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Article

# Definition of a Threshold on the Plasma A $\beta$ 42/A $\beta$ 40 Ratio Measured by Single-Molecule Array to Predict the Amyloid Status of Individuals without Dementia

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**Abstract:** Alzheimer's disease (AD) is characterized by abnormal amyloid beta (A $\beta$ ) plaques and hyperphosphorylated tau in the brain. A $\beta$  plaques precede cognitive impairments and can be detected through amyloid-PET or in cerebrospinal fluid (CSF). Assessing the plasma A $\beta$ 42/A $\beta$ 40 ratio seems promising for non-invasive and cost-effective detection of brain A $\beta$  accumulation. This approach involves some challenges, including the accuracy of blood-based biomarker measurements and the establishment of clear, standardized thresholds to categorize the risk of developing brain amyloid pathology. Plasma A $\beta$ 42/A $\beta$ 40 ratio was measured in 277 volunteers without dementia, 70 AD patients and 18 non-AD patients using single-molecule array. Patients (n=88) and some volunteers (n=66) were subject to evaluation of amyloid status by CSF A $\beta$  quantification or PET analysis. Thresholds of plasma A $\beta$ 42/A $\beta$ 40 ratio were determined based on a Gaussian mixture model, a decision tree, and the Youden index. The 0.0472 threshold, the one with the highest sensitivity, was retained for general population without dementia screening, and the 0.0450 threshold was retained for research and clinical trials recruitment, aiming to minimize the need for CSF or PET analyses to identify amyloid-positive individuals. These findings offer a promising step towards a cost-effective method for identifying individuals at risk of developing AD.

**Keywords:** Alzheimer's disease 1; plasma amyloid 2; SIMOA 3; plasma A $\beta$ 42/A $\beta$ 40 ratio 4; amyloid prediction 5; biomarkers 6; Alzheimer's disease screening 7

## 1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia [1]. AD is characterized by the abnormal accumulation of amyloid beta (A $\beta$ ) peptides in the brain, and the aggregation of hyperphosphorylated tau deposits in neurons, forming neurofibrillary tangles. A $\beta$  peptides are naturally released in the brain by the proteolytic cleavage of the amyloid- $\beta$  precursor protein (APP) [2]. In AD, there is an imbalance between production and clearance of A $\beta$  which leads to abnormal levels of long A $\beta$  forms (e.g. A $\beta$ 42), which aggregate to form plaques in the brain [3]. This aggregation process results in decreased A $\beta$ 42 concentrations in cerebrospinal fluid (CSF) [4]. The shorter and more abundant A $\beta$ 40 form, also produced from APP cleavage, is less prone to aggregation, and its concentration is assumed to distinguish high and low  $\beta$ -amyloid producers. The CSF A $\beta$ 42/A $\beta$ 40 ratio decreases in AD, and the ratio may be more relevant to the diagnosis of AD than A $\beta$ 42 or A $\beta$ 40

levels alone [5], because it normalizes the A $\beta$ 42 concentration to intra-individual A $\beta$  production reflected by A $\beta$ 40 concentration.

Positron emission tomography (PET) imaging studies showed that A $\beta$  plaques precede the formation of tau neurofibrillary tangles and cognitive decline [6]. Individuals with abnormal amyloid deposits and no cognitive impairment can be characterized as having preclinical AD [7]. These clinically normal individuals with abnormal amyloid deposits have a higher risk to develop cognitive decline than individuals without amyloid deposition [8]. Determining amyloid status in clinically normal individuals thus allows identification of a population at risk to develop AD. Amyloid status (positive or negative) is currently determined by measuring A $\beta$ 42 in the CSF or by PET-based radiotracers detecting amyloid deposits in the brain parenchyma. However, their widespread use is restricted because they are invasive (CSF sampling) or expensive (PET).

Blood biomarkers including A $\beta$  measurement have recently been developed [9], thanks to novel technologies allowing protein detection in the femtomolar range, typically around or below 1 pg/ml. Indeed, concentrations of A $\beta$  are 50–100 times lower in the plasma than in the CSF [10]. These low concentrations of plasma A $\beta$  can now be measured with single-molecular array (SIMOA) [11,12] designed for multiplex A $\beta$ 42 - A $\beta$ 40 measurement. Because A $\beta$  deposition in the brain is the earliest pathological signature of AD, measuring the A $\beta$ 42/A $\beta$ 40 ratio appears promising to detect AD [13], even at preclinical stages. Blood measurement of A $\beta$  has the advantage of being minimally invasive compared to lumbar puncture, and less expensive than PET-imaging. Several studies have already shown that a decrease in the plasma A $\beta$ 42/A $\beta$ 40 ratio was an indicator of brain amyloidosis [14–17] and could predict cognitive decline [18,19]. Existing tools include mass spectrometry and SIMOA assays (Quanterix®), with SIMOA being easier to implement in clinical research settings.

Even though accurate measurement of the plasma A $\beta$ 42/A $\beta$ 40 ratio has been reported as a promising tool for the detection of early AD stages, no cut-off value of this ratio has been clearly established in the literature to define amyloid status (positive or negative). The aim of our study was to determine a threshold of the plasma A $\beta$ 42/A $\beta$ 40 ratio measured by SIMOA in order to use this plasmatic measure as a screening tool for amyloid pathology, and to identify individuals at risk to develop AD before dementia. Individuals with plasma A $\beta$ 42/A $\beta$ 40 ratios within the normal range would be characterized as free of amyloid pathology, and individuals with abnormal biomarker values would be recommended toward further confirmatory examination, including CSF biomarker assessments or PET imaging. This screening tool also holds promise for potential utilization in forthcoming treatments for AD, such as Lecanemab or Donanemab, both of which have demonstrated improved efficacy in mitigating cognitive decline in patients with prodromal AD or mild AD dementia [20,21]. The plasma A $\beta$ 42/A $\beta$ 40 ratio could be used to measure the effects of these treatments.

## 2. Materials and Methods

### 2.1. Participants

We recruited 277 volunteers in Belgium, all aged over 50 years and without known cognitive impairment, through local advertisements. In order to validate the absence of cognitive impairment, volunteers completed the Mini-Mental State Examination (MMSE) [22]. All volunteers had a MMSE  $\geq$  24/30 and were thus without dementia. We also enrolled 88 patients from the Memory Clinic at Saint-Luc University Hospital, all of whom had reported memory complaints during a prior consultation. Patients had been clinically diagnosed with AD (n=70) or non-AD (n=18) neurodegenerative diseases. The non-AD pathologies included: frontotemporal lobar degeneration, normal pressure hydrocephalus, microvascular disease, corticobasal degeneration, Lewy body dementia, or primary progressive aphasia.

The study was conducted in accordance with the Declaration of Helsinki and approved by the institution's Ethical Committee. All participants provided written informed consent (UCL-2022-473; UCL-2016-121; UCL-2018-119).

All participants (volunteers and patients) undertook a blood test in which DNA and plasma were extracted. The plasma A $\beta$ 42/A $\beta$ 40 ratio was measured using SIMOA (Neurology 3 plex A, Quanterix). The genetic risk for AD was estimated by genotyping the apolipoprotein E gene (*APOE*).

The cohort of 88 patients, along with a subset of volunteers (n=73), also underwent CSF analysis or an amyloid PET scan for validation of their amyloid status. The final analysis included only individuals with a maximum time delay of 2 years between the blood test and PET/CSF amyloid status measurement. The blood test could have been performed before or after the PET/CSF amyloid status measurement. Participants were excluded if more than 2 years had elapsed between the two exams, unless the PET/CSF amyloid measurement was done before the blood test and gave an amyloid positive result (n=21). Since none of the individuals in our cohort received anti-amyloid therapy, those who were previously identified as amyloid-positive will continue to retain their amyloid-positive status [23]. Six individuals were excluded who had a negative CSF/PET amyloid measurement done more than 2 years before the blood test, and four were excluded who had a blood test done more than 2 years before the CSF/PET amyloid status measurement. The final sample comprised 66 volunteers, 69 AD patients, and 16 non-AD patients.

Individuals were classified as amyloid positive (A $\beta$ +) when the CSF amyloid measurement was lower than 438 pg/ml [24], or when the amyloid PET Centiloid score was higher than 26 [25]. Otherwise, they were categorized as amyloid-negative (A $\beta$ -). Both volunteers and patients underwent an MMSE evaluation on the same day as the blood test. They were classified as having dementia when their MMSE was below 24/30, and without dementia when MMSE was higher or equal to 24/30.

## 2.2. Blood drawing and plasma preparation

A standard venipuncture procedure was performed using a 21 g needle and blood was collected in EDTA polypropylene K2 tubes (K2E K2EDTA, Vacuette Tube, REF:455045). Immediately after collection, the tube was placed on ice and plasma isolation was performed within 2h. Blood was centrifuged at 2000  $\times$  g for 10 min at 4°C, and plasma was aliquoted by 500 $\mu$ l in cryotubes and stored at -80°C until further analysis.

## 2.3. APOE genotyping

DNA was extracted from blood samples. Participants were analyzed for *APOE* polymorphisms rs429358 (which is a [T/C] substitution on chr19:44908684 (GRCh38.p14) of the sequence GCTGGGCGCGGACATGGAGGACGTG[T/C]GCGGCCGCCTGGTGCAGTACCGCGG), and rs7412 (which is a [C/T] substitution on chr19:44908822 of the sequence CCGCGATGCCGATGACCTGCAGAAG[C/T]GCCTGGCAGTGTACAGGCCGGGGC). Based on two single-nucleotide polymorphisms, alleles were assigned as  $\epsilon$ 2,  $\epsilon$ 3, or  $\epsilon$ 4. The *APOE*  $\epsilon$ 4 allele represents a major risk factor of AD [26–28]. We classified participants into two groups: “ $\epsilon$ 4 carriers” ( $\epsilon$ 3 $\epsilon$ 4,  $\epsilon$ 4 $\epsilon$ 4, and  $\epsilon$ 2 $\epsilon$ 4) and “ $\epsilon$ 4 noncarriers” ( $\epsilon$ 2 $\epsilon$ 2,  $\epsilon$ 2 $\epsilon$ 3 and  $\epsilon$ 3 $\epsilon$ 3);  $\epsilon$ 4 carriers have a higher risk of developing AD compared to  $\epsilon$ 4 noncarriers.

## 2.4. Quantification of plasma A $\beta$ 40 and A $\beta$ 42

Quantification of soluble A $\beta$ 40 and A $\beta$ 42 was performed using the Neurology Plex A kit from Quanterix (Neurology 3 Plex A, REF:101995) in strict accordance with the manufacturer’s protocol. Each plasma sample was thawed to room temperature for 1h before processing for SIMOA. The same experimenter and same duration of experiment were used for all assay runs.

## 2.5. CSF analysis

A $\beta$ 42 measurements in CSF were done with Lumipulse® (G  $\beta$ -Amyloid 1-42, REF: 230336) in the Biology Lab of the Saint-Luc University Hospital, Brussels, Belgium. Participants were classified as A $\beta$ + when CSF A $\beta$ 42 was below 438 pg/ml, and A $\beta$ - otherwise [24].

## 2.6. Amyloid PET-CT acquisition and computing

Two amyloid PET-radiotracers were used in this study: [18F]flutemetamol and [11C]Pittsburg Compound B ([11C]PiB). An anatomical 3D-T1 MRI was acquired for each participant on a 3T MRI (GE Signa Premier).

For both radiotracers, semi-quantitative PET data were computed using PNEURO software (v4.1) (PMOD LLC Technologies, Zurich, Switzerland) following the previously developed Centiloid pipeline [25] to return a Centiloid value for each participant. A threshold of Centiloid = 26 was used to discriminate participants considered A $\beta$ + (Centiloid > 26) and A $\beta$ - (Centiloid  $\leq$ 26). This threshold was previously shown to be the most useful for classifying patients that would progress to dementia from those who would remain clinically stable six years after PET imaging [25].

### 2.6.1. [18F]flutemetamol PET-CT

Ninety minutes after [18F]flutemetamol (GE Healthcare, Chicago IL) intravenous injection (target activity  $185 \pm 5$  MBq), a 30-min list-mode PET/CT acquisition was performed on a Philips Gemini TF (Philips Healthcare, Amsterdam Netherlands). The images were reconstructed as a dynamic scan of  $6 \times 5$  minutes frames with 2 mm isometric voxels including attenuation, scatter and decay corrections, and time-of-flight information using the manufacturer's standard reconstruction algorithm. No partial volume correction was applied to the data.

### 2.6.2. [11C]PiB PET-CT

Forty minutes after [11C]PiB intravenous injection (target activity 500MBq), a 20 min list-mode PET/CT acquisition was performed on a Philips Vereos digital PET (Philips Healthcare, Amsterdam, Netherlands). Images were reconstructed in  $4 \times 5$  minutes frames with 2 mm isometric voxels using the manufacturer's reconstruction algorithm which includes attenuation, scatter, and decay corrections, and time-of-flight information.

## 2.7. Basic characteristics

### 2.7.1. Characteristics of volunteers and patients

Baseline characteristics were compared between volunteers and patients, using Chi-square tests for gender and *APOE*  $\epsilon$ 4 status, and ANOVA for age and MMSE, with posthoc t-tests and Holms correction for multiple testing where appropriate.

### 2.7.2. Characteristics of participants with amyloid measured through CSF or PET analysis

A subgroup of volunteers (n=66) and patients (n=85) benefited from an amyloid measurement through either amyloid PET or CSF analysis, and were classified as A $\beta$ + or A $\beta$ -. The subgroup of volunteers to whom we proposed PET or CSF analysis was enriched in *APOE*  $\epsilon$ 4 carriers, matching the frequency of  $\epsilon$ 4 carriage observed in AD patients. We compared characteristics of A $\beta$ + and A $\beta$ - individuals, using t-tests for age and MMSE, and Chi-squared tests for *APOE*  $\epsilon$ 4 status and gender repartition.

## 2.8. Statistical analysis

The threshold for plasma A $\beta$ 42/A $\beta$ 40 ratio, to classify individuals as plasmatic A $\beta$ + or A $\beta$ -, was determined using three methods: an unsupervised learning method (Gaussian mixture model, GMM), a supervised learning method (decision tree), and the Youden's index calculated on the Receiver Operating Characteristic (ROC) curve. The unsupervised method was applied to all participants, including those with no amyloid measure done using CSF or PET. The supervised method included only participants with a known amyloid status using CSF or PET. The ROC curve was estimated on individuals with a known amyloid status and without dementia. Youden's index was calculated to maximize the sum of sensitivity and specificity. Finally, we computed the posttest

probabilities resulting in a positive or negative result of the plasma measurement, with the cutoffs giving the best sensitivity or specificity for clinically normal adults aged 60 or 80.

### 2.8.1. Gaussian mixture model (GMM)

A GMM was applied on the distribution of the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio of all participants (277 volunteers, 70 AD patients, and 18 non-AD patients) to approximate the distribution as two Gaussians. The GMM analysis was implemented in R (version 4.2.1) using the *mclust* package (version 6.0.0) [29]. First, outliers were removed from the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> distribution of all participants (n=8, 2.2%). Outliers were defined according to the interquartile range (IQR) criterion; values were excluded if they fell above  $q_{0.75} + 1.5 \text{ IQR}$ , or below  $q_{0.25} - 1.5 \text{ IQR}$ , where  $q_{0.75}$  and  $q_{0.25}$  corresponded to first and third quartile respectively, and IQR was the difference between the third and first quartile.

The plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> distribution, without outliers, was then modeled using a two-component GMM with unequal variance, as our aim was to classify the participants into two groups (plasmatic A $\beta$ <sup>+</sup> or A $\beta$ <sup>-</sup>). The threshold value of the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio, used to categorize participants, corresponds to the point of intersection between the two Gaussian distributions. Participants with plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio below the threshold were considered plasmatic A $\beta$ <sup>+</sup> and those with a ratio above the threshold were considered plasmatic A $\beta$ <sup>-</sup>.

The plasmatic amyloid classification of participants was then compared across clinical diagnoses (AD, non-AD, and volunteers). Among the volunteers, plasmatic amyloid classification was also compared between  $\epsilon$ 4-carriers and noncarriers using a Chi-squared test.

We also evaluated the sensitivity and specificity of the plasma test using the 151 participants (66 volunteers, 69 AD patients, and 16 non-AD patients) for whom amyloid status was determined by PET or CSF analysis. Finally, as we were specifically interested in the ability of the plasmatic measure to determine the amyloid status of individuals before dementia, we evaluated the sensitivity and specificity of the test based only on participants without dementia (n=126) with a known amyloid status.

### 2.8.2. Decision tree

The 151 participants with a known amyloid status were classified by a supervised algorithm. A decision tree was trained in MATLAB using the Classification Learner app, with a maximum of one split to classify individuals as plasmatic A $\beta$ <sup>+</sup> or plasmatic A $\beta$ <sup>-</sup> based on one feature: the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio. We trained the model using all data. The decision tree split nodes were based on the Gini diversity index. This decision tree provided a classification threshold. The sensitivity and specificity of this method were estimated.

Finally, sensitivity and specificity of the test were evaluated based only on individuals without dementia with a known amyloid status.

### 2.8.3. ROC curve

We generated the ROC curve for the 126 non-demented individuals with confirmed amyloid status. In this analysis, we exclusively considered individuals without dementia to assess the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio capability to accurately discriminate amyloid status as a screening tool among individuals before dementia. We computed the area under the curve (AUC) and its 95% confidence interval (CI). We determined the Youden's index. We evaluated the sensitivity and specificity of test at this cutoff value.

### 2.8.4. Posttest probabilities

Posttest probabilities were computed at two different ages 60 and 80 years, with the likelihood ratio for two cutoff values, one giving the best sensitivity and one giving the best specificity. The prevalence of amyloid pathology in the clinically normal population at these two ages (15.8% at 60, 32.6% at 80) were used to estimate the pretest probabilities [30].

The likelihood ratio for a positive test result (LR+) was defined as the sensitivity divided by one minus the specificity ( $LH+ = Sn/(1-Sp)$ ), and the likelihood ratio for a negative test result (LR-) as one minus the sensitivity divided by the specificity ( $LH- = (1-Sn)/Sp$ ). Then, the pretest odds were calculated as the pretest probability divided by one minus the pretest probability. Posttest odds were obtained by multiplying the pretest odds by the likelihood ratio. Finally, posttest odds were converted into the posttest probability.

### 3. Results

#### 3.1. Participants

Demographic characteristics of the participants are presented in Table 1. AD patients were significantly older than volunteers ( $p < 0.0001$ ) and non-AD patients ( $p = 0.017$ ). Age was not different between volunteers and non-AD patients. As expected, MMSE scores were higher among volunteers than AD ( $p < 0.0001$ ) and non-AD patients ( $p < 0.0001$ ); MMSE scores were also higher among non-AD patients compared to AD patients ( $p = 0.0011$ ). As expected, there were more  $\epsilon 4$  carriers among AD patients than volunteers ( $p < 0.0001$ ), and among AD than non-AD patients ( $p = 0.0006$ ). Indeed, the  $\epsilon 4$  allele of the *APOE* gene increases the risk to develop AD [26–28]. The *APOE*  $\epsilon 4$  status was not different between non-AD patients and volunteers. The gender repartition was not different between groups. Among AD patients, 23 individuals had dementia; among non-AD patients, 2 individuals had dementia.

**Table 1.** Demographic characteristics of volunteers and patients. SD: standard deviation.

	Volunteers	AD patients	Non-AD patients	p-value
n	277	70	18	
Age: mean (SD)	66.5 (7.78)	71.1 (8.05)	65.6 (9.55)	0.00044 <sup>a</sup>
MMSE: mean (SD)	28.5 (1.27)	24.1 (4.59)	26.4 (2.12)	10 <sup>-9a</sup>
<i>APOE</i> *: n $\epsilon 4$ -/n	192/83	21/40	14/2	10 <sup>-7b</sup>
$\epsilon 4+$ (% $\epsilon 4$ -/% $\epsilon 4+$ )	(70%/30%)	(34%/66%)	(87.5%/12.5%)	
Gender: male/female	98/179	32/38	7/11	0.28 <sup>b</sup>

<sup>a</sup>ANOVA. <sup>b</sup>Chi-squared test. \**APOE*: 2 missing among volunteers, 9 missing among AD patients, 2 missing among non-AD patients;  $\epsilon 4$ - = non-carriers,  $\epsilon 4+$  = carriers.

#### 3.2. Amyloid status

Amyloid PET or CSF analysis classified 74 participants as  $A\beta+$  and 77 participants as  $A\beta-$  (Table 2).  $A\beta+$  individuals were significantly older than  $A\beta-$ . Gender repartition did not differ between these two groups. As expected, MMSE scores were significantly lower for  $A\beta+$  participants than  $A\beta-$ , and there were more  $\epsilon 4$  carriers among  $A\beta+$  participants than  $A\beta-$ . Table 2 also shows the clinical diagnosis of participants (volunteers, AD, or non-AD patients), and the way that amyloid was measured (CSF, [18F]flutemetamol PET, or [11C]PiB).

**Table 2.** Characteristics of participants with confirmed amyloid status. SD: standard deviation.

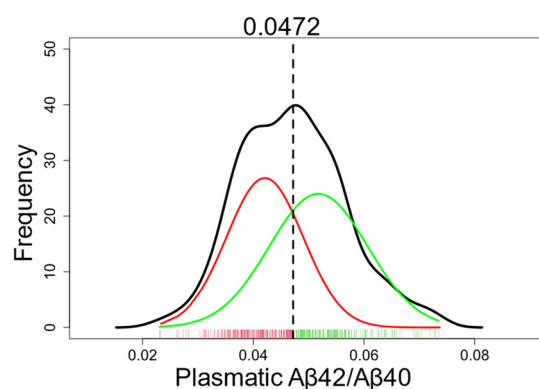
	CSF/PET $A\beta+$	CSF/PET $A\beta-$	p-value
n	74	77	
Age: mean (SD)	70.8 (8.11)	67.8 (8.67)	0.03 <sup>a</sup>
MMSE: mean (SD)	24.6 (4.57)	27.8 (2.15)	10 <sup>-7a</sup>
<i>APOE</i> *: n $\epsilon 4$ - / n $\epsilon 4+$ (% $\epsilon 4$ -/% $\epsilon 4+$ )	20/47 (30%/70%)	49/23 (68%/32%)	10 <sup>-5b</sup>
Gender: male/female	33/41	35/42	0.99 <sup>b</sup>
Non-demented/demented	53/21	73/4	
Volunteers	13	53	

<i>AD patients</i>	60	9
<i>Non-AD patients</i>	1	15
<i>Measure of amyloid:</i>		
CSF	54	24
[18F]flutemetamol PET	17	51
[11C]PIB PET	3	2

<sup>a</sup>t-test, <sup>b</sup>Chi-squared test. \*APOE: 7 missing among A $\beta$ +, 5 missing among A $\beta$ -;  $\epsilon$ 4- = non-carriers,  $\epsilon$ 4+ = carriers.

### 3.3. GMM-based classification of plasma A $\beta$

All participants (volunteers, AD, and non-AD patients) were included in the GMM analysis, except for 8 individuals (2.2%) whose plasma A $\beta$ 42/A $\beta$ 40 values were outliers. The GMM was modeled with two components (Figure 1). The intersection of the two gaussians was localized at 0.0472; participants with a plasma A $\beta$ 42/A $\beta$ 40 ratio  $\leq$ 0.0472 were classified as plasmatic A $\beta$ +, and those with a ratio  $>$ 0.0472 were classified as plasmatic A $\beta$ -.



**Figure 1.** The full distribution of the plasma A $\beta$ 42/A $\beta$ 40 ratio is shown in black. This distribution was then approximated as two gaussians, represented in red (plasmatic A $\beta$ +) and green (plasmatic A $\beta$ -). Each tick on the x-axis represents one individual. The threshold at the intersection of the two gaussians is 0.0472.

Using this threshold, 95.7% of AD patients were accurately categorized as plasmatic A $\beta$ + (Table 3), suggesting the presence of amyloid pathology. This specific threshold aligns with the 95<sup>th</sup> percentile of AD patients. Conversely, 44.4% of non-AD patients were appropriately classified as plasmatic A $\beta$ -, suggesting the absence of amyloid pathology. Finally, 58.5 % of the volunteers were classified as plasmatic A $\beta$ -, suggesting the absence of amyloid pathology. The true amyloid status of volunteers remains unknown, but it is possible that some volunteers would have amyloid brain deposits, corresponding to individuals with preclinical AD.

**Table 3.** Number of volunteers, AD, and non-AD patients classified as plasmatic A $\beta$ + or plasmatic A $\beta$ - using the threshold of 0.0472, determined by GMM.

	<b>Volunteers</b>	<b>AD</b>	<b>Non-AD</b>
Plasmatic A $\beta$ +	115	67	10
Plasmatic A $\beta$ -	162	3	8

Among the volunteers who were  $\epsilon$ 4-carriers, 49.4% (41/83) were classified as plasmatic A $\beta$ ++; among the volunteers who were  $\epsilon$ 4-noncarriers 38.5% (74/192) were classified as plasmatic A $\beta$ +. The difference in the proportion of plasmatic A $\beta$ ++ and plasmatic A $\beta$ - volunteers was not significantly different between  $\epsilon$ 4-carriers and noncarriers ( $p = 0.12$ ).

Sensitivity and specificity of the plasma A $\beta$ 42/A $\beta$ 40 ratio were evaluated using a subgroup of participants with known amyloid status. Table 4 shows the number of participants classified as

plasmatic A $\beta$ <sup>+</sup> or plasmatic A $\beta$ <sup>-</sup> according to their CSF or PET amyloid status, using the 0.0472 threshold, determined by GMM. Sensitivity was 87.8% and specificity was 55.8%.

**Table 4.** Number of participants classified as plasmatic A $\beta$ <sup>+</sup> or plasmatic A $\beta$ <sup>-</sup> based on the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio, using the 0.0472 threshold determined by GMM, and according to their amyloid status determined by PET or CSF analysis.

All individuals	CSF/PET A $\beta$ <sup>+</sup>	CSF/PET A $\beta$ <sup>-</sup>
Plasmatic A $\beta$ <sup>+</sup>	65	34
Plasmatic A $\beta$ <sup>-</sup>	9	43

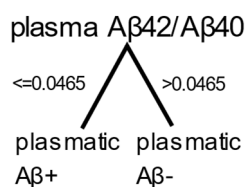
Since our focus lies in ascertaining the amyloid status of individuals without documented cognitive impairment, we conducted an evaluation of the test's sensitivity and specificity for classifying participants without dementia (Table 5), using the same threshold value of 0.0472. The sensitivity was 83%, and the specificity was 57.5%.

**Table 5.** Number of participants without dementia classified as plasmatic A $\beta$ <sup>+</sup> or plasmatic A $\beta$ <sup>-</sup> based on the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio, using the 0.0472 threshold determined by GMM, and according to their amyloid status determined by PET or CSF analysis.

Non-demented individuals	CSF/PET A $\beta$ <sup>+</sup>	CSF/PET A $\beta$ <sup>-</sup>
Plasmatic A $\beta$ <sup>+</sup>	44	31
Plasmatic A $\beta$ <sup>-</sup>	9	42

### 3.4. Decision tree

A second independent method was used to determine the threshold on the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio. A decision tree with one split was trained on one feature, the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio. The threshold determined by the decision tree was 0.0465 (Figure 2) and yielded a sensitivity of 86.5% and specificity of 58.4% (Table 6).



**Figure 2.** Result of the decision tree. Participants were classified as plasmatic A $\beta$ <sup>+</sup> if their plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio was  $\leq$ 0.0465, and as plasmatic A $\beta$ <sup>-</sup> otherwise.

**Table 6.** Number of participants classified as plasmatic A $\beta$ <sup>+</sup> or plasmatic A $\beta$ <sup>-</sup> based on the plasma A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio, using a threshold of 0.0465, as determined by decision tree, and according to their amyloid status determined by PET or CSF analysis.

All individuals	CSF/PET A $\beta$ <sup>+</sup>	CSF/PET A $\beta$ <sup>-</sup>
Plasmatic A $\beta$ <sup>+</sup>	64	32
Plasmatic A $\beta$ <sup>-</sup>	10	45

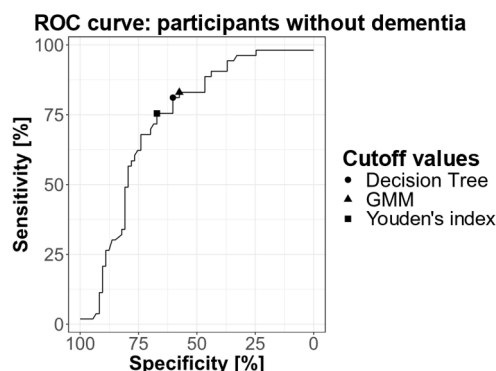
Subsequently, individuals without dementia were analyzed to determine whether those classified as plasmatic A $\beta$ <sup>+</sup> were indeed A $\beta$ <sup>+</sup> and those classified plasmatic A $\beta$ <sup>-</sup> were indeed A $\beta$ <sup>-</sup> (Table 7). For participants without dementia, the threshold value of 0.0465 yielded a sensitivity of 81.1% and a specificity of 60.3%.

**Table 7.** Number of participants without dementia classified as plasmatic A $\beta$ + or plasmatic A $\beta$ - based on the plasma A $\beta$ 42/A $\beta$ 40 ratio, using a threshold of 0.0465 as determined by decision tree, and according to their amyloid status determined by PET or CSF analysis.

Non-demented individuals	CSF/PET A $\beta$ +	CSF/PET A $\beta$ -
Plasmatic A $\beta$ +	43	29
Plasmatic A $\beta$ -	10	44

### 3.5. ROC curve

We plotted a ROC curve using only individuals without dementia for whom the amyloid status was defined by CSF or PET examination (Figure 3). The AUC was 0.728 (CI: 0.642 – 0.815). Youden's index was calculated and corresponded to a cutoff value of 0.0450. At the Youden's index, sensitivity was 75.4% and specificity 67.1%, for individuals without dementia.



**Figure 3.** ROC curve representing the sensitivity and specificity of the plasma A $\beta$ 42/A $\beta$ 40 ratio for different cutoff values, calculated on participants without dementia. The cutoff values obtained by GMM (0.0472) and decision tree (0.0465) are indicated on the curve, as well as the Youden's index (0.0450).

### 3.6. Posttest probabilities

Two cutoff values were retained: the one giving the best sensitivity (0.0472) and the one giving the best specificity (0.0450). The posttest probabilities of having brain amyloidosis were calculated according to the plasma A $\beta$ 42/A $\beta$ 40 ratio at these two cutoff values, at two different ages (60 and 80). The prevalence of amyloid pathology among the clinically normal population was 15.8% at 60 [30], corresponding to pretest odds of 0.187. The prevalence of amyloid pathology among the clinically normal population at 80 was 32.6% [30], corresponding to pretest odds of 0.484.

The threshold determined by GMM (0.0472) gave the best sensitivity. At this threshold, among the population without dementia, the sensitivity was 83.0%, specificity was 57.5%, LH+ was 1.95, and LH- was 0.29. At 60, if the plasmatic test gave a positive result, the posttest odds was 0.365, corresponding to a posttest probability of 26.8%; if the plasmatic test gave a negative result, the posttest odds was 0.055, corresponding to a posttest probability of 5.2%. At this same threshold, at 80, a positive plasmatic test gave a posttest probability of brain amyloidosis of 48.6%, and a negative plasmatic test gave a posttest probability of 12.5%.

The Youden's index (0.0450) gave a specificity of 67.1%, a sensitivity of 75.4%, LH+ of 2.30, and LH- of 0.36. With this threshold, at 60, if the plasmatic test gave a positive result, the posttest probability of having brain amyloidosis was 30.0%; if the plasmatic test gave a negative result, the posttest probability was 6.4%. At this same threshold, at 80, a positive plasmatic test gave a probability of brain amyloidosis of 52.6%, and a negative plasmatic test of 15.0%.

#### 4. Discussion

The present study confirmed that plasmatic measures of A $\beta$  are useful for predicting cerebral A $\beta$  deposition [12,15,17,31,32], as measured using either CSF or PET analysis. SIMOA is a promising technique, easier to implement and with a better throughput for measurement over a wider sample range than mass spectrometry. However, biomarker concentration values alone are of limited use for classifying individuals in a cohort and predicting their clinical status, as no clear threshold was available in the literature. The aim of this work was to determine a threshold to classify participants as amyloid positive or negative based on the plasma A $\beta$ 42/A $\beta$ 40 ratio obtained by SIMOA [14]. We determined two different thresholds, according to the context in which the plasmatic test would be used. The first threshold, which minimizes false negatives, is intended to be used as an AD exclusion tool; the second, which maximizes accuracy, is intended to be used to improve research enrollment in AD studies.

In this study, a threshold of 0.0472 was used for the plasma A $\beta$ 42/A $\beta$ 40 ratio measured by SIMOA, with the specific aim of applying it as a screening test to exclude an AD diagnosis in clinical practice. This threshold yielded the best sensitivity (83.0%), and a specificity of 57.5%, when tested on adults without dementia. This threshold corresponds to the 95th percentile among AD patients. In clinical conditions, the selection of a cutoff value should prioritize sensitivity because missing any diagnosis is undesirable. The blood test would be the first examination performed, and more specific examinations would be ordered only if necessary. A sensitivity of 83.0% would reassure individuals with a negative plasma test that they are not at risk for developing AD. This threshold allows to decrease the probability of having brain amyloid deposit in case of negative result to 5% at 60, and to 12% at 80. Therefore, in case of negative blood test result, individuals can be reassured in the sixties, and quite reassured in the eighties. Conversely, the low specificity means that specific assessments, such as amyloid-PET scans or CSF analysis, will be necessary follow-up for positive cases.

The threshold of 0.0450, obtained by the Youden's index, improved specificity to 67.1% but reduced sensitivity to 75.4%. It gave the best accuracy. A threshold with higher specificity could be useful for research applications, especially for enrollment in AD studies. It could be used to classify a large cohort of individuals without requiring PET imaging or CSF analysis. It could also be a helpful inclusion criterion in clinical trials studying amyloid in early stages of AD. Such prescreening for clinical trials recruitment would reduce the cost of clinical studies. To include one amyloid-positive individual aged 60 years old, an average of 6.3 amyloid-PET scans or CSF analyses are required given the known prevalence of amyloid positivity at this age in the general population (15.8%) [30]. In practice, using PET-scan for screening is not recommended. With the availability of a prescreening blood test, the frequency of amyloid-PET scans or CSF analyses could be substantially reduced by limiting the use of these examinations to at-risk individuals. Adults aged 60 years old with a positive blood test defined by our criteria have a 26.8% probability of cerebral amyloid deposit. Therefore, if we aim to recruit one amyloid-positive individual aged 60 years old in a clinical trial, we need to realize 3.73 PET amyloid-PET scans (or CSF analyses) among individuals with a positive blood test, which is half the number required without blood test (6.3 PET/CSF analysis).

The present findings expanded previous results on plasma amyloid measurements [12,15,17,32], and defined thresholds for the plasma A $\beta$ 42/A $\beta$ 40 ratio measured by SIMOA. Other authors have reported correlation between PET or CSF amyloid status and the plasma A $\beta$ 42/A $\beta$ 40 ratio measured by SIMOA [12,14,33]. Verberk et al. showed that the plasma A $\beta$ 42/A $\beta$ 40 ratio could serve as a prescreening test of AD pathology, and that a lower plasma A $\beta$ 42/A $\beta$ 40 ratio was associated with an increased risk of clinical progression to mild cognitive impairment or dementia [12]. These studies found that the best cutoff values to predict amyloid status varied from 0.038 [33] to 0.056 [34], without consensus. They focused on clinically unimpaired individuals as in our study. However, participants' mean age was higher than the present cohort in two of the studies (mean age: 77) [33,34] and younger than our cohort in one study (mean age: 61) [12]. Additional studies investigating the effect of age on plasma A $\beta$ 42/A $\beta$ 40 ratio should be carried out in the future. Our results confirmed a cutoff value between 0.038 and 0.056 on an independent cohort and extended it to a general population without

dementia aged over 50. We retained a threshold of 0.0472 for exclusion of AD, and a threshold of 0.0450 for research applications.

A previous study evaluated plasma biomarkers measured by SIMOA against brain autopsy [35]. The plasma A $\beta$ 42/A $\beta$ 40 ratio decreased with brain amyloid deposits, but with only modest accuracy (AUC = 0.60). Immunoprecipitation followed by spectrometry analysis also allowed to measure the plasma A $\beta$ 42/A $\beta$ 40 ratio, and gave better sensitivity and specificity (AUC = 0.75–0.89) than immunoassays for plasma [13,15,31,33,36–39]. Although mass spectrometry could appear as future gold standard, some hurdles remain for its implementation as a routine technique in comparison to SIMOA.

The present study evaluated plasma A $\beta$ 42/A $\beta$ 40 ratio, but not other AD blood biomarkers such as total-tau or neurofilament light chains, as our aim was to have a measure the brain amyloidosis. Blood-based biomarkers of phosphorylated tau (p-tau231, p-tau181, and p-tau217) have recently become available on SIMOA. P-tau181 and p-tau231 showed good accuracy for predicting AD-related neuropathological changes, but it may reflect different brain processes, such as soluble tau pathology, and/or neural damage [35]. Plasma p-tau231 has been shown to increase at early stages of AD but recent studies showed that p-tau217 better captures the earliest cerebral changes related to AD [40]. Prior studies have also shown a relationship between plasma p-tau181 and amyloid PET, even in clinically unimpaired participants [35,41,42]. Plasma p-tau181 could be an accurate predictor of AD [41]. However, only the A $\beta$ 42/A $\beta$ 40 ratio relates purely to brain amyloidosis without considering tau pathology. Given our objective to predict brain amyloidosis rather than brain tauopathy in AD, our focus was solely on quantifying plasma amyloid levels. The associations between A $\beta$  and p-tau measured in plasma require further evaluation in future studies.

Not all plasma amyloid peptides come from the central nervous system, which could explain the limited ability of observing AD-related A $\beta$  pathology in plasma. The key components of A $\beta$ -processing pathways are expressed in diverse extracerebral tissues, including the pancreas, appendix, gastrointestinal tract, and both male and female reproductive organs [43]. This suggests a potential peripheral A $\beta$  production. Furthermore, compelling evidence links A $\beta$  with the vascular system: higher plasma levels of A $\beta$  have been associated with cerebral white matter lesions, cerebral microbleeds, hypertension, diabetes, and ischemic heart disease [14]. A $\beta$  is also synthesized by the skeletal muscles, platelets, and vascular walls [44]. In addition, plasma proteins such as immunoglobulins, albumin, and complement are known to bind to and mask A $\beta$  peptides [45]. Plasma A $\beta$  is excreted by the kidneys, meaning that its level will rise in cases of renal dysfunction [46]. All these parameters influencing amyloid in plasma could explain the challenges in achieving high specificity and sensitivity in blood-based amyloid tests.

One limitation of the present study is that the thresholds it defines were established in participants older than 50. Because amyloid pathology is rare before age 50 [47], we do not recommend to test individuals for plasmatic amyloid before this age. Furthermore, aging has been reported to increase plasma A $\beta$  in both humans [46], and nonhuman primates [48]. Additional studies are needed to determine a threshold for the plasma amyloid level on a population younger than 50 years old, and to evaluate whether the threshold should be adapted according to age.

Another limitation is that amyloid status was confirmed using two different methods, PET and CSF analysis. Although it would have been more consistent to use a single reference technique, it would have restricted the number of participants eligible for the study. Finally, A $\beta$ 40 concentration was not measured in the CSF of our participants, in accordance with hospital protocols at the recruitment site. Given that the CSF A $\beta$ 42/A $\beta$ 40 ratio enhances the accuracy of AD diagnosis by adjusting A $\beta$ 42 values based on whether the patient is a high or low amyloid producer (with a high or low quantity of A $\beta$ 40) [5], the absence of this measurement in our study may contribute to the fact that some patients received a clinical diagnosis of AD while being classified as A $\beta$ - in CSF assessments.

## 5. Conclusions

The present findings build upon and refine previous work on plasma amyloid measurements. More specifically, this study established two plasma A $\beta$ 42/A $\beta$ 40 ratio thresholds measured by SIMOA, evaluated on a population without dementia aged over 50. The first threshold was 0.0472, offering higher sensitivity to minimize the risk of missing AD cases. In cases with positive results, individuals would benefit from additional exams such as PET or CSF analysis to confirm their amyloid status. The second threshold, 0.0450, may be more suitable for research and clinical trial recruitment to streamline processes and reduce costs. The significance of this threshold lies in its potential to facilitate early detection of amyloid pathology. These findings offer a promising step towards more accessible and cost-effective methods for identifying individuals at risk of developing AD, with potential implications for early intervention and clinical trial recruitment.

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** All data collected in the context of this study will be made available in an anonymized form upon the publication of the article.

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