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Evaluation of Blood-Based Plasma Biomarkers as Potential Markers of Amyloid Burden in Preclinical Alzheimer's Disease

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Abstract.
Background: Participant eligibility for the A4 Study was determined by amyloid PET imaging. Given the disadvantages of amyloid PET imaging in accessibility and cost, blood-based biomarkers may serve as a sufficient biomarker and more cost-effective screening tool for patient enrollment into preclinical AD trials.
Objective: To determine if a blood-based screening test can adequately identify amyloid burden in participants screened into a preclinical AD trial.
Methods: In this cross-sectional study, 224 participants from the A4 Study received an amyloid PET scan (^{18}Florbetapir) within 90 days of blood sample collection. Blood samples from all study participants were processed within 2 h after phlebotomy. Plasma amyloid measures were quantified by Shimazdu and C2 N Diagnostics using mass spectrometry-based platforms. A corresponding subset of blood samples ($n = 100$) was processed within 24 h after phlebotomy and analyzed by C2 N.

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Results: Plasma $A\beta_{42}/A\beta_{40}$ demonstrated the highest association for Aβ accumulation in the brain with an AUC 0.76 (95% CI = 0.69, 0.82) at C2 N and 0.80 (95% CI = 0.75, 0.86) at Shimadzu. Blood samples processed to plasma within 2 h after phlebotomy provided a better prediction of amyloid PET status than blood samples processed within 24 h (AUC 0.80 versus 0.64; $p < 0.001$). Age, sex, and *APOE* ε4 carrier status did not the diagnostic performance of plasma $A\beta_{42}/A\beta_{40}$ to predict amyloid PET positivity in A4 Study participants.

Conclusion: Plasma $A\beta_{42}/A\beta_{40}$ may serve as a potential biomarker for predicting elevated amyloid in the brain. Utilizing blood testing over PET imaging may improve screening efficiency into clinical trials.

Keywords: A4, Alzheimer's disease, amyloid-β, biomarkers, clinical trial, mass spectrometry, PET

INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disease associated with aging. In the United States, more than 6.2 million individuals over the age of 65 are affected by AD dementia, a number that is expected to increase to 13.8 million by the year 2050 [1]. AD is thought to begin with a predementia stage that slowly progresses to mild cognitive impairment (MCI) then to symptomatic AD [2]. Older clinically normal (CN) individuals with evidence of amyloid pathology (Aβ+) demonstrate more rapid declines on longitudinal neurophysiological testing results; more rapid decline in cognitive function; and at a greater risk of progression to mild cognitive impairment (MCI) and dementia [2].

The current state of AD diagnostics allows for asymptomatic or CN individuals to be diagnosed with preclinical AD by examining the presence of Aβ, now detectable with neuroimaging modalities such amyloid positron emission tomography (PET) and cerebrospinal fluid (CSF) analysis. These methods are traditionally employed to indicate the presence of brain amyloid plaques in CN individuals. Numerous studies have correlated elevated brain Aβ levels and lower CSF $A\beta_{42}/A\beta_{40}$ levels with cognitive decline [3–5]. However, these methods are expensive, not readily accessible in primary care settings, time consuming, and invasive [6–8]. These limitations have sparked researchers to examine the feasibility of using blood biomarker measures (e.g., $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$, tau, phospho-tau) to detect amyloid pathology as blood sample collection is procedurally simple and readily accessible in primary care settings to minority populations in rural areas, the elderly, and those of diverse socioeconomic groups [9, 10]. Moreover, blood-based biomarkers may serve as a more cost-effective screening tool for patient enrollment into preclinical AD trials. Early detection is considered critical, in that AD-modifying interventions designed to stop or slow disease progression during asymptomatic stages are likely to be most effective [11]. Lastly, identifying and treating a cognitively impaired patient at earlier and more treatable time points may alleviate the financial hardships and caregiver burden imposed on close relatives and on the healthcare system [12].

In 2014, Anti-Amyloid Treatment in Asymptomatic Alzheimer's Disease (A4) Study, a first-of-its-kind secondary prevention trial was initiated to assess the efficacy of a preclinical drug intervention in CN older individuals who were "at risk" for progression to AD [13, 14]. Participant eligibility into the A4 Study was determined by amyloid PET imaging (elevated Aβ+versus non elevated Aβ-). Given the advantages of using blood-based biomarkers over PET imaging [9, 15–17], we investigated whether blood-based biomarker screening tests can predict brain amyloid pathology in the pre-randomization A4 participants. Quantifying plasma amyloid biomarkers has the potential to improve the efficiency of screening and enrolling participants into clinical trials similar to the A4 Study [11, 18, 19]. Over the past few years, accumulating evidence has demonstrated that lower plasma $A\beta_{42}/A\beta_{40}$ ratios, as measured using mass spectrometry (MS)-based methods, are associated with brain amyloid pathology, accelerated cognitive decline, and increased risk of developing AD dementia in CN, older adults [20, 21].

In the current study, we examined whether plasma $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42}/A\beta_{40}$ levels, as measured by two different MS platforms (MALDI-TOF-MS and LC-MS/MS), can predict brain amyloid PET positivity in cognitively normal, A4 Study participants. Secondly, we compared the impact of two different plasma processing protocols (2 h versus 24 h) on the plasma $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42}/A\beta_{40}$ levels to predict amyloid PET positivity.

Table 1
Demographic and clinical data for the 224 participants with plasma samples collected using Protocol 1 that were analyzed using both MALDI-TOF-MS and LC-MS/MS platforms. Categorical variables are summarized using counts and percentages (%); continuous variables summarized using means and standard deviations (SD). MMSE, Mini-Mental State Exam; CFI, Cognitive Function Index

Patient Demographic Data ($n = 224$)				
Characteristic		$A\beta^-$ ($N = 112$)	$A\beta^+$ ($N = 112$)	Combined ($N = 224$)
APOE ε4	Negative	79 (35.27%)	57 (25.45%)	136 (60.71%)
	Positive	31 (13.84%)	54 (24.11%)	85 (37.95%)
	(Missing)	2 (0.89%)	1 (0.45%)	3 (1.34%)
Ethnicity	Hispanic or Latino	3 (1.34%)	1 (0.45%)	4 (1.64%)
	Not Hispanic or Latino	108 (48.21%)	110 (49.11%)	218 (97.32%)
	(Missing)	1 (0.45%)	1 (0.45%)	2 (0.89%)
Race	American Indian or Alaska Native	1 (0.45%)	0 (0.00%)	1 (0.45%)
	Asian	4 (1.79%)	0 (0.00%)	4 (1.79%)
	Black or African American	8 (3.57%)	0 (0.00%)	8 (3.57%)
	White	98 (43.75%)	112 (50.00%)	210 (93.75%)
	(Missing)	1 (0.45%)	0 (0.00%)	1 (0.45%)
Sex	Female	71 (31.70%)	57 (25.45%)	128 (57.14%)
	Male	41 (18.30%)	55 (24.55%)	96 (42.86%)
Age, mean (SD)		72.08 (5.27)	73.29 (4.86)	72.69 (5.10)
MMSE, mean (SD)		28.83 (1.11)	28.54 (1.43)	28.69 (1.29)
CFI, mean (SD)		3.83 (3.82)	3.97 (3.75)	3.90 (3.78)
Clinical Biomarker Summaries				
C2 N – Protocol 2		Amyloid protein concentrations, mean (SD)		
$A\beta_{40}$ (pg/mL)		508.07 (102.24)	499.92 (69.44)	504.00 (87.29)
$A\beta_{42}$ (pg/mL)		47.15 (10.26)	42.61 (6.49)	44.88 (8.86)
$A\beta_{42}/A\beta_{40}$		0.093 (0.008)	0.085 (0.007)	0.089 (0.009)
Shimadzu – Protocol 2				
$A\beta_{40}$ (pg/mL)		8.92 (2.61)	8.31 (1.93)	8.61 (2.31)
$A\beta_{42}$ (pg/mL)		0.49 (0.14)	0.40 (0.10)	0.45 (0.13)
$A\beta_{42}/A\beta_{40}$		0.055 (0.01)	0.048 (0.01)	0.052 (0.01)

METHODS

Patient characterization, blood sample testing, and amyloid PET imaging

Blood plasma samples from 224 A4 trial screened participants were included in this study [13, 14]. The A4 Study was approved by the independent ethics committee and registered at Clinicaltrials.gov (identifier: NCT02008357). All participating subjects signed the study's informed consent form which had also been approved by the independent ethics committee.

Patient characterizations and demographics are described in Table 1. Briefly, A4 trial participants received amyloid PET scan (^{18}Florbetapir) within 90 days of blood sample collection, as previously described [13, 14]. Amyloid status (elevated $A\beta^+$ or non-elevated $A\beta^-$) was defined using a combination of both quantitative mean cortical standard uptake value ratio (SUVr) and qualitative visual inspection. A quantitative SUVr threshold of ≥ 1.15 was utilized to define brain amyloid positivity, the primary criterion. An SUVr between 1.10 and 1.15 was considered amyloid positive ($A\beta^+$) only when the corresponding visual read was also considered positive by a two-reader consensus determination. One half of the 224 screened A4 trial participants were classified as $A\beta^+$ ($n = 112$) and the other half of the A4 trial participants were classified as $A\beta^-$ ($n = 112$). $A\beta^+$ PET positive participants were stratified equally based on gender and *APOE* genotype (Table 1) while $A\beta^-$ PET negative participants were majority female and APOE ε4 non-carriers (Table 1). Average age for all participants across both groups is 72.7 years of age while the average MMSE score for all participants across both groups is 28.7 (Table 1).

Blood plasma samples from 224 A4 trial screened participants were collected into K_2 EDTA tubes and processed using one of two protocols: blood samples were collected and express shipped on cold packs to the Rissman lab Biomarker Core at the University of California, San Diego (UCSD), where they were processed to plasma (centrifuged at 3000 rpm for 10 min

at room temperature) approximately 24 h after phlebotomy. Under Protocol 1, plasma processed within 24 h were frozen in 0.5 mL aliquots at -80°C and stored at the Rissman lab Biomarker Core. Under Protocol 2, blood samples were processed to plasma at the collection site, frozen in 0.5 mL aliquots within 2 h after phlebotomy, and the frozen plasma was shipped on dry ice to UCSD for storage at -80°C.

Two hundred and twenty-four (224) duplicate frozen plasma samples, processed within 2 h (Protocol 2) were shipped on dry ice to Shimadzu and C_2 N labs. Plasma amyloid measures $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42}/A\beta_{40}$ were quantified in singlicate using state-of-the-art mass spectrometry-based analytical platforms (Shimadzu Techno-Research Inc., MALDI-TOF-MS and C_2 N Diagnostics, LC-MS/MS) as previously described [19, 21, 22]. Shimadzu measures also included a Composite score that was generated by averaging the normalized scores (z-score to NCGG database) of APP669–711/$A\beta_{1–42}$ and $A\beta_{1–40}/A\beta_{1–42}$ as previously described [19, 23]. A corresponding subset of 100, randomly selected, frozen plasma samples processed within 24 h (Protocol 1) were shipped only to C_2 N Diagnostics where plasma $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42}/A\beta_{40}$ were quantified using LC-MS/MS. Both labs were blinded to participant demographics and meta-data during plasma sample analysis. After analysis, plasma biomarker values were transferred from the two MS labs to Rissman lab Biomarker Core (UC San Diego) for statistical analysis. *Data integration*

C_2 N Diagnostics and Shimadzu plasma biomarker values were transferred to ATRI statisticians and merged with A4 pre-randomization study data that was frozen on June 25, 2019. The C_2 N Diagnostics biomarker measures were transferred to Rissman lab Biomarker Core in April 2020. The Shimadzu lab provided biomarker measures in two formats: initial measures from the original MALDI-TOF-MS analysis; and a second dataset of measures that used available QC data to recalibrate the biomarker values derived from the original MALDI-TOF-MS analysis. Since the QC recalibration approach is meaningful for comparisons among multiple sites, but less meaningful for single site data such as this, we have presented biomarker data from the initial MALDI-TOF-MS analysis.

Statistical analysis

Receiver operating characteristic (ROC) curve analysis was conducted to test the ability of plasma amyloid biomarkers to identify brain amyloid PET status in A4 participants. ROC curves were calculated for each biomarker and paired statistical comparisons of analytical platform (MALDI-TOF-MS vs LC-MS/MS), and Rissman lab Biomarker Core sample collection, processing, and transport protocols (24 h versus 2 h) were performed using Sun and Xu [24] algorithm of the DeLong and Clarke-Pearson [25] method. All ROC curves were summarized using the area under the curve (AUC), AUC 95% confidence intervals (CI), Youden's Index [25] and corresponding sensitivity, specificity, accuracy for the optimal plasma $A\beta_{42}/A\beta_{40}$ cut off value. Multivariable logistic regression model with a log link function was used to determine if age, sex, and/or *APOE* genotype influenced the ability of plasma amyloid biomarkers to predict amyloid PET status in all participants. For this analysis, age was categorized into four groups (50-65, 71-75, 76-80, and 81+) and stratified bootstrapping resampling was performed using 5-fold cross-validation within each age group. The predictive performance for each iteration (500 iterations total) was then estimated by predicting the out-of-fold sample, for each fold, and then performing ROC analysis. An AUC of 0.8 was predetermined to be clinically important and results are reported using point estimates and corresponding 95% CI. All statistical analyses were conducted using the statistical software R.

Exploratory PET scan savings analysis

An exploratory analysis was conducted to estimate the potential impact of using plasma amyloid biomarker testing as a screening tool prior to amyloid PET. We estimated the number of PET scans that would be required to achieve 1,000 amyloid PET positive screens along with the number of participants requiring an initial plasma screening sample (pool size). For the purposes of these analyses, a prevalence level of 0.3 was used [13, 26].

RESULTS

Two hundred and twenty-four EDTA plasma samples processed within 2 h (Protocol 2) were shipped frozen and analyzed for $A\beta_{40}$ and $A\beta_{42}$ concentrations (pg/mL) and $A\beta_{42}/A\beta_{40}$ concentration ratio at the Shimadzu and C_2 N labs. Participants' baseline demographics and amy-

Table 2
Demographics for the subset of 100 patient's blood samples collected and processed using Protocols 1 and 2 and analyzed by C_2 N labs using LC-MS/MS. Categorical variables are summarized using counts and percentages (%) with continuous variables summarized using means and standard deviations (SD)

Demographic Data ($n = 100$)				
Characteristic		$A\beta^-$ ($n = 50$)	$A\beta^+$ ($n = 50$)	Combined ($n = 100$)
APOE ε4	Negative	39 (39.00%)	27 (27.00%)	66 (66.00%)
	Positive	11 (11.00%)	23 (23.00%)	34 (34.00%)
Ethnicity	Hispanic or Latino	0 (0.00%)	0 (0.00%)	0 (0.00%)
	Not Hispanic or Latino	50 (50.00%)	50 (50.00%)	100 (100.00%)
Race	American Indian or Alaska Native	0 (0.00%)	0 (0.00%)	0 (0.00%)
	Asian	2 (2.00%)	0 (0.00%)	2 (2.00%)
	Black or African American	6 (6.00%)	0 (0.00%)	6 (6.00%)
	White	42 (42.00%)	50 (50.00%)	92 (92.00%)
Sex	Female	35 (35.00%)	32 (32.00%)	67 (67.00%)
	Male	15 (15.00%)	18 (18.00%)	33 (33.00%)
Age, mean (SD)		73.21 (6.05)	73.47 (4.78)	73.34 (5.43)
MMSE, mean (SD)		28.88 (0.94)	28.40 (1.53)	28.64 (1.28)
CFI, mean (SD)		3.64 (4.05)	3.89 (3.92)	3.76 (3.97)

Clinical Biomarker Summaries			
Protocol 1	Amyloid protein concentrations (pg/mL), mean (SD)		
$A\beta_{40}$	426.12 (109.63)	408.41 (64.22)	417.26 (89.83)
$A\beta_{42}$	31.42 (7.92)	28.84 (6.90)	30.13 (7.50)
$A\beta_{42}/A\beta_{40}$	0.074 (0.011)	0.070 (0.010)	0.072 (0.011)
Protocol 2			
$A\beta_{40}$	514.94 (116.58)	502.47 (66.46)	508.70 (94.62)
$A\beta_{42}$	48.60 (11.40)	42.91 (7.27)	45.76 (9.93)
$A\beta_{42}/A\beta_{40}$	0.095 (0.008)	0.085 (0.008)	0.090 (0.009)

loid biomarker protein concentrations determined using both analytical platforms are summarized in Tables 1 and 2, overall and by amyloid PET status.

The ability to identify brain amyloid status using plasma $A\beta_{42}/A\beta_{40}$ quantified using MALDI-TOF-MS (Shimadzu) or LC-MS/MS (C_2 N) was evaluated using ROC curve analysis (Fig. 1). Plasma $A\beta_{42}/A\beta_{40}$ quantified using MALDI-TOF-MS identified brain amyloid PET status with AUC-ROC=0.80 (95% CI = 0.75 – 0.86), and LC-MS/MS analysis identified brain amyloid PET status with AUC-ROC=0.76 (95% CI = 0.69 – 0.82) ($p = 0.13$ for ROC comparison by DeLong). Individual concentrations of plasma $A\beta_{42}$ and $A\beta_{40}$ were quantified using both analytical platforms. MALDI-TOF-MS analysis identified brain amyloid PET status with AUC-ROC=0.70 ($A\beta_{42}$) and 0.56 ($A\beta_{40}$) (95% CI = 0.64 – 0.77). LC-MS/MS analysis of $A\beta_{42}$ and $A\beta_{40}$ identified brain amyloid PET status with AUC-ROC=0.64 ($A\beta_{42}$) and 0.51 ($A\beta_{40}$) (95% CI = 0.57 – 0.71). Shimadzu lab also provided an additional marker; the Composite blood plasma marker score (APP669–711/$A\beta_{42}$ and $A\beta_{40}/A\beta_{42}$) which returned an AUC of 0.82 (95% CI 0.76 - 0.87).

Aβ cut points identified from the Youden Index (see Table 3) were used to generate scatter plots of Aβ against PET SUVr for C2 N and Shimadzu under Protocol 2 (2 h processing) (Fig. 2). Shaded regions represent the areas where the plasma biomarker correctly identifies patients as amyloid negative (green) and amyloid positive (orange). The lighter color shaded between 1.11 and 1.15 SUVr is the region where a participant was considered amyloid positive only when the corresponding visual read was also considered positive by a two-reader consensus determination.

Plasma $A\beta_{42}/A\beta_{40}$ was also quantified by LC-MS/MS in a subset ($n = 100$) of the 224 participants. These randomly selected blood samples were processed using both protocols (Protocol 1: blood processed to plasma 24 h after phlebotomy versus Protocol 2: blood processed to plasma within 2 h after phlebotomy; Table 2) and results were compared to examine the effects of blood processing and handling, prior to biomarker analysis. Samples processed within 2 h after phlebotomy returned a significantly higher AUC-ROC (0.80 (95% CI = 0.71 – 0.89) as compared to samples processed within 24 h after phlebotomy (AUC-ROC=0.64 (95% CI = 0.52 – 0.75) (Fig. 3, $p < 0.001$).

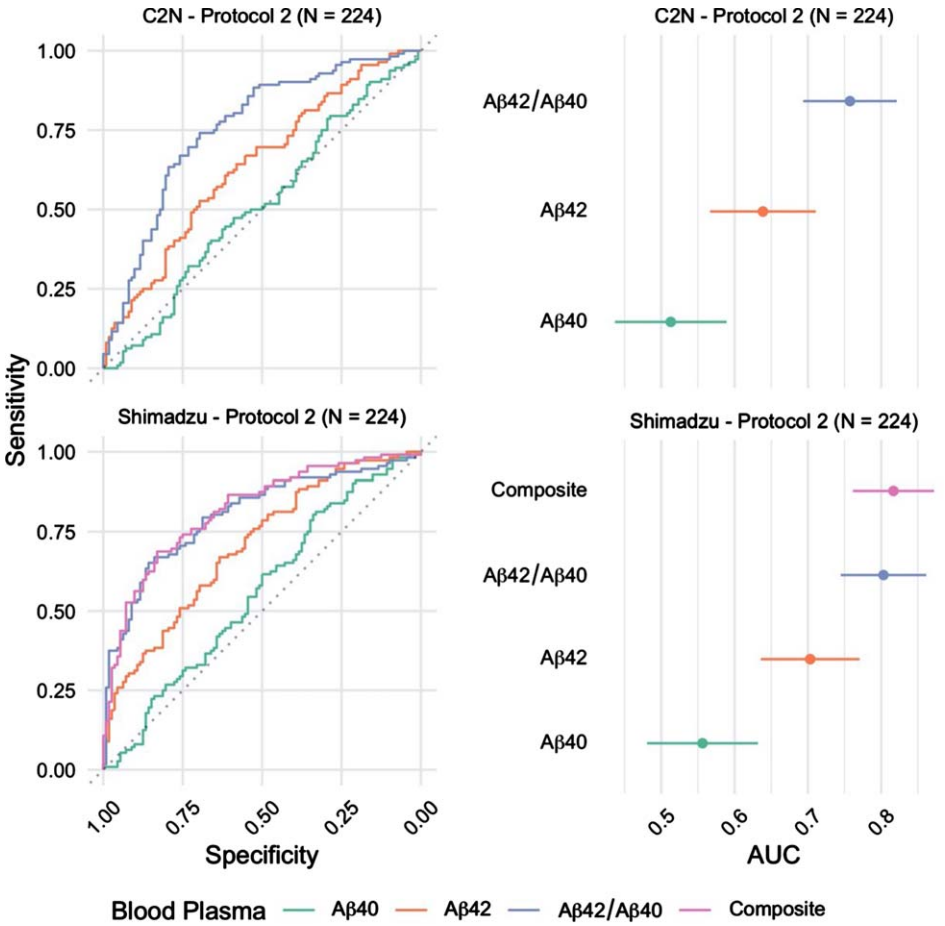

Fig. 1. Receiver operating characteristic (ROC) curves for the blood plasma samples ($n = 224$) Processed using Protocol 2 (2 h post phlebotomy) and analyzed at C_2 N and Shimadzu laboratories (left). The corresponding area under curve (AUC) estimates and 95% confidence intervals (right).

Biomarker predictability with the inclusion of age, sex, and APOE status

Multivariable analysis was conducted on the 224 A4 trial plasma samples (Protocol 2 – 2 h) to determine whether demographic covariates (age, sex, and/or *APOE* genotype) improved ability of plasma $A\beta_{42}/A\beta_{40}$ to predict amyloid PET status, as measured by AUC. Odds ratio (OR) estimates determined that participants aged 76-80 were approximately 2.5 times more likely to be amyloid PET positive than participants aged 65-70 (Table 3). When *APOE* genotype (having one or two ε4 alleles) was considered, *APOE* $\varepsilon4^+$ carriers were approximately 2 times more likely to be amyloid PET positive as compared to non-carriers (Table 3). The out-of-fold sample was then used to calculate the ROC estimates at each iteration for all plasma amyloid predictors. Here, we determined that the addition of these pre-specified covariates (age, sex, and *APOE* genotype) did not improve the predictive ability of plasma $A\beta_{42}/A\beta_{40}$, as measured by the AUC score (Table 3).

Implications for reducing reliance on amyloid PET scans

An exploratory analysis was conducted to determine the potential impact of using plasma testing as the primary screening tool for enrollment into a clinical trial similar to the A4 Study. We determined that approximately 6,7000 trial participants would need to have an initial plasma screening in order to identify 1,000 amyloid PET positive participants. No statistically significant differences were observed in the estimated sample size and number needed to screen

Table 3
Table summarizes Area Under the Curve (AUC); AUC 95% confidence intervals; cut point, and corresponding accuracy, Youden index value, sensitivity, and specificity estimates for each blood plasma biomarker analyzed at C2 N lab, and Shimadzu. The cut point was determined by maximizing the Youden Index value. Table summarizes ROC estimates and odds ratio (OR) estimates for $A\beta_{42}/A\beta_{40}$ with additional covariates (sex, age, and APOE $\varepsilon 4+/-$) for samples analyzed by C2 N and Shimadzu labs. PACC, Preclinical Alzheimer's Cognitive Composite score. CFI, cognitive function index

Process	Biomarker	AUC (95% CI)	Cut Point	Accuracy	Youden	Sensitivity	Specificity
C_2 N LC-MS/MS ($n=224$)	$A\beta_{40}$	0.51 (0.44, 0.59)	453.19	0.54	0.08	0.80	0.29
	$A\beta_{42}$	0.64 (0.57, 0.71)	43.73	0.61	0.22	0.64	0.58
	$A\beta_{42}/A\beta_{40}$	0.76 (0.69, 0.82)	0.088	0.72	0.44	0.74	0.70
Shimadzu MALDI-TOF-MS ($n=224$)	$A\beta_{40}$	0.56 (0.48, 0.63)	9.74	0.57	0.14	0.81	0.33
	$A\beta_{42}$	0.70 (0.64, 0.77)	0.42	0.65	0.30	0.67	0.63
	$A\beta_{42}/A\beta_{40}$	0.80 (0.75, 0.86)	0.05	0.75	0.51	0.67	0.84
C2 N LC-MS/MS; Protocol 1 ($n=100$)	$A\beta_{40}$	0.50 (0.39, 0.62)	390.49	0.60	0.20	0.72	0.48
	$A\beta_{42}$	0.58 (0.47, 0.70)	39.42	0.59	0.18	0.96	0.22
	$A\beta_{42}/A\beta_{40}$	0.64 (0.52, 0.75)	0.075	0.66	0.32	0.76	0.56
C2 N LC-MS/MS; Protocol 2 ($n=100$)	$A\beta_{40}$	0.55 (0.44, 0.67)	500.58	0.60	0.20	0.56	0.64
	$A\beta_{42}$	0.65 (0.54, 0.76)	45.03	0.63	0.26	0.70	0.56
	$A\beta_{42}/A\beta_{40}$	0.80 (0.71, 0.89)	0.094	0.76	0.52	0.92	0.60

ROC Estimates for $A\beta_{42}/A\beta_{40}$ ($n=224$) with Additional Covariates (sex, age, and *APOE* $\varepsilon 4+/-$)							
	Biomarker	AUC (95% CI)	Cut Point	Accuracy	Youden	Sensitivity	Specificity
C_2 N	$A\beta_{42}/A\beta_{40}$	0.72 (0.67, 0.82)	0.323	0.67	0.35	0.89	0.46
Shimadzu	$A\beta_{42}/A\beta_{40}$	0.78 (0.72, 0.86)	0.528	0.73	0.46	0.68	0.78

Odds Ratio (OR) Estimates for $A\beta_{42}/A\beta_{40}$ ($n=224$)							
	$A\beta_{42}/A\beta_{40}$	Age (y) 71 –75	Age (y) 76 – 80	Age (y) 81+	*APOE* $\varepsilon 4^+$	PACC	CFI
C2 N (95% CI)	2.32 (1.67, 3.72)	1.73 (0.80, 3.99)	2.41 (0.89, 7.12)	0.98 (0.32,3.10)	1.76 (0.83, 4.50)	0.98 (0.85, 1.10)	0.99 (0.91, 1.08)
Shimadzu (95% CI)	1.52 (1.31, 1.95)	1.56 (0.61, 4.08)	2.77 (0.97, 8.07)	1.11 (0.33, 4.54)	1.96 (0.93, 4.27)	1.00 (0.89, 1.16)	0.97 (0.90, 1.04)

(NNS) as measured by both analytical platforms used (MALDI or LC-MS/MS). Additional work is ongoing to obtain an understanding of the potential financial savings from the two approaches.

DISCUSSION

Plasma Aβ biomarkers were quantified by two independent laboratories in samples collected from screened, cognitively normal, A4 study participants with brain amyloid PET status determined by central readers. Our findings show that plasma $A\beta_{42}/A\beta_{40}$ quantified using either of the available MALDI-TOF-MS or LC-MS/MS analytical platforms can predict amyloid PET positivity among asymptomatic participants enrolled in the A4 trial. Blood samples were collected and processed to plasma within 2 h after phlebotomy (Protocol 2) provided a better prediction of amyloid PET status than blood samples that were collected and shipped on cold packs to the labora-

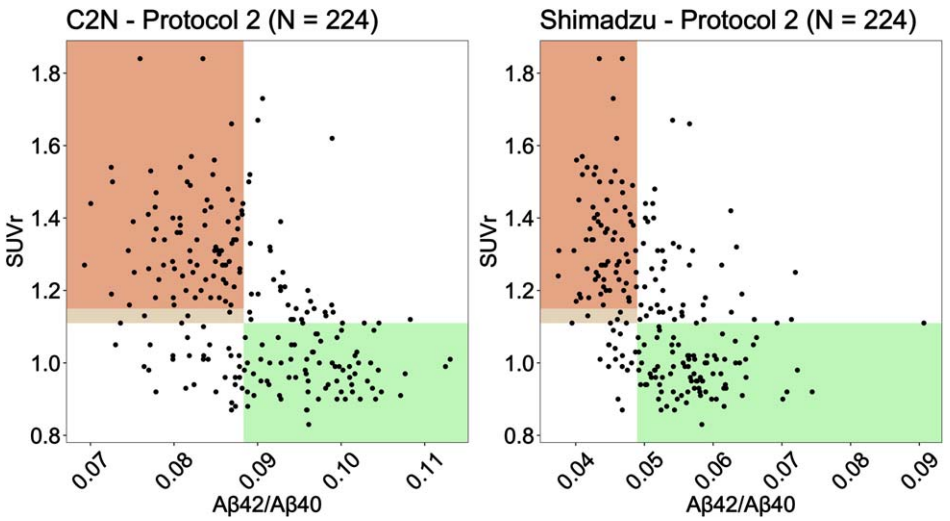

Fig. 2. Scatter plots of Aβ against PET SUVr for C2 N and Shimadzu under Protocol 2 (2 h post phlebotomy). Shaded regions represent the areas where the plasma biomarker correctly identifies patients as amyloid negative (green) and amyloid positive (orange).

tory, where they were processed to plasma within 24 h after phlebotomy (Protocol 1). The findings imply that these plasma $A\beta_{42}/A\beta_{40}$ measures and analytical platforms can be used to pre-screen cognitively normal volunteers for eligibility into AD prevention trials; reduce traditional screen-failure rates; and save time and funds when used to screen for enrollment into prevention trials.

In 224 participant samples that were processed within 24 h, we determined that plasma $A\beta_{42}/A\beta_{40}$ quantified using the Shimadzu MALDI-TOF-MS platform provided a numerically better AUC = 0.80 than the C_2 N LC-MS/MS platform (AUC = 0.76), however these AUC values were not statistically different ($p = 0.13$). Conversely, the C_2 N LC-MS/MS platform provided an AUC = 0.80 for plasma $A\beta_{42}/A\beta_{40}$ in a subset of 100 participant plasma samples that were processed within 2 h. This was an improvement in AUC that was significantly better than what was achieved in the same 100 participants' samples that were processed within 24 h (AUC = 0.64). Finally, Shimadzu calculated an additional Composite plasma biomarker that was generated by averaging the normalized scores of APP669–711/$A\beta_{42}$ and $A\beta_{40}/A\beta_{42}$ score [19, 23]. With an AUC of 0.82, we determined that measuring the Composite plasma marker could serve as a potential biomarker for predicting elevated brain amyloid, however further investigation is required. Previous studies have reported the combination of APP669-711/$A\beta_{42}$ ratio and $A\beta_{40}/A\beta_{42}$ ratio can serve as highly predictive surrogates for brain amyloid pathology [27]. We were not able to compare the biomarker performance of the Composite score between the MALDI-TOF-MS and LC-MS/MS methods as this marker is not measured by the C_2 N LC-MS/MS assay.

Plasma $A\beta_{42}/A\beta_{40}$ performed similarly between the two MS–based methods; however, we observed that the AUCs for plasma $A\beta_{42}/A\beta_{40}$ ratios were significantly lower than previously published reports (AUC 0.88 – 0.97) [21, 27–30]. Moreover, the sensitivity (SN) and specificity (SP) varied significantly across individual plasma amyloid measures and across both analytical platforms. Plasma $A\beta_{42}/A\beta_{40}$ measures generated by C_2 N were highly sensitive (0. 74 – 0.92) yet poorly specific (0.56 – 0.70) in its ability to identify a participant who was not amyloid PET positive. While plasma $A\beta_{42}/A\beta_{40}$ measures generated by Shimadzu was highly specific (0.84) yet lack sensitivity (0.67) to identify an amyloid PET positive participant. Given the variance in sensitivity and specificity across both MS-based planforms, its plausible that our data may better interpreted as a measure to screen out those who are amyloid negative versus predicting those who are actually amyloid positive. Rabe and colleagues [31] determined that pre-analytical errors of plasma $A\beta_{42}/A\beta_{40}$ measures may lead to patient misclassifications (i.e., incorrectly ruling out amyloid pathology or vice versa) thus impacting sensitivity or specificity. As researchers continue to interpret their findings based on AUCs, additional clinical performance measures including the number of false positive and false

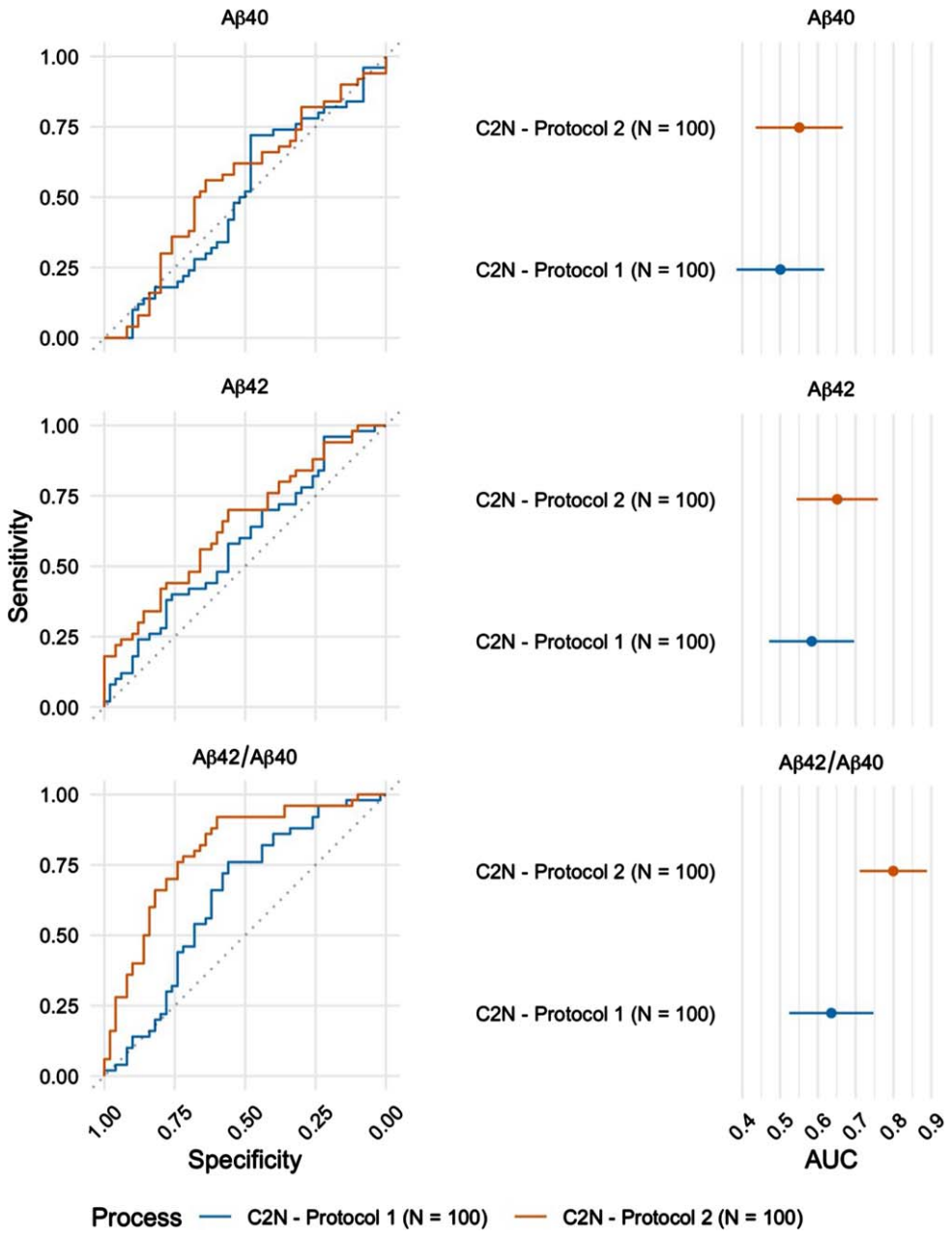

Fig. 3. ROC curves for the blood plasma samples ($n = 100$) collected using Protocol 1 (24 h post phlebotomy) and Protocol 2 (2 h post phlebotomy) and analyzed at C_2 N laboratory (left). The corresponding AUC estimates and 95% confidence intervals (right).

negative [21] robustness [31–33] and population performance [34] must also need to be considered to support the use of plasma amyloid measures as reliable and accurate biomarkers for AD.

In general, MS-based assays are thought to have superior performance to detect amyloid pathology in brain as compared to immunoassays [35]. Recently, it has been reported that their level of performance as measured by AUC differed substantially among several MS-based and immunoassay-based analytical platforms that quantify plasma $A\beta_{42}/A\beta_{40}$ concentration ratio and identify brain amyloid status [35]. In particular, the immunoprecipitation LC-MS/MS assay (as performed by Bateman et al.) outperformed seven other analytical assays for detecting plasma $A\beta_{42}/A\beta_{40}$ [35]. The C_2 N LC-MS/MS analytical

used herein is based on the Bateman laboratory assay. In addition, some disparities among analytical platform performance observed by Janelidze et al. [35] were attributed to differences in patient cohort characteristics and variations in sample processing. The current study supports prior reports [36] that sample handling procedures prior to analysis can significantly impact the sensitivity and specificity of plasma $A\beta_{42}/A\beta_{40}$ as biomarker for predicting amyloid PET positivity and this topic requires further investigation.

The interpretation of our data may be further complicated by the imaging protocols used to establish amyloid PET positivity. The existence of multiple imaging tracers with varying amyloid positivity (SUVr) cutoffs could have a direct impact on the ability of plasma $A\beta_{42}/A\beta_{40}$ to predict amyloid burden in the brain [37, 38]. Clinical trials, including the A4 Study, are opting to use newer generation, FDA approved, ^{18}F-based radioligand tracers, such as florbetapir over the commonly used Pittsburgh compound B (PiB) amyloid tracer for imaging. Florbetapir enables a wider application due it its longer half-life (110 min) as compared to PiB (20 min) [38]. However, studies report that florbetapir-based amyloid measurements had higher variability due to nonspecific white matter deposition in the brain [37]. Because of this, tracers like florbetapir are likely to be less suitable for detecting subtle amyloid deposition in the brain [37, 38]. Future studies are needed to assess how amyloid tracer sensitivity influences the blood amyloid measurements across the spectrum of disease states. Understanding this will be vital for AD prevention trials where subtle amyloid deposition is more likely to occur in CN adults.

Despite age and *APOE* genotype being considered as risk factors for AD, we determined that these covariates included sex did not improve the ability of plasma $A\beta_{42}/A\beta_{40}$ to predict amyloid PET positivity. While previous studies have shown significant improvements in the sensitivity of plasma amyloid biomarkers when age, sex, and *APOE* ε4 carrier status are accounted for [19, 21, 29], several others have demonstrated similar results to ours. Keshavan and colleagues demonstrated that sex and *APOE* ε4 carrier status did not improve plasma $A\beta_{42}/A\beta_{40}$ ability to predict PET amyloid positivity in CN older adults [39] while Yamashita and colleagues [30] observed a slight numerical increase in the performance of the plasma $A\beta_{42}/A\beta_{40}$ ratio when incorporating the *APOE* ε4 status. Further investigation is required to reconcile these conflicting reports.

One major limitation of the current study is our relatively small sample number and the lack of racial and ethnic diversity among the trial participants. Blacks/African Americans and Hispanics/Latino(s) have a higher prevalence of AD as compared to non-Hispanic whites; yet they are consistently underrepresented in clinical trials for AD and other dementias [40]. A recent cross-sectional study found that blacks, Hispanics, and Asian participants were more likely to be ineligible for the A4 Study after the first screening visit [40]. In our sample, out of the 224 trial participants we analyzed, only 14 (9%) screened participants belonged to a minoritized ethnic group. Given that our data was generated from a relatively homogenous population, this may explain why our covariate analysis did not show any significant differences in AUC. Recent observational studies have revealed notable differences in the performance of AD biomarkers among different racial groups [41–43], therefore it is imperative that efforts are made to increase the diversity among trial participants. We anticipate that blood testing will increase accessibility to diverse minority and socioeconomic groups as well as improve screening and retention of trial participants [44].

In summary, our work and work of several others [21, 27–30] determined that plasma $A\beta_{42}/A\beta_{40}$ may serve as a potential biomarker for predicting elevated brain amyloid in cognitively normal adults. Additionally, the combination of APP669-711/$A\beta_{42}$ ratio and $A\beta_{42}/A\beta_{40}$ ratio may also serve as predictive surrogate for brain amyloid pathology as more studies are investigating a multi-biomarker approach for predicting cognitive decline and amyloid PET positivity [45]. Plasma p-tau181/$A\beta_{1\text{-}42}$ ratio [46] and t p-tau 217 [47] has recently emerged as alternative biomarker for predicting cognitive decline and amyloid PET positivity. A head-to-head comparison is necessary to determine which biomarker would be the most effective at predicting cognitive decline and amyloid PET positivity in older, CN adults.

Lastly, our exploratory analysis reveals the potential savings associated with using a blood test as a screening tool for enrollment into secondary prevention clinical trials like the A4 Study. Previous analysis has suggested that a blood test can reduce the use of amyloid PET scans by 50% [39]; however, future studies are still needed to understand how sample *APOE* status, race and ethnicity, and amyloid tracer sensitivity impacts plasma $A\beta_{42}/A\beta_{40}$ ability to predict amyloid brain pathology in normal controls.

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CONFLICT OF INTEREST

Kevin Yarasheski and Tim West are employees of C2 N Diagnostics.

Colin L. Masters and Robert A. Rissman are Editorial Board members of this journal but were not involved in the peer-review process nor had access to any information regarding its peer-review.

All other authors have no conflict of interest to report.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available on request. Please contact the corresponding author for access to all datasets for re-analysis.

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