Featured Article

Plasma amyloid β 40/42 ratio predicts cerebral amyloidosis in cognitively normal individuals at risk for Alzheimer's disease

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Abstract

Introduction: Blood-based biomarkers of pathophysiological brain amyloid β (Aβ) accumulation, particularly for preclinical target and large-scale interventions, are warranted to effectively enrich Alzheimer's disease clinical trials and management.

Methods: We investigated whether plasma concentrations of the $A\beta_{1-40}/A\beta_{1-42}$ ratio, assessed using the single-molecule array (Simoa) immunoassay, may predict brain Aβ positron emission tomography status in a large-scale longitudinal monocentric cohort (N = 276) of older individuals with subjective memory complaints. We performed a hypothesis-driven investigation followed by a no-a-priori hypothesis study using machine learning.

Results: The receiver operating characteristic curve and machine learning showed a balanced accuracy of 76.5% and 81%, respectively, for the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio. The accuracy is not affected by the apolipoprotein E (*APOE*) ε4 allele, sex, or age.

Discussion: Our results encourage an independent validation cohort study to confirm the indication that the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio, assessed *via* Simoa, may improve future standard of care and clinical trial design.

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Keywords: Alzheimer's disease; Plasma amyloid β; Simoa immunoassay; Machine learning; Subjective memory complainers; Amyloid PET; Classification and regression trees (CART)

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1. Introduction

Blood-based biomarkers are expected to facilitate critical clinical solutions catalyzed by the global threat of the evolving Alzheimer's disease (AD) epidemic [1–3]. They will support early screening and identification of individuals who are very unlikely to develop AD-related pathophysiology and increase the probability that individuals with AD pathophysiology are being selected for further investigations using more specific, expensive, and/or more invasive methods with reduced accessibility (e.g., positron emission tomography [PET] imaging or cerebrospinal fluid [CSF] assessment). The broad availability of blood-based biomarkers will also generate a critical step toward a cost-, resource-, and time-effective multistep diagnostic workup and facilitate the reengineering of drug Research & Development pipelines, from proof of pharmacology to clinical trial design [4–8].

Brain accumulation of amyloid β (Aβ) and Aβ-induced neuronal and glial proteotoxicity is a core feature of the complex pathophysiological landscape of late-onset polygenic AD [7–14]. In this regard, growing evidence has shown that plasma Aβ concentrations can accurately predict cerebral amyloidosis in AD and mild cognitive impairment (MCI). Previous population-based studies reported that the $A\beta_{1-40}/A\beta_{1-42}$ ratio, assessed using mass spectrometry, demonstrated an accuracy of approximately 90% using Aβ-PET imaging as a standard of truth in two pooled cohorts (either combined or alone) of cognitively normal, prodromal (MCI), and mild dementia stage AD participants [9]. In patients with MCI or dementia, the strength of the association (concordance) between plasma Aβ and Aβ-PET imaging (or CSF) was assessed using both a dichotomized composite status (i.e., Aβ "positivity" or "negativity") and quantitative threshold measures, such as the standardized uptake value ratio (SUVR) [10–13]. Plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio was also suggested to constitute surrogate biomarkers of cortical Aβ deposition in cognitively normal subjects without suffering from any condition at risk for AD [14]. However, the predictive value of plasma Aβ levels has not been investigated in cognitively normal individuals facing subjective memory complaint (SMC) or subjective cognitive decline with or without memory impairment, which are conditions at risk for MCI or dementia within the clinical *spectrum* of AD [15,16]. It has also been shown that SMC and subjective cognitive decline with or without memory impairment may be associated with an increased risk of being positive when assessed for core biomarkers of AD [15,16]. Here, we investigated the performance of plasma $A\beta_{1-42}$ and of the $A\beta_{1-40}/A\beta_{1-42}$ ratio in the "INveStIGation of AlzHeimer's PredicTors in Subjective Memory Complainers" (INSIGHT-preAD) cohort, which provides a high-quality framework for a standardized large-scale, observational, monocentric, university-based expert-center longitudinal study of cognitively intact individuals with SMC [17]. We analyzed the accuracy of $A\beta_{1-42}$, $A\beta_{1-40}$, ratio $A\beta_{1-40}/A\beta_{1-42}$, and four other pathophysiological plasma biomarkers (total tau [t-Tau], neurofilament light chain [NFL], human cartilage glycoprotein-39 and chitinase-like protein 1 [YKL-40], and β-site amyloid precursor protein cleaving enzyme 1 [BACE1]) in predicting brain amyloidosis in 276 cognitively intact individuals with SMC through the analysis of measures gathered across three timepoints over a 3-year follow-up to test whether the prediction might be timepoint independent. For statistical modeling, we first applied a hypothesis-driven statistical approach commonly used for biomarker studies with univariate and receiver operating characteristic curve (ROC) analyses. We then carried out an unbiased machine learning approach involving random forest analysis (RFA) and classification and regression trees (CART) analysis in which we made no *a priori* hypothesis on the presence of a predictive variable in the data and in which we evaluated for the effect of the time at which the measures are collected. As developed below, these statistical models identify the $A\beta_{1-40}/A\beta_{1-42}$ ratio as the only one variable that may predict brain Aβ amyloidosis. These models systematically yielded a balanced accuracy comprised between 71% and 81% for the $A\beta_{1-40}/A\beta_{1-42}$ ratio to predict brain Aβ amyloidosis, which represents high accuracy considering the relatively small sample. These results call for independent validation cohort studies to test if the $A\beta_{1-40}/A\beta_{1-42}$ ratio may constitute a cost-effective biomarker useful for assessing brain amyloidosis in individuals who are at risk for AD.

2. Materials and methods

2.1. Study participants

We designed a large-scale monocentric research program using a cohort of SMC recruited from the INSIGHT-preAD study [17], a French academic university-based cohort, which is part of the Alzheimer Precision Medicine Initiative Cohort Program (APMI-CP) [18,19]. Participants were enrolled at the Institute of Memory and Alzheimer's disease (*Institut de la Mémoire et de la Maladie d'Alzheimer*, IM2A) at the Pitié-Salpêtrière University Hospital in Paris, France. The main objective of the INSIGHT-preAD study is to explore the earliest preclinical stages of AD through intermediate to later stages until conversion to first cognitive symptoms, using clinical parameters and biomarkers associated with cognitive progression.

The INSIGHT-preAD study includes 318 cognitively and physically normal Caucasian individuals, recruited from the community in the wider Paris area, France, aged 70 to 85 years, with SMC. The status of SMC was confirmed as follows: (1) participants gave an affirmative answer ("YES") to both questions: "Are you complaining about your memory?" and "Is it a regular complaint that has lasted for more than

6 months?" (2) participants showed intact cognitive functions based on the Mini–Mental State Examination score ≥ 27, Clinical Dementia Rating scale score = 0, and Free and Cued Selective Rating Test total recall score ≥ 41. Aβ-PET investigation was performed at baseline visit, as mandatory inclusion criterion. Thus, all subjects enrolled into the study have SMC and are stratified as either positive or negative for cerebral Aβ deposition. Time-series data for plasma concentrations of $A\beta_{1-42}$ and $A\beta_{1-40}$ were collected at three timepoints (within a 3-year follow-up) using a highly sensitive in-house immunoassay (see Section 2.3, "Immunoassays for plasma biomarkers"). In addition, four novel candidate surrogate markers of AD-related pathways were longitudinally assessed in plasma, including upstream regulators of amyloidogenic pathways and axonal sprouting, such as BACE1, astrocytes, and microglia overactivation, neuroinflammation (YKL-40), and damage of large-caliber myelinated axons (NFL), and t-Tau [8,20,21]. At the point of the study inclusion, several data were collected, namely demographic and clinical data and apolipoprotein E (*APOE*) genotype. Exclusion criterion was having a history of neurological or psychiatric diseases, including depressive disorders. The study was conducted in accordance with the tenets of the Declaration of Helsinki of 1975 and approved by the local institutional review board at the participating center. All participants or their representatives gave written informed consent for use of their clinical data for research purposes.

2.2. Blood sampling and collection tube storage

Blood samples were taken in the morning, after a 12-hour fast, handled in a standardized way, and centrifuged for 15 minutes at 2000 G-force at 4°C. Per sample, plasma fraction was collected, homogenized, aliquoted into multiple 0.5-mL cryovial-sterilized tubes, and finally stored at −80°C within 2 hours from collection.

2.3. Immunoassays for plasma biomarkers

All analyses of plasma $A\beta_{1-42}$ and $A\beta_{1-40}$, t-Tau, NFL, and YKL-40 concentrations were performed at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Sweden [22–25]. In particular, a volume of 0.5 mL of plasma for each subject was required for performing the analyses using the aforementioned platforms.

Plasma BACE1 concentrations were measured at ADx NeuroSciences, Belgium, using a research prototype ELISA, based on the commercially available ELISA for CSF measurements (EQ 6541-9601-L; Euroimmun AG, Lübeck, Germany) [26]. The measurements of each biomarker were performed in one round of experiments, using the same batch of reagents, by board-certified laboratory technicians who were blinded to the clinical data (for more details see Supplementary Material).

2.4. PET data acquisition and processing

All ^{18}F-florbetapir-PET scans are acquired in a single session on a Philips Gemini GXL CT-PET scanner 50 (±5) minutes after injection of approximately 370 MBq (333-407 MBq) of ^{18}F-florbetapir (for imaging acquisition, reconstructions as well as for SUVR calculation, and threshold identification see Supplementary Material) [17].

2.5. Statistical modeling

2.5.1. Comparisons between Aβ-PET–stratified groups, univariate correlation analysis, and diagnostic performance ROC test of the plasma Aβ ratio

The χ^2 test was used to study categorical variables in the sample stratified for Aβ-PET burden. Independent sample *t* tests (corrected for multiple comparisons) were carried out to compare continuous variables (clinical demographic data) in the sample stratified for brain amyloidosis. Normality of variables and residuals and heteroskedasticity were checked visually. However, the samples size was large enough for the *t* test to be valid even if all variables were not normally distributed.

To evaluate the performance of plasma Aβ markers in predicting the individual Aβ-PET status, we carried out ROC tests (*a priori* statistical modeling). ROCs were built up only for those plasma markers significantly different between Aβ-PET groups. A confidence interval of 95% was chosen. The area under the curve (AUC) and the representative best values for the sensitivity (sensitivity*100) and specificity (1-specificity*100) were used for evaluating the performance of the model. AUCs were compared with each other using the DeLong test [27]. For each ROC, the best cutoff point was determined by using Youden's index, which optimizes biomarker performance when equal weight is given to sensitivity and specificity and which represents the likelihood of a positive test result in subjects with the condition versus those without the condition [28]. We elected to not apply any correction for random measurements error at our ROCs as we used a highly reproducible protocol that minimizes intra-assay variability [28]. We tested the association between the plasma concentrations of $A\beta_{1-42}$ and $A\beta_{1-40}$ at the three timepoints, by performing Pearson's correlation coefficient (ρ) adjusting for the timepoint-related age, if appropriate. To follow, we investigated the association between plasma Aβ markers and the global/regional Aβ-PET SUVRs, at timepoint 1 (T1, the study inclusion time-point), by performing ρ between residuals. Residuals were recovered through carrying out a linear regression analysis between each dependent variable (either plasma markers or regional SUVRs) and age plus sex as independent variables.

All tests were two-tailed, and *P* values < 0.05 were considered statistically significant. The Bonferroni correction was applied to adjust the false discovery rate for multiple comparisons. Data analysis was performed using the SPSS 23.0 IBM software for macOS.

2.5.2. Tree-based analysis of time-series data

We applied a machine learning approach to test, in a *no-a-priori* hypothesis manner, whether the brain amyloidosis status might be explained by candidate classifiers grouped into a panel of plasma biomarkers reflecting distinctive pathophysiological mechanisms of AD (herein $A\beta_{1-42}$, $A\beta_{1-40}$, t-Tau, NFL, BACE1, YKL-40) and whether the performance of such classifier(s) might be conserved across subjects and timepoints. Specifically, we applied a tree-based approach involving the use of RFA for variable selection [29,30] and that of CART [30] analysis for selecting optimal classifiers (pruned trees) from deep trees [31]. RFA, a nonparametric statistical method, is amenable to analyzing small data sets [32], and it may perform better than logistic regression, a parametric statistical method, as previously observed in 69% of the cases in a large benchmarking study [33]. RFA and CART analysis have been successfully used for biomarker-based research on neurodegenerative disease (ND), including AD [34–37]. RFA and CART analysis were performed after oversampling of T1 data to obtain a well-balanced training set, as well as on the true data. To perform oversampling, we used the Synthetic Minority Oversampling Technique (SMOTE) as implemented in the R package DMwR (https://cran.rproject.org/web/packages/DMwR/index.html). The resulting data set is herein referred to as the training set. We also evaluated whether the classifier is time dependent, i.e., whether the performance of the classifier might collapse for the biomarker data collected at T2 and T3. To this end, we built a test set including 140 subjects at T2 and 56 additional subjects at T3.

RFA was performed by using the library randomForest for R (https://CRAN.R-project.org/package=randomForest). CART analysis was performed using the library rpart for R (https://CRAN.R-project.org/package=rpart). Briefly, CART analysis was performed to generate an explicit model of the data set containing the most important predictors resulting from RFA. To test the influence of age and other plasma markers included in our panel on the performance of the classifier, we used a logistic regression analysis as implemented in the R package (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html).

3. Results

3.1. Association between plasma Aβ levels and brain amyloidosis: Univariate and ROC analysis

At T1, 276 subjects had plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations available with a sex ratio (F/M) of 170/107. In this sample set, 74 subjects were Aβ-PET positive, whereas 56 subjects showed at least one *APOE ε4* allele (see Table 1 for clinical and demographic baseline data). These two SMC subgroups (Aβ-PET–positive vs. Aβ-PET–negative and *APOE ε4* carriers vs. *ε4* noncarriers) differed regarding plasma $A\beta_{1-42}$ concentrations and $A\beta_{1-40}/A\beta_{1-42}$ ratio. Aβ-PET–positive subjects exhibited lower plasma $A\beta_{1-42}$ concentrations ($P < .001$) and a higher $A\beta_{1-40}/A\beta_{1-42}$ ratio ($P < .001$) than Aβ-PET–negative subjects (Supplementary Fig. 1). We observed a significant difference between *APOE ε4* carriers and noncarriers regarding plasma $A\beta_{1-42}$ concentrations, with the former group characterized by decreased concentrations of this marker ($P = .013$; see Supplementary Fig. 2A). However, plasma $A\beta_{1-40}/A\beta_{1-42}$ ratios were similar between *APOE ε4* carriers and noncarriers (Supplementary Fig. 2B). No sex differences were found, either in the whole sample of SMC individuals or in the two Aβ-PET status subgroups separately. No significant differences across Aβ-PET, sex, and *APOE ε4* groups were found for the other blood-based candidate biomarkers: t-Tau, NFL, BACE1, and YKL-40 (see Supplementary Table 1). A trend was found at T1 between age and plasma $A\beta_{1-42}$ (ρ: 0.117; $P = .052$), whereas age and plasma $A\beta_{1-40}$ were correlated (ρ: 0.135; $P < .023$). There was no effect of age on plasma $A\beta_{1-42}$, $A\beta_{1-40}$, and the composite ratio at baseline and over time (data not shown). The plasma concentrations of $A\beta_{1-42}$ and $A\beta_{1-40}$ were positively correlated at each timepoint (ρ: 0.807; $P < .0001$ T1 [N = 276]; ρ: 0.723; $P < .0001$ T2 [N = 215], ρ: 0.518; $P < .0001$ T3 [N = 134]).

Baseline correlation analysis between global/regional Aβ-PET SUVR and plasma $A\beta_{1-42}$ presented a significant negative association in all tests performed even after correction for multiple-comparisons analysis (false discovery rate value of 0.0038) with the exception of three brain regions

Table 1
Demographic and clinical data of subjects at timepoint 1

	Positive Aβ-PET status (N = 73)	Negative Aβ-PET status (N = 203)	Statistics (df), *P* value*
Sex (F/M)	47/27	123/80	$\chi^2 = 0.19$ (1), $P = .67$
Age (years)	77.3; SD 3.2	76.6; SD 3.4	F = −1,5 (275), $P = .13$
APOE ε4 (n/p)	46/27	176/27	$\chi^2 = 18.5$ (1), $\boldsymbol{P < .001}$
$A\beta_{1-42}$ (pg/mL)	15.1; SD 4.0	18.4; SD 5.8	F = 5,2 (188, 4), $\boldsymbol{P < .001}$
$A\beta_{1-40}$ (pg/mL)	295.5; SD 75.4	301.9; SD 87.8	F = 0,5 (274), $P = .58$
$A\beta_{1-40}/A\beta_{1-42}$	19.4; SD 3.3	16.7; SD 5.2	F = −4,0 (274), $\boldsymbol{P < .001}$
Mean Aβ-PET SUVR	1.07; SD 0.27	0.608; SD 0.05	F = −11,7 (74, 8), $\boldsymbol{P < .001}$

NOTE. Quantitative demographic and clinical characteristics (at timepoint 1) are expressed as mean (SD).

Abbreviations: $A\beta_{1-40}$, plasma concentrations of 40 amino acid–long amyloid-β; $A\beta_{1-42}$, plasma concentrations of 42 amino acid–long Aβ; $A\beta_{1-40}/A\beta_{1-42}$, plasma concentrations of ratio $A\beta_{1-40}/A\beta_{1-42}$; Aβ-PET, amyloid-β positron emission tomography; SUVR, standardized uptake value ratio; *APOE ε4*, apolipoprotein E ε4 allele; n/p, ε4 allele negative/positive; χ^2, χ^2 test; F, Fisher's F-test.

*Statistical tests are presented as test value (degree of freedom), *P* value (significant level $P < .05$, two tailed).

Table 2
Correlation between regional Aβ-PET SUVRs and plasma Aβ markers

Regional Aβ-PET SUVRs	$A\beta_{1-42}$ (pg/mL)
Cingulum posterior R	$\rho = 0.189$* $P = .0015$
Cingulum posterior L	$\rho = 0.183$* $P = .0022$
Cingulum anterior R	$\rho = 0.198$* $P < .001$
Cingulum anterior L	$\rho = 0.210$* $P < .001$
Frontal superior orbital L	$\rho = 0.183$* $P = .0024$
Frontal superior orbital R	$\rho = 0.189$* $P = .0015$
Parietal inferior L	$\rho = 0.155$ $P = .0099$
Parietal inferior R	$\rho = 0.188$* $P = .0016$
Precuneus L	$\rho = 0.198$* $P < .001$
Precuneus R	$\rho = 0.168$ $P = .0051$
Temporal middle L	$\rho = 0.157$ $P = .0088$
Temporal middle R	$\rho = 0.173$ $P = .0039$
Mean cortical binding	$\rho = 0.181$* $P = .0025$

NOTE. Pearson's correlation coefficients seek through residuals (original value cleaned from the potential effect of age and sex).

P value significant level $\alpha < 0.05$, two tailed. False discovery rate: $P = .0038$.

Abbreviations: $A\beta_{1-40}$, plasma concentrations of 40 amino acid–long amyloid-β; $A\beta_{1-42}$, plasma concentrations of 42 amino acid–long Aβ; Aβ-PET, amyloid-β positron emission tomography; SUVR, standardized uptake value ratio; ρ, Pearson's correlation coefficient; P: uncorrected P value; R, right; L, left.

*Significant correlations after correction for multiple comparisons.

Fig. 1. ROC of plasma $A\beta_{1-42}$ levels and of the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio in predicting the individual Aβ-PET status. Baseline diagnostic performance test ROCs show for $A\beta_{1-42}$ at the best cutoff point an AUC 68.1% with a sensitivity of 52.3% and a specificity of 79.7% (balanced accuracy of 66 %), whereas the ratio $A\beta_{1-40}/A\beta_{1-42}$ shows an AUC of 0.794 with a sensitivity of 78.1% and a specificity of 74.9% (balanced accuracy of 76.5%), at the best cutoff point (17.82). The DeLong's test discloses a statistically significant difference between the two ROCs ($P = .006$). Abbreviations: AUC, area under the curve; $A\beta_{1-40}$, plasma concentrations of 40 amino acid–long amyloid-β; $A\beta_{1-42}$, plasma concentrations of 42 amino acid–long Aβ; Aβ-PET, amyloid-β positron emission tomography; ROC, receiver operating characteristic curve.

(Table 2). All results were controlled for age and sex by using residuals to perform these correlation tests. When we examined the diagnostic performance test at T1, the ROCs displayed an AUC of 0.680 for $A\beta_{1-42}$, at the best cutoff point, with a sensitivity of 52% and a specificity of 80% (balanced accuracy of 66 %). The $A\beta_{1-40}/A\beta_{1-42}$ ratio exhibited an AUC of 0.794 with a sensitivity of 78.1% and a specificity of 74.9% (balanced accuracy of 76.5%), at the best cut-off point (17.82). DeLong's test indicated a statistically significant difference between the two ROCs ($P = .006$, Fig. 1).

3.2. RFA and CART analysis identify plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio as the best predictive variable of brain amyloidosis

Next, we applied an unbiased statistical modeling approach to determine whether any of seven biomarker variables, including $A\beta_{1-40}$, $A\beta_{1-42}$, ratio $A\beta_{1-40}/A\beta_{1-42}$, t-Tau, YKL-40, NFL, and BACE1, might accurately predict the brain Aβ-PET status in SMC subjects, either alone or in combination. To this end, we carried out RFAs followed
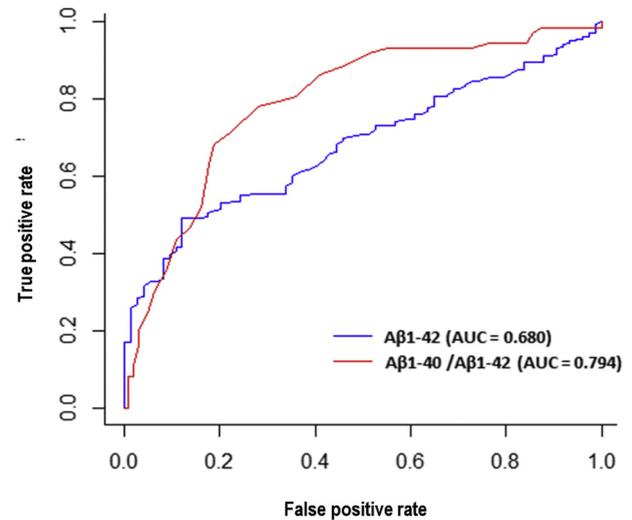
by CART analyses in which classifiers were constructed in training sets and tested in replication sets, taking advantage of the data collected across three timepoints (T1, T2, and T3) over a time period of 3 years.

Two hundred sixty-four subjects exhibited no missing values at T1, and 212 subjects and 126 subjects showed no missing values at T2 and T3, respectively. Sixty-six subjects showed no missing values for the three timepoints. There was no significant difference in the distribution of plasma $A\beta_{1-42}$ and ratio $A\beta_{1-40}/A\beta_{1-42}$ across timepoints.

The group with no missing value at T1 showed an excess of Aβ-PET–negative subjects (196 subjects are Aβ-PET negative and 68 subjects are Aβ-PET positive), which could impair the performance of the RFA, leading to high accuracy for the majority class but low prediction accuracy for the minority class. To overcome this problem, we applied the SMOTE before performing RFA (see Methods). We set SMOTE parameters so that, for each subject in the minority class, SMOTE creates an Aβ-PET–positive subject using the information from the 5 nearest neighbors [38]. With these settings, 68 synthetic Aβ-PET–positive subjects are generated by SMOTE. Then, 60 Aβ-PET–negative subjects were removed to obtain a balanced data set with 136 Aβ-PET–positive and 136 Aβ-PET–negative subjects. On over-sampling, RFA indicated that plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio may best explain cerebral Aβ deposition out of 7 variables initially included into the analysis ($A\beta_{1-40}/A\beta_{1-42}$ ratio, $A\beta_{1-40}$, $A\beta_{1-42}$, t-Tau, YKL-40, NFL, and BACE1). The

A

Random forest variable selection

$A\beta_{1-40}/A\beta_{1-42}$, $A\beta_{1-42}$, $A\beta_{1-40}$, YKL40, Tau, BACE1, NFL

MeanDecreaseGini (0, 10, 20, 30, 40)

Training set: over-sampling, T1 data

Predicted status

True status		$A\beta PET-$	$A\beta PET+$
	$A\beta PET-$	125	11
	$A\beta PET+$	19	117

- Specificity: 92%
- Sensitivity: 86%
- **Balanced accuracy: 89%**

Test set: true subjects, T2 and T3 data

Predicted status

True status		$A\beta PET-$	$A\beta PET+$
	$A\beta PET-$	113	33
	$A\beta PET+$	12	38

- Specificity: 77%
- Sensitivity: 76%
- **Balanced accuracy: 76%**

B

Decision tree

$\dfrac{A\beta_{1-40}}{A\beta_{1-42}} < 17.88$ (yes / no)

0: 106 21   1: 30 115

Training set: over-sampling, T1 data

Predicted status

True status		$A\beta PET-$	$A\beta PET+$
	$A\beta PET-$	106	30
	$A\beta PET+$	21	115

- Specificity: 78%
- Sensitivity: 85%
- **Balanced accuracy: 81%**

Test set: true subjects, T2 and T3 data

Predicted status

True status		$A\beta PET-$	$A\beta PET+$
	$A\beta PET-$	110	36
	$A\beta PET+$	16	34

- Specificity: 75%
- Sensitivity: 68%
- **Balanced accuracy: 71%**

C

Decision tree

$\dfrac{A\beta_{1-40}}{A\beta_{1-42}} < 17.84$ (yes / no)

0: 148 12 $A\beta 42 \geq 18.87$

0: 16 7   1: 32 49

Training set: true subjects, T1 data

Predicted status

True status		$A\beta PET-$	$A\beta PET+$
	$A\beta PET-$	164	32
	$A\beta PET+$	19	46

- Specificity: 84%
- Sensitivity: 72%
- **Balanced accuracy: 78%**

Test set: true subjects, T2 and T3 data

Predicted status

True status		$A\beta PET-$	$A\beta PET+$
	$A\beta PET-$	121	25
	$A\beta PET+$	20	30

- Specificity: 83%
- Sensitivity: 60%
- **Balanced accuracy: 71%**

Fig. 2. RFA and CART analysis retain the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio as a predictive classifier of the brain amyloid status in the INSIGHT-preAD cohort. (A) RFA identifies the ratio $A\beta_{1-40}/A\beta_{1-42}$ as the best variable explaining the brain amyloidosis status (i.e., positive, negative) out of seven biomarkers ($A\beta_{1-40}$, $A\beta_{1-42}$, ratio $A\beta_{1-40}/A\beta_{1-42}$, t-Tau, YKL-40, NFL, and BACE1). The performance of the model is preserved between the training set (build from oversampled data at timepoint 1) and the test set (build using unseen data from true subjects at time-point 2, and time-point 3). (B) CART analysis identifies the ratio $A\beta_{1-40}/A\beta_{1-42}$ as the only predictive variable. The threshold selected for the classification is the same as the one retained via the ROC-AUC analysis (see Fig. 1). The performance of the model is preserved between the training set (built from oversampled data at timepoint 1) and the test set (same test set as in A). (C) CART analysis identifies the ratio $A\beta_{1-40}/A\beta_{1-42}$ and $A\beta_{1-42}$ levels as predictive variables in true subjects. The performance of the model is preserved between the training set (built from data at timepoint 1) and the test set (same test set as in A). Abbreviations: $A\beta_{1-40}$, plasma concentrations of 40 amino acid–long amyloid-β; $A\beta_{1-42}$, plasma concentrations of 42 amino acid–long Aβ; $A\beta_{1-40}/A\beta_{1-42}$, plasma concentrations of ratio $A\beta_{1-40}/A\beta_{1-42}$; Aβ-PET, amyloid-β positron emission tomography; SUVR, standardized uptake value ratio; APOE ε4, apolipoprotein E ε4 allele; INSIGHT-preAD, INveStIGation of AlzHeimer's PredicTors in Subjective Memory Complainers; t-Tau, total tau; YKL-40, chitinase-like protein 1; NFL, neurofilament light chain; BACE1, β-site amyloid precursor protein cleaving enzyme 1; RFA, random forest analysis; CART, classification and regression trees.

model (training set) showed 92% specificity and 86% sensitivity with a balanced accuracy of 89% (Fig. 2A, left panel). Of note, predictive performance was preserved in the test set (i.e., true subjects and biomarker data for T1 and T2), showing a balanced accuracy of 76% (Fig. 2A, right panel).

Next, we used CART for analyzing the oversampled data and original data, using the two best explanatory variables (i.e. plasma $A\beta_{1-42}$ and $A\beta_{1-40}/A\beta_{1-42}$ ratio) obtained through RFA. In the oversampled data set, the resulting decision tree only retained the ratio $A\beta_{1-40}/A\beta_{1-42}$ as a discriminative variable (Fig. 2B, left panel). In so far, the ratio $A\beta_{1-40}/A\beta_{1-42}$ kept discriminating Aβ-PET–positive and Aβ-PET–negative subjects with a performance similar to that found when carrying out the ROC-AUC analysis

(Fig. 1). The sensitivity was 85% and the specificity was 78%, with a balanced accuracy of 81% (Fig. 2B, middle panel). These performances were well preserved in the test set, showing a sensitivity of 68%, a specificity of 75%, and a balanced accuracy of 71% (Fig. 2B, right panel). On CART analysis of the original data (true subjects), the final output is changed compared with the oversampled model because the resulting decision-tree retained both the $A\beta_{1-40}/A\beta_{1-42}$ ratio and $A\beta_{1-42}$ concentrations (Fig. 2C, left panel). The performance of the classifier built for this latter data set shows a sensitivity of 72% and a specificity of 84%, for a balanced accuracy of 78% (Fig. 2C middle panel). As expected, these performances slightly decreased, being nonetheless well preserved considering the sample size, in

the test set for true subjects because the sensitivity was 60% and specificity was 83% with a balanced accuracy of 71% (Fig. 2C, right panel).

Finally, we tested whether age, *APOE ε4*, and sex might influence the performance of the classifier built using the training set (entire population of SMC individuals at time-point 1 with oversampling). To this end, we analyzed the variation of the performances of this classifier on the test set (i.e., true subjects at T2 and T3) across the subjects. The age distribution was homogeneous and centered onto 80 years (see Supplementary Fig. 3). To test whether the age has an influence on the performance of the classifier, we used the fact of being well classified or miss classified as a dependent variable and the age as an explanatory variable. Logistic regression analysis ($P < .05$ was considered significant) indicated no significant association between age and the performance of the classifier in predicting brain amyloidosis ($P > .1$), suggesting that the performance of the global CART classifier in predicting brain amyloidosis is independent of age.

Logistic regression using the same criterion as mentioned previously ($P < .05$) also indicated no significant association between any of the other candidate biomarkers (t-Tau, NFL, BACE1, and YKL-40) considered individually and the performance of the global CART classifier in predicting brain amyloidosis (all P values > 0.09). These results indicate that the prediction of Aβ status, using a global classifier in which the plasma $Aβ_{1-40}/Aβ_{1-42}$ ratio is the discriminative feature, is not influenced by the plasma concentrations of other AD-related plasma biomarkers.

Regarding a putative effect of the *APOE ε4* allele, we compared the performances of the CART classifiers between the population carrying at least one *APOE ε4* allele and the population without *APOE ε4* allele in the test set. We observe no differences of performance between both populations, suggesting that the performance of the CART classifier is independent of the *APOE ε4* status, suggesting the global CART classifier in predicting brain amyloidosis is independent of the *APOE ε4* status (data not shown). Regarding a putative effect of sex, 31% of the women and 21% of the men were incorrectly classified as false positive or false negative in the test. This effect was not statistically significant, as indicated by the $χ^2$ test ($P > .1$), thus suggesting that the performance of the CART classifier might be equally well in both populations. However, there is a trend toward females to be more often misclassified than males, which led us to further explore this question as developed below.

Next, we tested whether the predictive performances of the CART classifiers might be altered when separating male and female subjects for constructing these classifiers. To this end, we carried out RFA and CART analysis in each of these subgroups of SMC subjects, considering only the subjects with no missing value at T1 (see Supplementary Fig. 4 A–D).

Taken together, these data suggest that the performances of the CART classifier in predicting the Aβ-PET status in the INSIGHT-preAD cohort were sex independent, corroborating the conclusion obtained for the classifier built using the entire population of SMC individuals.

4. Discussion

Robust translational evidence suggests that disease-modifying therapies for NDs, including AD, are more likely to achieve significant efficacy if initiated as early as possible in the early preclinical, asymptomatic stages of the disease process, when neuronal networks homeostasis may be relatively well preserved and pathomechanistic alterations are potentially reversible [6,39,40]. There is compelling demand for blood-based biomarker-guided investigations in cognitively normal individuals at risk for AD. Blood-based biomarkers are anticipated to help improve early detection and stratification of individuals according to their underlying individual pathophysiology, at certain time-points, during disease progression and to enable a precise biological staging [6,8,40]. Herein, our data show that the plasma $Aβ_{1-40}/Aβ_{1-42}$ ratio has good diagnostic performance in predicting cerebral Aβ deposition, as determined using the Aβ-PET status as standard of truth (see Fig. 1 and 2A) within a population of cognitively normal individuals at risk for AD. Our results indicate that the plasma $Aβ_{1-40}/Aβ_{1-42}$ ratio is the best CART predictor of cerebral Aβ amyloidosis in the INSIGHT-preAD cohort, using both ROC test and tree-based modeling. RFA showed an AUC of 0.794 with a sensitivity of 78.1% and a specificity of 74.9% (balanced accuracy of 76.5%), at the best cutoff point (17.82), and CART analysis retained a predictor that yielded a 78%-81% balanced accuracy at T1 (Fig. 2B, C, middle columns). Interestingly, this performance is well preserved (71% balanced accuracy) when using data collected at different timepoints (T2 and T3) over a period of time of 3 years (see Fig. 2 B, C, right columns), thus suggesting the CART predictor is not affected by the time at which marker data were collected. Of note, this statement should be taken carefully because no follow-up Aβ-PET data are available and thus the number of SMC subjects who converted to a positive Aβ-PET status is unknown.

Our findings extend to SMC individuals the recent data reported for prodromal and dementia populations in which the $Aβ_{1-40}/Aβ_{1-42}$ ratio replaced the $Aβ_{1-42}/Aβ_{1-40}$ ratio in the prediction of cerebral Aβ deposition [9]. However, we observed that the distributions of Aβ-PET–positive subjects are similar for both ratios in the INSIGHT-preAD cohort. We also observed a similar performance of the global CART classifier in predicting brain amyloidosis when using both ratios (data not shown), suggesting that the two ratios may be equally suitable for predicting brain amyloidosis.

From a practical and translational standpoint, our results indicate that only two plasma biomarkers ($Aβ_{1-40}$

and $A\beta_{1-42}$) and the related $A\beta_{1-40}/A\beta_{1-42}$ ratio are adequate for predicting cerebral $A\beta$ deposition with a good accuracy. These observations provide an encouraging basis for prospective studies aimed at developing a blood-based analysis platform that may increase global accessibility as it would be cost- and time-effective compared with the assessment of brain amyloidosis using $A\beta$-PET scanning technology. For instance, our data raise the possibility for the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio to support baseline screening to rule out individuals who do not need $A\beta$-PET scanning in scenarios other than confirmatory diagnosis cases. This possibility is supported by the observation that the predictive performance of the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio does not appear to be affected by age, sex, and the *APOE ε4 allele* (although the number of *APOE ε4*–positive individuals is relatively small in our study).

Moreover, we found a strong positive correlation between the concentrations in plasma of $A\beta_{1-42}$ and $A\beta_{1-40}$ at all the timepoints. This observation is consistent with previous studies investigating the association of the two $A\beta$ peptides in plasma in individuals with MCI (both converters and stables) and AD dementia [13,41].

Thus, the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio can be considered a robust candidate for *in vivo* prediction of brain amyloidosis across the large biological *spectrum* of AD that shows high interindividual heterogeneity along its clinical *continuum*, including preclinical phases.

Our results support the development and usefulness of blood-based biomarkers because they may inform all contexts of use (1) in clinical research studies investigating large-scale heterogeneous populations of cognitive normal individuals at risk of AD and (2) when time series are part of the study design. The collection, processing, and storage of blood are minimally invasive, easy to perform, and widely accessible to biotech and pharmaceutical industry, academic research, and primary health care facilities [1–3]. Blood-based biomarkers are currently on the way to optimize clinical decision-making and therapeutic practice in advanced fields of biomarker-based medicine, such as oncology and immunology [42], and have gained *momentum* to be introduced in the area of neuroscience, including NDs such as AD.

The amyloid/tau/neurodegeneration (A/T/N) system is an agnostic hypothesis-independent biomarker-driven classification system that has been proposed to stratify individuals, from cognitively normal to MCI and demented ones, according to core AD-related pathological and pathophysiological hallmarks [43]. The A/T/N system is based on CSF, MRI, and PET core biomarkers of AD that have been validated, qualified, and integrated into current diagnostic research criteria.

The present study is in line with the worldwide aim of expanding the unbiased A/T/N biomarker classification system to the blood matrix level, making the real-world application of the ATN system more feasible, cost- and time-effective, and globally accessible [3,44].

Moreover, although the ATN system provides key pathophysiological insights, it only partially reflects the expanding spectrum of pathomechanistic alterations occurring in AD.

On these conceptual bases, we built up a panel encompassing novel candidate biomarkers, capable of charting a wider set of age- and AD-related pathophysiological pathways. In particular, we included plasma NFL, which is a candidate marker of axonal damage of myelinated large-caliber fibers and potentially reliable predictor of neurodegeneration. Besides plasma $A\beta$ plasma, NFL is the most investigated candidate blood-based marker for several neurological conditions, including AD. Plasma t-Tau has been recently proposed as a marker of axonal damage of unmyelinated small-caliber fibers, thus suggesting that t-Tau and NFL may also provide different information over the course of NDs, including AD. BACE1 is a crucial candidate drug target for treating AD in preclinical stages. Thus, the development of blood-based biomarkers of BACE1 concentrations and enzymatic activity has increasingly been speed up. Indeed, a reliable marker for in vivo demonstration of proof of mechanism needs urgently to be integrated in clinical trials for BACE1-targeted therapies. YKL-40 is to date the strongest candidate to chart in vivo microglial overactivation, which is a key component of the complex neuroinflammatory dynamics occurring during the earliest pathophysiological events of AD. After having identified, in a *no-a-priori* hypothesis manner, the best predictor in plasma of cerebral amyloidosis, we sought to explore whether the performance might have been influenced by the expression levels of other pathomechanistic alterations as reflected by related candidate markers. We outline that there was no influence of the levels of neurodegeneration, neuroinflammation, or BACE1-related pathways on the performance of the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio in predicting cerebral amyloidosis. We firmly think that this finding is encouraging for the development of a future blood-based A/T/N system. A growing number of working groups under the umbrella of the precision medicine paradigm have been funded to support the discovery, validation, and development of blood-based biomarkers in the field of AD [3,6,16,17]; to this end, the choice of the assay is of primary importance. Simoa is a sensitive candidate assay for the assessment of comprehensive blood-based biomarker panels to be used in future large-scale population studies targeting the earliest phase of disease as it makes use of the same reagents of conventional ELISAs to simultaneously detect thousands of single-protein molecules in different matrices—blood (plasma/serum) and CSF—at femtomolar (fg/mL) concentrations. Simoa provides about a 1000-fold improvement in terms of sensitivity and is cost-effective. As a result, a more sensitive assessment will likely be produced to get an earlier detection of the disease [45].

In summary, our data suggest that the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio, examined using the Simoa immunoassay, is a reliable predictor of brain amyloidosis, which may open the gate

toward the development of (1) a feasible prescreening tool to evaluate risk of developing AD in individuals with SMC in a multistage diagnostic process and (2) a valuable selection and enrichment tool for amyloid targeted clinical trials.

The relatively small size of the INSIGHT-preAD cohort, especially with regard to participants longitudinally assessed with plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio measurement, represents a limit inherent to the study that deserves to be outlined.

We have already planned to extend the clinical and biological follow-up of the INSIGHT-preAD study to explore whether certain longitudinal trajectories of plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio may predict the development of objective cognitive impairment (either MCI or overt dementia). A longitudinal study design will be necessary to test the association between the slope of $A\beta$-PET SUVRs (i.e., the rate of brain $A\beta$ accumulation over time) and the temporal dynamics of the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio, at the individual level and in asymptomatic individuals, to establish the positive predictive performance. This step will support the understanding of the question whether plasma $A\beta$ ratios may represent a practical, accurate, and robust prescreening tool, besides being a diagnostic test, for early identification of preclinical individuals with biomarker trajectories reflecting incipient cerebral amyloidosis. Indeed, longitudinal studies on the rate of changes in $A\beta$-PET SUVRs coupled with temporal changes in neurodegeneration biomarkers and psychometric measures, suggested that higher subtle (i.e., below the threshold for assessing $A\beta$-PET positivity) increases in $A\beta$-PET SUVRs predict faster neuronal loss and cognitive decline [46,47]. Thus, we expect that changes over time of plasma $A\beta$ ratios, rather than a single assessment, may serve as a peripheral surrogate biomarker of early phases of brain $A\beta$ accumulation that are likely to progress toward overt cerebral amyloidosis (accumulators vs. nonaccumulators). The validation of plasma $A\beta$ ratios according to $A\beta$-PET rates of accumulation, rather than the $A\beta$-PET status, represents a paradigm shift toward the investigation of anti-$A\beta$ disease-modifying compounds in large-scale populations of very early preclinical individuals, which represent the most suitable target.

We deductively argue that a longitudinal study design, carried out in cognitively healthy individuals, will substantially help overcome the limitations affecting findings of cross-sectional pooled cohort-based studies. In fact, such studies have shown (1) remarkable overlapping in terms of individual assessments of plasma $A\beta$ ratios among cognitively healthy individuals as well as subjects with MCI and AD dementia and (2) the problem to consider the individual vulnerability to early brain regional accumulation of $A\beta$.

We would like to add a comment regarding the use of machine learning techniques for the evaluation of biomarker performance. We recommend the use of double statistical approaches (hypothesis-driven ROCs and non-a-priori approaches such as RFA and CART) in further biomarker validation studies. Machine learning analysis may untangle, in an unbiased manner, multiple genetic and biological factors

that may account for interindividual variability in terms of biomarkers trajectories and endophenotypes [48]. Accomplishing such a map of pathophysiological interactions represents a key challenge along the path to develop effective and safe drugs for precision medicine treatments [18].

To conclude, we suggest a large-scale longitudinal multicenter study including at least one discovery and one independent validation cohort of cognitively healthy individuals both at risk for AD and not, to optimize the standardization and harmonization of preanalytical and analytical protocols, and at the same time, to investigate key predictive biomarker performance parameters. We assume that such study design will ultimately fill the void along the roadmap of qualification of the plasma $A\beta_{1-40}/A\beta_{1-42}$ (or $A\beta_{1-42}/A\beta_{1-40}$) ratio as a peripheral surrogate biomarker of early cerebral amyloidosis, thus enriching both pharmacological trials and clinical management during early stages of preclinical AD.

Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jalz.2019.03.009.

RESEARCH IN CONTEXT

1. Systematic review: We first delivered a brief state of art on the development of blood-based biomarkers tracing brain amyloidosis. The following words were used for a key-based search strategy: "plasma," "blood-based," "amyloid β," and "Alzheimer's disease". We used a unique time-series data for plasma concentrations of $A\beta_{1-42}$ and $A\beta_{1-40}$ that were collected at three timepoints (within a 3-year follow-up) in a monocentric cohort of cognitively normal individuals at risk for Alzheimer's disease (AD). To substantially rule out *the limitations of a proposed biomarker based on or derived from cross-sectional data* and to overcome the limitations inherent to traditional statistical modeling (i.e., receiver operating characteristic curves), for the first time in the field of blood-based biomarkers for detecting cerebral amyloidosis, we carried out no-a-priori hypothesis approach. In particular, we performed a nonparametric machine learning approach looking for the best predictor of amyloid positron emission tomography status (positive *versus* negative) among a panel of 5 strong candidate biomarkers of AD pathophysiology. We found that the plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio is significantly and largely the best predictor of cerebral amyloidosis (best balanced accuracy: 81%. at baseline). We also show that such a performance is well preserved (balanced accuracy: 71%) when using data collected at the subsequent time-points (after 1-year follow-up and 3-year follow-up).

2. Interpretation: The same statistical modeling allowed us to show that the performance of plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio is not affected by the apolipoprotein E (*APOE*) *ε4* allele, sex, or age. This original finding is of primary importance for the qualification of the marker as a (1) prescreening tool to evaluate risk of developing AD in individuals at risk in a multistage diagnostic process and a (2) valuable selection and enrichment tool for amyloid targeted clinical trials.

3. Future directions: We call for independent validation cohort studies to further investigate the value of plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio. On the flip side, we do inform the reader with our intention to extend the clinical and biological follow-up of the INSIGHT-preAD study to explore whether certain longitudinal trajectories of plasma $A\beta_{1-40}/A\beta_{1-42}$ ratio may predict the development of objective cognitive impairment (either mild cognitive impairment or overt dementia).

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