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RESEARCH ARTICLE

Clinical utility of an antibody-free LC-MS method to detect brain amyloid deposition in cognitively unimpaired individuals from the screening visit of the A4 Study

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Abstract

INTRODUCTION: This study explored the ability of plasma amyloid beta (Aβ)42/Aβ40 to identify brain amyloid deposition in cognitively unimpaired (CU) individuals.

METHODS: Plasma Aβ was quantified with an antibody-free high-performance liquid chromatography tandem mass spectrometry method from Araclon Biotech (ABtest-MS) in a subset of 731 CU individuals from the screening visit of the Anti-Amyloid Treatment in Asymptomatic Alzheimer's (A4) Study, to assess associations of Aβ42/Aβ40 with Aβ positron emission tomography (PET).

RESULTS: A model including Aβ42/Aβ40, age, apolipoprotein E ε4, and recruitment site identified Aβ PET status with an area under the curve of 0.88 and an overall accuracy of 81%. A plasma-based pre-screening step could save up to 42% of the total number of Aβ PET scans.

DISCUSSION: ABtest-MS accurately identified brain amyloid deposition in a population of CU individuals, supporting its implementation in AD secondary prevention trials to reduce recruitment time and costs. Although a certain degree of heterogeneity is inherent to large and multicentric trials, ABtest-MS could be more robust to pre-analytical bias compared to other immunoprecipitation mass spectrometry methods.

KEYWORDS
Anti-Amyloid Treatment in Asymptomatic Alzheimer's (A4) Study, Alzheimer's disease, amyloid beta, Aβ42/Aβ40, blood biomarkers, clinical trials, mass spectrometry, pre-screening

HIGHLIGHTS

- Plasma amyloid beta (Aβ)42/Aβ40 accurately identified brain Aβ deposition in cognitively unimpaired individuals from the Anti-Amyloid Treatment in Asymptomatic Alzheimer's (A4) Study.
- The inclusion of the recruitment site in the predictive models has a non-negligible effect.
- A plasma biomarker-based model could reduce recruitment costs in Alzheimer's disease secondary prevention trials.
- Antibody-free liquid chromatography mass spectrometry methods may be more robust to pre-analytical variability than other platforms.

1 | BACKGROUND

Alzheimer's disease (AD), the most common form of dementia, is characterized by a long preclinical stage in which individuals do not show clinical symptoms though neuropathological alterations (amyloid plaques and neurofibrillary tangles) are already present.[1–4] It is widely accepted that disease-modifying therapies are likely more effective during these very first stages of AD before extensive neurodegeneration and overt clinical symptoms arise. Accordingly, clinical trials in AD are currently being conducted in earlier stages of the disease,[5–7] that is, in preclinical individuals with evidence of brain amyloid deposition.

Accurate screening procedures for brain amyloidosis, such as positron emission tomography (PET) imaging or cerebrospinal fluid (CSF) measurements, are expensive and invasive, and impose a significant monetary burden for study sponsors. This is especially relevant in clinical trials targeting preclinical populations in which the prevalence of brain amyloid deposition is estimated to be ≈25%.[8] On the other hand, blood assays are gaining increasing popularity in the clinical research framework.[4,9–14] These blood tests are non-invasive, cost effective (compared to CSF and PET), and represent limited physical risk for the patient. Some of these blood biomarkers also show high accuracy for the discrimination of amyloid beta (Aβ) or tau abnormalities in the brain, years before symptoms start. All these characteristics make blood assays useful tools for participant pre-screening in clinical trials, along with other applications, significantly reducing the necessary number of PET scans or lumbar punctures.

Plasma Aβ42/Aβ40 ratio is a well-established marker for amyloidosis.[15–17] However, the measurement of this ratio is not straightforward due to the chemical nature of both peptides. Some recent work calls plasma Aβ42/Aβ40 ratio robustness into question due to the small differences between Aβ PET positive (+) and negative (–) individuals and the inherent overlap in the distribution of the biomarker values between groups.[18,19] According to this, it seems clear that high accuracy and robust methods should be used for Aβ42 and Aβ40 quantitation in plasma. This is particularly important in large and multicentric studies in which pre-analytical factors may significantly contribute to overall variability. Even after the implementation of standardized operating protocols for sample collection, handling, and storage, a certain degree of pre-analytical variability is always present.[20,21]

Araclon Biotech developed an antibody-free high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method (ABtest-MS) for the quantification of plasma Aβ42/Aβ40. This innovative procedure includes a direct extraction of Aβ peptides from plasma without involving any immunoprecipitation (IP) or digestion step, which significantly reduces time and costs, resulting in a more affordable and accessible assay.[11,22,23]

In this work, we investigate the ability of plasma Aβ42/Aβ40 ratio measured with ABtest-MS to identify brain amyloid deposition in a subset of cognitively unimpaired (CU) individuals from the second screening visit of the Anti-Amyloid Treatment in Asymptomatic Alzheimer's (A4) Study. Moreover, because recent findings have revealed the impact of blood processing protocols on the ability of plasma Aβ42/Aβ40 to predict amyloid PET positivity in samples from the same study,[24] we aim to expand this observation and highlight the influence of pre-analytical variables in large multicentric studies.

2 | METHODS

2.1 | Study participants

The A4 Study (ClinicalTrials.gov identifier: NCT02008357) is a 240-week trial testing whether solanezumab (Eli Lilly & Company) can slow cognitive decline associated with AD at the preclinical stage of the disease. Detailed information describing the study design can be found elsewhere.[25–27] Briefly, participants eligible for screening were 65 to 85 years of age, assessed to be cognitively normal, living independently, and have a study partner to provide information on daily life cognitive function on an annual basis. More than 4400 participants were subjected to amyloid PET to select a final subgroup of 1169 Aβ PET(+) individuals. The study is being conducted at 68 sites in the United States, Canada, Australia, and Japan. For the present study, a subset of

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed literature on plasma biomarkers in Alzheimer's disease (AD) using PubMed. This search focused on the performance of plasma amyloid beta (Aβ)42/Aβ40 in large multicentric studies. Though several efforts are made to harmonize plasma-obtaining procedures, information about the impact of pre-analytical variability on the performance of plasma Aβ42/Aβ40 to predict brain Aβ positivity is lacking.
2. **Interpretation**: Findings suggest that plasma Aβ42/Aβ40 accurately identifies brain amyloid deposition in cognitively unimpaired individuals from the multicentric Anti-Amyloid Treatment in Asymptomatic Alzheimer's (A4) Study. These results highlight the potential value of a plasma biomarker-based models to reduce recruitment costs in AD secondary prevention trials. Nevertheless, pre-analytical variability could have a platform-dependent influence in the performance of Aβ42/Aβ40.
3. **Future directions**: Further research is needed to validate these findings in other multicentric studies and to elucidate the unequal impact of pre-analytical variability on different technologies. Blood biomarkers monitoring under real-life settings will require highly robust analytical procedures.

731 participants screened at 60 sites in the United States and Canada for inclusion in the A4 trial were considered.

2.2 | Amyloid PET imaging

PET imaging was acquired 50 to 70 minutes after an injection of 10 mCi of ^{18}F-Florbetapir and measured using a mean cortical standardized uptake value ratio (SUVR) with a whole cerebellar reference region.[25] A SUVR threshold of ≥ 1.15 was used to define elevated amyloid.

2.3 | Pre-analytical sample handling

Plasma samples were derived from whole blood samples collected at the site in 10 mL K2-EDTA tubes, and shipped overnight on cold packs to Rissman Laboratory Biomarker Core at the University of California, San Diego (UCSD). Whole blood samples were centrifuged (2000 × g, 10 minutes, room temperature) within 24 hours, to separate the plasma. Plasma was then aliquoted and frozen at −80°C. Plasma samples from the second screening visit arrived frozen at Araclon Biotech (Zaragoza, Spain).

2.4 | Plasma Aβ measurements

Plasma Aβ40 and Aβ42 concentrations were measured using ABtest-MS, an antibody-free HPLC-differential mobility spectrometry-triple quadrupole mass-spectrometry (HPLC-DMS-MS/MS) method (Araclon Biotech, Zaragoza, Spain).[28] Calibration curves were prepared in human plasma after spiking with ^{15}N-Aβ40 and ^{15}N-Aβ42 (rPeptide) at seven concentration levels. Quality control samples were also prepared in human plasma at three concentration levels (150/30, 400/75, and 750/150 pg/mL for ^{15}N-Aβ40 and ^{15}N-Aβ42, respectively). Calibration ranges were 50 to 1000 pg/mL for ^{15}N-Aβ40 and 10 to 200 pg/mL for ^{15}N-Aβ42. Two calibration curves were used in each analytical run, one at the beginning and one at the end of the sequence. Additionally, six quality control samples (three concentration levels per duplicate), uniformly distributed along the sequence, were analysed in each run. Analytes were extracted directly from plasma as no IP procedure was followed. Intact Aβ40 and Aβ42 species were measured as no enzymatic digestion was performed. Deuterated internal standards (^{2}H-Aβ40 and ^{2}H-Aβ42, Bachem AG) were spiked in all samples and response ratios corresponding to the endogenous species in study samples (^{14}N-Aβ40/^{2}H-Aβ40 and ^{14}N-Aβ42/^{2}H-Aβ42) were interpolated in the calibration curves. Plasma samples were analyzed in 13 analytical runs, between March 19, 2021 and May 25, 2021. Accuracy and precision values for back-calculated concentrations of calibrators and quality control samples are shown in Tables S1 and S2 in supporting information. Further details about the analytical procedure and instrumental acquisition parameters are described in the literature.[28] The specifics of the method are subject matter of patent application (EP2020382352).

2.5 | Cognitive testing

Participants underwent a neuropsychological test battery as part of the clinical evaluations. For this study, data regarding performance in the Mini-Mental State Examination (MMSE) and the Preclinical Alzheimer Cognitive Composite (PACC) were examined. The PACC is a well-validated composite and sensitive to the earliest signs of cognitive decline. It includes four components: MMSE, Digit Symbol Substitution Test, Logical Memory IIa Delayed Recall, and Free and Cued Selective Reminding Tests.[29–31]

2.6 | Statistical analysis

All statistical analyses and data visualizations were carried out using GraphPad Prism v5.03 (GraphPad Software), SPSS v18 (IBM), or R (www.r-project.org). Group differences were examined using the Mann–Whitney or Kruskal–Wallis, and chi-square tests for continuous and categorical variables, respectively. Spearman correlation coefficient (rho) was used to investigate correlations between continuous variables. Logistic regression models and receiver operating characteristic (ROC) curves were constructed to evaluate the discriminative accuracy of Aβ PET status. Logistic regression model selection was

performed based on Akaike information criterion (AIC)[32] values, that is, the most parsimonious nested model was selected. Aβ PET status (dichotomized) was used as the dependent variable. A reduction of two units in the AIC values was considered significant.[33] Reductions of less than two units in the AIC were further reconfirmed by the likelihood-ratio test. Area under the curve (AUC) values, obtained after ROC analyses, between selected models were compared using the DeLong test. Significance level was set at $P < 0.05$.

3 | RESULTS

3.1 | Participant characteristics

Demographic, clinical, as well as plasma biomarker data of study participants are presented in Table 1. Among the 731 individuals included in this study, 241 (33%) were classified as Aβ PET(+) according to the established cutoff of SUVR ≥ 1.15 for ^{18}F-Florbetapir. Median age for the studied population was 70.4 years (interquartile range [IQR] = 67.5–74.5) and no statistically significant difference was found between Aβ PET(+) and Aβ PET(−) individuals ($P = 0.123$). Number of *APOE ε4* alleles (from now on, *APOE ε4*) was differentially distributed between Aβ PET(+) and Aβ PET(−) groups ($P < 0.001$). MMSE scores did not differ between Aβ PET status ($P = 0.094$). PACC values differed between groups, with Aβ PET(+) individuals performing worse ($P = 0.009$).

3.2 | Association of plasma biomarkers with brain amyloid deposition

Logarithmic transformations of plasma biomarker values were used in the analyses due to the absence of normality in the distributions. No statistically significant differences were found in Aβ40 plasma levels between Aβ PET(+) and Aβ PET(−) groups ($P = 0.130$, Figure 1A). Significant differences were found for Aβ42 and Aβ42/Aβ40 between the Aβ PET(+) and Aβ PET(−) groups ($P < 0.001$, Figure 1B and C). According to the median values, plasma Aβ42/Aβ40 was 13.3% lower in the Aβ PET(+) than in the Aβ PET(−) group ($P < 0.001$). Both Aβ42 and Aβ42/Aβ40 showed significant negative correlations with ^{18}F-Florbetapir-PET SUVR values (rho = −0.35 and −0.44 respectively, $P < 0.001$; Figure 1D–F).

3.3 | Effect of recruitment site in Aβ42/Aβ40 measures

Taking into account that blood samples were collected, and initially stored, in multiple recruitment centers across the United States and Canada, we evaluated if this site multiplicity could produce a significant degree of pre-analytical variability, affecting Aβ42/Aβ40 measures. Site information was missing for 10 participants, and centers contributed with uneven number of participants (range 1 to 49, Table S3 in supporting information). Kruskal–Wallis analysis was carried out excluding sites contributing with only one ($n = 4$) or two ($n = 2$) volunteers. Data from 713 out of 731 participants were used in the calculations. Significant differences ($P = 0.034$) were found in Aβ42/Aβ40 values between different recruitment sites (Figure S1 in supporting information). This observation led us to include this variable in the regression models to account for this variability.

3.4 | Logistic regression and ROC analysis

Logistic regression models for predicting Aβ PET status were built. The following predictors (independent variables) were used: Aβ42/Aβ40, age, *APOE ε4*, sex, and MMSE and PACC scores. Multiple models were developed (Table 2 and Table S4 in supporting information). The most parsimonious model yielding a minimum AIC value (709.120) was composed of Aβ42/Aβ40 ratio, age, *APOE ε4*, and site (Model 7). The inclusion of PACC in the model produced a non-significant decrease in AIC (0.54 units, Model 12) and therefore this neuropsychological test was removed from the final model. A baseline (demographic) model containing all of the above predictors with the exception of Aβ42/Aβ40 ratio yielded an AIC value of 838.082 (Model 4).

ROC analysis was performed to assess the predictive ability of the different models to identify brain amyloid deposition (Figure 2 and Table 2; selected models are shown in Figure 2). Baseline model, including age, *APOE ε4*, and site yielded an AUC value of 0.80 (95% confidence interval [CI] 0.77–0.84). The inclusion of Aβ42/Aβ40 ratio in the model (Full model, Model 7 in Table 2) increased the AUC to 0.88 (95% CI 0.86–0.91). This increase in AUC is considered statistically significant according to the DeLong test. Applying a cutoff value of 0.303 (calculated at maximum Youden index), this model yielded a sensitivity of 87%, a specificity of 78%, and an overall accuracy of 81% (Figure 3A). Median probability scores derived from the full predictive model were highly different between Aβ PET(+) and Aβ PET(−) individuals (0.636 vs. 0.102, $P < 0.001$, Figure 3B).

3.5 | Heat maps

Heat maps showing the predicted probability of being Aβ PET(+) based on plasma Aβ42/Aβ40 measures (y axis) and age (x axis), stratified by *APOE ε4* genotype, were generated (Figure 4). As an example, a 75-year-old individual bearing no *APOE ε4* allele and with an Aβ42/Aβ40 value of 0.25, has a 42.7% probability of being Aβ PET(+), while the same individual bearing at least one *APOE ε4* allele has a probability of 66.6%.

3.6 | Savings in the number of PET scans

The feasibility of applying ABtest-MS as a pre-screening tool in clinical trials was also evaluated. As an example, to obtain 1000 Aβ PET(+) CU individuals as in the A4 Study, 3030 PET scans should be necessary

TABLE 1 Participant characteristics.[a]

	Aβ PET(−)[b]	Aβ PET(+)[b]	Total	*P* value
Participants, *N* (%)	490 (67)	241 (33)	731	
Age (years)				0.123
Median	70.3	70.6	70.4	
Q1–Q3	67.5–74.3	67.8–75.22	67.5–74.5	
Sex, *N* (%)				0.899
Male	207 (42.2)	103 (42.7)	310 (42.4)	
Female	283 (57.8)	138 (57.3)	421 (57.6)	
APOE ε4, *N* (%)				<0.001
0 alleles	371 (75.7)	99 (41.0)	470 (64.3)	
1 allele	110 (22.4)	120 (49.8)	330 (45.1)	
2 alleles	9 (1.8)	22 (9.1)	31 (4.2)	
MMSE				0.094
Median	29	29	29	
Q1–Q3	28–30	28–30	28–30	
PACC				0.009
Median	0.36	−0.19	0.16	
Q1–Q3	−1.48–2.00	−2.12–1.55	−1.58–1.86	
Florbetapir-PET				<0.001
SUVR				
Median	1.03	1.29	1.08	
Q1–Q3	0.97–1.08	1.21–1.40	1.00–1.21	
Aβ40 (pg/mL)				0.130
Median	201.5	209.2	204.3	
Q1–Q3	181.9–230.9	180.8–236.2	181.1–232.6	
Aβ42 (pg/mL)				<0.001
Median	61.2	52.9	58.0	
Q1–Q3	53.7–69.1	46.8–59.4	51.0–67.1	
Aβ42/Aβ40				<0.001
Median	0.30	0.26	0.28	
Q1–Q3	0.27–0.33	0.23–0.28	0.25–0.32	

Abbreviations: Aβ, amyloid beta; *APOE*, apolipoprotein E; MMSE, Mini-Mental State Examination; PACC, Preclinical Alzheimer Cognitive Composite; PET, positron emission tomography; Q, quartile; SUVR, standardized uptake value ratio.

[a]Data are median values (interquartile range) except for sex and *APOE* ε4 number of alleles, which are number of cases and percentages. Differences between groups Aβ PET(−) and Aβ PET(+) groups were tested using Mann–Whitney and chi-square test, as appropriate.

[b]Aβ-PET status was defined using the cutoff of SUVR ≥ 1.15 for ^{18}F-Florbetapir.

according to a population with a prevalence for Aβ PET(+) of 33%. A predictive model taking into account plasma Aβ42/Aβ40 ratio, number of *APOE* ε4 alleles, and the age of the patient could significantly reduce the number of PET scans. Using data from this work, the predictive model shown above (Model 6 in Table 2; Figures 3C and 3D) yields a sensitivity of 81%, a specificity of 70%, an accuracy of 74%, a positive predictive value (PPV) of 57%, and a negative predictive value (NPV) of 88%. Considering these figures, 3741 individuals should be necessary for blood analysis, of which 1754 should undergo PET imaging (instead of 3030), resulting in a net savings of 42% of the scans. Assuming an average cost of $3000 to $6000 per PET scan,[34,35] several million dollars could be saved in large studies such as A4. In addition, recruitment time could be greatly reduced.

4 | DISCUSSION

In this work, we have evaluated the performance of ABtest-MS, a direct extraction protocol coupled to MicroLC-DMS-MS/MS for the quantitation of Aβ40 and Aβ42 in plasma, in a large international and

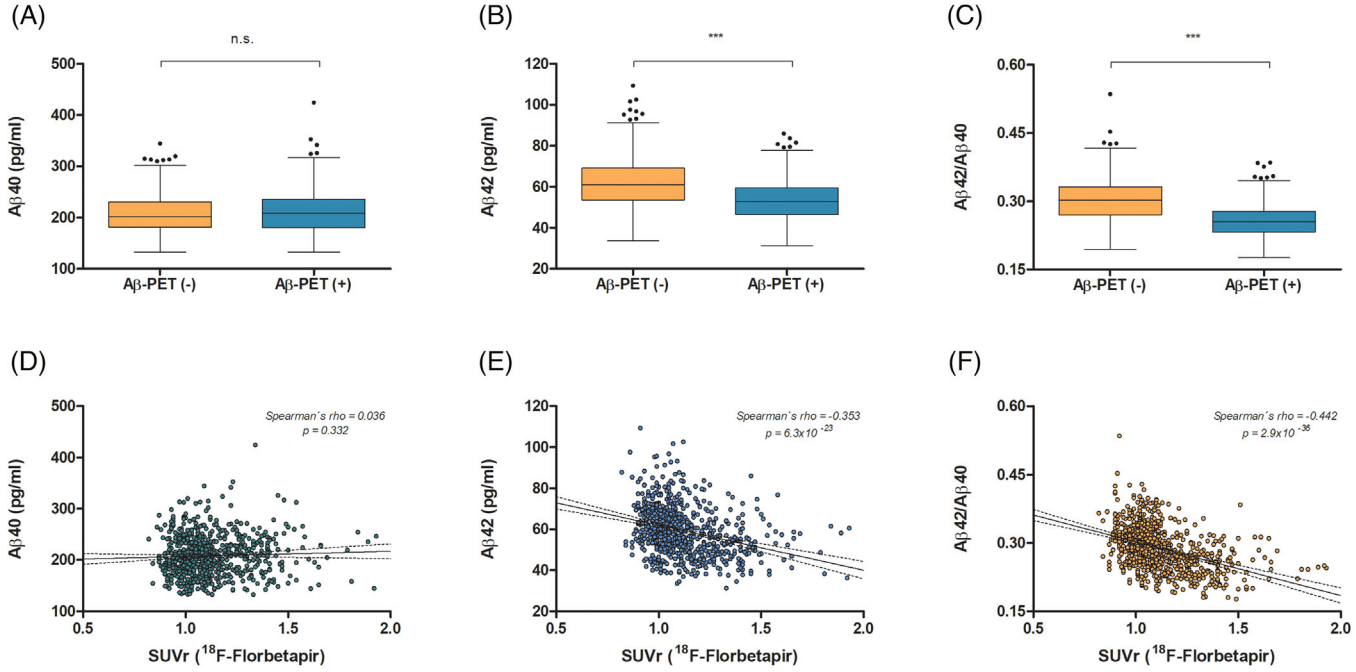

FIGURE 1 Distribution of plasma biomarkers between Aβ PET(−) and Aβ PET(+) groups and correlation of plasma biomarkers with brain amyloid deposition. Distribution of plasma Aβ40 (A), Aβ42 (B), and Aβ42/Aβ40 (C) between Aβ PET(−) and Aβ PET(+) groups. Group comparisons were carried out using a Mann–Whitney test. Correlations for Aβ40 (D), Aβ42 (E), and Aβ42/Aβ40 (F) and ^{18}F-Florbetapir SUVR values. Dashed lines represent 95% confidence intervals. ***$P < 0.001$. Aβ, amyloid beta; n.s., non-significant; PET, positron emission tomography; SUVR, standardized uptake value ratio.

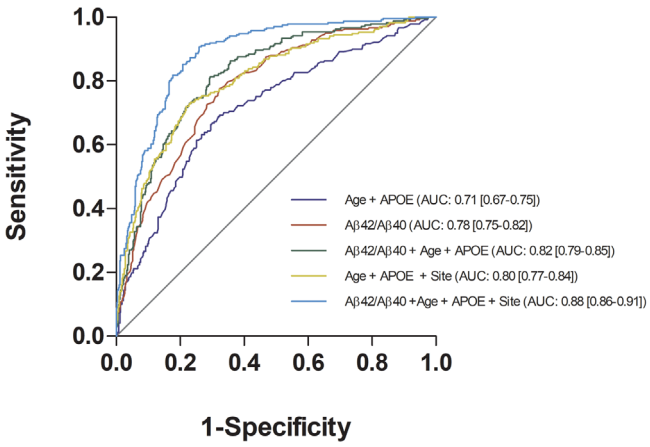

FIGURE 2 Predictive ability of different regression models for identifying Aβ PET status. ROC curves for discriminating Aβ PET status. Five regression models from the 12 shown in Table 2 are selected for representation. The model with highest AUC and accuracy, as well as lowest AIC, includes Aβ42/Aβ40, age, number of *APOE* ε4 alleles, and recruitment site. AIC, Akaike information criterion; *APOE*, apolipoprotein E; AUC, area under the ROC curve; PET, positron emission tomography; ROC, receiver operating characteristic.

multicentric study, an environment that much better reflects real-world conditions than small, single-center studies in which variability associated with sample handling, storage, and transportation should be lower.

As expected, plasma Aβ42/Aβ40 was reduced in Aβ PET(+) individuals, being 13.3% lower than in Aβ PET(−) counterparts. This observed reduction is in good agreement with previously reported works.[18] The small differences in plasma Aβ42/Aβ40 values between Aβ PET(+) and Aβ PET(−) groups suggest that the use of high reliability analytical procedures is critical. Method robustness and analytical repeatability are needed for the analysis of amyloid peptides in plasma; otherwise, small analytical drifts could dramatically change the outcome of the assay.[18,19]

The full descriptive model (including Aβ42/Aβ40, age, *APOE* ε4, and recruitment site), outperformed the most complete demographic model, which included all the above covariates, with the exception of plasma Aβ42/Aβ40 ratio. The best model, in terms of AIC, yielded the highest AUC value and the highest accuracy. The inclusion of the number of *APOE* ε4 alleles and age in any predictive model for identification of brain amyloid status is well justified,[36–41] as these are two well-known risk factors for AD and additive to plasma Aβ42/Aβ40 ratio. The obtained predictive model (Aβ42/Aβ40, age, and *APOE* ε4) is frequently reported in the literature,[42] identifying age and the presence of *APOE* ε4 alleles as risk factors for brain amyloid deposition. An AUC value of 0.82 obtained in this study is in good agreement with those recently reported in a comparative study for IP-MS (0.78–0.84) and immunoassays (0.77–0.81) in an Alzheimer's Disease Neuroimaging Initiative dataset.[43]

The inclusion of recruitment site in the model corrects, at least partially, the heterogeneity introduced by such a large number of sites. Other authors have adopted similar approaches in multi-cohort

TABLE 2 Logistic regression model diagnostics.

Model	Covariates	AIC	pseudo-r^2	AUC	95% CI
1	Age, sex, *APOE* ε4, MMSE	836.984	0.180	0.72	0.68–0.76
2	Age, *APOE* ε4, MMSE	835.131	0.180	0.72	0.68–0.76
3	Age, *APOE* ε4	837.008	0.174	0.71	0.67–0.75
4	Age, *APOE* ε4, site	838.082	0.337	0.80	0.77–0.84
5	Ratio	763.842	0.284	0.78	0.75–0.82
6	Ratio, age, *APOE* ε4[a]	716.767	0.362	0.82	0.79–0.85
7	Ratio, age, *APOE* ε4, site[b]	709.120	0.513	0.88	0.86–0.91
8	Age, sex, *APOE* ε4, PACC	832.916	0.188	0.72	0.68–0.76
9	Age, *APOE* ε4, PACC	831.425	0.187	0.72	0.68–0.76
10	Ratio, age, *APOE* ε4, PACC	714.964	0.367	0.82	0.79–0.85
11	Age, *APOE* ε4, site, PACC	834.027	0.346	0.81	0.77–0.84
12	Ratio, age, *APOE* ε4, site, PACC	708.578	0.516	0.88	0.86–0.91

Abbreviations: AIC, Akaike information criterion; *APOE*, apolipoprotein E; AUC, area under the curve; CI, confidence interval; MMSE, Mini-Mental State Examination; PACC, Preclinical Alzheimer Cognitive Composite; Site, recruitment site.

Note: Logistic regression model fit was evaluated according to AIC, where a lower value means a better fit after a correction for overparameterization. Pseudo-r^2 values correspond to Nagelkerke r^2 values. 95% CI for each AUC value are also shown. ΔAIC between Model 12 and Model 7 (0.542) is not considered significant according to a likelihood-ratio test ($P = 0.05$ and 1 degree of freedom), so PACC was not included in the final model.

[a]AUC from Model 6 is statistically different from that of Model 3, according to the DeLong test ($P < 0.001$).

[b]AUC from model 7 is statistically different from that of Model 4, according to the DeLong test ($P < 0.001$).

studies.[44,45] Accordingly, the full model, which includes this covariate, yields a much better separation between Aβ PET(+) and Aβ PET(−) subjects, improving clinical robustness of the plasma biomarker. Contribution of this covariate in the performance of regression models could be impacted by the unequal distribution of participants per center, as well as by different factors that are part of pre-analytical variability.

Regarding this last point, a recent study has revealed large differences in the predictive value of plasma Aβ42/Aβ40 when two different plasma collection protocols were applied in A4 samples.[24] Although a direct comparison is not possible, because the same samples have not been analyzed, when plasma samples were processed within 24 hours from blood extraction, the C2N's IP-LC-MS/MS technology identified amyloid-PET status with an AUC of 0.64, notably worse than the 0.78 reported by ABtest-MS in the present study in plasma samples processed with the same protocol. On the other hand, in the study by Winston et al.,[24] a dataset of 224 plasma samples was processed within 2 hours from blood extraction and analyzed by Shimadzu's and C2N's platforms. In this case, the AUCs for amyloid-PET status were 0.80 and 0.76, respectively, which were comparable to the performance reported by ABtest-MS in plasma samples processed within 24 hours. These results reflect that the impact of pre-analytical variables on the final performance of the biomarker is highly dependent of the analytical methodology used, and consequently, suggest that ABtest-MS may be a more robust approach for the analysis of plasma Aβ42/Aβ40 under a variety of pre-analytical conditions such as delayed blood processing.

The impact of pre-analytical variables on Aβ40 and Aβ42 levels has been investigated in other studies. Unlike Winston et al.,[24] Verberk et al.[21] observed that storage of blood samples up to 24 hours at 4°C before centrifugation does not have an impact on Aβ levels. Further studies should address the potential causes of these observed variations and compare the sensitivity of different platforms, for example, platforms that include an immunocapture step versus platforms such as ABtest-MS that does not, to this pre-analytical variable.

According to the concordance plot, it is observed that there were many more false positive than false negative predictions. Most of the discordant cases (78%) were plasma Aβ42/Aβ40(+), according to a threshold of 0.303, but Aβ PET(−). This observation has been interpreted as the plasma ratio being an earlier biomarker of brain amyloid deposition than PET itself, that is, CSF and plasma ratio values begin to decrease before amyloid accumulation is detected by PET imaging.[46]

There is a common agreement in the difficulty of the analysis of Aβ peptides in human plasma.[47,48] Hydrophobicity and self-aggregating properties of these peptides suppose an analytical challenge. Besides this, interferences with plasma components (the so-called matrix effect) may impact analytical reproducibility. Methods including an immunocapture step are more prone to show matrix effects than those procedures which avoid it. These effects may be more detrimental in immunoassays than in MS-based methods, as the latter usually include a solid phase extraction step for analyte purification. In our opinion, antibody-free, direct extraction methods are faster and more robust in terms of sample preparation. Moreover, a significant increase in sample throughput and a reduction in cost per sample is achieved, making the assay much more affordable. All these results demonstrate that ABtest-MS is a useful and accurate tool for the identification of brain amyloid deposition in CU individuals, even in large and heterogeneous datasets.

In the last few years, trials are being conducted in the early stages of the disease, even in asymptomatic individuals, when the prevalence of amyloid positivity is very low. The use of an accurate blood test

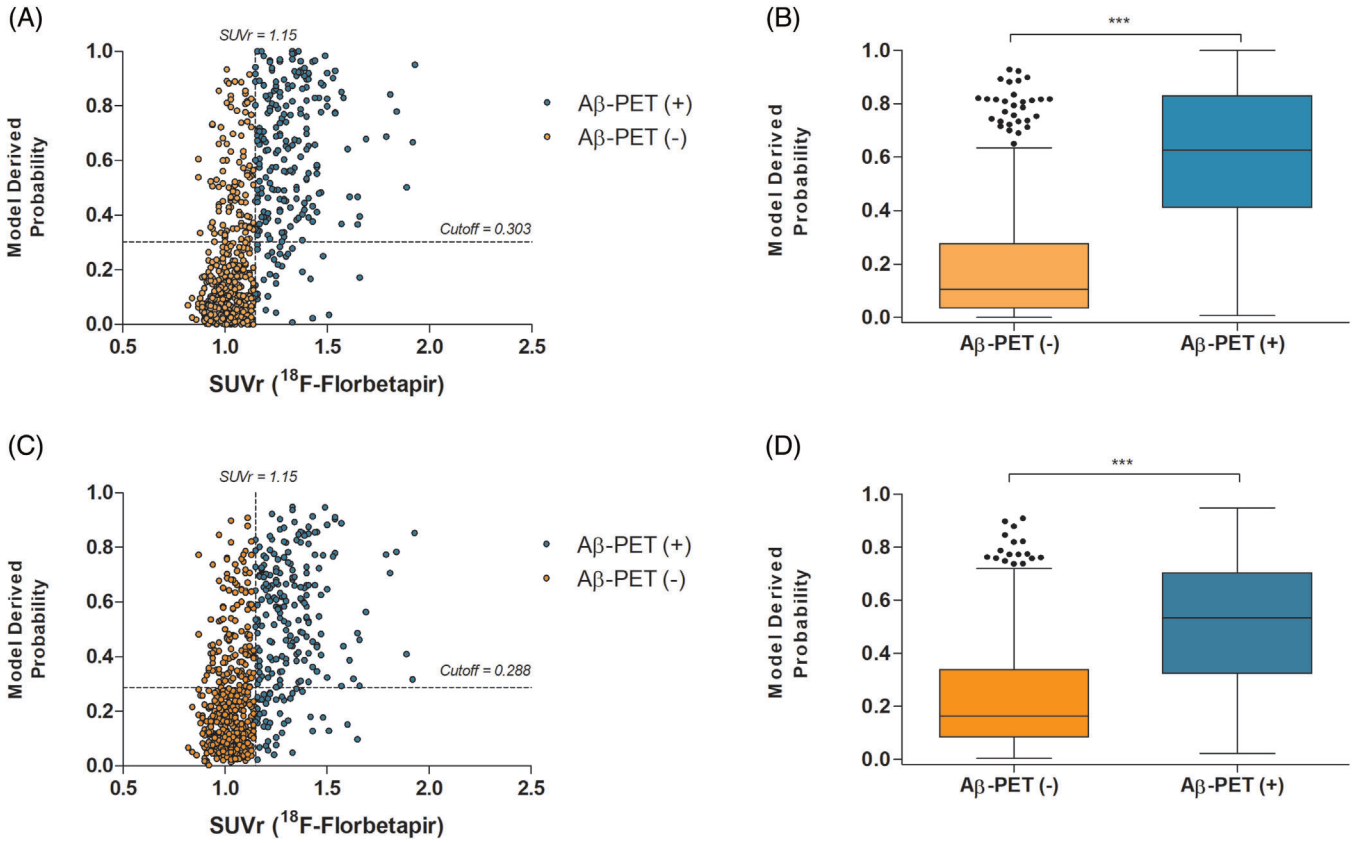

FIGURE 3 Concordance plots and distributions of model-derived probabilities between Aβ PET(−) and Aβ PET(+) groups. A, Concordance plot between ^{18}F-Florbetapir SUVR and derived probabilities of the regression model including Aβ42/Aβ40, age, number of *APOE* ε4 alleles, and recruitment site. Dashed lines represent cutoffs for SUVR (vertical) or probability at Youden maximum index (horizontal). B, Distribution of predicted probabilities between Aβ PET(−) and Aβ PET(+) groups for the model described above (A). Group comparisons were carried out using a Mann–Whitney test (*** $P < 0.001$). Values exceeding median value ± 1.5 x interquartile range (IQR) are displayed as outliers. C, Concordance plot between ^{18}F-Florbetapir SUVR and derived probabilities of the regression model including Aβ42/Aβ40, age, and number of *APOE* ε4 alleles. Dashed lines represent cutoffs as in (A). D, Distribution of predicted probabilities between Aβ PET(−) and Aβ PET(+) groups for the model described above (C). Group comparisons were carried out using a Mann–Whitney test (*** $P < 0.001$). Values exceeding median value ± 1.5 x IQR are displayed as outliers. Aβ, amyloid beta; *APOE*, apolipoprotein E; PET, positron emission tomography; SUVR, standardized uptake value ratio.

applying cutoff values which maximize PPV could provide a significant reduction of the number of PET scans or lumbar punctures[34,49,50] needed to select the desired population of Aβ PET(+) individuals. Additionally, recruitment time (3.5 years for the A4 Study[6]) would be dramatically reduced, providing a clear benefit not only for study sponsors, but mainly patients, relatives, and caregivers.

The main limitation of this work is the availability of data from a single blood biomarker. Though it is commonly accepted that Aβ peptides show one of the earliest changes among the current biomarker panel, combinations of several biomarkers (i.e., Aβ42/ Aβ40 and phosphorylated tau) could be of great help for the identification of CU individuals who are in the initial stages of brain amyloid accumulation.

Another limitation is the overwhelming majority of White participants (92% of the 4486 participants in the second screening visit and 94% in this dataset), which can add complexity to the generalization of the results to real-world settings. Further studies should address potential differences in the performance of blood tests across different racial groups.

To conclude, in this work, we have demonstrated that ABtest-MS, a direct extraction protocol coupled to LC-MS for the quantitation of Aβ40 and Aβ42 in plasma samples, accurately identifies brain amyloid deposition in a population of CU individuals from the second screening visit of the A4 Study, despite the high heterogeneity inherent to large and multicentric clinical trials. Unlike other platforms, ABtest-MS has demonstrated high robustness for plasma Aβ42/Aβ40 performance when plasma is obtained up to 24 hours after blood extraction, outperforming IP-MS methodology. A predictive model based on plasma Aβ42/Aβ40, number of *APOE* ε4 alleles, and the age of the patient, could provide significant savings in time and cost for the clinical trial recruitment step.

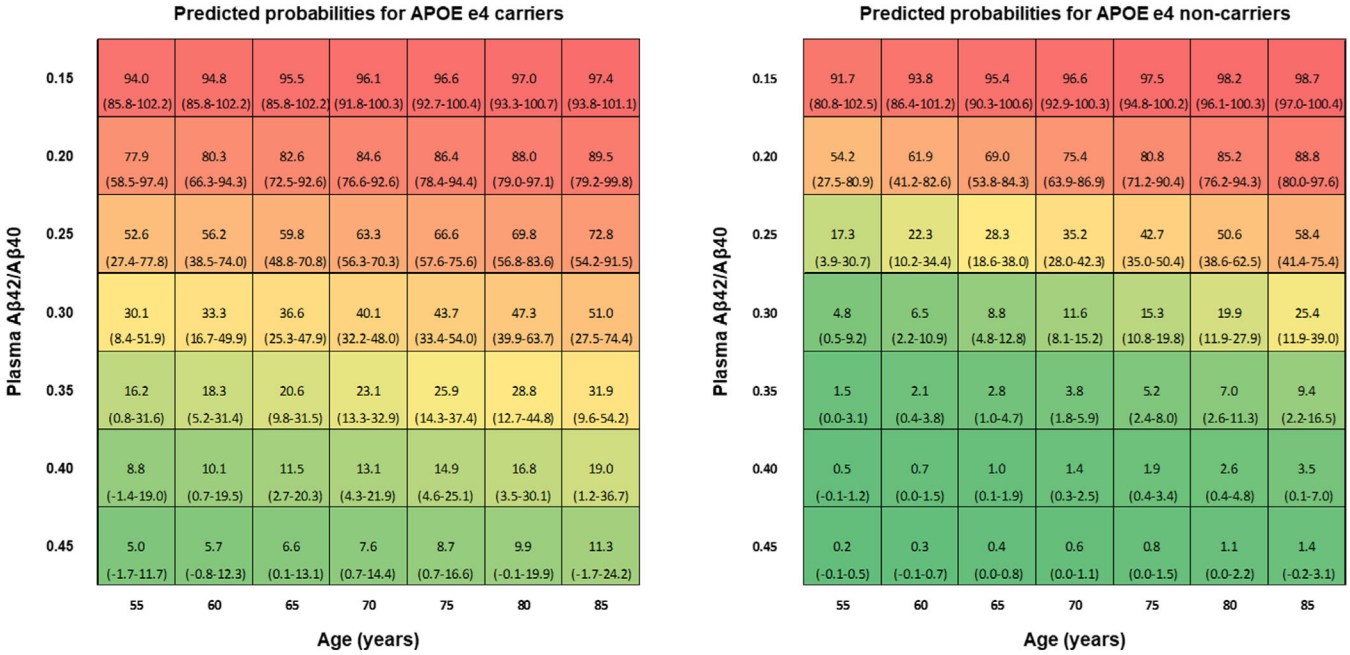

FIGURE 4 Heat maps showing predicted probability of being Aβ PET(+) according to age (horizontal axis) and Aβ42/Aβ40 plasma values (vertical axis) for those subjects bearing at least one *APOE* ε4 allele (left) and *APOE* ε4 non-carriers (right). Predicted probabilities are displayed in percentages. 95% confidence intervals are indicated between brackets. Aβ, amyloid beta; *APOE*, apolipoprotein E; PET, positron emission tomography.

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CONFLICT OF INTEREST STATEMENT

J.A.A., M.P.L., L.S., S.C., and J.T. are full-time employees at Araclon Biotech-Grifols. M.E.S. is a statistical consultant at Caebi. R.A.R. and S.A.L. have no conflicts of interest relevant to this study. Author disclosures are available in the supporting information.

CONSENT STATEMENT

All human subjects provided informed consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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