiple mechanisms likely contributing at different stages of the disease. Expanding the scope beyond neuron-centric conceptualizations of AD, astrocytes are tightly linked to amyloid- β (A β) degradation and clearance but may serve different functions as the disease progresses [1, 2]. Proposed roles for astrocytes in AD include A β degradation and clearance [3], indirect neurotoxicity [2], and even A β production [4]. Astrocytic changes along the spectra of A β deposition and clinical disease stage remain poorly understood despite significant interest in disentangling both neuronal and glial contributions to AD pathophysiology [1].

Advances in molecular biomarker measurement create opportunities for clarifying associations between dynamic neuronal and glial physiologies. Neurofilament light chain (NfL), an increasingly studied biomarker of neuronal injury [5] that correlates with neurodegeneration and brain hypometabolism [6, 7], has shown diagnostic and prognostic utility in older adults at-risk for AD dementia and a possible synergistic relationship with cortical A β burden [7–10]. Complementary biomarkers reflecting astrocytic pathophysiology have thus far been less frequently studied. Reactive astrocytes overexpress glial fibrillary acidic protein (GFAP), a core intermediate filament protein of the astrocytic cytoskeleton [11]. GFAP expression is consistently elevated in AD brains and colocalizes with A β plaques in autopsy studies [12]. GFAP levels, like NfL, can be

measured in blood and correlate well with cerebrospinal fluid (CSF) levels in healthy controls [13], but fluid-based GFAP findings are inconsistent across AD studies. A meta-analysis identified two studies showing no clear difference in CSF GFAP between AD patients and controls [14], but recent work demonstrated significantly elevated plasma GFAP in both early-onset and late-onset AD dementia groups [15]. More granular consideration of both degree of amyloidosis and clinical disease stage might be essential given the likely fluctuating astrocytic response at different points in the disease process [16, 17].

Quantifying GFAP in blood as a proxy for astrocytic changes is a convenient and noninvasive way of capturing the relationship between astrocytic integrity and A β deposition. Coupling GFAP and A β biomarkers may help to shed light on astroglial contributions to AD pathophysiology. This study examined associations between plasma GFAP and cortical A β deposition in two independent cohorts of older adults across the typical aging-to-AD dementia spectrum. We uniquely leveraged complementary plasma and PET modalities to capture markers of astrocytic changes along the spectrum of cortical amyloid burden. We evaluated this relationship in older adults with A β -PET deposition ranging from undetectable to severe, and clinical disease status ranging from normal to dementia. We further assessed how a marker of neurodegeneration (plasma neurofilament light) and sex might influence observed A β -GFAP associations.

METHODS

Study participants

We cross-sectionally sampled participants at the UCSF Memory and Aging Center from larger ongoing studies of typical aging and AD. Participants received diagnoses via multidisciplinary consensus conference as clinically normal, mild cognitive impairment (MCI), or dementia based on neurologic exam, cognitive testing, and Clinical Dementia Rating (CDR). All participants completing an A β -PET scan did so within one year of blood draw and clinical diagnosis. The sample was separated into two cohorts (Table 1) based on plasma analytic batches. Plasma from both cohorts were analyzed about one year apart using different analytic kits on the same analytic platform. Due to well-described batch and lot effects [18], absolute concentrations are not comparable. As independent samples, we aimed to increase the rigor of our clinical study by testing the reliability of the evaluated relationships. Models demonstrating similar effect size and directionality across both cohorts may therefore be interpreted as more generalizable and robust.

Clinical disease staging

We leveraged the CDR Sum of Boxes (CDR-SB) score as a continuous index of clinical disease stage (range from 0 to 18). The CDR-SB is derived from a semi-structured interview with study partners that captures multiple aspects of cognitive and functional abilities and is most applicable for suspected AD populations. CDR-SB was grouped as CDR-SB = 0, CDR-SB = 0.5–4.0, and CDR-SB > 4.0 to generally correspond with “normal,” “MCI,” and “dementia” subgroups for *post hoc* comparisons and figure plots [19].

Cohort 1

Cohort 1 (N = 50) was identified to capture older adults early in the spectrum of A β -PET positivity and clinical functioning. Thirty-nine Cohort 1 participants were diagnosed via consensus conference as clinically normal (age 72.7 ± 6.3 years; N = 13 with positive A β -PET scan), and 11 were diagnosed with MCI (age 70.7 ± 8.6 years; all with a positive A β -PET scan). Cohort 1 largely represented a later-life amyloidosis sample with mild to no cognitive changes and preserved independent functioning.

Cohort 2

Cohort 2 (N = 75) was a non-overlapping sample of older adults diagnosed as clinically normal, MCI, or dementia via consensus conference, 37 of whom completed an A β -PET scan and were included in primary analytic models: clinically normal (N = 14, age 75.2 ± 5.2 years), MCI (N = 11, age 66.8 ± 9.9 years), and dementia (N = 12, age 65.9 ± 10.8 years). In this N = 37 subset, MCI and dementia participants met NIA-AA diagnostic criteria for “MCI due to AD” [20] or “Probable AD” [21], had a positive A β -PET scan, and included both early-onset (N = 16) and late-onset (N = 7) AD spectrum based on reported onset of symptoms before or after age 65, respectively. Cohort 2 represented a wider range of cognitive and functional abilities along the typical aging-to-Alzheimer’s dementia spectrum than Cohort 1 (Fig. 1). *APOE* genotype was unknown for 1 case. The remaining 38 participants in Cohort 2 who had available GFAP values but without A β -PET were included only for visualization of GFAP by clinical disease severity (CDR-SB) figures (see Results).

Amyloid PET imaging

Cortical A β deposition was defined by PET completed with either ^{11}C -Pittsburgh compound B (PiB; N = 22 [60%] of Cohort 2 participants) or ^{18}F -florbetapir (N = 50 [100%] of Cohort 1, N = 15 [40%] of Cohort 2). Standardized uptake value ratios (SUVR) were calculated and then converted to the Centiloids (CLs) scale to harmonize data across the two tracers [22]. The CLs conversion is used to calibrate A β -PET measurements acquired with different tracers and analytic pipelines to a common scale. A value of 100 CLs corresponds with the average degree of A β deposition observed on PET imaging in patients diagnosed with mild-moderate AD dementia, while a value of 0 CLs corresponds to mean uptake in healthy young controls devoid of A β pathology [22]. We used continuous CL values for regression analyses, but report A β -PET positivity frequencies based on processing pipeline- and tracer-specific thresholds: PiB SUVR > 1.21 (20.9 CLs); florbetapir SUVR > 1.11 (22.5 CLs). See Supplementary Methods for more details.

Plasma GFAP and NfL quantification

We assessed plasma GFAP as a proxy for astrocyte integrity. We additionally quantified plasma NfL, which was used as a control biomarker intended to be an index of severity of neurodegeneration [7] and to evaluate specificity of significant plasma GFAP findings. Venous blood was collected and stored at -80°C until analysis (1 thawing only). For Cohort 1, GFAP and NfL were measured via multiplex single molecule arrays on an HD-1 analyzer (Simoa, Quanterix Neurology 4-Plex A). For Cohort 2, GFAP and NfL were

measured using single analyte Simoa assays. All analyses were performed in duplicate according to manufacturer's published protocols. We only included sample concentrations with coefficients of variance (CV) <20% (Cohort 1, N = 0; Cohort 2, N = 7 GFAP and N = 5 NfL excluded from larger batch prior to dataset aggregation for the current study). Mean $\pm$ SD CV% for included samples was $4.1\% \pm 3.3\%$ (GFAP) and $5.1\% \pm 4.3\%$ (NfL) for Cohort 1 and $4.6\% \pm 4.2\%$ (GFAP) and $6.4\% \pm 6.3\%$ (NfL) for Cohort 2. Laboratory technicians were blinded to clinical diagnoses. See Supplementary Methods for more details.

Statistical analyses

Analyses were identical for Cohort 1 and Cohort 2 and were completed using IBM SPSS Statistics v.25 (IBM; Armonk, NY). Plasma GFAP and NfL were log transformed to better approximate a normal distribution. Age at blood draw and sex were covariates in all initial analyses. *APOE* genotype ($\epsilon 4$ carrier or non-carrier), and plasma NfL were then added as a covariates to assess stability of significant findings while controlling for genetic A β burden risk and neurodegeneration, respectively. We first evaluated the association between cortical A β burden (A β -PET CLs) and plasma GFAP concentrations using linear regression. Based on resulting scatterplots, we assessed nonlinear associations and statistically compared model fit to the linear trends (R^2 change). Relevant analyses were repeated with plasma NfL as the dependent variable to assess specificity of findings to plasma GFAP.

Based on evidence of a nonlinear association between plasma GFAP and A β -PET CLs, we then evaluated clinical factors that may be contributing to this effect. To do so, we tested the interactions between amyloid and clinical severity (A β -PET CLs $\times$ CDR-SB) and between amyloid and sex (A β -PET CLs $\times$ Sex) on plasma GFAP. All models with interaction terms also covaried for age, sex, *APOE* genotype, and plasma NfL. Statistical significance was defined a priori as $p < 0.05$.

RESULTS

Cohort demographics

Participant characteristics stratified by study cohort are summarized in Table 1. There were no statistically significant differences between cohorts in age, sex, education, race, or frequency of *APOE* $\epsilon 4$ carriers. As expected based on inclusion of more MCI and dementia participants, Cohort 2 had higher average A β -PET CLs (72.2 versus 36.6 CLs, $p < 0.001$), was more likely to be A β -PET positive (73% versus 50%, $p = 0.03$), had worse global cognition (Mini-Mental State Examination; median score 27 versus 29, $p < 0.001$), and had higher CDR-SB scores (Fig. 1; $p < 0.001$). Plasma GFAP was not significantly associated with plasma NfL after controlling for age and sex in Cohort 1 ($\beta = 0.175$, $p = 0.19$) but showed a significant positive association in Cohort 2 ($\beta = 0.584$, $p < 0.001$). There was no significant difference in plasma GFAP concentrations between *APOE* $\epsilon 4$ carriers and non-carriers in either Cohort 1 ($t = 0.978$, $p = 0.33$) or Cohort 2 ($t = -1.078$, $p = 0.29$).

A β -PET association with plasma GFAP

In Cohort 1, higher A β -PET CLs was associated with higher plasma GFAP ($\beta = 0.324$, $p = 0.01$), with a slightly stronger effect seen when controlling for *APOE* $\epsilon 4$ carrier status (β

= 0.417, $p = 0.005$). Including the quadratic A β -PET CLs term did not improve model fit (quadratic model R^2 change = 0.002, $p = 0.75$; Fig. 2A). In Cohort 2, there was a statistically significant, negative curvilinear association between A β -PET CLs and plasma GFAP (Fig. 2B). Including the quadratic A β -PET CLs term improved model fit (quadratic model R^2 change = 0.165, $p = 0.009$) with both the linear ($p = 0.002$) and quadratic ($p = 0.009$) A β -PET CLs effects remaining statistically significant. The Cohort 2 scatterplot showed that plasma GFAP increased until around 75 CLs before plateauing and then decreasing beyond 100 CLs. Associations remained statistically significant when additionally controlling for *APOE* $\epsilon 4$ carrier status and plasma NfL concentration. For completeness, we also evaluated the A β -PET CLs $\times$ plasma NfL and A β -PET CLs $\times$ *APOE* status interactions on plasma GFAP. There was no significant A β -PET CLs $\times$ plasma NfL (Cohort 1: R^2 change = 0.042, $p = 0.10$; Cohort 2: R^2 change = 0.005, $p = 0.57$) or A β -PET CLs $\times$ *APOE* (Cohort 1: R^2 change = 0.006, $p = 0.54$; Cohort 2: R^2 change = 0.001, $p = 0.88$) association with plasma GFAP.

A β -PET association with plasma NfL

In contrast, we did not observe a significant linear or nonlinear association between A β -PET CLs and plasma NfL in either Cohort 1 (linear $\beta = 0.035$, $p = 0.78$, quadratic R^2 change = 0.008, $p = 0.48$; Fig. 2C) or Cohort 2 (linear $\beta = 0.228$, $p = 0.22$, quadratic R^2 change = 0.060, $p = 0.14$; Fig. 2D).

Interactions of A β -PET and CDR-SB

Given the observed nonlinear association between A β -PET CLs and plasma GFAP in Cohort 2, we investigated whether this was related to clinical disease stage by testing the A β -PET CLs $\times$ CDR-SB interaction effect on plasma GFAP. In Cohort 2 there was a statistically significant A β -PET CLs $\times$ CDR-SB interaction on plasma GFAP (R^2 change = 0.164, $p = 0.007$; Fig. 3B), which remained after controlling for *APOE* $\epsilon 4$ carrier status (R^2 change = 0.199, $p = 0.004$). Cohort 2 participants with CDR-SB=0 showed a strong, positive association of higher A β -PET CLs with higher plasma GFAP (linear model $R^2 = 0.426$), while Cohort 2 participants with CDR-SB > 4.0 showed a strong, *negative* association of higher A β -PET CL with *lower* plasma GFAP (linear model $R^2 = 0.294$). Cohort 2 participants with intermediate CDR-SB = 0.5–4.0 showed a weak (negative) association between A β -PET CL and plasma GFAP (linear model $R^2 = 0.043$). The interaction did not reach statistical significance in Cohort 1 (R^2 change = 0.023, $p = 0.23$) but showed similar directionality (Fig. 3A). Cohort 1 participants with CDR-SB=0 showed a positive association of higher A β -PET CLs with higher plasma GFAP (linear model $R^2 = 0.090$) while participants with CDR-SB = 0.5–4.0 showed little-to-no meaningful association (linear model $R^2 < 0.000$). Controlling for plasma NfL somewhat attenuated the A β -PET CL $\times$ CDR-SB interaction on plasma GFAP in Cohort 2 (R^2 change = 0.061, $p = 0.05$).

Again, in contrast, there was no significant A β -PET CLs $\times$ CDR-SB interaction on plasma NfL in either Cohort 1 (R^2 change = 0.005, $p = 0.59$; Fig. 3C) or Cohort 2 (R^2 change = 0.075, $p = 0.10$; Fig. 3D; see also Supplementary Figure 1). Exploratory decomposition of the interaction terms suggested A β -PET CLs may be positively associated with plasma NfL in Cohort 2 participants with CDR-SB = 0 (Pearson's $r = 0.40$).

We plotted A β -PET CLs and plasma GFAP as a function of CDR-SB score in both Cohort 1 and Cohort 2 to help visualize potential divergence of these biomarkers across the functional spectrum. Regression analyses controlling for age and sex supported a positive, linear association between A β -PET CLs and CDR-SB (Cohort 1 linear model $R^2 = 0.367$, $p < 0.001$; Cohort 2 linear model $R^2 = 0.597$, $p < 0.001$) and curvilinear association between plasma GFAP and CDR-SB across a wider clinical disease spectrum (Cohort 2, linear model $R^2 = 0.112$, quadratic model $R^2 = 0.246$; R^2 change $p = 0.001$; Fig. 4).

Sex as a biologic variable

There was no significant main effect of sex on plasma GFAP, controlling for age, in either Cohort 1 ($\beta = 0.123$, $p = 0.32$) or Cohort 2 ($\beta = 0.121$, $p = 0.49$). There also was no significant A β -PET CLs $\times$ Sex interaction on plasma GFAP in either Cohort 1 (R^2 change = 0.001, $p = 0.77$) or Cohort 2 (R^2 change = 0.040, $p = 0.22$; Supplementary Figure 2). We found a trend towards statistical significance for a nonlinear A β -GFAP association in Cohort 2 females (R^2 change = 0.175, $p = 0.07$).

DISCUSSION

Synopsis

We evaluated the association between markers of astrocytic changes and cortical A β deposition in older adults using plasma-based quantification of GFAP and A β -PET. Our study involved two independent cohorts with a wide range of A β deposition and functional abilities. In both cohorts, we found that plasma GFAP increased linearly with A β -PET CLs in older adults with mild or no functional changes (CDR-SB < 4.0), which appeared largely driven by functionally intact participants (CDR-SB = 0). Within Cohort 2, which included older adults with dementia and more severe A β deposition, we observed that the A β -GFAP association became curvilinear—plasma GFAP concentrations decreased as A β -PET exceeded 100 CLs. Interestingly, Cohort 2 participants with dementia exhibited a strong, negative association between plasma GFAP and A β -PET CLs. The relatively few cases in Cohort 1 with >100 CLs ($N = 4$) may have limited the ability to detect the curvilinear association seen in Cohort 2. Notably, these findings were not observed in plasma NfL and remained significant after controlling for plasma NfL as an index of neurodegeneration.

The positive association between plasma GFAP and A β burden at the mildest ends of the A β spectrum in cognitively normal older adults may be unique among other commonly measured AD biomarkers. Prior work using a different A β -PET tracer (flutemetamol) and a marker of astroglial activation (YKL-40) found only modest associations across the A β SUVR spectrum [9]. However, our findings are remarkably consistent with two prior PET studies investigating ^{11}C -DED tracer binding to the monoamine oxidase-B (MAO-B) enzyme, another indicator of astrocyte integrity. These previous studies demonstrated higher MAO-B binding in presymptomatic subjects with familial AD [17] and in MCI subjects with suspected AD [16] than in those with dementia. Our results collectively support the concepts that 1) the signal for astrocytic integrity may be strongest in the pre-dementia phase of AD, 2) biomarkers of astrocytic integrity may diverge from A β biomarkers in later disease

stages, and 3) astrocyte changes may precede or at least show some independence from frank neurodegeneration.

Dynamics of astrocyte changes in AD

These data extend current knowledge by demonstrating that astrocytic integrity measured by plasma GFAP may not track linearly with A β burden measured with PET, though the temporal sequencing of these changes remains unclear. Human neuropathology studies and mouse models compellingly demonstrate that astrocytes play a direct role in A β plaque degradation and clearance [23–26]. Some theories posit that A β plaque deposition results from upstream dysfunction of the lifelong role of astrocytes in A β processing and clearance [27–29], including mechanisms associated with the glymphatic system [27, 30]. One hypothesis is that plasma GFAP elevations early in AD may therefore reflect a compensatory response to increased demand for A β plaque clearance, though it is conceivable that astrocyte changes even precede (or promote) A β plaque deposition. Lower plasma GFAP levels in the setting of severe amyloidosis may indicate dampened astrocytic response, though our study design precludes directional mechanistic conclusions.

On the other hand, increased A β *production* and neurotoxicity also is tied to astrocytes. For example, apolipoprotein (*APOE*) is predominantly produced by astrocytes and could be a mechanism contributing to A β metabolism [31]. Astrocytes also respond to oxidative stress induced by cortical A β accumulation by either releasing antioxidants (neuroprotective) or proinflammatory factors and reactive oxygen species (neurotoxic) [2]. These conflicting roles have led to proposed nomenclature for a biphasic model of toxic (“A1 astrocytes”) and protective (“A2 astrocytes”) reactive astrocytes [2, 32]. It is unclear if or how plasma GFAP concentrations may reflect these various astrocytic states, but our finding of fluctuations across the spectra of amyloidosis and clinical disease stage suggest plasma GFAP could be a proxy for more than one aspect of astrocyte changes. We did not find a significant difference in plasma GFAP concentration between *APOE* $\epsilon 4$ carriers and non-carriers, and *APOE* $\epsilon 4$ carrier status did not explain the observed GFAP-A β associations. However, more nuanced examination of *APOE* genotype effects on astrocyte and neurodegenerative disease biomarkers is needed to clarify a potential moderating role.

Diverse roles in A β degradation, clearance, production, and inflammatory response strongly implicate astrocytes as key contributors to AD pathophysiology, and our observed nonlinear association between plasma GFAP and A β -PET highlights the complex dynamics of AD pathophysiology and the relevance of disease severity. Our study was limited to A β -PET as a biomarker for AD pathology but we suspect that the older adults with MCI and dementia in this sample also had significant cortical phosphorylated tau deposition [33, 34]. Astrocytes are involved with tau processing across multiple tauopathies [35] and future work integrating astrocytic and tau biomarkers will help clarify their interplay in AD.

Plasma GFAP as an AD biomarker

Our findings support the potential utility of plasma GFAP as an early-identification biomarker for neurodegenerative diseases, though likely nonspecific to AD [36]. The nonlinear association of plasma GFAP with A β -PET has significant implications for

interpreting plasma GFAP findings in the clinic and determining appropriate contexts of use in AD research. For example, low or “normal” plasma GFAP levels could signal later-stage disease process rather than poor sensitivity to disease per se. Conversely, abnormally high levels, particularly preceding or early in the clinical manifestation of disease, may have utility in disease staging. Therefore, plasma GFAP may serve both diagnostic and prognostic roles if sampled at different points in the disease trajectory. Clinical interpretation of plasma GFAP concentrations should occur in the context of severity of cortical A β burden and/or functional status.

Alternate interpretations for plasma GFAP fluctuations

The convenience and feasibility of plasma biomarker collection is counterbalanced by limitations associated with quantifying brain-based changes in blood, including non-brain sources of proteins like GFAP and unknown spatial distributions of astrocyte changes in the cortex. GFAP is expressed by enteric nervous system glial cells [37] and in non-myelinating Schwann cells of peripheral nerves [38]. Certain GFAP isoforms (e.g., GFAP- α) are predominant in the brain, but blood-based GFAP assays are expected to identify all GFAP isoforms [39]. Aging also is associated with loss of blood-brain barrier integrity [40, 41] potentially leading to elevated brain-based proteins in the blood that is disproportionate to their production rate in the brain. These confounds are intertwined with glymphatic system functioning and its relationship with highly variable behaviors like sleep [30]. Inefficient glymphatic clearance, regardless of the reason, may result in “lower” plasma proteins in later disease stages. However, divergent findings between plasma GFAP and plasma NfL in this study argue against a completely nonspecific association with all blood-derived proteins.

Key populations for future research

Important subgroups warrant deeper study. In Cohort 1, plasma GFAP concentrations appeared *higher* in clinically normal older adults than in those with MCI in a similar A β -PET CL range, but we were insufficiently powered to compare statistically. This subgroup is highly relevant because a better understanding of mechanisms underlying variable cognitive function in the face of neurodegenerative pathology has significant implications for developing novel treatment therapies.

We also noted that A β -PET correlated strongly with CDR-SB in both cohorts, which complicates the ability to determine whether A β -PET or CDR-SB more strongly drives the variance in plasma GFAP concentrations. A targeted study of plasma GFAP concentrations in asymptomatic older adults matched to cognitively impaired older adults on A β burden could test hypotheses about the role of astrocyte changes in either manifestation of cognitive symptoms (i.e., resilience) or development of additional pathologies (i.e., resistance) [42].

Another key subgroup from Cohort 1 included individuals in the CDR-SB 0.5–2.0 range (largely absent from Cohort 2). We observed potential nonlinear variability even within this narrow “early MCI” range but had too few cases to assess polynomial trends appropriately (i.e., risk for model overfitting). Targeting older adults at the mildest end of the clinical disease spectrum along with clinically normal older adults with elevated GFAP for

longitudinal tracking would clarify this phenomenon (e.g., is presymptomatic elevation in plasma GFAP a harbinger of disease and cognitive decline?).

Limitations

Appropriately investigating the complex disease mechanisms and pathways discussed here requires longitudinally following large samples of sociodemo-graphically diverse older adults. Both cohorts were almost exclusively white/Caucasian so findings may not generalize well to other racial/ethnic groups. The limited sample size in both cohorts likely impacted identification of significant interactions, particularly for sex-specific differences in the associations among plasma GFAP, A β -PET, and functional status. Our cohorts contained a subset of older adults with early-onset cognitive changes that may have different disease characteristics than later-onset samples [43], and it is unknown whether the described relationships are consistent across all clinical manifestations of AD. Plasma GFAP alone is insufficient for fully characterizing astrocyte changes. We cannot draw firm conclusions about mechanism or temporal dynamics because of our observational, cross-sectional study design. History of head trauma was not comprehensively evaluated in this sample and may influence biomarker outcomes independent of aging and AD processes [41, 44]. While A β -PET imaging informs whether participants may exist along the Alzheimer's continuum, we did not have tau biomarkers or ways of directly accounting for the high likelihood of mixed pathology in our sample. We also relied on a proxy indicator of neurodegeneration (NfL), which may not precisely correlate with brain atrophy or structural neuroimaging measures. Nonetheless, based on using plasma NfL as a covariate and comparative outcome, we found that the relationships between GFAP and A β remained significant and showed a distinct pattern. Despite converting A β -PET SUVRs to the standardized Centiloid scale, it is possible that the use of two different amyloid tracers between our cohorts somewhat limits pure comparisons of the data. Lastly, beyond the need to analyze our cohorts separately because of completing biomarker assays at different times, our study was subject to limitations common to blood-based biomarker studies, such as variability in assay kit performance (measurement precision and accuracy).

Conclusions

Plasma GFAP is nonlinearly associated with A β -PET and may depend on both severity of cortical A β deposition and functional status. Serial quantification of plasma GFAP considered alongside A β biomarkers might ultimately inform disease stage and prognosis in older adults at-risk for AD. Examination of nonlinear relationships among AD biomarkers appears highly warranted given potentially differing dynamics across disease and clinical stage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the patients and their families whose help and participation made this work possible. We also thank the National Centralized Repository for Alzheimer's Disease and Related Dementias (NCRAD) for sample storage,

which receives government support under a cooperative agreement grant (U24 AG21886) awarded by the National Institute of Aging (NIA). We thank contributors who collected samples used in this study, Dr. Adam Boxer for giving us access to the HD1 Analyzer by Quanterix (Lexington, MA), as well as the following funding sources that have supported our work: NIH-NIA ADRC (P30 AG062422) and PPG (P01 AG019724) to BLM, R01 AG045611 to GDR, R01(s) AG032289 and AG048234) and Larry L. Hillblom Network Grant (2014-A-004-NET) to JHK, American Academy of Neurology grant to FME, NIH-NIA (K23 AG058752) to KBC, NIH-NIA (K23 AG061253) and Larry L. Hillblom Fellowship (2018-A-025-FEL) to AMS, NIH-NIA (K23AG059888) to JCR, NIH-NIA (U19AG063911, U54NS092089, R01AG038791) to ALB, and NIH-NIA (K99AG065501) and Alzheimer's Association (AARF-16-443577) to RLJ.

REFERENCES

- [1]. Carter SF, Herholz K, Rosa-Neto P, Pellerin L, Nordberg A, Zimmer ER (2019) Astrocyte biomarkers in Alzheimer's disease. *Trends Mol Med* 25, 77–95. [PubMed: 30611668]
- [2]. Liddel SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Münch AE, Chung WS, Peterson TC (2017) Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* 541, 481–487. [PubMed: 28099414]
- [3]. Nalivaeva NN, Fisk LR, Belyaev ND, Turner AJ (2008) Amyloid-degrading enzymes as therapeutic targets in Alzheimer's disease. *Curr Alzheimer Res* 5, 212–224. [PubMed: 18393806]
- [4]. Leuba G, Wernli G, Vernay A, Kraftsik R, Mohajeri MH, Saini KD (2005) Neuronal and nonneuronal quantitative BACE immunocytochemical expression in the entorhinohippocampal and frontal regions in Alzheimer's disease. *Dement Geriatr Cogn Disord* 19, 171–183. [PubMed: 15677864]
- [5]. Gaetani L, Blennow K, Calabresi P, Di Filippo M, Parnetti L, Zetterberg H (2019) Neurofilament light chain as a biomarker in neurological disorders. *J Neurol Neurosurg Psychiatry* 90, 870–881. [PubMed: 30967444]
- [6]. Zetterberg H (2016) Neurofilament light: A dynamic cross-disease fluid biomarker for neurodegeneration. *Neuron* 91, 1–3. [PubMed: 27387643]
- [7]. Mattsson N, Andreasson U, Zetterberg H, Blennow K (2017) Association of plasma neurofilament light with neurodegeneration in patients with Alzheimer disease. *JAMA Neurol* 74, 557–566. [PubMed: 28346578]
- [8]. de Wolf F, Ghanbari M, Licher S, McRae-McKee K, Gras L, Weverling GJ, Wermeling P, Sedaghat S, Ikram MK, Waziry R, Koudstaal W, Klap J, Kostense S, Hofman A, Anderson R, Goudsmit J, Ikram MA (2020) Plasma tau, neurofilament light chain and amyloid- β levels and risk of dementia; a population-based cohort study. *Brain* 143, 1220–1232. [PubMed: 32206776]
- [9]. Palmqvist S, Insel PS, Stomrud E, Janelidze S, Zetterberg H, Brix B, Eichenlaub U, Dage JL, Chai X, Blennow K, Mattsson N, Hansson O (2019) Cerebrospinal fluid and plasma biomarker trajectories with increasing amyloid deposition in Alzheimer's disease. *EMBO Mol Med* 11, e11170. [PubMed: 31709776]
- [10]. Wang SY, Chen W, Xu W, Li JQ, Hou XH, Ou YN, Yu JT, Tan L (2019) Neurofilament light chain in cerebrospinal fluid and blood as a biomarker for neurodegenerative diseases: A systematic review and meta-analysis. *J Alzheimers Dis* 72, 1353–1361. [PubMed: 31744001]
- [11]. Wilhelmsson U, Bushong EA, Price DL, Smarr BL, Phung V, Terada M, Ellisman MH, Pekny M (2006) Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury. *Proc Natl Acad Sci U S A* 103, 17513–17518. [PubMed: 17090684]
- [12]. Kamphuis W, Middeldorp J, Kooijman L, Sluijs JA, Kooi EJ, Moeton M, Freriks M, Mizee MR, Hol EM (2014) Glial fibrillary acidic protein isoform expression in plaque related astrogliosis in Alzheimer's disease. *Neurobiol Aging* 35, 492–510. [PubMed: 24269023]
- [13]. Abdelhak A, Huss A, Kassubek J, Tumani H, Otto M (2018) Serum GFAP as a biomarker for disease severity in multiple sclerosis. *Sci Rep* 8, 14798. [PubMed: 30287870]
- [14]. Olsson B, Lautner R, Andreasson U, Öhrfelt A, Portelius E, Bjerke M, Hölttä M, Rosén C, Olsson C, Strobel G (2016) CSF and blood biomarkers for the diagnosis of Alzheimer's disease: A systematic review and meta-analysis. *Lancet Neurol* 15, 673–684. [PubMed: 27068280]
- [15]. Elahi FM, Casaletto KB, La Joie R, Walters SM, Harvey D, Wolf A, Edwards L, Rivera-Contreras W, Karydas A, Cobigo Y, Rosen HJ, DeCarli C, Miller BL, Rabinovici GD, Kramer

- JH (2020) Plasma biomarkers of astrocytic and neuronal dysfunction in early-and late-onset Alzheimer's disease. *Alzheimers Dement* 16, 681–695. [PubMed: 31879236]
- [16]. Carter SF, Schöll M, Almkvist O, Wall A, Engler H, Långström B, Nordberg A (2012) Evidence for astrocytosis in prodromal Alzheimer disease provided by 11C-deuterium-L-deprenyl: A multitracers PET paradigm combining 11C-Pittsburgh compound B and 18F-FDG. *J Nucl Med* 53, 37–46. [PubMed: 22213821]
- [17]. Rodriguez-Vieitez E, Saint-Aubert L, Carter SF, Almkvist O, Farid K, Schöll M, Chiotis K, Thordardottir S, Graff C, Wall A (2016) Diverging longitudinal changes in astrocytosis and amyloid PET in autosomal dominant Alzheimer's disease. *Brain* 139, 922–936. [PubMed: 26813969]
- [18]. Casaleto K, Elahi F, Fitch R, Walters S, Fox E, Staffaroni A, Bettcher B, Zetterberg H, Karydas A, Rojas J (2018) A comparison of biofluid cytokine markers across platform technologies: Correspondence or divergence? *Cytokine* 111, 481–489. [PubMed: 29908923]
- [19]. O'Bryant SE, Waring SC, Cullum CM, Hall J, Lacritz L, Massman PJ, Lupo PJ, Reisch JS, Doody R (2008) Staging dementia using Clinical Dementia Rating Scale Sum of Boxes scores: A Texas Alzheimer's research consortium study. *Arch Neurol* 65, 1091–1095. [PubMed: 18695059]
- [20]. Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, Gamst A, Holtzman DM, Jagust WJ, Petersen RC, Snyder PJ, Carrillo MC, Thies B, Phelps CH (2011) The diagnosis of mild cognitive impairment due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 7, 270–279. [PubMed: 21514249]
- [21]. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR Jr, Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, Weintraub S, Phelps CH (2011) The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 7, 263–269. [PubMed: 21514250]
- [22]. Klunk WE, Koeppe RA, Price JC, Benzinger TL, Devous MD Sr, Jagust WJ, Johnson KA, Mathis CA, Minhas D, Pontecorvo MJ (2015) The Centiloid Project: Standardizing quantitative amyloid plaque estimation by PET. *Alzheimers Dement* 11, 1–15.e1–4. [PubMed: 25443857]
- [23]. Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, Silverstein SC, Husemann J (2003) Adult mouse astrocytes degrade amyloid- β *in vitro* and *in situ*. *Nat Med* 9, 453–457. [PubMed: 12612547]
- [24]. Katsouri L, Birch AM, Renziehausen AW, Zach C, Aman Y, Steeds H, Bonsu A, Palmer EO, Mirzaei N, Ries M (2020) Ablation of reactive astrocytes exacerbates disease pathology in a model of Alzheimer's disease. *Glia* 68, 1017–1030. [PubMed: 31799735]
- [25]. Thal DR, Schultz C, Dehghani F, Yamaguchi H, Braak H, Braak E (2000) Amyloid β -protein (A β)-containing astrocytes are located preferentially near N-terminal-truncated A β deposits in the human entorhinal cortex. *Acta Neuropathol* 100, 608–617. [PubMed: 11078212]
- [26]. Wegiel J, Wang KC, Tarnawski M, Lach B (2000) Microglial cells are the driving force in fibrillar plaque formation, whereas astrocytes are a leading factor in plaque degradation. *Acta Neuropathol* 100, 356–364. [PubMed: 10985692]
- [27]. Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, Benveniste H, Vates GE, Deane R, Goldman SA (2012) A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β . *Sci Transl Med* 4, 147ra111.
- [28]. Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, Jacobs AH, Wyss-Coray T, Vitorica J, Ransohoff RM (2015) Neuroinflammation in Alzheimer's disease. *Lancet Neurol* 14, 388–405. [PubMed: 25792098]
- [29]. Heneka MT, Golenbock DT, Latz E (2015) Innate immunity in Alzheimer's disease. *Nat Immunol* 16, 229–236 [PubMed: 25689443]
- [30]. Jessen NA, Munk ASF, Lundgaard I, Nedergaard M (2015) The glymphatic system: A beginner's guide. *Neurochem Res* 40, 2583–2599. [PubMed: 25947369]

- [31]. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small G, Roses A, Haines J, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 261, 921–923. [PubMed: 8346443]
- [32]. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, Barres BA (2012) Genomic analysis of reactive astrogliosis. *J Neurosci* 32, 6391–6410. [PubMed: 22553043]
- [33]. Mitchell TW, Mufson EJ, Schneider JA, Cochran EJ, Nissarov J, Han LY, Bienias JL, Lee VMY, Trojanowski JQ, Bennett DA (2002) Parahippocampal tau pathology in healthy aging, mild cognitive impairment, and early Alzheimer's disease. *Ann Neurol* 51, 182–189. [PubMed: 11835374]
- [34]. Jack CR, Bennett DA, Blennow K, Carrillo MC, Dunn B, Haeberlein SB, Holtzman DM, Jagust W, Jessen F, Karlawish J (2018) NIA-AA research framework: Toward a biological definition of Alzheimer's disease. *Alzheimers Dement* 14, 535–562. [PubMed: 29653606]
- [35]. Kovacs GG (2020) Astroglia and Tau: New perspectives. *Front Aging Neurosci* 12, 96. [PubMed: 32327993]
- [36]. Heller C, Foiani MS, Moore K, Convery R, Bocchetta M, Neason M, Cash DM, Thomas D, Greaves CV, Woollacott IO, Shafei R, Van Swieten JC, Moreno F, Sanchez-Valle R, Borroni B, Laforce R Jr, Masellis M, Tartaglia MC, Graff C, Galimberti D, Rowe JB, Finger E, Synofzik M, Vandenberghe R, de Mendonca A, Tagliavini F, Santana I, Ducharme S, Butler CR, Gerhard A, Levin J, Danek A, Frisoni G, Sorbi S, Otto M, Heslegrave AJ, Zetterberg H, Rohrer JD (2020) Plasma glial fibrillary acidic protein is raised in progranulin-associated frontotemporal dementia. *J Neurol Neurosurg Psychiatry* 91, 263–270. [PubMed: 31937580]
- [37]. Gulbransen BD, Sharkey KA (2012) Novel functional roles for enteric glia in the gastrointestinal tract. *Nat Rev Gastroenterol Hepatol* 9, 625–632. [PubMed: 22890111]
- [38]. Mokuno K, Kamholz J, Behrman T, Black C, Sessa M, Feinstein D, Lee V, Pleasure D (1989) Neuronal modulation of Schwann cell glial fibrillary acidic protein (GFAP). *J Neurosci Res* 23, 396–405. [PubMed: 2769798]
- [39]. Yang Z, Wang KK (2015) Glial fibrillary acidic protein: From intermediate filament assembly and gliosis to neurobiomarker. *Trends Neurosci* 38, 364–374. [PubMed: 25975510]
- [40]. Popescu BO, Toescu EC, Popescu LM, Bajenaru O, Muresanu DF, Schultzberg M, Bogdanovic N (2009) Blood-brain barrier alterations in ageing and dementia. *J Neurol Sci* 283, 99–106. [PubMed: 19264328]
- [41]. Plog BA, Dashnaw ML, Hitomi E, Peng W, Liao Y, Lou N, Deane R, Nedergaard M (2015) Biomarkers of traumatic injury are transported from brain to blood via the glymphatic system. *J Neurosci* 35, 518–526. [PubMed: 25589747]
- [42]. Arenaza-Urquijo EM, Vemuri P (2018) Resistance vs resilience to Alzheimer disease: Clarifying terminology for preclinical studies. *Neurology* 90, 695–703. [PubMed: 29592885]
- [43]. Jacobs D, Sano M, Marder K, Bell K, Bylsma F, Lafleche G, Albert M, Brandt J, Stern Y (1994) Age at onset of Alzheimer's disease: Relation to pattern of cognitive dysfunction and rate of decline. *Neurology* 44, 1215–1215. [PubMed: 8035918]
- [44]. Sullan MJ, Asken BM, Jaffee MS, DeKosky ST, Bauer RM (2018) Glymphatic system disruption as a mediator of brain trauma and chronic traumatic encephalopathy. *Neurosci Biobehav Rev* 84, 316–324. [PubMed: 28859995]

Fig. 1.
Clinical disease stage breakdown of Cohort 1 and Cohort 2.

Fig. 2.

Main effect of A β -PET CLs on plasma GFAP (A-B) and plasma NfL (C-D) concentrations. All figures show standardized model-predicted plasma biomarker concentrations on the Y-axis and both linear (dashed lines) and quadratic (solid lines) associations with amyloid PET. For plasma GFAP, Cohort 1 (A) showed a linear association between higher A β -PET CLs and higher plasma GFAP while Cohort 2 (B) showed a curvilinear association. Cohort 2 notably differed from Cohort 1 in the range of clinical disease stage (see Fig. 1) and the frequency of cases at the severe end of the A β -PET CLs spectrum (red dashed boxes). There were no statistically significant associations between A β -PET CLs and plasma NfL in either Cohort 1 (C) or Cohort 2 (D).

Fig. 3.

Interaction between A β -PET and CDR-SB on plasma GFAP (A-B) and NfL (C-D) concentrations. All figures show standardized model-predicted plasma biomarker concentrations on the Y-axis. In both Cohort 1 (A) and Cohort 2 (B) higher A β -PET CLs was associated with higher plasma GFAP in cases with CDR-SB = 0 (yellow line) but not CDR-SB = 0.5–4.0 (orange line). For cases in the dementia range (CDR-SB > 4.0, red line, Cohort 2 only), higher A β -PET was associated with *lower* plasma GFAP. The A β -PET CLs $\times$ CDR-SB interaction was statistically significant only in Cohort 2 for plasma GFAP. These associations largely were not observed for plasma NfL (C and D) except for an apparent association of higher A β -PET CLs with higher plasma NfL in Cohort 2 cases with CDR-SB = 0 (Pearson's $r = 0.40$).

Fig. 4.

Plots depicting plasma GFAP (blue) and Aβ-PET (pink) as a function of CDR-SB score.

In Cohort 1, plasma GFAP was not significantly associated with CDR-SB after controlling for age and sex ($\beta = 0.189$, $p = 0.16$). In Cohort 2, we observed potential divergence of plasma GFAP and Aβ-PET in the older adults with CDR-SB > 8.0 , though the Aβ-PET data in this CDR-SB range largely was extrapolated (red box). Cohorts notably differed in representation of older adults with CDR-SB > 4.0 ($N = 0$ in Cohort 1) and in the mildest clinical disease stage (CDR-SB = 0.5–1.0; gold boxes). An additional 38 older adults from Cohort 2 with available plasma GFAP and CDR-SB (without Aβ-PET scan) were included in this plot to better represent the spectrum of clinical disease stage, particularly in the dementia range (total $N = 75$, age = 71.9 ± 9.1 years old, 52% female, 17.2 ± 3.0 years of education, 91.5% white).

Table 1

Sample Characteristics

	Cohort 1	Cohort 2	Sig. (p)	Effect Size
N	50	37	–	–
Age, y	72.3 (6.9)	69.8 (9.6)	0.19	$d = 0.30$
Sex, % Female	52%	57%	0.59	$V = 0.05$
Education, y	17.5 (1.9)	17.4 (2.4)	0.80	$d = 0.05$
Race, % White	92%	91%	0.89	$V = 0.02$
APOE, % ε4 carriers	42%	41%	0.98	$V < 0.01$
MMSE	29 (28, 30)	27 (23, 29)	<0.001	$d = 0.96$
CDR-SB	0.0 (0.0, 0.0)	3.0 (0.0, 5.0)	<0.001	$d = 1.30$
Clinical Diagnosis, N(%)				
Normal	39 (78%)	14 (38%)	<0.001	$V = 0.49$
MCI	11 (22%)	11 (30%)		
Dementia	0 (0%)	12 (32%)		
Aβ-PET				
Centiloid	36.6 (40.5)	72.2 (52.0)	0.001	$d = 0.76$
% Positive	50%	73%	0.031	$V = 0.23$
Plasma Biomarkers				
GFAP, pg/mL	190 (143, 241)	169 (98, 244)	[not comparable]	–
Normal	183 (140, 242)	113 (79, 184)		
MCI	213 (168, 254)	180 (139, 315)		
Dementia	–	207 (123, 295)		
Aβ-PET (+)	206 (154, 251)	184 (136, 283)		
Aβ-PET (–)	161 (131, 213)	92 (77, 132)		
NfL, pg/mL	19.6 (16.4, 27.4)	24.3 (16.9, 31.9)	[not comparable]	–
Normal	21.7 (16.4, 29.2)	21.5 (15.5, 31.0)		
MCI	19.4 (18.3, 25.6)	31.3 (23.7, 34.8)		
Dementia	–	20.6 (15.2, 28.6)		
Aβ-PET (+)	19.6 (17.8, 26.6)	26.5 (18.5, 33.7)		
Aβ-PET (–)	18.3 (14.3, 29.4)	21.0 (15.5, 25.2)		

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Data presented as mean (standard deviation) or median (lower quartile, upper quartile) depending on the data distribution, or frequency (percent), and compared using independent samples *t*-test (parametric), Mann-Whitney U (nonparametric), or chi-square (categorical). Effect size estimates provided either as Cohen's *d* or Cramer's *V*. Clinical diagnosis reflected multidisciplinary consensus determination of cognitive and functional abilities. Aβ-PET, amyloid-β positron emission tomography; *APOE*, apolipoprotein E; CDR-SB, Clinical Dementia Rating Sum of Boxes; GFAP, glial fibrillary acidic protein; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; NFL, neurofilament light; pg/mL, picograms per milliliter; y, years.