FULL TITLE
Plasma biomarkers, memory impairment, and MRI measures of Alzheimer's disease in American Indians: the Strong Heart Study

SHORT TITLE

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INTRODUCTION

Early identification of Alzheimer's disease (AD) is critical to advancements in prevention and treatment efforts. The 2018 joint statement by National Institute on Aging and Alzheimer's Association recommends a research framework for defining AD using objective, quantifiable features reflecting presence and degree of amyloid deposition (A), pathologic tau (T), and neuronal injury (N).[1] This "AT(N) framework" aims to distinguish AD pathology from the cognitive and functional symptoms comprising the resulting clinical syndrome, as a more sensitive, more specific approach. In proof of concept, cerebrospinal fluid (CSF) biomarkers have high sensitivity and specificity for AT(N) pathology, even in advance of detectable changes on imaging, and long before detectable changes in cognitive sequelae.[2] However, collection of CSF involves training and technichal skills, may be regarded as invasive and costsvary across healthcare systems.[3] The alternative, PET imaging, is expensive, involves exposure to radiation, and unrealistic for settings with poor resource or specialty care access, such as rural American Indian communities or other underserved groups. Also, although structural MRI reasonably reflects N markers in this framework, even MRI can be unattainably expensive, and still does not provide insight into A or T markers. Finally, prior research has shown that established AD risk markers, such as *APOE*ε4 carrier status, may not be epidemiologically associated with risk in American Indians, suggesting potential for novel population stratification, risk saturation, or latent resilience features.[4] Consequently, circulating biomarkers for A, T, and N pathology may offer a viable low-cost measure,[5] an especially pragmatic innovation in the community clinic setting. Candidate circulating AT(N) markers include amyloid β, phosphorylated tau, glial fibrillary protein, and neurofilament light chain. Our study aims to evaluate these markers in a large cohort of aging American Indians, in association with clinical, MRI, and cognitive features.

Amyloid-β, or beta amyloid (Aβ) protein fragments form oligomers in the context of AD by abnormal cleavage of the amyloid precursor protein. Aβ oligomers (and/or higher order assemblies of Aβ fibrils, so-called plaques) are neurotoxic,[6] and requisite for subsequent accumulation of pathologic tau, inducing misfolding of tau in a prion-like disease pathogenesis.[7] Plasma concentration of $A\beta_{42}$, especially when considered in a ratio of the shorter and less aggregation-prone and $A\beta_{40}$, are differentially dilute in AD and vascular dementias,[8] obstructive sleep apnea,[9] traumatic brain injury;[10] correlate with Aβ positivity by PET,[11,12] lower grey matter density in the medial temporal lobe, precuneus, and frontal lobe;[13] and 78-80% sensitive and 70-87% specific differentiating AD cases from controls.[14,15]

Tau is a microtubule-binding protein important for neuronal axon stability; in AD, tau is abnormally truncated, phosphorylated, and aggregated into helical fragments ("tangles") in the proximal axoplasm. Plasma total tau is increased in several neurological diseases, including hypoxic brain injury[16] Creutzfeldt-Jakob disease[17], multiple sclerosis,[18] acute pediatric neurological disorders,[19] concussion,[20,21] traumatic brain injury,[10,22,23] and Down syndrome.[24] Phosphorylated tau, specifically at threonine 181 ($ptau_{181}$), is increased in CSF[25] and in plasma of AD patients,[26,27] correlating with PET and MRI imaging,[28] with density loss in the medial temporal lobe, precuneus, thalamus, striatum, hippocampus, and amygdala;[13,29] and increases dose-dependently with AD cognitive symptoms,[30] including decline in global cognition, general and logical memory, attention, visuospatial ability, and verbal fluency.[29,31]

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein released by disintegrating astrocytes, with isoforms including mutations resulting in fibrillary deposits disrupting myelin formation, and post-translational modifications that interrupt astrocytic activation.[32] GFAP is elevated in blood for several neurological disorders including vascular injury, multiple sclerosis, frontotemporal degeneration, Parkinson's disease, epilepsy, schizophrenia, autism, major depressive disorder, traumatic injury, and AD. [33,34] and is positively associated with higher glucose consumption in imaging studies of the whole brain, probably reflecting increased energetic demands of reactive astrocytes and microglial activation.[35,36] GFAP is emerging as an important AD predictor and risk stratifier, outperforming many extant markers, either alone[37,38] or in combination with Aβ[37,38] or tau.[39]

Finally, neurofilament light chain (NFL) is a cytoplasmic protein highly expressed in myelinated axons[40] and an established marker for acute neuroaxonal injury.[41-43] As a CSF or plasma marker,[44] NFL is associated with primary ataxias,[45] primary progressive aphasias,[46] frontotemporal dementia,[47,48] and AD,[49,50] and correlates with extent of damage in clinical and subclinical vascular events.[51-53] However, NFL performance as an AD biomarker is likely to be stronger for familial than for sporadic AD, and due to acute upregulation may be more reflective of active or ongoing pathological changes than of prevalent pathology.[36]

Overall, multiple candidate plasma ATN biomarkers ($A\beta_{42/40}$, $ptau_{181}$, GFAP, NFL) may present sensitive, specific, objective AT(N) markers for a biological framework by which to define presence of AD pathology *in vivo*, with especial utility in circumstances wherein other markers are not available, such as rural communities. In the Strong Heart Study, older American Indians living in communities across 3 regions have been previously examined for prevalent vascular brain injury (VBI),[54,55] stroke,[56-58] and risk factors[59-62] associated with AD and dementia. However, availability of AT(N) markers in this population has been thus far limited to structural imaging (1.5T MRI); no measure of A or T is yet available. Furthermore, 1.5T structural imaging is limited in its ability to specifically characterize neurodegenerative-type atrophy from others types of atrophy. Therefore, in this study, we collected and evaluated plasma biomarkers for A ($A\beta_{42}$ and $A\beta_{40}$), T (p-tau/tau), and N (NFL) as well as key novel markers related to Aβ response (GFAP), to characterize these measures in this population and to contextualize these measures with existing data on VBI and AD markers and sequelae.

This work contributes novel *in vivo* disease characterization in American Indians, especially in relation to AD, potentially providing researchers and clinicians better information on how to characterize, diagnose, and evaluate AD in American Indian patients and communities. Such an opportunity also has potential to support future researchers and public health practitioners to develop clinical and public health programs to improve detection, reduce risk, and identify treatment opportunities for American Indian elders and all peoples. Further, this proposed work will contribute objective information on these diseases of brain aging, allowing more direct inter-population comparisons, enabling researchers and clinicians to understand similarities and differences in how brain aging differs among all peoples. This type of knowledge will support development of new methods to evaluate and treat individual patients may be more culture, language, and context-appropriate. Finally, this work will allow investigators to understand these biomarkers, as a basic scientific understanding, so that future researchers and clinicians may have stronger tools to address these significant public health needs, for the benefit of American Indian elders and all peoples.

METHODS

Setting: The Strong Heart Study (SHS) began recruitment of American Indian adults from communities and tribes in the U.S. Northern Plains, Southern Plains, and Southwest in 1989-1991. From 2010-2019, survivors from the initial SHS study cohort were recruited for examinations related to cognitive aging, stroke, and Alzheimer's disease ((N=818 in 2010-13, N=403 in 2017-2019)). Full description of recruitment and examination protocols including CONSORT diagrams, ethical approvals, and registrations have been previously published.[63,64] All participating institutional, Indian Health Service, and tribal review boards approved study protocols; all participants provided written, informed consent.

Plasma Assays: Five AT(N) plasma biomarkers ($pTau_{181}$, $A\beta_{40}$, $A\beta_{42}$, GFAP, NFL) were measured in all participants who had available, stored samples from the second visit (2017-2019, N=401). Standard sample handling for ethylene diamine tetraacetic acid (EDTA) plasma samples included: multiple inversion; centrifugation at 1000G at 4°C; immediate separation from buffy coat and aliquot into 2mL cryovial tubes; and -80°C storage. Assays were done by the Clinical Neurochemistry Laboratory at the University of Gothenburg, according to standard protocols evaluated by the Standardization of Alzheimer's Blood Biomarkers workgroup of the Global Biomarker Standardization Consortium of the Alzheimer's Association.[65] Assays were Simoa Neurology 4-Plex E kit (Quanterix) for $A\beta_{42\ \&\ 40}$, GFAP, and NFL, which has good test-retest performance metrics.[66] Separately, $ptau_{181}$ was measured using the Simoa prototype two-step assay, as previously described in detail.[26] The preparation protocol included thaw at room temperature, vortex, centrifuge 5 min at 10,000G (4-plex kit) or centrifuge 10 min (two-step assay). The resulting data concentrations are in pg/mL, except for $A\beta_{42/40}$ which is calculated as a ratio. All measurements were performed in one round of experiments, using one batch of reagents, by board-certified laboratory technicians, who were blinded to clinical data. Intra-assay coefficients of variation were below 10% for all of the markers.

Imaging data: The 1.5T MRI collection and processing protocols, briefly, included six sequences obtained in contiguous slices--sagittal T1-weighted localizer, co-registered axial-T1, axial-T2, axial-T2* susceptibility-weighted images, axial fluid-attenuated inversion recovery, and sagittal T1-weighted volumetric gradient echo. [55,67] We estimated structural tissue volumes for features particularly associated with AD, such as hippocampus[68] and entorhinal cortex,[69] via semi-automated software processing using version 5.3 of the FreeSurfer image analysis suite,[70-73] after skull stripping with cortical reconstruction via voxel-based

parcellation of T1 images.[74] Neuroradiologists coded infarcts (>3mm in maximum dimension) and hemorrhages (any size), as well as white matter hyperintensity grade by comparison to standard image templates (grade 0-9).

Cognitive definitions: Neuropyschological tests included the California Verbal Learning Test II –Short Form (CVLT II-SF), with several indices of immediate and delayed verbal learning and memory including 4 trials of 9 words, followed by a free recall trial after a brief (30 second) interference task, and both free and cued recall trials after another 10-minute delay.[75] Memory impairment was operationalized using an algorithmic method,[76,77] as previously reported, and structured as follows: an index of percent loss on short delay free recall was compared to the best of the latter two learning trials, with those who were <1.5 SD below mean (poor performance) considered as having an encoding memory deficit. Then, short recall scores were compared to long (10 min) free recall, with those who lost >50% between these trials considered as having either a storage or a retrieval memory deficit. To distinguish between these, the free recall score was compared with the score, after provision of semantic cues. Those who had a cued score >50% higher than their free score were considered as having a retrieval memory deficit, with the remainder considered as having a storage memory deficit. All remaining participants, namely those with adequate or good performance in trials, short, and long free recall tasks were considered as having intact memory.

Other data: Field center staff collected self-reported age (years), sex (male, female), years of formal education. Apolipoprotein E ε4 (*APOE*ε4*)* carrier status was measured by standard genotyping procedures[78,79] using blood samples collected at the baseline SHS visit.

Statistical Analyses: We summarized or tabulated participant characteristics for the study population overall and by *APOE*ε4 carrier status using standard metrics, including mean and standard deviation (normal, continuous), median and interquartile range (skewed, continuous), or count and percent (dichotomous). Percent difference in median, mean, or count for these features was caculated by comparing *APOE*ε4 carriers to non-carriers. Bar and scatter plots visually summarized distribution and range of plasma marker measures, for *APOE*ε4 carriers and non carriers. Linear or logistic regressions estimated associations of MRI features with plasma markers, adjusted for intracranial volume, age, sex, *APOE*ε4 status, and eGFR, with robust (sandwich) errors estimation to account for misspecification, and with calculation of false discovery rate to control for multiple comparisons. We did not log-transform skewed factors because no evidence suggested that skews resulted from any biasing factor, such as measurement error, but rather reflected the underlying true population values,[80] and our aim was to evaluate directly interpretable, *a priori* specified comparisons, rather than *ad hoc*, best-fit models. Age was also evaluated as a primary (sensitivity) exposure, for comparison purposes. Values of plasma marker measures and select participant characteristics were summarized as above, by memory categories, including memory intact or impaired, as well as by categories of impaired (retrieval, storage, encoding). Graphical scatterplots and histograms were conducted by memory impairment status. Discriminant capacity of plasma markers in categorizing participants correctly as either memory intact or impaired was evaluated using receiver operating characteristic (ROC) analysis, including estimation of area under the curve (AUC), empirical cut point based on Liu product maximization method with bootstrap inference (50 iterations), and sensitivity and specificity calculated at that cut point. To idenitify possible patterns of the five biomarkers, unsupervised k-means and hierarchical cluster analysis with bootstrap (100 iterations) included evaluation of Gap statistic for optimal k, principal components analysis and shield plot for visualizing clusters in the two primary orthogonal latent estimated features, silhouette plot to visualize cluster membership by individual participant, and dendrogram to visualize participant similarities. Cluster assignments were then evaluated in terms of participant characteristics as described above. All statistics were conducted using Stata v17 (College Station, TX) and R v. 4 (R Foundation for Statistical Computing, Vienna Austria).

RESULTS

Our analysis included 401 (of 403, 99.5%) participants from the 2017-2019 examination visit who contributed viable plasma samples (**Table 1**). This study population was generally elderly (mean age 78, range 70-94), with mean 13 years education, and with 20.9% *APOE*ε4 allele carriers. Memory impairment assessments for this population-based study sample suggested an estimated 31% with some memory impairment, including 13.8% retrieval type deficit, 6.5% storage type deficit, and 10.7% encoding type deficit. Mean cortical volume was approximately 24.9% (SD 2.4) of intracranial volume, and mean total brain volume was 63.8% (SD 5.5) of intracranial volume. Mean $pTau_{181}$ was 8.6 pg/mL, $A\beta_{40}$ 144.5 pg/mL, $A\beta_{42}$ 8.4 pg/mL,

$A\beta_{42/40}$ ratio 0.06, GFAP 178.2 pg/mL, and NFL 41.1 pg/mL, with wide ranges and heavy right-skew for most markers.

Because of distribution skew, we subsequently examined median plasma marker values, comparing clinical or cognitive features. Median values were: $pTau_{181}$ 5.0 pg/mL, $A\beta_{40}$ 128 pg/mL, $A\beta_{42}$ 8.2 pg/mL, $A\beta_{42/40}$ ratio 0.1, GFAP 150 pg/mL, and NFL 31.6 pg/mL (**Table 2**). Comparing *APOE*ε4 carriers to non-carriers, $pTau_{181}$ and GFAP were substantively higher (12%, 16%, repsectively) and $A\beta_{42}$ and $A\beta_{42/40}$ ratio substantively lower (-8.4%, -16.7%, respectively), however only the difference for $A\beta_{42/40}$ was statistically significant after correction for multiple testing. Bar and scatter plots of plasma marker measures (**Supplemental Figure 1**) illustrate a heavy distribution skew for each of the plasma marker measures, with long right tails corresponding to high concentration of plasma proteins.

Multivariate regressions estimated associations of each plasma marker measure with imaged brain findings, adjusted for intracranial volume, *APOE*ε4, age, sex, and MDRD eGFR and for multiple testing (**Table 3**). Significant associations for ventricle volume, cortical volume, and overall (grey & white matter) volume were detected for $A\beta_{40}$, $A\beta_{42}$, GFAP, and NFL, but not for $A\beta_{42/40}$ or for $pTau_{181}$. Significant findings for $pTau_{181}$ included presence of infarcts. No associations were detected for entorhinal or hippocampal volumes or for white matter grade, except for age.

Comparison of plasma marker measures and other characteristics across memory impairment categories identified significant differences comparing participants who were memory intact vs impaired, with those who were impaired having higher $pTau_{181}$, higher NfL, older age, and male sex (**Table 4**). In graphical examination of distributions of plasma markers, comparing participants who were memory intact vs. impaired, the statistically significant differences in $pTau_{181}$ appear to be driven by a few individuals in the higher value above 25 pg/mL. The significant differences in NfL appear to be a true shift in mean, but with substantive overlap between the two groups (**Supplemental Figure 2**). Comparing across types of memory impairment, significant associations for $pTau_{181,}$ NfL, age, and male sex were worst among those with encoding type memory impairment. Those with retrieval type impairment had intermediate values of NfL. Insignificant findings included $A\beta_{42,}$ with highest values among those with retrieval deficits; and GFAP with highest values among those with encoding deficits. *APOE*ε4 was most common among those with retrieval and encoding deficits, but these associations were not significant. No associations were detected for $A\beta_{42/40}$ ratio or education.

Evaluation of performance for individual plasma markers to discriminate memory impaired from memory intact (**Table 5**), adjusted for age, sex, and *APOE*ε4, demonstrated poor to moderate performance for all markers. Empirical estimation of the optimal cut-point for discrimination of memory impairment, using Liu sensitivity-specificity product maximization, were: $pTau_{181}$>6.5 pg/mL, $A\beta_{40}$>128.5 pg/mL, $A\beta_{42}$>8.0 pg/mL, $A\beta_{42/40}$ ratio>0.06, GFAP> 187.5 pg/mL, NfL>30.5 pg/mL. These cut points performed with poor to moderate sensitivity and specificity, ranging from 0.4-0.7.

Unsupervised hierarchical and K-means cluster analysis of the plasma marker measures as a group empirically established an optimal cluster solution of of k=2 (**Table 6, Supplemental Figure 3**). Comparing participant characteristics across the two clusters, all 6 plasma markers were significantly associated with cluster assignment, with higher $pTau_{181}$, NfL in cluster 1; and higher GFAP and lower $A\beta_{40}$ and $A\beta_{42}$ in cluster 2. Comparing risk characteristics, cluster 1 was older, with lower education, had smaller entorhinal hippocampal, crotical, and overall brain volume, larger ventricles, and more extreme white matter hyperintensity grade. Cluster 1 also had worse memory, with fewer intact, and more encoding and storage type impairment. Cluster 2 had no significant differences in imaging findings, but worse retrieval type memory impairment.

DISCUSSION

Overall, this is the first report on plasma markers related to Alzheimer's and other brain diseases—including $pTau_{181}$, $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42/40}$ ratio, GFAP, and NfL—among older American Indians, describing associations with clinical, imaging, and cognitive findings. Our findings include association of *APOE*ε4 with $A\beta_{42/40}$ ratio only; associations of imaged volumes with $A\beta_{40}$, $A\beta_{42}$, GFAP, and NfL but imaged brain infarcts with $pTau_{181}$; and associations of memory impairments with $pTau_{181}$, NfL, age, and sex. We established optimal cutoff values to discriminate memory impairment in this population. Our unsupervised cluster analysis identified cluster 1 compared to cluster 2 with higher $pTau_{181}$, higher GFAP, higher NfL, older age, lower education,

greater brain atrophy, worse vascular brain injury, and encoding and storage type memory impairment. In contrast, cluster 2 compared to cluster 1 had lower $A\beta_{40,}$ lower $A\beta_{42,}$ and worse memory impairment.

Comparisons with other populations: In this study of American Indians, our marker of $pTau_{181}$ (mean 8.6 pg/mL; range 1.5-442), had a lower mean but a larger variance, compared with NHW (16, 10-23), African American (14, 9.4-22.9), and Hispanic/Latino (18.0 (11.3-25.0). Values >40-60 are often considerd consistent with Alzheimer's disease.[81,82] Thus, aside from a few outliers, this population of American Indians aged >70 years did not appear to have evidence of excessively common pathologic tau (T). In contrast, two of our markers of amyloid (A) pathology—$A\beta_{42}$ and $A\beta_{42/40}$—were much lower than in NHW or other populations, and consistent with 3X more pathology, especially among the larger, more insoluble, more AD-specific $A\beta_{42}$.[83] In cognitive intact NHW, $A\beta_{40}$ is estimated to have mean 95 pg/mL, $A\beta_{42}$ 22 pg/mL, and $A\beta_{42/40}$ ratio 0.23; for cognitive impaired, these numbers are 98 pg/mL, 20 pg/mL, 0.2 ratio (respectively).[84] In our study, mean $A\beta_{40}$ was 140.7 pg/mL, $A\beta_{42}$ 8.3 pg/mL, and $A\beta_{42/40}$ ratio 0.06 among memory intact participants and $A\beta_{40}$ 150.2 pg/mL, $A\beta_{42}$ 8.5 pg/mL, and $A\beta_{42/40}$ ratio 0.06 in cognitive impaired participants. Thus, compared with NHW of either cognitive status, American Indians had 47-52% higher $A\beta_{40}$ (less abnormal), but 60-64% lower $A\beta_{42}$ and 70-74% lower $A\beta_{42/40}$ ratio (both more abnormal). These findings suggest the possibility that American Indians may have earlier, faster, or more common accumulation of amyloid plaques than in NHWs.[83] GFAP and NfL, were similar in this study as in other populations, especially in consideration of age-comparable standards.[82,84-88]

Feature associations: Our imaging and cognitive findings confirm prior reports that these plasma protein concentrations may serve as biomarkers for MRI findings, menory impairment, or both in American Indians, as in other populations.[2,5,26,36,89,90] Although some findings were not statistically significant, overall a pattern of association appeared of volumetric loss on MRI in association with abnormal values in $pTau_{181}$, GFAP, NfL, $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42/40}$. Also, a $pTau_{181}$ was significant associated with MRI-defined brain infarcts, consistent with prior findings in White Europeans.[91] For cognitive outcomes, $pTau_{181}$ and NfL were especially associated with encoding and storage type memory deficits, suggesting neurodegenerative impact; whereas $A\beta_{40}$, $A\beta_{42}$ and GFAP were more closely associated with retrieval type memory deficits, suggesting a more vascular-dominant mechanism.

Some of these non-significant findings may have been unable to excude the role of chance due to relatively low statistical power. Post-hoc analyses for associations with entorhinal, hippocampus, and whole brain volumes (respectively) estimated $pTau_{181}$ had 0.11, 0.06, and 0.10 power, with needed sample size 5-32K. Amyloid measures were somewhat better, with power for $A\beta_{40}$ 0.06, 0.22, and 0.80; for $A\beta_{42}$ 0.05, 0.05, 0.65; for $A\beta_{42/40}$ 0.14, 0.05, 0.05; with sample size estimations as little as 331 or 474 and as high as >10K. Estimates of power/needed sample size for GFAP were (respectively) 0.35/n=1070, 0.41/n=865, 0.49/n=703; and for NfL 0.39/n=923, 0.41/n=884, 0.94/n=209. Overall, these serial power calculations suggest that due to the heavy skew, $pTau_{181}$ and Aβ are likely underpowered in our analyses. Coupled with our observations of extreme values and ranges in these markers, and the older age of our population, it logically follows that selective survival may have removed some of the most strongly affected from our observations, resulting in invariance in the exposures. Future research should include younger individuals, in order to evaluate true population variance, as well as whether markers are associated with imaging features in preclinical phases.

Discriminant performance: Prior reports considered these plasma markers as individual or collective (panel) measures in their ability to discriminate AD pathology or predict cognitive decline, mostly in European or Canadian NHWs. In such studies, $pTau_{181}$, $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42/40}$ have the best performance with both PET[92] and with cognitive outcomes,[26] in a dose-dependent manner, that is improved with inclusion of *APOEε*4. AUC for $pTau_{181}$ for dementia is 90-99%, depending on the comparison group, and for PET features 76-88%. $A\beta_{42/40}$ has somewhat lower performance, with AUC 0.6 for cognitive outcomes and 0.8-0.9 for PET.[26,90,93] In our study on memory status or imaging features, biomarker performance was somewhat consistent, albeit with poorer performance, for markers related to amyloid pathology, but was not at all consistent with prior research on markers for pathologic tau. As noted earlier, it is possible that selective survival has removed many of the affected individuals from the observable set, with relatively healthier individuals remaining. The extreme outliers and the long right-skew in $pTau_{181}$ warrant further consideration, especially among younger groups who are less prone to selective survival and who are more likely to represent a preclinical population group.

Despite these comparisons, true normative ranges and diagnostic cutoffs are not definable without PET imaging for validation. Prior studies have examined plasma markers in comparison with serum, [94] with CSF,

[89,95] and with PET in white Europeans and Canadians.[26,93] However, lab standards for plasma measures of plasma biomarkers in AD are still forthcoming. Because studies using non-standardized assay methods may be subject to technologic and batch-based errors, comparison of non-standardized, unvalidated measures across cohorts can bias interpretation of results. Therefore, future research should involve direct validation of plasma and other markers with gold standards, such as PET, in all minoritized populations.

Prior null findings for APOEε4: In the context of prior reports from our research group that *APOE*ε4 had no detectable association with imaging, cognitive, or memory features in this population,[4] these current findings suggest that *APOE*ε4 is likely to influence amyloid pathology, but–due to its continued lack of association with other plasma markers or any other comparisons—such a function may not mediate other imaging or memory associations in this population. However, it should be noted that possibilities of selective survival, risk saturation in unexposed, or latent resilience factors may also contribute to these null associations, and future research should continue to examine these and other risk exposures that may be unique to this population.

Cluster analyses: Given prior reports that these plasma features may have improved discriminant performance as a panel, rather than individual features, and the still uncertain role of *APOE*ε4 in evaluating AD and dementia risk in this population, we conducted agnostic, unsupervised cluster analyses for empirical evaluation of co-occurring and associating features. . These analyses identified two distinct clusters, the first with features related to small vessel disease, axon remodeling, neurodegenerative atrophy, tau pathology, and encoding impairments (cluster 1); and the other with astrocytic remodeling, amyloid pathology, and retrieval impairments. . Similar analyses have been undertaken in NHWs,[96-98] and have detected similar associations, most notably NfL with white matter disease.[99] Futhermore, traumatic brain injury is common in this population,[100,101] which carries relevance to both tau[21,102] and NfL levels,[103] and for risk of AD.[104-106] Given these epidemiologic obvservations, in combinations with these empirically detected clusters, NfL and GFAP may be important features in accurate determinance of AD in American Indians, and in other similarly-exposed populations.

Strengths and limitations: These analyses include data from comprehensive, standardized collection protocols in a well-characterized cohort of an understudied population. The novel biomarker assays have potential to inform better cognitive impairment and dementia case definitions, as well as guide future research for the purposes of evaluating diagnostic, therapeutic, and prevention efforts. Furthermore, these analyses are theory-driven, and not empirical, with the strong potential to provide novel information both about this population as well as about the underlying neurology. As mentioned, because this population represents a survival cohort, differential selection may influence our findings, if likelihood of participation is associated with the outcome. Previous reports in this cohort have found little evidence of selective survival using indirect analysis.[61] However, future research should focus on younger population strata in order to include preclinical groups in the study sampling frame.

In this study, we adjusted for eGFR because renal filtration losses have been reported to increase plasma marker concentrations. However, the association of plasma markers with renal dysfunction may not be mediated by filtration function, as measured by eGFR. Proteins are not cleared by the glomerular basement membrane, where defects result in proteinuria or lower plasma protein concentrations. Therefore, alternative mechanisms accounting for observed renal associations, such as proximal tubular secretion (dys)function or hormone (dys)production, may be needed in future research on plasma biomarkers of brain injury. Furthermore, we did not evaluate associations for endocrine features, which may further serve as mediating or modifying clinical features in these associations, and represents an important avenue for future investigation.

Conclusion: In summary, this report contains seminal evaluation of blood biomarkers for brain injury, especially AD, in American Indian elders, using sociodemographics, clinical features, imaging, and cognitive evaluations. Future research to validate these measures using PET or other gold standards and to examine these measures in younger groups are needed. However, our findings establish these measures, and their coinciding features, as potential markers in determinance of brain injury, including AD, with implications for researchers and clinicians developing understanding of this complex and devastating condition in this unique population.

TABLE 1 : Selected characteristics from American Indian participants of the Strong Heart Study (2017-2019)

Available sociodemographics, clinical data	*N=401*
Age (years)	78.1 (4.7)
Male sex, n (%)	118 (29.4%)
Years education	13.0 (2.5)
APOEε4 status, n (%)	83 (20.9%)
Available plasma biomarkers data	*N=401*
$pTau_{181}$ pg/mL, mean (SD); range	8.6 (25.6); 15-442
$A\beta_{40}$ pg/mL, mean (SD); range	144.5 (48.4) 6-518
$A\beta_{42}$ pg/mL, mean (SD); range	8.4 (2.8); 0-21
$A\beta_{42/40}$ ratio, mean (SD); range	0.06 (0.01); 0.0-0.2
GFAP pg/mL, mean (SD); range	178.2 (96.1); 0-651
NFL pg/mL, mean (SD); range	41.1 (30.5) 10-343
Available MRI data	*N=334*
Entorhinal volume, mm^3	2532.9 (776.3)
Hippocampal volume, mm^3	6521.6 (860.3)
Ventricle volume, mm^3	34543.1 (17084.5)
Cortical volume, mm^3	342432.6 (41295.8)
Cortical volume, % IC volume	24.9 (2.4)
Overall (grey & white) volume, mm^3	878445.3 (113069.3)
Overall (grey & white) volume, % IC volume	63.8 (5.5)
WMH grade (0 best to 9 worst)	2.5 (1.3)
Infarct (cortical or subcortical, >3mm), n(%)	108 (32.3%)
Hemorrhage (parenchymal hematoma), n(%)	21 (6.3%)
Available CVLT data	*N=385*
Memory intact, n (%)	264 (68.9%)
Memory impaired, n (%)	119 (31.0%)
Retrieval impaired, n (%)	53 (13.8%)
Storage impaired, n (%)	25 (6.5%)
Encoding impaired, n (%)	41 (10.7%)

Values provided as mean(SD) unless otherwise specified; IC = intracranial

TABLE 2 : Plasma marker measures, overall and by APOEε4 status, in American Indian participants of the Strong Heart Study (2017-2019)

	Overall N=401	No APOEε4 allele n=314	APOEε4 carrier n=83	% difference (ε4 vs not)	*P-value*	*FDR Q-value*
$pTau_{181}$ pg/mL	5.0 (3.5, 7.4)	4.9 (3.4, 7.2)	5.5 (3.9, 8.3)	+ 12%	*0.067*	*0.182*
$A\beta_{40}$ pg/mL	128 (116, 175)	128 (116, 175)	127 (113, 177)	-0.8%	*0.780*	*0.780*
$A\beta_{42}$ pg/mL	8.2 (6.7, 9.7)	8.3 (6.8, 9.8)	7.6 (6.2, 9.4)	-8.4%	*0.140*	*0.210*
$A\beta_{42/40}$ ratio	0.06 (0.05, 0.07)	0.06 (0.05, 0.07)	0.05 (0.04, 0.06)	- 16.7%	*0.001*	*0.006*
GFAP pg/mL	150 (114, 218)	149 (112, 215)	173 (121, 240)	+ 16.1%	*0.091*	*0.182*
NFL pg/mL	31.6 (23.0, 48.9)	31.5 (22.9, 48.6)	31.8 (23.4, 49.6)	+ 3.2%	*0.470*	*0.564*

Values provided as med (IQR) unless otherwise indicated

TABLE 3: Regression coefficient estimates of association between plasma marker measures and volumetric imaging markers, in American Indian participants of the Strong Heart Study (2017-2019)

N=334 with MRI data	$pTau_{181}$, pg/mL β coefficient P-value	$A\beta_{40}$, pg/mL β coefficient P-value	$A\beta_{42}$, pg/mL β coefficient P-value	$A\beta_{42/40}$, ratio β coefficient P-value	GFAP, pg/mL β coefficient P-value	NfL, pg/mL β coefficient P-value	Age, years β coefficient P-value
Entorhinal volume, mm3	-1.19 p=*0.088*	-0.32 p=*0.743*	-3.11 p=*0.858*	-376.35 p=*0.891*	-0.69 p=*0.122*	-2.34 p=*0.051*	-17.7 *p=0.049*
Hippocampal volume, mm3	-0.52 p=*0.507*	-1.41 p=*0.062*	-1.81 p=*0.890*	-376.35 p=*0.891*	-0.85 p=*0.058*	-2.65 p=*0.040*	-37.8 *p<0.001**
Ventricle volume, mm3	20.23 p=*0.170*	55.90 p=*0.003**	799.71 p=*0.010**	-41467.64 p=*0.456*	29.16 p=*0.008**	65.90 p=*0.013**	579.3 *p=0.001**
Cortical volume, mm3	-56.48 p=*0.510*	-130.41 p<*0.001**	-1886.97 p<*0.001**	18182.46 p=*0.856*	-45.24 p=*0.013**	-259.21 P<*0.001**	-896.6 *p=0.031**
Overall volume, mm3	-124.95 p=*0.460*	-208.32 p=*0.013**	-2982.53 p=*0.022**	-2706.75 p=*0.992*	-120.77 p=*0.014**	-468.84 p=*0.002**	-2637.6 *p=0.003**
WMH grade, scale 0-9	0.00 p=*0.163*	0.00 p=*0.240*	0.01 p=*0.750*	-2.83 p=*0.626*	0.00 p=*0.268*	0.01 p=*0.043*	0.06 *p=0.001**
Infarct, presence †	0.00 p=*0.001**	0.00 p=*0.753*	0.01 p=*0.173*	1.12 p=*0.545*	0.00 p=*0.816*	0.00 p=*0.143*	0.04 *p=0.180*
Hemorrhage, presence †	0.00 p=*0.215*	0.00 p=*0.802*	-0.004 p=*0.358*	-0.58 p=*0.434*	0.00 p=*0.825*	0.00 p=*0.975*	0.001 *p=0.983*

Regressions adjusted for IC volume, APOEε4, age, sex, eGFR. Coefficient interpretation: estimate of corresponding absolute change in volumetric feature, per unit increase in plasma marker (or year of age).

† Logistic regression used in place of linear regression because of binary exposure variable

* designates significant finding (FDR-calculated Q-value <0.1)

TABLE 4 : Plasma marker measures, comparing memory impairment categories with memory-intact, among American Indian participants of the Strong Heart Study (2017-2019)

N=385 with CVLT data	Memory intact N=264	Memory impaired N=119	Impaired (retrieval) N=53	Impaired (storage) N=25	Impaired (encoding) N=41	*P-value*	*FDR Q-value*
$pTau_{181}$ pg/mL	4.8 (3.2, 6.6)	5.6 (3.9, 8.3) *	4.9 (3.9, 8.4)	4.6 (3.6, 7.1)	7.1 (5.0, 8.9)	*0.002*	*0.020*
$A\beta_{40}$ pg/mL	126 (116, 171)	133 (119, 178)	134 (123, 176)	131 (114, 178)	133 (117, 182)	*0.120*	*0.171*
$A\beta_{42}$ pg/mL	8.1 (6.6, 9.6)	8.3 (6.9, 9.8)	8.6 (6.8, 10.0)	8.1 (7.1, 9.8)	8.0 (6.8, 9.8)	*0.550*	*0.611*
$A\beta_{42/40}$ ratio	0.1 (0.1, 0.1)	0.1 (0.1, 0.1)	0.1 (0.0, 0.1)	0.1 (0.1, 0.1)	0.1 (0.1, 0.1)	*0.710*	*0.710*
GFAP pg/mL	148 (116, 212)	173 (115, 254)	151 (119, 218)	124 (95, 260)	207 (148, 265)	*0.029*	*0.70*
NFL pg/mL	29.5 (21.8, 46.7)	36.2 (26.4, 54.8) *	35.9 (24.1, 51.6)	31.5 (22.5, 51.6)	36.3 (30.8, 63.7)	*0.008*	*0.040*
Age years, m(SD)	77.7 (4.5)	78.8 (5.1) *	78.4 (4.9)	77.8 (5.2)	80.0 (5.1)	*0.032*	*0.070*
Male sex, n(%)	64 (24.2%)	46 (38.7%) *	20 (37.7%)	9 (36.0%)	17 (41.5%)	*0.035*	*0.070*
Years education, m(SD)	13.1 (2.5)	12.7 (2.5)	12.9 (3.1)	12.9 (2.0)	12.2 (2.0)	*0.190*	*0.237*
APOE ε4 carrier, n(%)	57 (21.9%)	24 (20.2%)	15 (28.3%)	1 (4.0%)	8 (19.5%)	*0.110*	*0.171*

Values given as med(IQR) unless otherwise specified. Statistical test comparing intact with impaired (t-test): pTau P=0.002; AB40 p=0.028, AB42 p=0.210, AB42/40 P=0.240; GFAP P=0.044; NFL P=0.003; Age P=0.028, sex P=0.004, edu P=0.110, Apoε4 P=0.70.

TABLE 5:Performance metrics for individual plasma marker measures in discriminating between memory impaired and intact, among American Indian participants of the Strong Heart Study (2017-2019)

N=378	ROC regression AUC (95% CI)	Empirical, optimal cut point	Sensitivity, specificity (AUC) at cut point
$pTau_{181}$ pg/mL	0.63 (0.57, 0.69)	6.5 pg/mL	0.42, 0.72 (0.57)
$A\beta_{40}$ pg/mL	0.56 (0.48, 0.63)	128.5 pg/mL	0.57, 0.55 (0.56)
$A\beta_{42}$ pg/mL	0.51 (0.44, 0.57)	8.0 pg/mL	0.59, 0.49 (0.54)
$A\beta_{42/40}$ ratio	0.44 (0.37, 0.52)	0.06 ratio	0.40, 0.57 (0.49)
GFAP pg/mL	0.59 (0.53, 0.64)	187.5 pg/mL	0.46, 0.71 (0.59)
NFL pg/mL	0.57 (0.50, 0.63)	30.5 pg/mL	0.64, 0.53 (0.58)

Regressed linear ROC model adjusted for age, sex, APOE4, with bootstrap inference (50 reps).

TABLE 6: Comparison of select participant characteristics by clusters from K-means cluster analysis of 6 plasma marker measures, with empirically defined, optimal k=2 cluster solution, among American Indian participants of the Strong Heart Study (2017-2019)

	Cluster 1 N=111	Cluster 2 N=285	*P-value*	*FDR Q-value*
$pTau_{181}$ pg/mL, med (IQR)	7.4 (5.1, 10.6)	4.2 (3.2, 6.3)	<0.001	0.003
$A\beta_{40}$ pg/mL, med (IQR)	167.0 (122.0, 202.0)	124.0 (113.0, 163.0)	<0.001	0.003
$A\beta_{42}$ pg/mL, med (IQR)	9.0 (7.3, 11.3)	7.8 (6.5, 9.2)	<0.001	0.003
$A\beta_{42/40}$ ratio, med (IQR)	0.1 (0.1, 0.1)	0.1 (0.1, 0.1)	0.024	0.030
GFAP pg/mL, med (IQR)	269.0 (234.0, 318.0)	130.0 (102.0, 158.0)	<0.001	0.003
NFL pg/mL, med (IQR)	46.7 (33.4, 67.4)	27.9 (21.4, 37.9)	<0.001	0.003
Age, years	80.3 (4.9)	77.2 (4.4)	<0.001	0.003
Male Sex, n(%)	23 (20.7%)	95 (33.3%)	0.014	0.190
Education, years	12.6 (2.5)	13.1 (2.5)	0.075	0.089
APOE ε4 carrier, n(%)	27 (24.5%)	55 (19.5%)	0.27	0.285
Entorhinal vol	2331.8 (742.6)	2608.1 (783.7)	0.007	0.010
Hippocampal vol	6294.6 (1001.8)	6612.3 (796.6)	0.005	0.010
Ventricle vol	39250.8 (20479.3)	33220.5 (15474.2)	0.007	0.010
Cortex vol	330958.9 (47003.6)	346727.6 (38518.0)	0.003	0.007
Graywhite vol	849724.0 (121019.3)	889966.9 (109038.8)	0.007	0.010
WMG	2.9 (1.4)	2.4 (1.2)	<0.001	0.003
Infarct, n(%)	32 (36.0%)	72 (30.3%)	0.32	0.320
Hemorrhage, n(%)	8 (9.1%)	11 (4.6%)	0.13	0.145
Cog category-intact, n(%)	61 (59.2%)	200 (72.7%)	0.005	0.010
Cog category-retreival, n(%)	13 (12.6%)	39 (14.2%)		
Cog category-storage, n(%)	9 (8.7%)	15 (5.5%)		
Cog category-encoding, n(%)	20 (19.4%)	21 (7.6%)		

Values provided as mean (%) unless otherwise indicated

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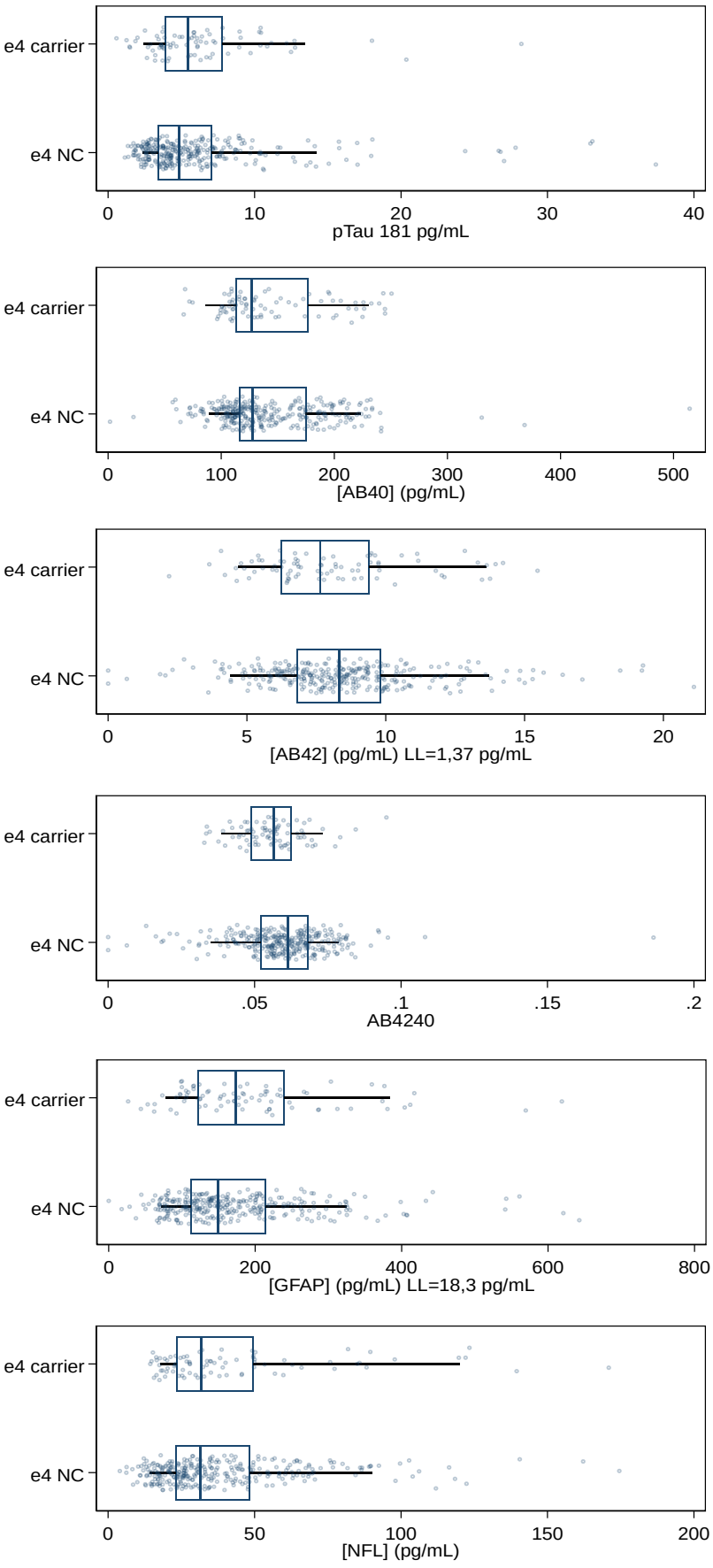
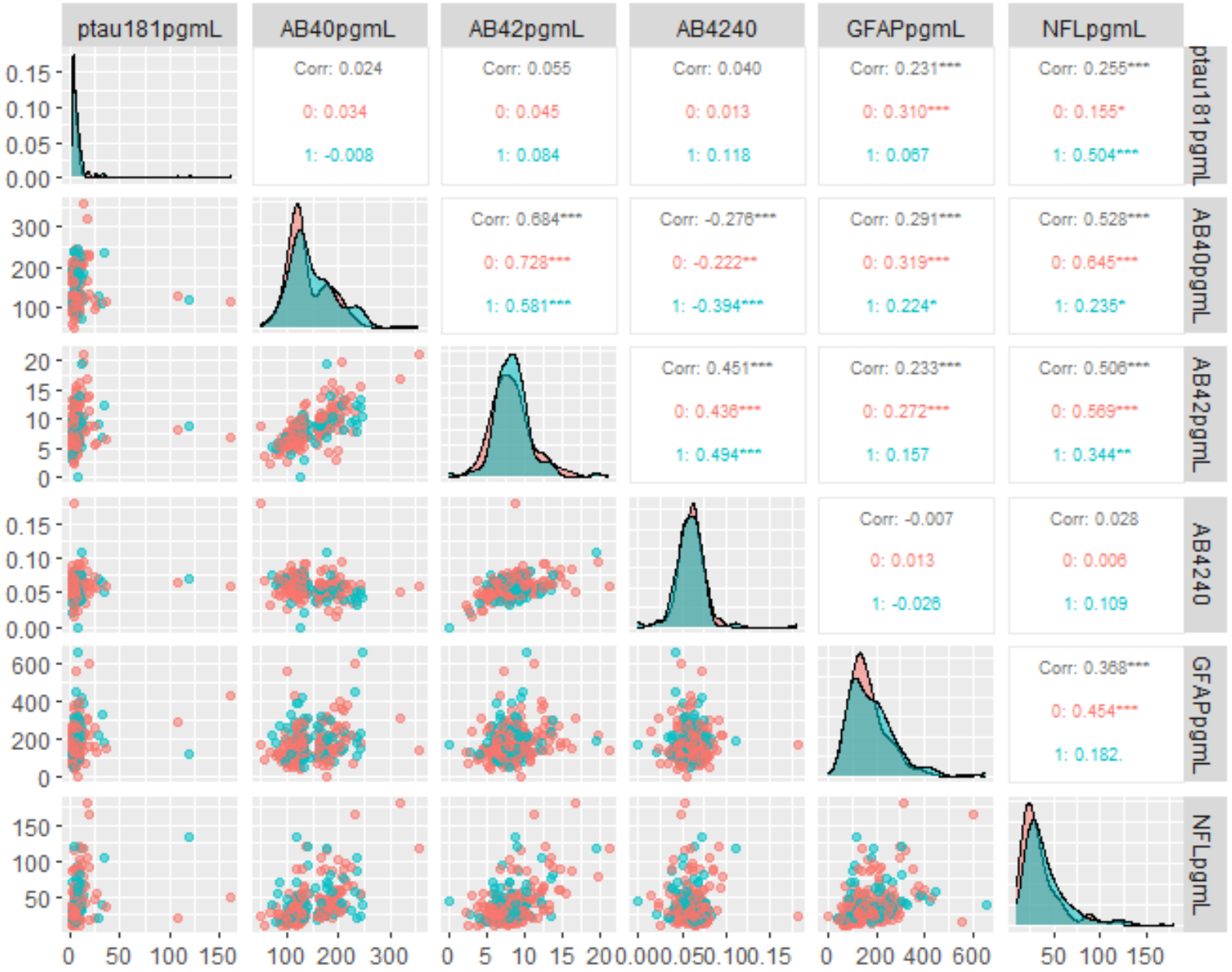
SUPPLEMENT FIGURE 1 LEGEND: Scatter + boxplots for plasma marker measures, including pTau181, AB40, AB42, AB42/40, GFAP, and NFL, separated by APOE ε4 carrier status. NOTE: graphs cut off for pTau at <100 pgmL (n=5 outliers, range: 100-500 pg/mL) and for NFL at <300 pg/mL (n=1 outlier, 343 pg/mL)



SUPPLEMENT FIGURE 2 LEGEND: Associations of individual plasma marker with each other, separated by cognitive imairment status. Excluding 1 extreme outlier in ptau (>400 pg/mL).



SUPPLEMENT FIGURE 3 LEGEND: KMEANS clustering (expectation maximization, bootstrapping 100 iterations for ideal solution: generates k=2 cluster solution. Gap statistic plot: shows optimal number of clusters (K=2) Shield plot: shows PCA dimension 1 vs dimension 2, colored by cluster assignment (k=2 & k=3 solutions) Silhouette plot, colored by cluster assignment, shows average silhouette width (0.38) Dendrogram: shows partitioning of individual observations, indicating degree of relationship, with 2 distinct, observable cl usters.