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RECEIVED 02 January 2024

ACCEPTED 08 March 2024

PUBLISHED 25 March 2024

CITATION

Weber DM, Taylor SW, Lagier RJ, Kim JC,
Goldman SM, Clarke NJ, Vaillancourt DE,
Duara R, McFarland KN, Wang W-e,
Golde TE and Racke MK (2024) Clinical utility
of plasma A β 42/40 ratio by LC-MS/MS in
Alzheimer's disease assessment.
Front. Neurol. 15:1364658.
doi: 10.3389/fneur.2024.1364658

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Clinical utility of plasma A β 42/40 ratio by LC-MS/MS in Alzheimer's disease assessment

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Introduction: Plasma A β 42/40 ratio can help predict amyloid PET status, but its clinical utility in Alzheimer's disease (AD) assessment is unclear.

Methods: A β 42/40 ratio was measured by LC-MS/MS for 250 specimens with associated amyloid PET imaging, diagnosis, and demographic data, and for 6,192 consecutive clinical specimens submitted for A β 42/40 testing.

Results: High diagnostic sensitivity and negative predictive value (NPV) for A β -PET positivity were observed, consistent with the clinical performance of other plasma LC-MS/MS assays, but with greater separation between A β 42/40 values for individuals with positive vs. negative A β -PET results. Assuming a moderate prevalence of A β -PET positivity, a cutpoint was identified with 99% NPV, which could help predict that AD is likely not the cause of patients' cognitive impairment and help reduce PET evaluation by about 40%.

Conclusion: High-throughput plasma A β 42/40 LC-MS/MS assays can help identify patients with low likelihood of AD pathology, which can reduce PET evaluations, allowing for cost savings.

KEYWORDS

Alzheimer's disease, beta-amyloid, blood biomarkers, LC-MS/MS, PET, prescreening

1 Introduction

Alzheimer's disease (AD) pathophysiology is characterized by cognitive impairment and the accumulation of extracellular beta-amyloid (A β) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein in brain tissue (1–3). Because A β aggregation and deposition occurs 10–20 years prior to clinical presentation, plaque detection reflects an underlying pathophysiologic process at a prodromal or pre-clinical disease stage (4–6) and a potential opportunity for early intervention and treatment. In addition, ruling out AD can prompt investigation of non-AD causes of dementia and cognitive decline (7).

Recently, the United States Food and Drug Administration (FDA) approved the first disease-modifying treatments for AD, using monoclonal antibodies targeting A β aggregates and removing them from the brain. Administering monoclonal antibodies that target A β slowed the rate of functional and cognitive decline among patients with AD in the mild cognitive impairment (MCI) or mild dementia stage. Presence of A β protein was assessed using positron emission tomography (PET) (8, 9). PET and measurement of cerebrospinal fluid (CSF) beta-amyloid 42 (A β 42) (10, 11) are methods that have been used as entry criteria for clinical trials and/or as outcome measures for disease-modifying AD treatments (12).

Blood-based biomarkers, including the plasma beta-amyloid ratio (A β 42/40), may guide, complement, or be alternatives to PET and CSF testing. However, despite its lower cost and decreased invasive nature, routine clinical use of plasma A β 42/40 testing has been suggested to have substantial challenges (13).

Accurately determining plasma A β 42/40 ratio depends on a high degree of assay robustness, which is characterized by minimal preanalytical errors, imprecision, and bias. Lack of robustness can potentially lead to a high risk for misclassification, particularly when differences in the A β 42/40 ratio are small between amyloid PET-positive and PET-negative individuals. Recently, the Elecsys electrochemiluminescence (ECL) immunoassay was evaluated for robustness using specimens from a cohort with about a 10% difference in A β 42/40 means between amyloid PET-positive and PET-negative individuals. A simulated 10% imprecision led to a misclassification rate of 26% and a simulated 10% bias (22% bias in total if A β 42 and A β 40 values drifted in opposite directions) led to almost all PET-positive individuals being reclassified as PET-negative (13). Despite these concerns, no liquid chromatography–tandem mass spectrometry (LC-MS/MS) platform has been fully evaluated for assay robustness (13), even though head-to-head comparisons have shown superior diagnostic accuracy for the best performing LC-MS/MS assays vs. immunoassays in identifying amyloid PET status (13, 14).

We developed and validated a protein immunoprecipitation (IP)-LC-MS/MS assay for the detection of plasma A β 40 and A β 42 (15). Here we assessed the clinical performance of using the A β 42/40 ratio to identify amyloid PET status in a well-characterized cohort that included age-matched A β PET-positive and A β PET-negative individuals characterized as AD, MCI, or healthy controls with *APOE* genotype inferred from apolipoprotein E (ApoE) proteoform. Part of the assessment included examining the effect of imprecision and bias estimates on classifying associated risk for PET positivity (13). Based on these performance characteristics, we evaluated incorporating A β 42/A β 40 results into AD assessment using a limited data set from 6,192 specimens submitted for A β 42/A β 40 ratio testing to Quest Diagnostics.

2 Methods

2.1 Study design and clinical assessments

This cross-sectional study involved 250 participants who provided plasma specimens at the 1Florida Alzheimer's Disease Research Center (ADRC cohort). The study was approved by the Mount Sinai Medical Center IRB, and all participants provided informed consent. Participants were between the ages of 50 and 95 years, had a minimum

6th grade reading comprehension level, and spoke English or Spanish as their primary language. Participants were excluded from the study if they had significant visual or auditory deficits or non-AD medical or psychiatric illness.

Information regarding concurrent medications for AD participants during initial and annual follow-up visits was obtained in accordance with the National Alzheimer's Coordinating Center's (NACC) Uniform Data Set version 3 (UDSv3) protocol.¹ Most were taking a cholinesterase inhibitor, namely donepezil, galantamine or rivastigmine. A few AD participants were also taking memantine. Anti-amyloid therapies were not available at time of specimen collection.

In the ADRC cohort, all participants underwent clinical assessments including an extensive medical, neurological, psychiatric, and neuropsychological evaluation. Assessments included the clinical dementia rating (CDR) (16) and the mini-mental state exam (MMSE) (17), and cognitive diagnoses were carried out using a previously reported algorithmic procedure (18). Participants were categorized as healthy controls (HC), having mild cognitive impairment (MCI), or AD (19). The cohort included 72 HC (ADRC-HC; 5 A β positive by PET [A β -PET+] and 67 negative [A β -PET-] by PET), 124 with MCI (ADRC-MCI; 42 A β -PET+ and 82 A β -PET-), and 54 with AD (ADRC-AD; all A β -PET+). Participant demographics are summarized in Table 1; Supplementary Table S1. AD participants were further subclassified using CDR (4.5 to 9, mild dementia, 9.5 to 15.5, moderate dementia; ≥ 16 , severe dementia) (16) and MMSE (≥ 25 , cognitively unimpaired; 20 to 24, mild cognitive impairment; 10 to 19, moderate cognitive impairment; 0 to 9, severe cognitive impairment) (17) score-based cutoffs for severity of cognitive dysfunction.

In addition, we performed a retrospective analysis of 6,192 consecutive plasma specimens submitted to Quest Diagnostics for A β 42/40 ratio testing. This was a limited data set² with only patient sex and age information retained. Participant demographics are summarized in Table 2.

2.2 Assessment of amyloid positivity from amyloid PET scans

Qualitative and quantitative analysis of amyloid positivity by visual reads and standardized uptake value ratio (SUVR) data have previously been described (19). All ADRC participants underwent an amyloid PET scan within 12 months of plasma collection. Of these individuals, 205 had SUVR computed using 2 different tracers: [¹⁸F]-florbetapir ($n=41$, cutoff >1.11) and [¹⁸F]-florbetaben ($n=164$, cutoff >1.40). SUVR values were transformed into a binary scale (A β -PET+ or A β -PET-) based on SUVR cutoffs. However, visual reads were used as the gold standard for amyloid positivity designations (19) whenever there were conflicting data between SUVR cutoffs and visual reads. Amyloid PET statuses for the remaining 45 individuals were determined by visual reads alone (19, 20).

1 <https://naccdata.org/data-collection/forms-documentation/uds-3>

2 https://privacyruleandresearch.nih.gov/pr_08.asp

2.3 Plasma beta-amyloid assay

Blood specimens were collected by venipuncture into 10-mL tubes containing EDTA as anticoagulant, kept on ice (<1 h) until

TABLE 1 Alzheimer’s Disease Research Center (ADRC) cohort participant characteristics by amyloid positron emission tomography (Aβ PET) status.

Characteristic	Aβ PET– [N = 149]	Aβ PET+ [N = 101]
Age (years) (p=0.150)		
Mean (SD) [N]	71.6 (7.5) [149]	73.1 (8.5) [101]
Sex (p=0.794)		
% female [N]	58.4% [87]	60.4% [61]
Ethnicity (p=1.000)		
% Hispanic [N]	59.1% [88]	58.4% [59]
Education (p=0.234)		
Mean years (SD)	15.6 (3.2)	15.1 (3.2)
MMSE (P<0.001)		
% MMSE ≥ 25 [N]	94.0% [140]	57.4% [58]
Mean (SD) [N]	28.4 (2.1) [149]	23.6 (6.2) [101]
CDR (P<0.001)		
% CDR>0 [N]	66.4% [99]	92.1% [93]
Plasma Aβ42/40 (P<0.001)		
Mean (SD) [N]	0.173 (0.029) [149]	0.141 (0.017) [101]
Plasma Aβ42 (P<0.001)		
Mean (SD) [N]	68.3 (17.4) [149]	58.4 (13.6) [101]
Plasma Aβ40 (p=0.079)		
Mean (SD) [N]	394.5 (74.7) [149]	411.5 (74.5) [101]
ApoE4+, % (P<0.001)		
% [N]	20.8% [31]	56.4% [57]
¹⁸ F-florbetaben SUVR (P<0.001)		
Mean (SD) [N]	1.020 (0.092) [93]	1.469 (0.187) [71]
¹⁸ F-florbetapir SUVR (P<0.001)		
Mean (SD) [N]	1.042 (0.068) [26]	1.455 (0.238) [15]

Aβ, beta-amyloid; ApoE4, apolipoprotein E4 proteoform; CDR, Clinical dementia rating; MMSE, Mini-mental state exam; SUVR, standardized uptake value ratio.

centrifugation at 1,200 relative centrifuge force for 12 min at room temperature. Aliquots of plasma (0.5mL) were transferred into polypropylene tubes and stored at –80°C until analysis. All plasma specimens were deidentified, and results were blinded during the analysis.

A detailed description of the development and validation will be reported elsewhere (15). Briefly, 0.5 mL of calibrators, quality control (QC) samples, and plasma specimens were diluted with 0.5 mL of PBS and 0.1 mL of 1% Tween-20/CHAPS (v/v). Internal standard for Aβ40 (uniformly ¹³C/¹⁵N labeled) and Aβ42 (uniformly ¹⁵N labeled) were added to each sample, and Aβ40 and Aβ42 were simultaneously immunoprecipitated, proteolytically digested using the enzyme Lys-C, and desalted and concentrated using a mixed-mode anion exchange solid-phase extraction (SPE) plate (Waters, Milford, MA). All sample preparation steps were automated using a Hamilton Star liquid handler (Hamilton, Reno, NV). Digested samples (70 μL) were injected onto a XBridge Protein BEH 300 Å C4 column (Waters, Milford, MA) and separated using a Transcend Vanquish TLX-4 TurboFlow UPLC (ThermoFisher Scientific, Waltham, MA) using a staggered 4-column configuration to facilitate high throughput. Both peptides were chromatographically separated using a 16-min gradient at a flow rate of 0.6 mL/min of solvent A (water with 0.15% formic acid) and solvent B (acetonitrile with 0.15% formic acid) with a 2-min acquisition window. Detection was achieved using a TSQ Altis Plus Triple Quadrupole MS (ThermoFisher Scientific, Waltham, MA) operated in multiple-reaction monitoring (MRM) mode, and data was collected every 4 min using the described staggered 4-column configuration. Each 96-well plate consisted of an 8-point calibration curve for Aβ40 and Aβ42, two sets of 4 quality control samples, and 79 patient samples. The ratio of the peak area of the analyte to the internal standard was used to calculate the concentrations from the standard curve using TraceFinder Clinical Research v5.1 software (ThermoFisher Scientific). The ratio of Aβ42 to Aβ40 was determined by taking the back-calculated value for Aβ42 and dividing it by the back-calculated value for Aβ40.

Analytical validation studies, including assay precision (within-run and between-run), analytical measurement range (AMR), analytical sensitivity (limit-of-blank [LOB], limit-of-detection [LOD], and limit-of-quantification [LOQ]), interference testing, and stability were conducted according to CLSI guidelines (21–25).

For this study, we determined average inter-assay (between-run) imprecision by taking the average of 4 quality control (QC) samples

TABLE 2 Demographics for 6,192 clinical specimens^a submitted for Aβ42/40 testing.

Age, years	N total, % female	Aβ42 mean, pg/mL (SD)	Aβ40 mean, pg/mL (SD)	Aβ42/40 mean (SD)	Aβ42/40 < 0.160, N, % (indeterminant/high risk of PET positivity)	Aβ42/40 ≥ 0.170, N, % (likely PET negative)
<40	83, 65%	38.7 (8.3)	207.3 (43.5)	0.188 (0.021)	3, 4%	69, 83%,
40 to 49	162, 62%	38.2 (8.6)	210.4 (42.4)	0.182 (0.023)	20, 12%,	116, 72%,
50 to 59	582, 63%	40.5 (10.6)	228.7 (57.2)	0.178 (0.024)	138, 24%,	369, 63%,
60 to 69	1,370, 58%	40.6 (10.5)	241.7 (58.5)	0.169 (0.023)	497, 36%	631, 46%,
70 to 79	2,451, 57%	42.9 (11.9)	264.7 (71.0)	0.163 (0.022)	1,157, 47%	863, 35%,
80 to 89	1,417, 54%	45.5 (12.5)	284.9 (77.8)	0.161 (0.021)	713, 50%	436, 31%,
≥ 90	127, 60%	48.1 (10.7)	304.5 (74.0)	0.159 (0.019)	68, 54%,	32, 25%,
All	6,192, 57%	42.7 (11.7)	259.4 (71.3)	0.166 (0.023)	2,596, 42%,	2,516, 41%,

Aβ, beta-amyloid; PET, positron emission tomography. ^aThis was a limited dataset with only patient age and sex available.

run in duplicate over 25 consecutive days (Weber et al. submitted; ranges were 163–619 pg/mL A β 40, 40–188 pg/mL A β 42, and 0.124–0.304 A β 42/40; a non-physiological high QC value was excluded) (15).

Analytical robustness and the effects of reclassification bias were experimentally determined by reanalyzing previously tested samples using a different operator and a different lot of reagents and calibrators. A total of 196 plasma specimens were randomly selected and reanalyzed over the course of 1 month. Both freeze/thaw and storage stability were considered when selecting specimens for reanalysis. Values for A β 40, A β 42, and the A β 42/40 ratio were compared as a percentile difference calculated between the mean values of the original results and the mean values of the retest results to establish “measured bias.”

2.4 Statistical analysis

Two-sample *t*-tests were used to evaluate differences in baseline continuous measures across PET status. Differences in categorical variables were evaluated by Fisher’s exact test. Comparisons between groups with more than two outcomes were performed using one-way analysis of variance (ANOVA) for continuous variables and Fisher’s exact tests for categorical variables. Effect size for means were determined by eta-square measurements. Statistically significant results from ANOVA were followed by post-hoc analysis using Tukey multiple pairwise-comparisons between group means. The performance of the A β 42/40 ratio on classification of amyloid PET status was evaluated by logistic regression and ROC curve analysis. ROC-AUC 95% confidence intervals (CI) and comparisons between ROC curves were determined using the DeLong method (26). Optimal cutoffs for sensitivity and specificity from ROC analyses were determined by Youden’s (27). Correlation of the A β 42/40 ratio and PET SUVR were assessed by Spearman’s rho. Robustness simulations were based on the methodology described in Rabe et al. (13).

To account for the potential underestimation of variability in the measure of the A β 42/40 ratio in this study, 10,000 simulations of the A β 42 and A β 40 responses were generated with an added 10% CV [per Rabe et al. (13)] and 6% measured CV from a scaled standard normal distribution. For each simulated pair of markers with added noise, an A β 42/40 ratio was calculated. The rate of reclassification around the 0.160 A β 42/40 threshold from the original observed A β 42/40 ratio to the simulated noise-added ratio was calculated. The average reclassification rate was estimated as the mean of the 10,000 simulated reclassification rates. A 95% CI for the mean reclassification rate was estimated by the 2.5th and 97.5th percentiles of the simulated reclassification rates.

To estimate the effects of potential bias in the measure of A β 42 and A β 40 on the performance characteristics of the A β 42/40 ratio, per Rabe et al. (13), we evaluated a 10% increase in A β 42 response and a 10% decrease in the A β 40 response for a total A β 42/40 bias of 22% (1.1/0.9 = 1.22). The measured bias was similarly assessed. Performance characteristics were calculated for both the observed A β 42/40 ratio and the biased A β 42/40 ratio. Joint 95% CI were obtained for sensitivity/specificity and PPV/NPV on classification of PET status (28). All CI for performance characteristics were obtained by non-parametric bootstrap. All analyses were conducted using R (version 4.2.1).

3 Results

3.1 ADRC cohort participant demographics: PET status and cognitive outcomes

Compared to ADRC A β -PET– individuals, A β -PET+ individuals were more likely to be *APOE4* carriers, have lower MMSE scores, and more likely to have CDR scores greater than 0 ($p < 0.001$ for all; Table 1). No statistically significant differences ($p > 0.05$) between the A β -PET– and the A β -PET+ group were observed in age, sex, percentage of individuals identifying as Hispanic, or years of education (Table 1).

When categorized by a cognitive diagnosis, from HC to MCI to AD (Supplementary Table S1; Supplementary Figure S1A), we observed lower MMSE scores among MCI and AD patients compared to HC ($p < 0.001$; Supplementary Table S1). In addition, the frequency of *APOE4* carriers was greater in the ADRC HC A β -PET+, MCI A β -PET+, and AD A β -PET+ groups compared to the HC A β -PET– and MCI A β -PET– group ($p < 0.001$). The ADRC-MCI A β -PET– group had a lower percentage of female participants relative to the other groups ($p = 0.039$; Supplementary Table S1). There were no statistically significant differences in PET status based on age, patient education, or percentage of individuals identifying as Hispanic.

In distinguishing a cognitive diagnosis of AD vs. HC, the AUC for the A β 42/40 ratio was 0.91 (95% CI = 0.86 to 0.97). At the A β 42/40 ratio classification threshold of 0.160, sensitivity was 96% (95% CI = 91 to 100%) and specificity was 83% (95% CI = 75 to 92%; Supplementary Figure S1B).

Among the 54 AD participants, MMSE scores increased as the A β 42/40 ratio increased, and CDR scores increased as A β 42/40 decreased; however, the trend did not reach statistical significance ($p > 0.05$; Supplementary Figure S2). To establish a stronger relationship between the A β 42/40 ratio and cognitive dysfunction, a more uniform distribution of AD subjects across the cognitive dysfunction classes, particularly those with severe dementia (represented by only 4 individuals), is required.

3.2 Performance and robustness of plasma A β 42/40 ratio for identifying amyloid PET status

Overall, for the 250 individuals with PET data (quantitative and qualitative reads), plasma A β 42 concentrations and A β 42/40 ratios were significantly lower ($p < 0.001$) in ADRC A β -PET+ individuals compared with A β -PET– individuals, with no significant differences in A β 40 concentrations (Table 1; Figure 1A). Using ROC analysis and the maximum of Youden’s J index, a plasma A β 42/40 cutoff ratio of 0.160 had an AUC of 0.84 (95% CI = 0.79 to 0.89, Figure 1B) with 91% sensitivity, 76% specificity, and overall accuracy of 82% (Table 3). Based on a 40% prevalence of amyloid positivity in the ADRC cohort (40.4% observed), we found a positive predictive value (PPV) of 72% and a negative predictive value (NPV) of 93% at the 0.160 cutpoint (Table 3). Based on a 34% prevalence of amyloid positivity in the patients in the ADRC cohort with MCI, which may be more reflective of the target population, PPV decreased to 56% and NPV decreased to 90% at the 0.160 cutpoint (Table 3) when HC and AD patients were excluded.

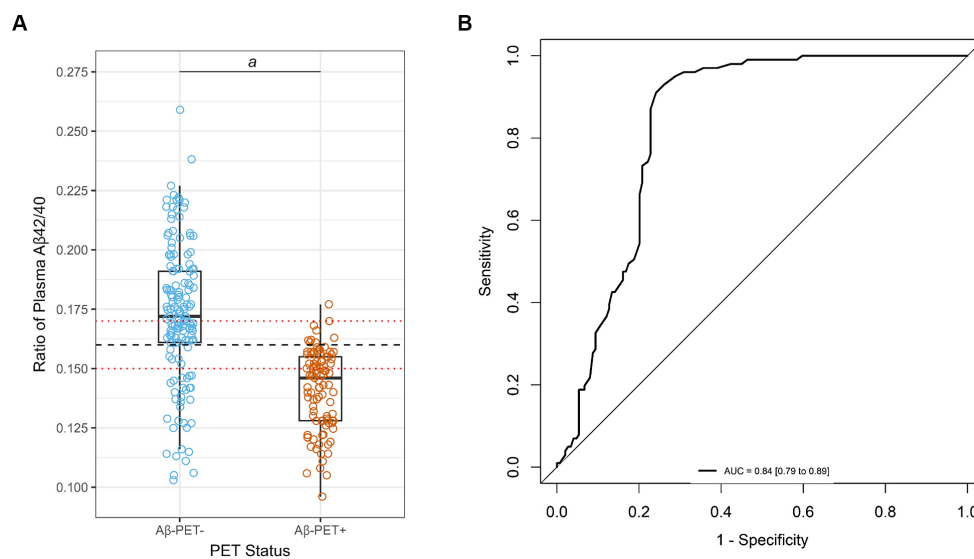


FIGURE 1 Correlation and diagnostic performance of the Aβ42/40 ratio and amyloid PET imaging. **(A)** Plasma Aβ42/40 ratio compared with amyloid PET status (Aβ-PET- and Aβ-PET+). Black dashed line denotes optimal Aβ42/40 ratio cutoff of 0.160. Red dotted lines denote Aβ42/40 ratio indeterminant risk cutoffs (0.150 and 0.170); a = significant at $p < 0.001$; **(B)** ROC-AUC of the plasma Aβ42/40 ratio for prediction of amyloid PET positivity. AUC, area under the curve; PET, positron emission tomography; ROC, receiver operating characteristic.

TABLE 3 Performance characteristics and the effects of prevalence and bias for detecting positron emission tomography (PET) status in the Alzheimer’s Disease Research Center (ADRC) cohort.

Plasma Aβ42/40 Cutpoint ^a	Prevalence (%)	Bias (%)	Sensitivity (95%, CI)	Specificity (95%, CI)	Accuracy (95%, CI)	PPV (95%, CI)	NPV (95%, CI)
0.160	40	None	0.911 (0.842–0.970)	0.758 (0.678–0.832)	0.820 (0.772–0.864)	0.719 (0.656–0.788)	0.926 (0.876–0.974)
0.160	34 ^b	None	0.857 (0.738–0.976)	0.659 (0.537–0.768)	0.726 (0.645–0.798)	0.563 (0.478–0.661)	0.900 (0.820–0.980)
0.160	34 ^b	22 ^c	0.333 (0.167–0.500)	0.854 (0.756–0.939)	0.677 (0.605–0.742)	0.538 (0.346–0.737)	0.714 (0.663–0.771)
0.160	34 ^b	2 ^d	0.762 (0.619–0.905)	0.671 (0.549–0.780)	0.702 (0.621–0.782)	0.542 (0.446–0.649)	0.846 (0.762–0.932)
0.170	40	None	0.990 (0.960–1.000)	0.537 (0.443–0.624)	0.720 (0.672–0.768)	0.592 (0.546–0.643)	0.988 (0.953–1.000)
0.170	34 ^b	None	0.976 (0.905–1.000)	0.476 (0.354–0.598)	0.645 (0.573–0.718)	0.488 (0.433–0.554)	0.975 (0.907–1.000)
0.170	34 ^b	22 ^c	0.429 (0.262–0.595)	0.829 (0.732–0.915)	0.694 (0.621–0.766)	0.563 (0.400–0.732)	0.739 (0.681–0.802)
0.170	34 ^b	2 ^d	0.929 (0.833–1.000)	0.537 (0.415–0.659)	0.669 (0.589–0.742)	0.506 (0.441–0.583)	0.936 (0.851–1.000)

Aβ, beta-amyloid; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value. ^a0.160 (maximum of Youden’s J index) and 0.170 (likely PET-negative), bold = performance based on measured prevalence of PET positivity in full ADRC cohort. ^bAmyloid positivity by PET in the ADRC cohort—MCI group only. ^c+10% bias in Aβ42 and –10% bias in Aβ40 = 22% total bias per Rabe, et al. (13). ^d+2% total measured bias in the LC-MS/MS assay.

The difference in means between the PET+ and PET– Aβ42/40 ratio was about 18% with substantial shift of Aβ PET+ to PET– status after applying 10% bias (22% total bias if Aβ42 and bias in Aβ40 shifted in opposite directions, Figure 2A) per Rabe et al. (13). This level of bias would substantially diminish the PPVs and NPVs of this assay (Table 3). However, the measured bias in this LC-MS/MS assay was 11.5% (95% CI = 9.5% to 13.6%) for Aβ42, 10.6% (95% CI = 9.0% to 12.6%) for Aβ40 (both in the same direction), and 0.7% (95% CI = –0.3% to 1.8%) for Aβ42/40 ratio. Accordingly, we used a higher estimate (upper 95% limit of the ratio CI rounded to 2%) to reflect worst-case effects of bias (Figure 2A). PPVs and NPVs are much less affected under these conditions (Table 3).

Assuming an estimated imprecision of 10% per Rabe et al. (13), we simulated a reclassification rate of 22% (95% CI = 18% to 27%) in

the ADRC cohort at a cutpoint of 0.160 (Figure 2B, upper scatterplot). However, measured mean inter-assay imprecision, calculated by taking the average imprecision across 5 study samples analyzed in duplicate over 25 days, was closer to 6% (15). Using this measured precision, the reclassification rate by simulation is 15% (95% CI = 11% to 19%; Figure 2B lower scatter plot).

Based on 6% assay imprecision, we propose cutpoints of 0.150 and 0.170 as defining indeterminate risk of amyloid PET positivity, constituting 30% of the ADRC cohort (Figure 2B; Table 4). The 0.170 cutpoint has an NPV of 99% in the ADRC cohort for ruling out amyloid positivity (98% in the target population with MCI, 94% when adding measured bias, Table 3). Large differences were observed within the ranges defined by indeterminate risk in the ADRC cohort including about 4-fold higher PET positivity and ApoE4 proteotype

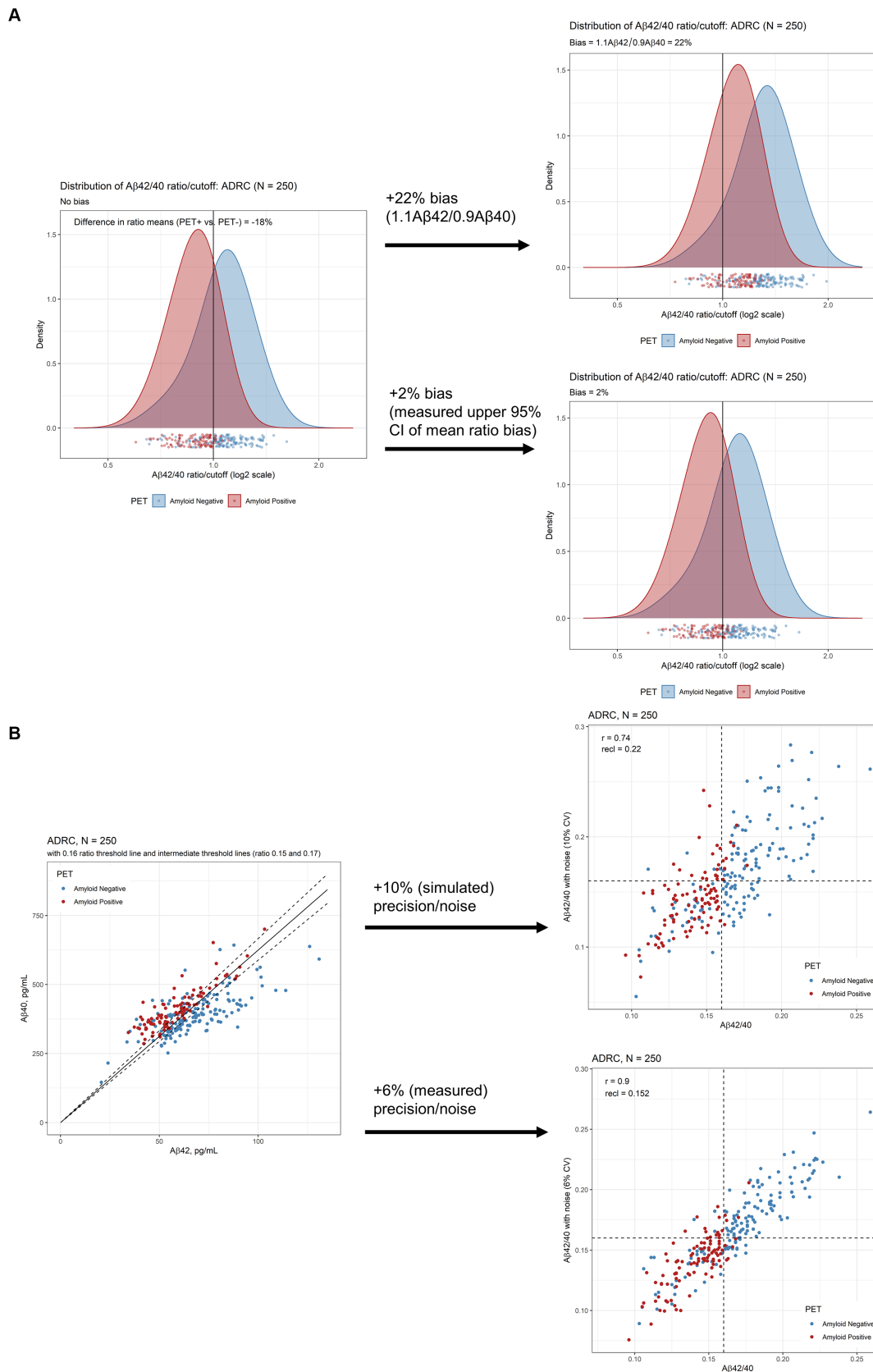
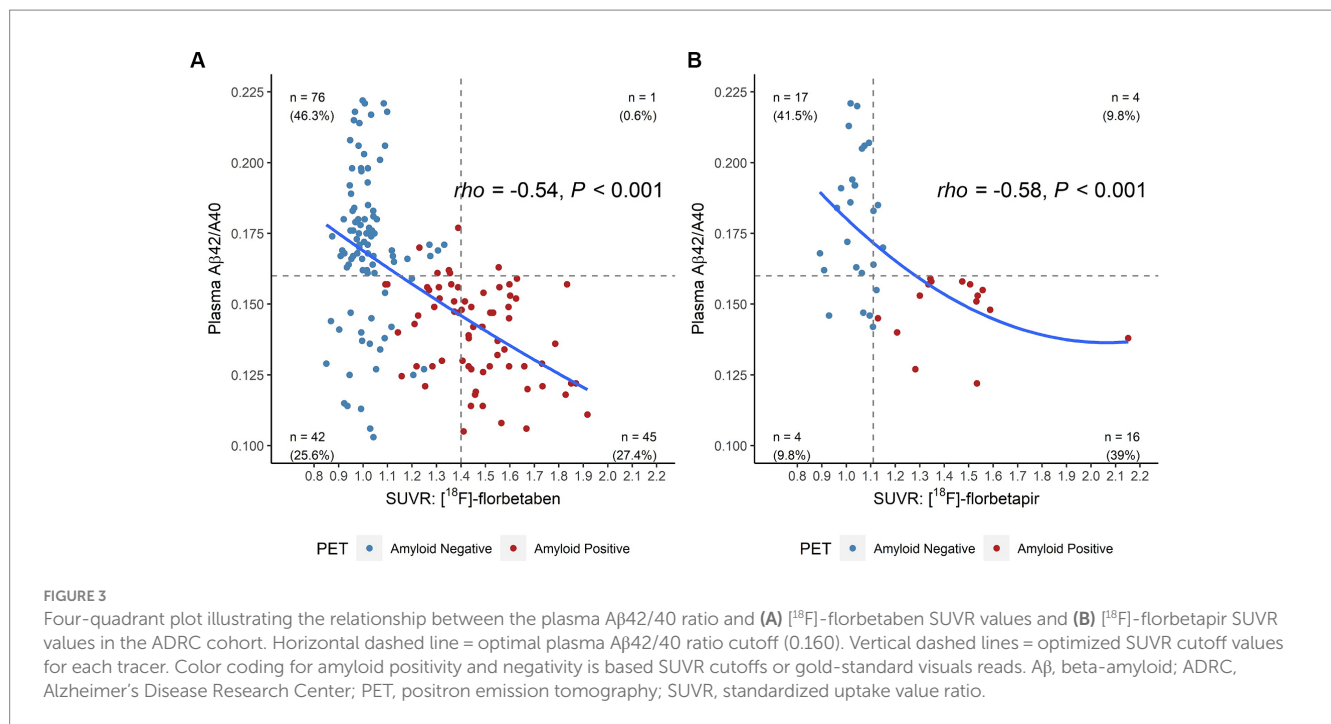


FIGURE 2
Robustness assessment of A β 42/40 ratio for predicting amyloid PET imaging results. **(A)** Densities of plasma A β 42/40 ratio by LC-MS/MS in ADRC cohort with and without 10% bias (22% total, upper plot) and 2% measured bias (lower plot) added. **(B)** Scatterplots of A β 42 and A β 40 to illustrate proximity to cutoffs defining indeterminate risk and scatterplots of A β 42/40 ratio with and without 10% CV added noise (upper plot) and 6% added CV (measured) noise (lower plot). A β , beta-amyloid; ADRC, Alzheimer's Disease Research Center; CI, confidence interval; PET, positron emission tomography.

TABLE 4 Proposed plasma Aβ42/40 ranges for clinical decision making^a.

Plasma Aβ42/40 range	Designation	ADRC, N (% of cohort)	PET positivity, N (%)	MCI, N (%)	AD, N (%)	ApoE4, N (%)	Real world, N (% of cohort)	Real world, projections of PET positivity, N ^b	Potential next evaluation and management considerations
<0.150	Positive (low ratio)	91 (36.4)	61 (67.0)	50 (54.9)	34 (37.4)	43 (47.3)	1,468 (23.7)	984	Follow up for AD workup (high risk)
0.150–0.159	Indeterminant (lower ratio)	37 (14.8)	31 (83.8) ^c	14 (37.8)	18 (48.7)	25 (67.6)	1,128 (18.2)	945	Follow up for AD workup (indeterminant risk)
0.160–0.169	Indeterminant (higher ratio)	38 (15.2)	7 (18.4)	19 (50.0)	2 (5.3)	7 (18.4)	1,080 (17.4)	199	Follow up for AD workup (low risk)
≥0.170	Negative (high ratio)	84 (33.6)	2 (2.4)	41 (48.8)	0 (0.0)	13 (15.5)	2,516 (40.6)	60	Follow up for non-AD etiology
All	All	250	101 (40.4)	124 (49.6)	54 (21.6)	88 (35.2)	6,192	2,188	

Aβ, beta-amyloid; AD, Alzheimer's disease; ApoE4, apolipoprotein E4 proteoform; MCI, mild cognitive impairment. ^aPercentage of individuals falling within a range unless otherwise specified. ^bReal world projections of positron emission tomography (PET) positivity are based on percent PET positivity in the Alzheimer's Disease Research Center (ADRC) cohort. ^cNo statistically significant difference was observed between the percentage of PET positivity in the <0.150 and 0.150–0.159 groups ($p=0.090$ by 2-sample test for equality of proportions with continuity correction).



(APOE4 genotype) and 9-fold higher AD diagnosis, in the 0.150–0.159 range vs. the 0.160–0.169 range (Table 4).

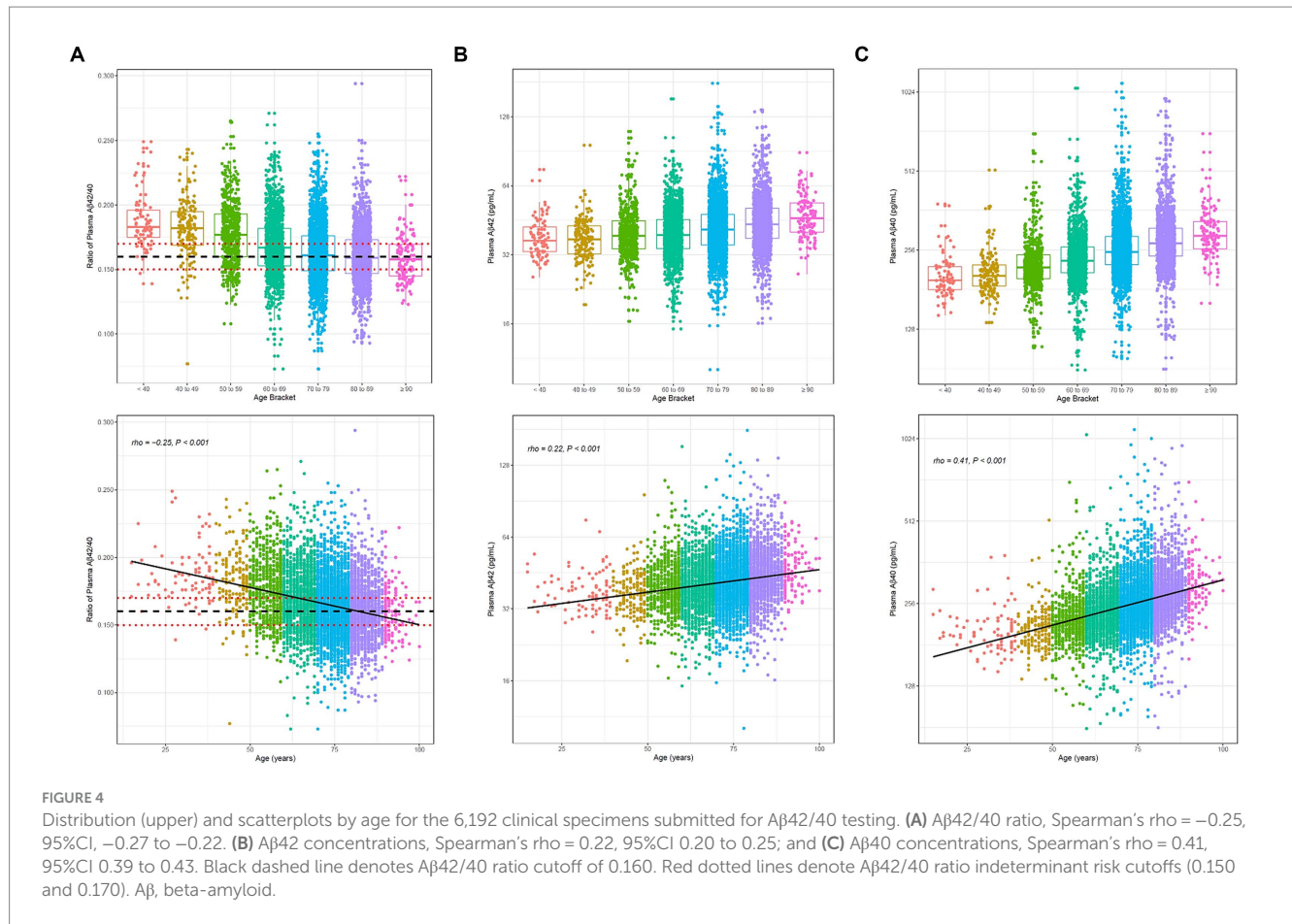
For the 205 individuals for whom we had quantitative PET data, 5 [18F]-florbetapir positive results (range 1.11 to 1.15) were reclassified as negative and 25 [18F]-florbetaben negative results (range 1.09 to 1.39) were reclassified as positive based on visual reads. After reclassification, the concordance of the Aβ42/40 ratio increased from 80 to 88% for [18F]-florbetapir and from 74 to 83% [18F]-florbetaben. Overall concordance between Aβ42/40 ratio and SUVR data changed from 75% before to 84% after reclassification based on visual reads.

Both tracers showed similar results when plotted against the Aβ42/40 ratio (Figures 3A,B). We observed a significant inverse relationship between the Aβ42/40 ratio and quantitative SUVR values

for [18F]-florbetaben PET (Spearman's rho of -0.54 [95% CI = -0.65 to -0.42 , $p < 0.001$], Figure 3A) and [18F]-florbetapir PET (Spearman's rho of -0.58 [95% CI = -0.76 to -0.32 , $p < 0.001$], Figure 3B).

3.3 Plasma Aβ42/40 ratio and clinical specimens

A significant inverse relationship between age and Aβ42/40 ratio was observed for the 6,192 clinical specimens (Spearman's rho = -0.25 , 95% CI = -0.27 to -0.23 , $p < 0.001$; Figure 4A; Table 2), contrasting with the increases of plasma Aβ42 and Aβ40 concentrations with age (Figures 4B,C; Table 2). Indeterminate results defined by cutpoints of



0.150 and 0.170 define 2,208 (35.7%) almost evenly split between individuals with ratios from 0.150 to 0.159 and individuals with ratios from 0.160 to 0.169, but with the former group potentially representing a much higher percentage of PET-positivity based on the ADRC cohort (Table 4). Using a 0.170 cutpoint to indicate the likelihood of PET negativity, we identified 2,516 individuals for whom a PET scan/CSF would be potentially unnecessary. As expected, among these individuals, the percentage who are likely PET negative decreases with age from <40 years (83%) to ≥90 years (25%; Table 2).

4 Discussion

4.1 Assay performance, robustness, and clinical decision making

Our data support the use of plasma Aβ42/40 ratio by LC-MS/MS to help predict a low likelihood of PET-positivity in AD assessment. In the ADRC group, with an Aβ42/40 ratio cutpoint of 0.170, we observed a 99% NPV. Although previous studies have cast doubt on the clinical utility of plasma Aβ42/40 assays based on misclassification potential, the only detailed analysis was provided for the Elecsys Aβ42 and Aβ40 electrochemiluminescence assay. LC-MS/MS assays, although acknowledged to have better discriminatory performance as assessed by ROC-AUC analysis, were said to have similar issues in terms of narrow analytical ranges and fold changes

between Aβ-PET+ and PET- individuals yielding insufficient robustness for clinical decision making (13).

We followed the example of Rabe et al. (13) by applying these tools to the results for the ADRC cohort obtained using our new LC-MS/MS assay. The ADRC cohort has a moderate prevalence of amyloid PET positivity (40%), within the range of 18% to 65% (Supplementary Table S2) previously reported for cohorts studied by LC-MS/MS (29–32).

Applying a 10% bias [in opposite directions, total 22% per Rabe et al. (13)] negatively impacted assay sensitivity, specificity, accuracy, NPV, and PPV (Table 3). However, applying a measured mean Aβ42/40 ratio bias (0.7%, worst case 2%) had a much lesser effect (Figure 2A), despite mean bias for each analyte being close in magnitude to the simulations for Aβ42 (~12%) and Aβ40 (~11%). Under real-world conditions, bias for each analyte drifted in the *same direction*, unlike the simulations, effectively canceling out its effect on the ratio.

Sources of bias that affect accurate determination of the Aβ42/40 ratio are those that negatively impact one analyte over the other, effectively skewing the ratio over time. For example, specimen storage at room temperature can result in differential loss of Aβ42 relative to Aβ40 (33), suggesting that Aβ42 is more susceptible to proteolytic cleavage, aggregation, or absorptive loss under these conditions. However, proper handling and storage mitigates these risks in the clinical laboratory. Immunoassays may be more susceptible to matrix effects that affect one analyte over the other compared with LC-MS/MS assays (14), wherein analytical losses and suppression are

corrected for using isotopically labeled internal standards. Errors in calibrator concentrations based on inaccurate supplier-provided peptide content can also cause bias, which we avoid by employing independent quantitative amino acid analysis of peptide calibrators (34). All these factors are impediments to standardization of these assays and may explain the high variability of A β 42/40 ratio across assays and laboratories (14).

Applying a simulated 10% imprecision per Rabe et al. (13) yielded a relatively high reclassification rate for individuals in our ADRC cohort (22%), similar to their immunoassay and the BioFINDER cohort (26%). However, measured imprecision was closer to 6%, yielding a reclassification rate of 15%. Although this still represents a substantial group of individuals, it should not unduly affect the clinical utility of this assay for identifying those with a low likelihood of PET-positivity.

Our clinical data set was obtained from 6,192 consecutively run A β 42/40 ratio specimens, presumably submitted by healthcare providers to help understand the cause of memory decline and dementia, and potentially help diagnose AD. The trends we observed in our data set, namely that plasma A β 42/40 decreased as plasma A β 42 and A β 40 concentrations increased with age, generally aligned with those recently reported in China for ~200 specimens obtained from apparently healthy, older (50–89 years) individuals (35). Age-dependent trends suggest that NPV could be influenced by age, especially for individuals under 60 years of age who tend to have higher A β 42/40 ratios (see below).

Based on cutpoints defining high, low, and indeterminant risk in the ADRC cohort, we suggest that plasma A β 42/40 ratio results can help guide clinical decision making (Table 4). Notably, the high cutpoint of 0.170 almost rules out PET-positivity with an NPV of almost 99% (acknowledging potential variations caused by prevalence and bias in Table 3). Projecting percentages of PET-positivity at this cutpoint in the ADRC cohort onto the 6,192 clinical specimens, the assay could potentially negate the need for PET testing in 2,516 patients (Table 4). Assuming a PET scan cost of \$5,000 in the United States (36), this approach saves \$12,580,000 before accounting for the cost of 6,192 LC-MS/MS analyses (at current list pricing ~\$550 USD) to identify these patients and 60 PET scans for the projected 2.4% patients with false negative results who would eventually need a PET scan. This represents a total savings of \$8.9 million or about \$1,432 per patient. In addition to younger (<60 years) patients, exceptions to this clinical decision making may include some patients with autosomal dominant AD and both amyloid precursor protein and presenilin-1 mutations who may have an elevated A β 42/40 ratio (37).

4.2 Assay performance comparisons

In keeping with previous studies, we found that the plasma A β 42/40 ratio was significantly lower in individuals who were A β -PET+ compared to those who were A β -PET-. Analytical performance characteristics were comparable to other mass spectrometry-based assays but with higher throughput on a lower-cost MS instrument and with a relatively large difference (18%) between A β 42/40 ratio means for PET+ and PET- individuals (Figure 2A) compared to most literature reports (Supplementary Table S2) (14, 30,

32, 38, 39). Robustness was comparable to the best performing LC-MS/MS assays for which precision and bias estimates were available (Supplementary Table S2) (13, 29).

Interestingly, the 0.160 A β 42/40 ratio cutoff optimized for maximum sensitivity and specificity for detecting PET positivity is identical to the optimized cutoff for differentiating HCs from individuals with AD, both in the current plasma study (Supplementary Figure S1) and in CSF (40). This suggests that the proportion of A β 40 and A β 42 is similar in CSF and plasma. Unfortunately, PET data were not available for the CSF study, and while both studies differentiated HC vs. AD participants, the specimens came from different cohorts and locations making performance comparisons difficult.

ROC-AUC analysis provides a means to make assay comparisons of clinical performance with other studies but with some major caveats. Head-to-head comparison using the same specimens from the same cohorts using the different methods are rare because they employ precious clinically characterized cohort specimens (14, 41). Otherwise, the cited performance is only relevant to the cohorts in which those assays were performed; differences in the prevalence of amyloid positivity, cognitive status, and PET-tracer cutoffs used among cohorts make these comparisons fraught as described by Brand et al.'s review of clinical performance of A β 42/40 in 21 publications (31). In addition, age, the number of APOE4 alleles, and race/ethnicity, can all impact the AUCs. In fact, these characteristics have been incorporated in an algorithmic approach to improve performance above and beyond the A β 42/40 ratio (discussed below).

Given the marked variation across cohorts in the literature, we compared our A β 42/40 clinical performance with recent studies having a similar prevalence of PET-positivity (~40%; Supplementary Table S3) (14, 30, 42). Our AUC of 0.84 compares favorably with the IP-LC-MS/MS assay of West et al. with an AUC of 0.81 (30) (best performance of 0.83 in a head-to-head comparison with other assays also at ~40% prevalence of PET-positivity) (14). This assay is similar to ours but employs prohibitively expensive high-resolution MS instrumentation that may not be accessible to many clinical laboratories; in contrast, our assay employs a low-resolution and relatively low-cost MS platform (Supplementary Table S2). In addition, concerns for assay robustness for the West et al. method comes from the small separation of A β 42/40 values between A β PET+ and A β PET- individuals in most reports using this methodology (8%–11%) (13, 14, 29, 30, 41); in contrast, we observed a larger 18% separation in the current study (Supplementary Table S2). However, measured bias (i.e., <1% "drift" A β 42/40 ratio values) was low and similar to the current study (29). Another important difference was that we used age-matched subjects, whereas the average age difference between A β PET+ and PET- cohorts in their study was about 6 years (30).

Similarly, Bun et al. recently used a chemiluminescence enzyme immunoassay to achieve an AUC of 0.95 (37% prevalence of PET-positivity) but again with a 6-year age difference between PET+ and PET- participants (Supplementary Table S3) (42). Age is a known risk factor for Alzheimer's disease (43), and in our clinical specimen cohort, we observed a significant difference in the mean A β 42/40 ratio between the 60- to 69-year age group and the 70- to 79-year age group (0.169 vs. 0.163, $p < 0.001$), irrespective of clinical outcomes or

amyloid PET status (Figure 4A). While age is known to improve model predictors for PET-positivity, this covariate is not reflected in the clinical performance for our aged-matched populations or, interestingly, Bun et al.'s more age-disparate populations. In addition, although the Bun et al. study had good precision (<4% for each analyte), the effects of bias on reclassification rates were not evaluated; (42) these effects can be marked in immunoassays, where separate A β 42 and A β 40 values can drift in opposite directions (13).

Similar to Bun et al. (42) but in contrast to many studies (29, 30, 32, 44), we found that the number of *APOE4* alleles did not substantially improve classification accuracy based on ROC analysis, possibly due to the demographics of our cohort. Previous studies included predominantly White and/or Asian populations (29, 30, 32, 44). Notably, the association between the *APOE4* allele and AD risk has been reported to be lower in Hispanic and Black non-Hispanic individuals than in White non-Hispanic individuals (45, 46). Our ADRC cohort had roughly 3-times the number of Hispanic individuals (59.1% of the A β PET+ and 58.4% of the A β PET- individuals) compared with similar studies that showed a significant improvement in AUC when *APOE4* status was included (30). Our findings are a cautionary note that algorithms incorporating A β 42/40 and *APOE4* allele status may not be generally applicable for all races and ethnicities.

4.3 Study limitations

We did not perform a detailed comparison with other biomarkers in the current study; instead, we focused on the utility of the plasma A β 42/40 ratio biomarker in isolation. However, in a separate investigation, we show that early AD-related pathological changes in the A β 42/40 biomarker were associated with quantifiable changes in brain microstructure and connectivity in A β -PET negative patients preceding deviations in other plasma biomarkers including t-tau, p-tau, neurofilament light chain (NFL), and glial fibrillary acidic protein (GFAP), and cortical atrophy (47).

We did not examine adding an additional plasma biomarker such as p-tau or GFAP. Some studies have combined plasma biomarkers to enhance performance in predicting clinical outcome. Addition of plasma p-tau with plasma A β 42/40 might enhance the diagnosis in patients with MCI, because plasma p-tau levels typically increase with disease progression (48). The combination of biomarkers could be used in patients with specific clinical or demographic characteristics to further help define individual outcomes (49). The combination of A β 42/40 and p-tau may better predict cognitive decline, but performance of the individual assays used would also play a role in how well the combination aids with this prediction (50). In addition, the combination of A β 42/40 and GFAP enhanced the likelihood of becoming p-tau positive, suggesting that this biomarker might distinguish AD patients with progressive features (51).

Other study limitations include a relatively small sample size for individuals with PET data and insufficient racial diversity (predominantly Hispanic) that may pose a potential bias to our results, especially when it comes to the effect of the *APOE4* allele on AD status. An unequal distribution of subjects across the AD dysfunctional classes (especially those with severe MMSE or CDR scores) prevented us from establishing firm A β 42/40 ratio cutpoints that may help

identify patients who may benefit from disease-modifying therapeutics. The lack of longitudinal plasma specimens from individuals that either converted from being A β -PET- to A β -PET+, or individuals that transitioned from being cognitively normal to MCI and AD limit our current understanding of the prognostic utility of the A β 42/40 ratio in monitoring disease progression.

5 Conclusion

Our IP-LC-MS/MS assay accurately identified individuals with positive amyloid PET and differentiated individuals diagnosed with AD from age-matched HC. These findings support the use of this blood-based assay for assessing presence of AD pathology in individuals with cognitive impairment and can help reduce PET evaluations of patients with low likelihood of AD pathology, allowing for more efficient allocation of limited imaging resources.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Mount Sinai Medical Center IRB. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

DW: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. ST: Writing – original draft, Writing – review & editing. RL: Formal analysis, Visualization, Writing – review & editing. JK: Methodology, Validation, Writing – review & editing. SG: Supervision, Writing – review & editing. NC: Conceptualization, Writing – review & editing. DV: Resources, Writing – review & editing. RD: Resources, Writing – review & editing. KM: Resources, Writing – review & editing. WW: Resources, Writing – review & editing. TG: Resources, Writing – review & editing. MR: Resources, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. Funding for

this work was provided by National Institutes of Health (NIH) Center Core Grant P30AG066506 (TG) and NIH Research Grants R01NS052318 and R01NS075012 (DV).

Acknowledgments

The authors acknowledge helpful comments from Lee Hilborne, Harvey Kaufman, Matt Stroh, and Andrew Hellman.

Conflict of interest

DW, ST, RL, JK, SG, NC, and MR were employees of Quest Diagnostics. DW and NC hold patents for the detection of beta-amyloid by mass spectrometry as well as for the detection of apolipoprotein E proteoforms by mass spectrometry.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fneur.2024.1364658/full#supplementary-material>

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