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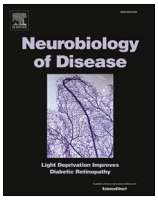

The free plasma amyloid $A\beta_{1-42}/A\beta_{1-40}$ ratio predicts conversion to dementia for subjects with mild cognitive impairment with performance equivalent to that of the total plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio. The BALTAZAR study

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ABSTRACT

Background and purpose: Blood-based biomarkers are a non-invasive solution to predict the risk of conversion of mild cognitive impairment (MCI) to dementia. The utility of free plasma amyloid peptides (not bound to plasma proteins and/or cells) as an early indicator of conversion to dementia is still debated, as the results of studies have been contradictory. In this context, we investigated whether plasma levels of the free amyloid peptides $A\beta_{1-42}$ and $A\beta_{1-40}$ and the free plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio are associated with the conversion of MCI to dementia, in particular AD, over three years of follow-up in a subgroup of the BALTAZAR cohort. We also compared their predictive value to that of total plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ levels and the total plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio.
Methods: The plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ peptide assay was performed using the INNO-BIA kit (Fujirebio Europe). Free amyloid levels (defined by the amyloid fraction directly accessible to antibodies of the assay) were obtained with the undiluted plasma, whereas total amyloid levels were obtained after the dilution of plasma (1/3) with a denaturing buffer. Free and total $A\beta_{1-42}$ and $A\beta_{1-40}$ levels were measured at inclusion for a subgroup of

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participants ($N = 106$) with mild cognitive impairment (MCI) from the BALTAZAR study (a large-scale longitudinal multicenter cohort with a three-year follow-up). Associations between conversion and the free/total plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ levels and $A\beta_{1-42}/A\beta_{1-40}$ ratio were analyzed using logistic and Cox Proportional Hazards models. Demographic, clinical, cognitive (MMSE, ADL and IADL), *APOE*, and MRI characteristics (relative hippocampal volume) were compared using non-parametric (Mann-Whitney) or parametric (Student) tests for quantitative variables and Chi-square or Fisher exact tests for qualitative variables.

Results: The risk of conversion to dementia was lower for patients in the highest quartile of free plasma $A\beta_{1-42}/A\beta_{1-40}$ ($\geq$ 25.8%) than those in the three lower quartiles: hazard ratio = 0.36 (95% confidence interval [0.15–0.87]), after adjustment for age, sex, education, and *APOE* ε4 (p-value = 0.022). This was comparable to the risk of conversion in the highest quartile of total plasma $A\beta_{1-42}/A\beta_{1-40}$: hazard ratio = 0.37 (95% confidence interval [0.16–0.89], p-value = 0.027). However, while patients in the highest quartile of total plasma $A\beta_{1-42}/A\beta_{1-40}$ showed higher MMSE scores and a higher hippocampal volume than patients in the three lowest quartiles of total plasma $A\beta_{1-42}/A\beta_{1-40}$, as well as normal CSF biomarker levels, the patients in the highest quartile of free plasma $A\beta_{1-42}/A\beta_{1-40}$ did not show any significant differences in MMSE scores, hippocampal volume, or CSF biomarker levels relative to the three lowest quartiles of free plasma $A\beta_{1-42}/A\beta_{1-40}$.

Conclusion: The free plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio is associated with a risk of conversion from MCI to dementia within three years, with performance comparable to that of the total plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio. Threshold levels of the free and total plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio could be determined, with a 60% lower risk of conversion for patients above the threshold than those below.

1. Introduction

There is a major interest in developing blood biomarkers for Alzheimer's disease (AD) and related dementias, as they offer a low-cost, non-invasive solution for longitudinal monitoring of ongoing pathological processes in the brain.

The plasma amyloid peptides $A\beta_{1-42}$ and $A\beta_{1-40}$ are biomarkers that have long attracted particular attention. Indeed, changes in brain amyloid peptides are among the first detectable signs of disease onset. An ordered clinico-biological sequence has been described in AD, with the development of abnormalities linked to cerebral amyloid peptides in the early stages of the disease: a decrease in $A\beta_{1-42}$ peptide concentrations (or in the $A\beta_{1-42}/A\beta_{1-40}$ ratio) in the CSF and positive amyloid PET tracers, making it possible to define the "Alzheimer's continuum" stage of AD, before the occurrence of Tau pathology (Jack et al., 2018).

Plasma amyloid $A\beta_{1-42}$ levels and the plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio have also been shown to be linked to Alzheimer's pathology on the basis of a significant correlation with CSF $A\beta_{1-42}$ levels or the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio (Teunissen et al., 2018; Hanon et al., 2018; Risacher et al., 2019) and PET amyloid positivity, with good sensitivity and specificity among elderly cognitively normal subjects, subjects with mild cognitive impairment (MCI) and AD patients (AUC from 0.72 to 0.98) (Risacher et al., 2019; Doecke et al., 2020; Lue et al., 2017; Hansson et al., 2010; Palmqvist et al., 2019a; Janelidze et al., 2016; Verberk et al., 2018; Vergallo et al., 2019; Ovod et al., 2017; Nakamura et al., 2018). These plasma concentrations are altered in the early stages of the disease, before positive PET-amyloid imaging and the alteration of Tau metabolism (for a review, see (Palmqvist et al., 2019b)).

Most studies have focused on the measurement of total amyloid forms using various analytical tools (Single Molecule Array [SIMOA], electrochemiluminescence, Immunomagnetic Reduction Assay [IMR], mass spectrometry, etc., for a review see (Del Prete et al., 2020) and (Leuzy et al., 2022)). Nevertheless, the differences in plasma amyloid concentrations observed between AD subjects and controls have been small in most studies using immunoanalytical techniques common in clinical practice. Thus, the fold change of plasma $A\beta_{1-42}$ concentrations between the AD group and controls has ranged from 0.28 to 4.73 by ELISA (Mean 0.98) and SIMOA (Mean 0.90) in a meta-analysis (Koychev et al., 2021). Similarly, the fold change of plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio ranged from 0.80 to 1.96 by ELISA (Mean 0.98) and SIMOA (Mean 0.72). There are various forms of total circulating plasma amyloid: free amyloid forms or amyloid linked to proteins (mainly sLRP, but also other plasma proteins such as albumin) or to circulating cells or platelets (Ullah et al., 2021), either as monomers or oligomers. It is not clear how the techniques currently used differ in their performance in measuring different forms ((Toombs and Zetterberg, 2020); (Hu et al., 2015); (Janssen et al., 2015)).

Several transversal studies have suggested that the concentrations of free amyloid forms (defined as those not bound to plasma proteins and/or cells) show greater differences between controls, MCI and AD patients. An initial study showed significant 3- to 4-fold higher free plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ levels (defined as those not bound to soluble LRP or other plasma proteins) in AD patients than controls (A. Sagare et al., 2007). Another study showed 1.8- and 4.3-fold higher free amyloid $A\beta_{1-42}$ and $A\beta_{1-40}$ levels for patients with MCI who converted to AD (MCI-AD) than for controls in the same group (A. P. Sagare et al., 2011). However, the method of assaying free forms by coimmunoprecipitation of sLRP-Aβ complexes is not well suited to clinical routine. Further studies defined free amyloid peptides as the"directly accessible" amyloid plasma fraction and measured them using classical Aβ immunoanalytical assays, but directly on undiluted plasma (UP) samples. This method is better adapted to routine clinical use and based on the principle that interactions between amyloid peptides and either the plasma matrix or within Aβ oligomers in the undiluted plasma mask epitopes from the antibodies of the assay. Dilution in a formulated buffer leads to demasking of these epitopes and recovery of the total amyloid fraction. In these studies, levels of UP amyloid were either significantly higher (Pesini et al., 2012) or, on the contrary, lower (Pérez-Grijalba et al., 2013) for patients with MCI than controls. In the most recent study, however, no significant differences in UP $A\beta_{1-42}$ or $A\beta_{1-40}$ levels or the $A\beta_{1-42}/A\beta_{1-40}$ ratio were observed between patients with MCI with negative neuroimaging (by MRI and FDG-PET) and those with positive neuroimaging. Finally, results in the literature are still scarce and contradictory and no study has compared the levels of total and free amyloid peptides.

The differences in plasma amyloid peptide concentrations observed in the cross-sectional studies cited above between control, MCI, and AD groups raise the question of their value as a prognostic marker.

Longitudinal studies on total plasma amyloid levels in large community-based cohorts have yielded contradictory results (Koyama et al., 2012). Low total plasma $A\beta_{1-42}$ or a low $A\beta_{1-42}/A\beta_{1-40}$ ratio have been found to be associated with conversion to dementia in some cohorts (van Oijen et al., 2006; de Wolf et al., 2020; Lambert et al., 2009; Chouraki et al., 2015; Janelidze et al., 2016), whereas this association was not confirmed in others (Hansson et al., 2010; Blasko et al., 2008; Oscar *L.* Lopez et al., 2019).

The prognostic relevance of total plasma amyloid levels in MCI has been less studied. Several longitudinal studies found an association between plasma amyloid levels and conversion to AD: association with a lower plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio in a large cohort ($n = 588$) of MCI

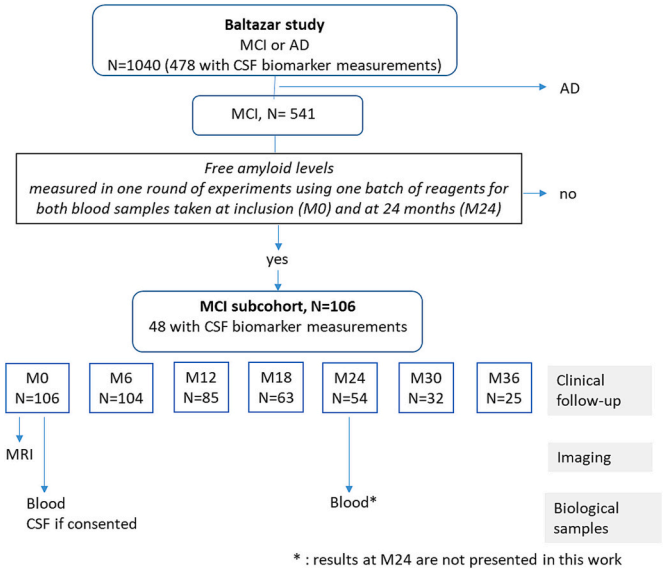

Fig. 1. Flow-chart of the study.
MCI: Mild Cognitive Impairment, AD: Alzheimer's Disease, CSF: cerebrospinal fluid, follow-up: MO, inclusion, M6: month 6, M12: month 12, M18: month 18, M24: month 24, M30: month 30, M36: month 36.

subjects (follow-up of 4 to 6 years, (Fei et al., 2011) and an association with higher $A\beta_{1-42}$ and $A\beta_{1-40}$ levels in amnesic MCI subjects (follow-up 2 years; (Cammarata et al., 2009)). Conversely, in other MCI cohorts, no association was found over a follow-up of 4 to 7 years, 2 to 4 years, and 8 years (Hansson et al., 2010; O. L. Lopez et al., 2008; Oscar *L.* Lopez et al., 2019). However, these cohorts were small (n = 120 to 200) and showed relatively low conversion rates.

There have been only two longitudinal studies that have studied the prognostic value of free plasma amyloid forms. The first was carried out by Sagare et al. on MCI subjects based on the sLRP co-immunoprecipitation technique, with a follow-up of 2 to 4 years. They showed MCI subjects who converted to AD to have equal free plasma $A\beta_{1-42}$ concentrations as those with stable MCI but higher levels of free $A\beta_{1-40}$ (Sagare et al., 2011). More recently, a much larger study did not find any significant difference between UP amyloid levels in preclinical AD patients (taken an average of 9.4 years before diagnosis) than in dementia-free controls (Lövheim et al., 2017)).

The large-scale longitudinal multicenter BALTAZAR (Biomarker of AmyLoid pepTide and ALZheimer's disease Risk) cohort, which included MCI and AD patients, is adapted for the evaluation and validation of prognostic biomarkers, with a three-year follow-up (Hanon et al., 2019: cohort description). In this cohort, we recently showed that MCI participants who converted to dementia had lower levels of total plasma $A\beta_{1-42}$ and a lower $A\beta_{1-42}/A\beta_{1-40}$ ratio than non-converters and identified a threshold for the total plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio that identified MCI patients with a 48% lower risk of developing dementia mainly AD within three years (Hanon et al., 2022).

To further test the value of amyloid peptides as an dementia progression marker, the aim of the present study was to investigate whether plasma concentrations of free $A\beta_{1-42}$ and $A\beta_{1-40}$ and the free plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio predict the conversion from MCI to dementia, in particular AD, over a three-year follow-up and compare, for the first time, the predictive performance of free and total amyloid peptides.

2. Methods

2.1. Participants

Participants were selected from the BALTAZAR (Biomarker of AmyLoid pepTide and AlZheimer's diseAse Risk) study, a multicenter prospective cohort study (ClinicalTrials.gov Identifier #NCT01315639) that enrolled patients with MCI or AD according to a previously published fully described protocol (Hanon et al., 2018). All participants gave written informed consent to participate in the study. All participants were Caucasian, community dwellers, and had caregivers. The BALTAZAR study was approved by the Paris ethical committee (CPP Ile de France IV Saint-Louis Hospital).

The MCI diagnosis was based on Petersen's criteria (Petersen et al., 1999)(Portet et al., 2006). Exclusion criteria were AD and non-AD dementia, major depression according to DSM IV-TR or the geriatric depression scale (GDS >20/30), other diseases that could interfere with cognition evaluation, diseases with short-term survival, the use of cholinesterase inhibitors or *N*-methyl-D-aspartate receptor partial antagonists before inclusion (for MCI participants) and being illiterate or with less than four years of education.

For this analysis, only a subgroup of the BALTAZAR cohort was selected based on specific criteria (Fig. 1). The exclusion criterion was an AD diagnosis. The inclusion criteria were an MCI diagnosis (N = 541) and available free amyloid levels measured in one round of experiments using one batch of reagents for both blood samples taken at inclusion (M0) and at 24 months (M24) (N = 106). The results at M24 are not presented in this study. Refusing a lumbar puncture was not an exclusion criterion. Thus, data on CSF biomarkers were available for only 48 of the 106 MCI participants.

At baseline (M0), all participants underwent clinical, neuropsychological, and biological assessments and for those without contra-indications, a brain MRI. CSF samples were collected from accepting participants. MCI participants were dichotomized into amnestic (aMCI) and non-amnestic (naMCI) phenotypes according to the presence of memory impairment on the free and cued selective reminding test (FCSRT) standardized for age, sex, and educational level.

Patients underwent visits every six months for three years: at six months (M6), 12 months (M12), 18 months (M18), 24 months (M24), 30 months (M30), and 36 months (M36). The duration of follow-up was censored at M36 as only one patient converted to AD later. MCI participants were reassessed at each visit for conversion to dementia. Cognitive evaluations were performed using an extensive neuropsychological battery by neuropsychologists after a training program to harmonize the results (Hanon et al., 2018).

2.2. Determination of Conversion from MCI to dementia

Conversions from MCI to AD dementia were reviewed by an adjudication committee based solely on clinical and neuropsychological characteristics using the NIA-AA criteria blinded to the CSF biomarker results (McKhann et al., 2011). The progression from MCI to dementia was determined based on a decline in cognitive functioning and disability, measured by changes from baseline in the mini mental state examination (MMSE), the activities of daily living (ADL) scale, the instrumental activities of daily living (IADL) scale, and the clinical dementia rating (CDR) sum of boxes ($\geq$ 1).

2.3. Biomarkers

The protocol used for the measurement of CSF and plasma biomarkers has already been detailed (Hanon et al., 2018). Briefly, analyses of plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ and CSF $A\beta_{1-42}$, Tau, and p-Tau levels were performed in a single centralized laboratory using the same collection tubes for all study centers (LoBind microtubes Eppendorf®, ref. 022431064, Hamburg, Germany)). All measurements were processed blind to the participants' diagnosis, MRI, and CSF results.

The plasma Aβ peptide assay was performed using the INNO-BIA kit (Fujirebio Europe NV, formerly Innogenetics NV, Belgium), based on the multiplex xMAP technique with a LABScan-200 system (Luminex BV, The Netherlands). The good analytical performance of this assay has

been previously reported (Lachno et al., 2012). Free amyloid levels were obtained with the undiluted plasma samples (UP) and total amyloid levels were obtained after dilution of plasma at 1/3, which allows to obtain the maximum peptide recovery (Lachno et al., 2012). The plasma amyloid $A\beta_{1-42}$ /$A\beta_{1-40}$ ratio is expressed as a percentage.

There were missing data for a number of samples. The ratio could not be calculated for 11 samples because the amount of either $A\beta_{1-42}$ or $A\beta_{1-40}$ was under the detection limit. The assay for total $A\beta_{1-42}$ and $A\beta_{1-40}$ failed for one plasma sample and it could not be re-assayed due to insufficient quantity. The corresponding free forms of $A\beta_{1-42}$ and $A\beta_{1-40}$ were detectable. Free $A\beta_{1-42}$ was undetectable in 10 samples, with the corresponding free $A\beta_{1-40}$ being detectable in all. Finally, free $A\beta_{1-40}$ was undetectable in one sample, with the corresponding free $A\beta_{1-42}$ being detectable.

CSF $A\beta_{42}$, total-tau, and phosphorylated-tau (p-tau_{181}) levels were measured using the commercially available Innotest® sandwich ELISA INNOTEST® hTAU Ag, INNOTEST® β-$Amyloid_{(1-42)}$, and INNOTEST® $Phospho\text{-}tau_{(181P)}$ according to the manufacturers' instructions (Fujirebio Europe NV, formally Innogenetics NV, Belgium). Cut-offs for AD were respectively: 834 pg/mL, 340 pg/mL and 62 pg/mL respectively for CSF $A\beta_{1-42}$, Tau and p-Tau (Lehmann et al., 2014).

APOE was genotyped in a single centralized laboratory (Hanon et al., 2018).

2.4. MRI brain imaging

The MRI protocol has already been detailed (Hanon et al., 2018). It included 3D volumetric T1 weighted, axial FLAIR T2W, axial EG T2W, axial T2W FSE images, axial blood oxygen-level dependence (BOLD) echo planar imaging EPI (10 min), axial diffusion tensor imaging (DTI), and arterial spin labeling imaging. After MRI was completed, the scans were sent for quality validation and post-processing. MRI analysis was centralized and performed at the CATI (Centre d'acquisition et de traitement d'images) (Operto et al., 2016).

2.5. Other patient characteristics

The standardized interview included questions on demographic and socioeconomic characteristics, health status, and medication use of the participants. Diabetes was defined as a self-reported diagnosis of diabetes, fasting blood glucose >7.0 mmol/L, or the use of glucose-modifying medication and hypertension, defined as a measured systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg or current antihypertensive treatment. Disability was assessed using the IADL (Instrumental Activities of Daily Living, normal score 14/14) and ADL (Activities of Daily Living, normal score 6/6) scales. Depressive symptoms were evaluated using the GDS (Geriatric Depression Scale).

2.6. Statistical analyses

Categorical variables are expressed as numbers (percentages). Quantitative variables are expressed as means with the standard deviation (SD) for data with a normal distribution or medians with the interquartile range for non-normally distributed data. The normality of the distribution of the variables was assessed graphically and using the Shapiro-Wilk test. The time of conversion is described using the Kaplan-Meier method. The time to conversion was defined as the time from baseline to the diagnosis of conversion. Data were censored at 36 months or at the last follow up visit. For the free $A\beta_{1-40}$ and free $A\beta_{1-42}$ biomarkers, non-detectable values were imputed as a value of 3.9, as the value for the limit of detection (LOD) value was 4. For total biomarkers, all values were above the LOD value. For other variables (including the free $A\beta_{1-42}/A\beta_{1-40}$ ratio), no imputation for missing data was performed.

An association between the levels of free and total plasma biomarkers and conversion to dementia was investigated using the Cox

Table 1
Characteristics of MCI patients of the study at inclusion for the whole group and according to the conversion to dementia status at three years.

General characteristics	MCI sample *	Conversion to dementia *			
		Yes	No		
	N = 106	N = 50	*N* = 56	Hazard Ratio + (95% CI)	p-value +
Age (years)	78.3 ± 5.4	78.9 ± 6.2	77.7 ± 4.6	1.03 (0.98–1.09)	0.26
Male	34 (32.1)	17 (34.0)	17 (30.4)	0.88 (0.49–1.60)	0.69
Education level					0.77
Primary	16 (15.1)	8 (16.0)	8 (14.3)	1 (reference)	–
Secondary	44 (41.5)	21 (42.0)	23 (41.1)	0.77 (0.34–1.75)	0.53
High school diploma or above	46 (43.4)	21 (42.0)	25 (44.6)	0.75 (0.33–1.68)	0.48
Tobacco [1]					0.80
Never	6 (5.9)	2 (4.3)	4 (7.4)	1 (reference)	–
Current	64 (63.4)	30 (63.8)	34 (63.0)	1.62 (0.39–6.79)	0.51
Former	31 (30.7)	15 (31.9)	16 (29.6)	1.52 (0.35–6.66)	0.58
BM I [2]	24.6 ± 3.4	25.0 ± 3.8	24.2 ± 3.1	1.03 (0.94–1.12)	0.58
Amnestic MCI	86 (81.1)	47 (94.0)	39 (69.6)	7.34 (1.78–30.3)	0.006
Comorbidity					
Hypertension	72 (67.9)	33 (66.0)	39 (69.6)	0.81 (0.45–1.46)	0.48
Mellitus Diabetes	10 (9.4)	5 (10.0)	5 (8.9)	1.14 (0.45–2.87)	0.78
Dyslipidemia	40 (37.7)	22 (44.0)	18 (32.1)	1.18 (0.67–2.08)	0.56
History of stroke or TIA	7 (6.6)	2 (4.0)	5 (8.9)	0.52 (0.13–2.14)	0.37
History of depression	27 (25.5)	12 (24.0)	15 (26.8)	0.80 (0.41–1.56)	0.51
Global cognitive assessment					
MMSE (/30) [3]	27.0 (25.0; 29.0)	26.0 (24.0; 27.0)	28.0 (26.0; 29.0)	0.85 (0.78–0.93)	0.0002
ADL score (/6)	6.0 (6.0; 6.0)	6.0 (6.0; 6.0)	6.0 (6.0; 6.0)	0.97 (0.76–1.23)	0.78
IADL score (/14)	14.0 (12.0; 14.0)	13.5 (12.0; 14.0)	14.0 (13.0; 14.0)	0.96 (0.88–1.04)	0.34
***APOE* ε4 carrier**	43 (40.6)	28 (56.0)	15 (26.8)	2.28 (1.29–4.02)	0.005
Hippocampal volume (R + L) (cm^3) [4]	4.4 ± 1.0	4.1 ± 1.0	4.7 ± 1.0	0.67 (0.52–0.86)	0.0021

MCI: Mild Cognitive Impairment; BMI: Body Mass Index in kg/m^2; TIA: Transient Ischemic Attack; MMSE: Mini Mental State Examination, ADL: Activities of Daily Living, IADL: Instrumental Activities of Daily Living (IADL), GDS: Geriatric Depression Scale, APOE: apolipoprotein E; *APOE* ε4: ε4 allele of *APOE*.

* Values are expressed as means ± SDs (standard deviations) or medians with interquartile ranges (IQRs) for quantitative variables or as counts (%) for categorical variables.

+ Hazard ratio and *p*-values derived from the CPH Model.

[1] 5 missing values.

[2] 2 missing values.

[3] 1 missing value.

[4] 15 missing value.

Table 2
Plasma and CSF biomarkers in the MCI patients at inclusion and according to the conversion to dementia status at 3 years.

Biomarkers	MCI sample*	Conversion to dementia*		*APOE* ε4 carrier		Not *APOE* ε4 carrier	
				Conversion to dementia		Conversion to dementia	
		Yes	No	Yes	No	Yes	No
	N = 106	N = 50	*N* = 56	N = 28	*N* = 15	*N* = 22	*N* = 41
Plasma biomarkers							
Free $A\beta_{1-42}$ (pg/mL)	12.2 ± 6.2	12.0 ± 6.4	12.5 ± 6.0	11.7 ± 5.4	13.6 ± 3.7	12.3 ± 7.6	12.1 ± 6.6
Free $A\beta_{1-40}$ (pg/mL)	63.1 ± 24.8	64.8 ± 26.1	61.6 ± 23.7	68.6 ± 23.8	53.4 ± 15.6	60.0 ± 28.6	64.5 ± 25.5
Free $A\beta_{1-42}/A\beta_{1-40}$ ratio (%)[a]	20.4 ± 7.3	19.5 ± 6.2	21.2 ± 8.0	18.0 ± 6.1	26.4 ± 5.8	21.7 ± 5.8	19.1 ± 7.9
Total $A\beta_{1-42}$ (pg/mL)[b]	38.1 ± 14.0	36.1 ± 14.1	39.9 ± 13.8	35.0 ± 13.4	39.8 ± 13.7	37.5 ± 15.2	39.9 ± 14.0
Total $A\beta_{1-40}$ (pg/mL)[b]	277.0 ± 68.0	278.4 ± 68.6	275.7 ± 68.1	277.8 ± 61.8	248.1 ± 87.8	279.2 ± 78.4	285.8 ± 57.3
Total $A\beta_{1-42}/A\beta_{1-40}$ ratio (%)[c]	13.9 ± 4.5	12.9 ± 4.0	14.7 ± 4.8	12.5 ± 3.8	16.6 ± 4.1	13.3 ± 4.3	14.1 ± 4.9
Free $A\beta_{1-42}/A\beta_{1-40}$ ratio quartiles (%)[a]							
Q1 < 14.90	19 (20.0)	9 (20.93)	10 (19.23)	6 (23.1)	0 (0.0)	3 (17.6)	10 (27.0)
Q2 [14.90; 19.25[	28 (29.47)	13 (30.23)	15 (28.85)	9 (34.6)	1 (6.7)	4 (23.5)	14 (37.8)
Q3 [19.25; 25.80[	24 (25.26)	14 (32.56)	10 (19.23)	9 (34.6)	6 (40.0)	5 (29.4)	4 (10.8)
Q4 ≥ 25.80	24 (25.26)	7 (16.28)	17 (32.69)	2 (7.7)	8 (53.3)	5 (29.4)	9 (24.3)
Total $A\beta_{1-42}/A\beta_{1-40}$ ratio quartiles (%)[c]							
Q1 < 11.40	26 (25.0)	14 (28.6)	12 (21.8)	8 (28.6)	0 (0.0)	6 (28.6)	12 (29.3)
Q2 [11.40; 13.70]	26 (25.0)	13 (26.5)	13 (23.6)	10 (35.7)	3 (21.4)	3 (14.3)	10 (24.4)
Q3 [13.70; 16.95]	26 (25.0)	16 (32.7)	10 (18.2)	8 (28.6)	5 (35.7)	8 (38.1)	5 (12.2)
Q4 ≥ 16.95	26 (25.0)	6 (12.2)	20 (36.4)	2 (7.1)	6 (42.9)	4 (19.0)	14 (34.1)
CSF biomarkers	N = 48	N = 25	*N* = 23	N = 12	N = 5	N = 13	N = 18
Aβ42 (pg/mL)	733 (535; 1226)	613.0 (452; 814)	1137 (697; 1424)	532.5 (425.0; 699.5)	1076 (557.0; 1386)	673.0 (532.0; 1079)	1162 (745.0; 1424)
Tau (pg/mL)[d]	401 (278; 604)	499 (386; 699)	312 (210; 542)	460.0 (288.0; 766.0)	312.0 (214.0; 384.0)	516.0 (401.0; 552.0)	305.5 (210.0; 602.0)
p-Tau (pg/mL)	60.5 (46.5; 90.5)	70.0 (54.0; 92.0)	53.0 (37.0; 78.0)	76.5 (47.0; 120.5)	47.0 (46.0; 56.0)	70.0 (56.0; 79.0)	54.0 (36.0; 97.0)
Aβ42/p-Tau ratio	13.8 (6.9; 25.0)	8.7 (6.6; 15.6)	25.2 (9.6; 31.3)	6.8 (4.2; 15.5)	29.1 (9.6; 29.5)	9.2 (7.6; 17.3)	24.8 (11.8; 32.5)

MCI: Mild Cognitive Impairment, Aβ: amyloid beta, CSF: cerebrospinal fluid, *APOE ε4*: ε4 allele of *APOE* For free $A\beta_{1-40}$ and free $A\beta_{1-42}$ biomarkers, non-detectable values were imputed by the value 3.9 since the limit of detection (LOD) value is 4. For the free $A\beta_{1-42}/A\beta_{1-40}$ ratio no imputation was performed. For total biomarkers, all values were greater than the LOD value.

[*] Values are expressed as means ± SDs (standard deviations) or medians with interquartile ranges (IQRs) for quantitative variables or counts (%) for categorical variables.

[a] 11 missing values.

[b] 1 missing value

[c] 2 missing values.

[d] 3 missing values.

proportional hazard model (CPH) with right censoring. The $A\beta_{1-42}/A\beta_{1-40}$ ratio (ratio of amyloid peptides Aβ; free and total) at baseline was categorized into quartiles (Hanon 2022). Log linearity assumptions were assessed for other quantitative biomarkers using Martingale residuals and proportional hazard assumptions during the follow-up were assessed using scaled Schoenfeld residual plots. The hazard ratio with a 95% confidence interval was computed as the effect size. Analyses were adjusted for predefined important confounders, namely age, sex, education level, and *APOE* ε4 risk alleles. As there was a large gradient for the effect size, as observed in Hanon et al. (2022) these analyses were repeated by considering the ratio of amyloid peptides (free and total) as binary variables (highest quartile versus three lower quartiles). The comparisons of patient characteristics at baseline between the upper and three lower quartiles of free and total forms in the MCI population were performed using the Chi-square test (or Fisher's exact test when the expected cell frequency was <5) for categorical variables and by the Student test for quantitative variables with a Gaussian distribution or the Mann-Whitney *U* test for those with a non-Gaussian distribution.

Statistical tests were performed at a two-tailed α level of 0.05. Data were analyzed using SAS software, release 9.4 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Characteristics of the participants at baseline and according to conversion during the follow-up

From the BALTAZAR cohort, 106 MCI patients met the inclusion criteria for this study (Fig. 1). The characteristics of the participants at baseline are presented in Table 1. At baseline, the 106 MCI participants had a mean (± standard deviation) age of 78.3 (± 5.4), 32.1% (*n* = 34) were men, and 43.4% (*n* = 46) had at least a high school diploma. The median (first quartile – third quartile) MMSE score was 27.0 (25.0–29.0) and 81% (*n* = 86) had the amnestic form. *APOE* ε4 carriers accounted for 40.6% (*n* = 43) of the participants.

During the clinical follow-up period of 6 to 36 months, 47.2% (*n* = 50) of the MCI participants converted to dementia and among them, 96% (*n* = 48) to probable AD. At one year, the rate of conversion was estimated to be 20%. During the three-year follow-up, 12.3% (*n* = 13) converted before the six-month visit (M6), 2.8% (n = 3) between M6 and M12, 7.5% (n = 8) between M12 and M18, 0.9% (n = 1) between M18 and M24, 26.4% (*n* = 28) between M24 and M30, and 4.7% (n = 5) after the 30-month visit. Moreover, one participant died during the three-year follow-up.

The characteristics of the participants at baseline according to conversion or not are presented in Table 1. Converters were more often amnestic (Hazard Ratio [HR] = 7.34, *p* = 0.006) and *APOE* ε4 carriers

(HR = 2.28, $p = 0.005$) and had a lower MMSE score (HR = 0.85, $p = 0.0002$) and hippocampal volume (HR = 0.67, $p = 0.0021$) than non-converters.

3.2. Plasma and CSF biomarkers at baseline and according to conversion during the follow-up

The baseline biomarker levels for all MCI and according to conversion are presented in Table 2 and compared in Table 3. There was a positive and significant correlation between free and total peptide isoforms for $A\beta_{1-42}$ ($r = 0.75$, $p < 0.0001$), $A\beta_{1-40}$ ($r = 0.36$, p = 0.0002), and the $A\beta_{1-42}/A\beta_{1-40}$ ratio ($r = 0.76$, p < 0.0001). Free and total $A\beta_{1-42}$ and $A\beta_{1-40}$ levels were not significantly different between MCI converters and non-converters. The free and total $A\beta_{1-42}/A\beta_{1-40}$ were significantly lower for converters: $p = 0.038$ and $p = 0.013$, respectively, after adjusting for age, sex, education, and *APOE* status (Table3). The fold-change for converters versus non-converters was 0.92 and 0.88 for the free and total $A\beta_{1-42}/A\beta_{1-40}$ ratio, respectively (Fig. 2).

Total and free amyloid peptide levels were then dichotomized based on quartiles, as in (Hanon et al., 2022). Of note, the lower limite value of the upper total $A\beta_{1-42}/A\beta_{1-40}$ quartile found in this study was 16.95% (Table 1), equal to that found previously (16.9%) in (Hanon et al., 2022) for the whole Baltazar MCI cohort. The relationship between conversion and the free plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio was not linear: 29.2% (7/24) of participants in the highest quartile converted to dementia (plasma $A\beta_{1-42}/A\beta_{1-40}$ (%) $\geq$ 25.8), 58.3% (14/24) in the 3rd quartile (25.8 > plasma $A\beta_{1-42}/A\beta_{1-40}$ (%) $\geq$ 19.25), 46.4% (13/28) in the 2nd quartile (14.9 > plasma $A\beta_{1-42}/A\beta_{1-40}$ (%) $\geq$ 19.25), and 42.3% (9/19) in the lowest quartile (plasma $A\beta_{1-42}/A\beta_{1-40}$ (%) < 14.9) (Table 2).

Among those with CSF measurements, converters had significantly lower CSF $A\beta_{1-42}$ levels (p = 0.002) and a lower $A\beta_{1-42}$/Tau ratio ($p = 0.001$) and significantly higher Tau levels ($p = 0.024$) than non-converters. Levels of pTau were also higher but did not reach significance ($p = 0.064$). However, the median value for non-converters (53 pg/mL) was under the cut-off for AD (62 pg/mL) and the median value of converters was above (70 pg/mL).

3.3. Association between free plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ levels and the $A\beta_{1-42}/A\beta_{1-40}$ ratio with conversion to dementia and comparison to the performance using total plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ levels and the $A\beta_{1-42}/A\beta_{1-40}$ ratio

The free and total $A\beta_{1-42}/A\beta_{1-40}$ ratio were significantly associated with conversion to dementia with Hazard ratios of 0.95 (p = 0.038) and 0.92 ($P = 0.013$) respectively (Table 3). When dichotomized in quartiles, we observed a significant association only between the upper quartile (the lowest quartile as reference) and conversion to dementia for both ratios after adjustment for confounding factors: HR = 0.31 (0.11–0.93), $p = 0.036$, for the free $A\beta_{1-42}/A\beta_{1-40}$ ratio and HR = 0.34 (0.13–0.90), $p = 0.030$, for the total $A\beta_{1-42}/A\beta_{1-40}$ ratio.

As quartiles Q2 and Q3 didn't show significant association with conversion (the lowest quartile as reference) and the three lowest quartiles had close conversion rates (42.3% to 58.3%), we grouped them together for further analysis. We observed a significant association between the upper quartile (the three lowest quartiles as reference) and conversion to dementia for both ratios after adjustment for confounding factors: HR = 0.36 (0.15–0.87), $p = 0.022$, for the free ratio and HR = 0.37 (0.16–0.89), $p = 0.027$, for the total ratio. The Kaplan Meier curves showed lower conversion rates to dementia in the highest quartile versus the three lower quartiles for both the free and total plasma $A\beta_{1-42}/$

Table 3
Association between conversion to dementia within three years and the baseline plasma levels of free and total amyloid $A\beta_{1-42}$, $A\beta_{1-40}$, and quartiles of the free $A\beta_{1-42}/A\beta_{1-40}$ and total $A\beta_{1-42}/A\beta_{1-40}$ ratio.

Biomarker	Hazard Ratio (95% CI) [+]	*p*-value [+]	Hazard ratio (95% CI)[*]	p-value[*]
Free $A\beta_{1-42}$ (per 10 pg/mL increase)	0.83 (0.52–1.34)	0.45	0.68 (0.40–1.17)	0.16
Free $A\beta_{1-40}$ (per 10 pg/mL increase)	1.04 (0.92–1.16)	0.53	1.03 (0.92–1.17)	0.59
Total $A\beta_{1-42}$[a] (per 10 pg/mL increase)[a]	0.87 (0.71–1.05)	0.15	0.85 (0.70–1.04)	0.12
Total $A\beta_{1-40}$[a] (per 10 pg/mL increase)[a]	1.00 (0.96–1.05)	0.94	1.01 (0.97–1.05)	0.69
Free $A\beta_{1-42}/A\beta_{1-40}$ ratio (%)[b]	0.97 (0.93–1.02)	0.21	0.95 (0.91–0.99)	**0.038**
Total $A\beta_{1-42}/A\beta_{1-40}$ ratio (%)[c]	0.94 (0.88–1.01)	0.053	0.92 (0.86–0.98)	**0.013**
Free $A\beta_{1-42}/A\beta_{1-40}$ ratio quartiles (%)[b]		0.24		0.13
Q1 < 14.90	1 (reference)	–	1 (reference)	–
Q2 [14.90; 19.25]	1.03 (0.44–2.42)	0.94	0.99 (0.41–2.41)	0.98
Q3 [19.25; 25.80]	1.32 (0.57–3.05)	0.52	0.75 (0.29–1.90)	0.54
Q4 ≥ 25.80	0.48 (0.17–1.36)	0.17	0.31 (0.11–0.93)	**0.036**
Total $A\beta_{1-42}/A\beta_{1-40}$ ratio quartiles (%)[c]		0.13		0.15
Q1 < 11.40	1 (reference)	–	1 (reference)	–
Q2 [11.40; 13.70]	1.07 (0.50–2.63)	0.87	0.95 (0.41–2.20)	0.91
Q3 [13.70; 16.95]	1.17 (0.56–2.43)	0.67	0.79 (0.35–1.76)	0.56
Q4 ≥ 16.95	0.39 (0.15–1.01)	0.05	0.34 (0.13–0.90)	**0.03**
∓Free $A\beta_{1-42}/A\beta_{1-40}$ ratio (%)[2] highest quartile and three lowest quartiles				
< 25.80 (Q1, Q2 and Q3)	1 (reference)		1 (reference)	
≥ 25.80 (Q4)	0.43 (0.18–1.03)	0.058	0.36 (0.15–0.87)	**0.022**
∓Total $A\beta_{1-42}/A\beta_{1-40}$ ratio[3] highest quartile and three lowest quartiles				
<16.95 (Q1, Q2 and Q3)	1 (reference)	0.019	1 (reference)	**0.027**
≥ 16.95 (Q4)	0.36 (0.15–0.85)		0.37 (0.16–0.89)	

∓ Three lower quartiles versus upper quartile.
[+] Hazard ratio and *p*-values derived from the CPH Model without adjustment.
[*] results are adjusted for age, sex, education, and *APOE* ε4 risk alleles. Aβ: amyloid beta
[a] 1 missing value both in univariate and multivariate.
[b] 11 missing values both in univariate and multivariate.
[c] 2 missing values both in univariate and multivariate.

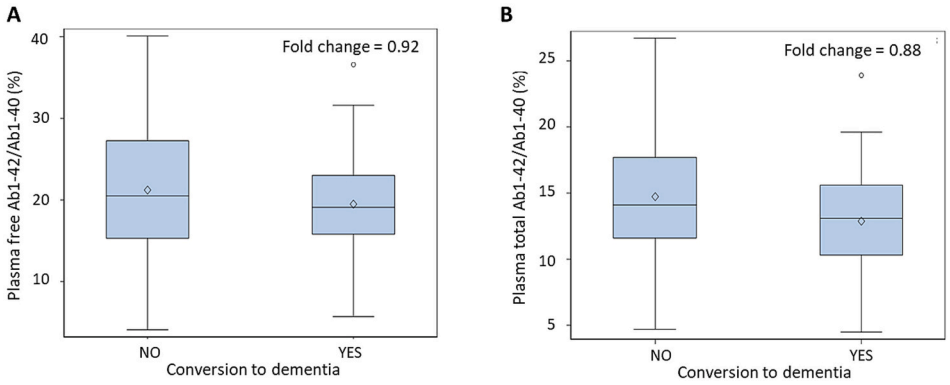

Fig. 2. Box plot analysis and Fold changes of plasma levels of free Aβ1–42/Aβ1–40 and total Aβ1–42/Aβ1–40 according to conversion to dementia or not.

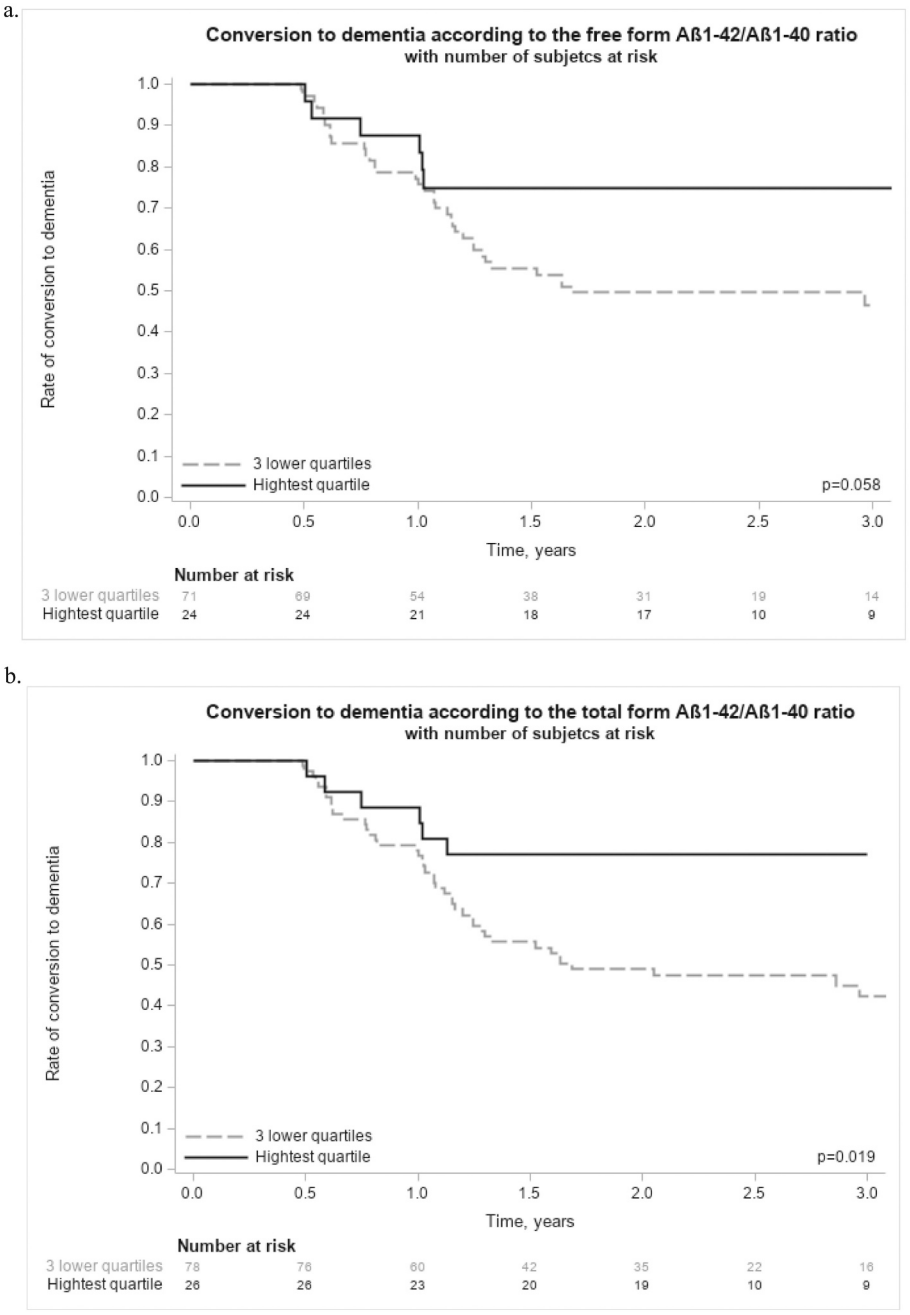

Fig. 3. Kaplan Meier curves for the conversion to dementia in MCI patients ($N = 106$) according to the quartiles of the free (3a) and total (3b) plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio measured at baseline.

Table 4
Characteristics of MCI patients in the highest quartile vs. three lower quartiles of the free and total plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio.

Characteristics	Free $A\beta_{1-42}/A\beta_{1-40}$ ratio			Total $A\beta_{1-42}/A\beta_{1-40}$ ratio		
	Highest quartile	3 lower quartiles	P*	Highest quartile	3 lower quartiles	P*
	***N* = 24**	***N* = 71**		***N* = 26**	***N* = 78**	
Age (years)	79.5 ± 5.7	77.9 ±5.	0.24	78.2 ± 4.8	78.3 ± 5.7	0.96
Male	8 (33.3)	22 (31.0)	0.83	7 (26.9)	26 (33.3)	0.54
Education level						
Primary	3 (12.5)	11 (15.5)	0.92	6 (23.1)	10 (12.8)	0.18
Secondary	11 (45.8)	30 (42.3)		7 (26.9)	36 (46.2)	
High school or above	10 (41.7)	30 (42.3)		13 (50.0)	32 (41.0)	
Tobacco[a]						
Never	1 (4.6)	4 (5.9)		3 (12.5)	2 (2.7)	
Current	17 (77.3)	41 (60.3)	0.34	15 (62.5)	49 (65.3)	0.15
Former	4 (18.2)	23 (33.8)		6 (25.0)	24 (32.0)	
BMI[b]	25.4 ± 3.8	24.3 ± 3.2	0.18	24.5 ± 4.1	24.6 ± 3.2	0.93
Amnestic MCI	17 (70.8)	60 (84.5)	0.23	19 (73.1)	65 (83.3)	0.25
Global cognitive assessment						
MMSE (/30)[b]	27 (25; 29)	27 (25; 29)	0.29	28 (26; 29)	27 (25; 29)	**0.046**
ADL score (/6)	6 (6; 6)	6 (6; 6)	0.76	6 (6; 6)	6 (6; 6)	0.41
IADL score (/14)	14 (11.5; 14)	14 (12; 14)	0.59	14 (13; 14)	14 (12; 14)	0.23
***APOE* ε4 carrier**	10 (41.7)	31 (43.7)	0.86	8 (30.8)	34 (43.6)	0.25
Hippocampal volume (R + L) (cm^3)	4.5 ± 0.8	4.4 ± 1.1	0.64	4.8 ± 0.9	4.3 ± 1.1	**0.033**
CSF biomarkers	*N* = 8	*N* = 33		N = 7	N = 41	
$A\beta_{42}$ (pg/mL)	1332 (547; 1487)	699 (508; 1079)	0.12	1345 (1076; 1489)	699 (532; 1137)	**0.029**
Tau (pg/mL)[2]	383 (293; 464)	432 (235; 649)	0.77	278 (191; 381)	434 (300; 649)	**0.044**
p-Tau (pg/mL)	58.5 (54.5; 75.5)	60 (42; 97)	0.71	46 (37; 61)	64 (52; 97)	0.077
$A\beta_{42}$/p-Tau ratio	22.7 (8.4; 25.0)	12.8 (6.7; 24.4)	0.48	29.08 (24.91; 32.5)	11.8 (6.65; 21.32)	**0.011**

The highest quartile is defined by a free $A\beta_{1-42}/A\beta_{1-40}$ ratio ≥ 25.80.
Values are expressed as means ± − SDs (standard deviations) or medians and interquartile ranges (Q1; Q3) for quantitative variables or counts (%) for categorical variables.
* p-values from non-parametric tests (Mann-Whitney) or parametric tests (Student) for quantitative variables and Chi-square or Fisher exact tests for qualitative variables. Aβ: amyloid beta, BMI: Body Mass Index in kg/m^2, MMSE: Mini Mental State Examination, ADL: Activities of Daily Living, IADL: Instrumental Activities of Daily Living (IADL), APOE: apolipoprotein E, *APOE* ε4: ε4 allele of *APOE*
[a] 5 missing values.
[b] 1 missing value.

$A\beta_{1-40}$ ratio (Fig. 3a and b, respectively).

The demographic, clinical, cognitive, *APOE*, and MRI characteristics of MCI participants in the highest quartile of the free plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio were not significantly different than those in the three lower quartiles (Table 4). There were no significant differences in CSF biomarker levels (Table 4). However, participants in the highest quartile of the total plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio had a significantly higher MMSE score ($p = 0.046$) and hippocampal volume ($p = 0.033$), significantly higher levels of CSF $A\beta_{1-42}$ (p = 0 0.029) and $A\beta_{1-42}$/Tau ratio ($p = 0.011$), and significantly lower CSF Tau levels ($p = 0.044$).

4. Discussion

In this sub-cohort of the large-scale multicenter longitudinal BALTAZAR cohort of clinically defined MCI (Hanon et al., 2022), MCI participants in the highest quartile of the free $A\beta_{1-42}/A\beta_{1-40}$ ratio (≥ 25.8%) showed a significant 64% reduction in the risk of developing dementia and AD independently of age, sex, education level, and *APOE* ε4 status.

The interest of measuring free circulating amyloid fractions was first reported by Sagare et al. (A. Sagare et al., 2007) as a potential diagnostic tool to differentiate patients with and without AD. In this transversal study, the free $A\beta_{1-42}$ and $A\beta_{1-40}$ amyloid fractions were measured using an ELISA assay (either $A\beta_{1-42}$ or $A\beta_{1-40}$) on sLRP-depleted plasma supernatants (to eliminate the sLRP-linked amyloid Aβ forms) after ultrafiltration with a Microcon (30 kDa cut-off, to eliminate other protein bound Aβ forms). In the AD group, 3- to 4-fold higher levels of free, protein-unbound $A\beta_{1-40}$ and $A\beta_{1-42}$ were observed than for non-demented controls. A few years later, the same group conducted a longitudinal study on MCI patients. They compared the baseline levels of free plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ in stable MCI (sMCI) and MCI that converted to AD (MCI-AD) during a follow-up period of 2 to 4 years. They found no significant differences, but the mean level of free $A\beta_{1-40}$ was 1.3-fold higher in MCI-AD than sMCI, whereas the mean levels of $A\beta_{1-42}$ were the same in both groups (A. P. Sagare et al., 2011). However, this study was limited to 14 MCI-AD and 24 sMCI participants, no calculation of the free $A\beta_{1-42}/A\beta_{1-40}$ ratio was performed, and total $A\beta_{1-40}$ and $A\beta_{1-42}$ levels were not measured.

Another transversal study found significantly higher levels of free $A\beta_{1-40}$ and $A\beta_{1-42}$ (15- and 12-fold higher, respectively) in MCI participants than healthy controls but no difference in the $A\beta_{1-42}/A\beta_{1-40}$ ratio (Pesini et al., 2012). The free Aβ pool was considered as Aβ forms detected in undiluted plasma, as in our study, but the levels were measured using a different ELISA sandwich assay (Araclon Biotech. Zaragoza, Spain). This group used the same method to compare MCI subjects with negative magnetic resonance imaging (Scheltens' score < 4) versus MCI with positive imaging (Scheltens' score > 4): neither free $A\beta_{1-40}$ or $A\beta_{1-42}$ levels nor the $A\beta_{1-42}/A\beta_{1-40}$ ratio showed significant differences between the two groups (Pérez-Grijalba et al., 2013). This study was limited to 12 participants in the negative imaging group and 15 in the positive imaging group.

Only one larger study in the literature has longitudinally evaluated the value of free amyloid peptides to identify individuals at risk of developing AD (Lövheim et al., 2017). As in the study of Pérez-Grijalba et al. (2013) and our study, the free Aβ pool was considered as Aβ forms detected in UP. Amyloid levels were measured using the same technology as ours (xMAP technology, using INNO-BIA plasma Aβ form assays). Plasma samples from 339 preclinical AD cases (76.4% women, mean age 61.3 years) and 339 controls free of dementia, and matched for age and sex, collected 9.4 years before AD diagnosis were analyzed. There was no difference in the free plasma $A\beta_{1-40}$ or $A\beta_{1-42}$ concentrations or the $A\beta_{1-42}/A\beta_{1-40}$ ratio between the preclinical AD cases and dementia-free controls in the full sample or in subgroups defined according to sex and age group (< 60 and ≥ 60 years). Analysis of the quartiles of free Aβ also showed no significant association with conversion. The results differences with our study can be explained by the differences in diagnostic groups: in this study the inclusion criteria of stable controls were to be

"non demented" and preAD were defined only on the criterion of subsequent conversion to AD which are less homogeneous groups than the stable and converted MCI groups of our study. Another possible explanation could be sampling time before conversion, which was much greater in the former study (mean 9.5 years) than in ours (mean 13.6 months) and suggests that the free $A\beta_{1-42}/A\beta_{1-40}$ ratio is a marker of conversion a little over one year before it occurs. The kinetics of free Aβ are not known. However the kinetic model proposed by Palmqvist et al. for total amyloid forms (Palmqvist et al., 2019b) showed that the total $A\beta_{1-42}/A\beta_{1-40}$ ratio decreases later than in CSF and reaches a plateau later than in CSF.

In undiluted neat EDTA plasma, binding to plasma proteins or oligomerization of Aβ gives rise to epitope masking and binding of the antibody of the assay kit becomes impossible for the bound Aβ forms. Given the relatively small size of Aβ (~4.5 kDa) relative to its major binding protein sLRP (~600 kDa, 70% bonding according to (A. Sagare et al., 2007)) or other plasma proteins, such as albumin (60 kDa), there is considerable steric hindrance. Only free Aβ forms are accessible to the antibodies of the kit and lead to a measurable signal. On the other hand, dilution of the plasma with sample buffer modifies the ionic strength and molecular interactions within the sample, resulting in the release of Aβ1–40 and Aβ1–42 bound to plasma proteins and other components (Kuo et al., 1999)(Sureshbabu et al., 2009) or the disassociation of Aβ oligomers (Janssen et al., 2015). Thus, hidden epitopes become available and measurements can be interpreted as an estimation of the total level of Aβ in plasma. Variations in free Aβ levels could therefore reflect mechanisms involved in the pathophysiology of AD, such as alterations in Aβ clearance via sLRP or Aβ oligomerisation.

Participants in the highest quartile of the free plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio were not significantly different than those in the three lower quartiles in terms of demographic, clinical, or neuropsychological characteristics (except for a marginal association with a lower hippocampal volume). This may be due to the 11 missing values relative to the total ratio, with the loss of statistical significance. This may also indicate that this ratio doesn't select the same patients than the total plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio. Data of CSF biomarkers levels, available for only a subgroup of subjects, are essentially of descriptive interest, due to the low number of individuals; the reproducibility of results should be validated on a larger study.

The difference in the plasma free $A\beta_{1-42}/A\beta_{1-40}$ ratio observed between MCI-AD and stable MCI subjects in our study was not greater than total $A\beta_{1-42}/A\beta_{1-40}$ ratio (0.92 versus 0.88) and comparable to the differences described in the metanalysis of (Koychev et al., 2021) for total $A\beta_{1-42}/A\beta_{1-40}$ (mean 0.98). It would be interesting to study whether the association of biomarkers with conversion depends on *APOE* status, but our sample size does not allow to perform subgroup analyses.

When we further compared the results between the free and total amyloid ratios, the performance was equivalent. Being in the highest quartile was associated with a similar reduction in the risk of conversion to dementia of 64 and 63%, respectively (the three lowest quartiles as reference). A possible explanation for the fact that the three lower quartiles showed an equivalent risk to convert to dementia and that it was significantly higher than that for the highest quartile is that the MCI subgroup of the BALTAZAR study was more homogeneous than other population-based cohorts, such as in the Rotterdam study or the AIBL or BioFinder cohorts, with only patients with cognitive impairment. Therefore, a large proportion were probably already engaged in the AD pathology, as suggested by the high level of conversion to dementia (30%) during the three-year follow-up.

The strength of our study lies in the well-characterized MCI participants, with repeated extensive standardized cognitive tests. All biochemical analyses were centralized in a single laboratory and the same pre-analytical protocol was followed throughout the study. The same lot was used for the measurement of plasma amyloid levels, thus limiting variability of the results. In addition, the brain MRI was analyzed in a single dedicated expert center (Operto et al., 2016). All conversions were adjudicated blind to the CSF and plasma biomarker results. In our study, the percentage of conversion was consistent with those already reported in published studies (Mitchell and Shiri-Feshki, 2009). In the CSF subgroup ($N = 25$), 80% ($N = 18$) had a biochemical profile in the AD continuum according to Jack et al. (2018) (A + T+ or A + T-) and the others were A-T+ which is a biochemical profile also prone to convert to dementia in MCI (Grøntvedt et al., 2020; Oberstein et al., 2022).

The present study had, however, several limitations. The diagnosis of AD was only based on clinical and brain MRI information and not on PIB-PET or pathological confirmation. The results for CSF biomarkers were not taken into consideration for the AD diagnosis to avoid a circular analysis due to the correlation between CSF and plasma biomarker levels. To increase the likelihood of conversion to AD, we excluded participants with Lewy Body, Parkinsonian, frontotemporal, or vascular MCI disorders. Therefore, 81% of subjects had the amnestic form of MCI and only 5% converted to a non-AD form of dementia. Moreover, a large proportion of participants had at least a high school diploma, as often observed in such longitudinal studies, and therefore our results may not be fully transposable to the general population. Only 106 MCI patients from the 541 of the BALTAZAR cohort could be included. Although the sample size is bigger than previously published studies, it is still limited, and results should be validated in a larger study. Nevertheless, the results found for the total $A\beta_{1-42}/A\beta_{1-40}$ ratio are comparable to those reported previously by Hanon et al. (Hanon et al., 2022), with the same lower limit value defining the upper total $A\beta_{1-42}/A\beta_{1-40}$ quartile, making the likelihood of a selection bias unlikely. Finally, the results obtained with this test should also be validated by other immunoassays. Indeed, the concentrations of free amyloid forms may differ according to the antibodies used, whose epitopes may or may not be masked by the binding proteins. As this is not a problem for the total form assay, the latter could provide more consistent results.

5. Conclusion

Our results show the relevance of the free plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio for identifying MCI patients at lower risk of conversion to dementia (mainly AD) within three years. Using the threshold of 25.8% for the free plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio, it was possible to identify MCI patients with an at least 60% lower risk of conversion to dementia. The performance of the free $A\beta_{1-42}/A\beta_{1-40}$ ratio in predicting conversion to dementia was similar to that of the total $A\beta_{1-42}/A\beta_{1-40}$ ratio.

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CRediT authorship contribution statement

S. Schraen-Maschke: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **A. Duhamel:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Formal analysis. **J.S. Vidal:** validation, Formal analysis, Data curation, methodology. **N. Ramdane:** Writing – original draft, Software, Formal analysis. **L. Vaudran:** Writing – original draft. **C. Dussart:** Writing – original draft. **L. Buée:** Validation, Supervision. **B. Sablonnière:** Validation. **C. Delaby:** Validation. **B. Allinquant:** Validation, Investigation, Conceptualization. **A. Gabelle:** Validation, Methodology, Investigation, Formal analysis. **S. Bombois:** Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis. **S. Lehmann:** Validation, Project administration, Methodology, Investigation, Formal

analysis, Conceptualization. **O. Hanon:** Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Olivier Hanon received personal payment from Bayer, Servier, AstraZeneca, Boston Scientific, Vifor, BMS, Boehringer-Ingelheim, and Pfizer for lectures and/or consulting services. Jean Sebastien Vidal received payment from Bayer for lectures made to non-profit medical association. Sylvain Lehmann received for his institution support from the following: H2020 MARIE SKŁODOWSKA-CURIE "MIRIADE Multiomics Interdisciplinary Research Integration to Address DEmentia diagnosis," ANR Flash Covid: "ProteoCOVID: Clinical proteomic characterization of the SARS-CoV-2 Spike protein to optimize its detection and the development of serological assays," ANR "Silk_road: The Stable Isotope Labeling Kinetics (SILK) road to investigate human protein turnover in blood and cerebrospinal fluid," EUROMET EMPIR "NeuroMet2 project: Metrology and Innovation for early diagnosis and accurate stratification of patients with neurodegenerative diseases." During the past 36 months, he had a patent issued for "Procédé de préparation d'un échantillon peptidique" Brevet INPI n°1,905,247 du 20/05/2019 du CHU DE MONTPELLIER, UNIVERSITÉ DE MONTPELLIER and SPOT TO LAB. He received personal payment for participating on the Roche Diagnostic board on CSF biomarkers. Stéphanie Bombois, Bernadette Allinquant, Christiane Baret-Rose, Jean-Marc Tréluyer, Hendy Abdoul, Patrick Gelé, Christine Delmaire, Jean-François Mangin, and Evelyne Galbrun have no conflicts of interest. Frederic Blanc received honoraria from Roche and Biogen for presentations. He received payment to his institution as the national coordinator for the clinical trial DELPHIA for patients with dementia with Lewy bodies (Eisai). He received payment to his institution as the national coordinator for the clinical trial GRADUATE for patients with Alzheimer's disease. Luc Buée received support for the present manuscript from LabEx DISTALZ. He received grants or contracts from the French National Research Agency (ANR) Fondation pour la Recherche Médicale (FRM). In the past 36 months, he had a patents on anti-tau therapy issued. Jacques Touchon received payment or honoraria as chairman of CTAD. He received contracts from Regenlife and consulting fees from Regenlife. He is Chairman of JT Conseil society. Jacques Hugon received grants or contracts from Protekt therapeutics, consulting fees from Protekt therapeutics. He is principal investigator of RECAGE project European Union H20/20 programme and he is member of the scientific board of Fondation Philippe Chatrier, Paris, France. Bruno Vellas received grants or contracts from Biogen, Roche, and Lilly; consulting fees from Roche, Lily, Biogen, and Cerellis; and is part of WHO's ICOPE program (unpaid position). AthanBase Benetos is the president of the European Geriatric Medicine Society (unpaid position). He received support for attending meetings and/or travel from Fukuda company, for the Congress of the European Society of Hypertension, and received royalties or licenses from Cambridge University Editions. Gilles Berrut received a grant from Boehringer Ingelheim and consulting fees from Boehringer Ingelheim, Smart macadam Institut, bien vieillir Korian. Elena Paillaud has no conflicts of interest. David Wallon, Giovanni Castelnovo, Lisette Volpe-Gillot, Marc Paccalin, Philippe Robert, and Vincent Camus have no conflicts of interest. Olivier Godefroy received support to his institution for attending meetings and/or travel from BRISTOL-MYERS SQUIBB, ROCHE SAS, BIOGEN FRANCE SAS. Joël Belmin received consulting fees from Pfizer and honoraria from Novartis Pharma. Pierre Vandel is the président of the "Société Francophone de Psychogériatrie et Psychiatrie de la Personne Agée" (SF3PA) and received consulting fees from Eisai. Jean-Luc Novella, Emmanuelle Duron, Anne-Sophie Rigaud, Susanna Schraen-Maschke, Alain Duhamel, Nassima Ramdane, Lucie Vaudran, Caroline Dussart, Bernard Sablonnière and Audrey Gabelle have no conflicts of interest.

Data availability

Data will be made available on request.

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