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## **Additional Use of A beta(42)/A beta(40) Ratio with Cerebrospinal Fluid Biomarkers P-Tau and A beta(42) Increases the Level of Evidence of Alzheimer's Disease Pathophysiological Process in Routine Practice**

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**Additional Use of A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> Ratio with Cerebrospinal Fluid Biomarkers P-Tau and A $\beta$ <sub>42</sub> Increases the Level of Evidence of Alzheimer's Disease Pathophysiological Process in Routine Practice**

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**Running title:** A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio in CSF AD biomarkers

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## **Abstract**

**Background.** Alzheimer cerebrospinal fluid (CSF) biomarkers have recently been included in the criteria for AD diagnosis. Since interpretation of CSF profile requires the combination of three parameters, biological data are not always conclusive and isolated elevation of P-Tau or reduction of  $A\beta_{42}$  alone can be observed. In these cases,  $A\beta_{42}/A\beta_{40}$  *ratio* could be more relevant than  $A\beta_{42}$  absolute values by considering inter-individual variations in the total amyloid load.

**Objective.** The objective of this study was to assess the use of  $A\beta_{42}/A\beta_{40}$  *ratio* to improve the accuracy of biological conclusions in the diagnosis of patients with ambiguous CSF  $A\beta_{42}$  or Tau results.

**Methods.** Among 386 lumbar punctures analyzed in the lab in 2 years, 122 showed ambiguous biological data that were completed by CSF  $A\beta_{40}$  quantification and  $A\beta_{42}/A\beta_{40}$  *ratio* calculation. A biological conclusion was then made using 0.05 as the  $A\beta_{42}/A\beta_{40}$  *ratio* cut-off.

**Results.** Our results showed that one-third of the biological profiles of patients with atypical dementia were ambiguous. The addition of  $A\beta_{42}/A\beta_{40}$  *ratio* increased the proportion of interpretable biological profiles from 69% to 87%, without changing the conclusion when usual biomarkers ( $A\beta_{42}$  and P-Tau) were concordant.

**Conclusion.** Our results support the use of the  $A\beta_{42}/A\beta_{40}$  *ratio* in addition to the usual CSF AD biomarkers for patients with ambiguous biological profiles. This method could be specifically directed to this population in order to improve the level of certainty for clinical routine practice.

**Key words:** Alzheimer's disease,  $A\beta_{40}$  peptide,  $A\beta_{40}/A\beta_{42}$  ratio,  $A\beta_{42}$  peptide, P-Tau, cerebrospinal fluid, diagnosis, differential diagnosis

## Introduction

Alzheimer's disease (AD) is the most prevalent cause of dementia leading to dramatic loss of cognitive and functional abilities [1]. AD diagnosis criteria, initially established by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) workgroup in 1984 [2], have been revised in 2011 [3] to assimilate the advances made in the understanding of the pathophysiological process of AD and related disorders. Although the clinical criteria constitute the core of the diagnosis, the revised criteria now include as complimentary parameters several biochemical cerebrospinal fluid (CSF) and imaging biomarkers, which allow reinforcing or reducing the evidence of AD pathophysiological process to which they are specifically related [4]. Biomarkers were related either to A $\beta$  peptide accumulation or to neuronal degeneration or injury [3, 5]. In the CSF, AD is biologically characterized by a reduction in CSF A $\beta$ <sub>42</sub> peptide levels which is correlated with the density of neuritic plaques [6] and by an increase in CSF Tau and P-Tau levels associated with neurodegeneration and neocortical neurofibrillary pathology [7]. This combination of parameters leads to high levels of sensitivity, specificity, and diagnostic accuracy for discrimination between AD and controls [8].

Thus, three levels of evidence of AD pathophysiological process have been proposed [3]: (1) a high probability level of AD process when biomarkers of both A $\beta$  brain accumulation and neuronal injury are positive, (2) an intermediate level when one marker type is positive and the other is negative (3) a low level when both are negative. Many investigators have pointed out that pre-analytical and analytical variations of these parameters could restrict their use in routine diagnosis. Nevertheless, nationally- and internationally-wide standardizations have been carried out and are still ongoing to minimize the inter-laboratory variations [9-13].

The measurement of CSF A $\beta$ <sub>42</sub> peptide levels appears to be consistent with the AD pathological process since this peptide is the major component of the amyloid plaques. However, the catabolism of the amyloid precursor protein (APP) by the  $\beta$ - and  $\gamma$ -secretases generates several A $\beta$  peptide species [14] and AD process involves very complex mechanisms such as shift of the secretase sites in APP sequence or alteration in the clearance of A $\beta$  through the blood brain barrier [15-17]. Indeed, in 1998, Shoji et al. [18] observed in a population of 157 Japanese AD and non-AD patients that A $\beta$ <sub>40</sub> did not decrease in AD patient CSF, unlike A $\beta$ <sub>42</sub>, and used the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio* to minimize the effect of A $\beta$ <sub>42</sub> variations. This result was confirmed later by Wiltfang et al. [19] who found that the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio*, but not the A $\beta$ <sub>42</sub> peptide levels, correlates with <sup>181</sup>P-Tau levels. At the same time, Hansson et al. [20] showed that the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio* reinforces the A $\beta$ <sub>42</sub> value to predict the conversion of MCI patients to AD. On the contrary, Parnetti et al. [21] stated that the A $\beta$ <sub>42</sub>/P-tau ratio is the best parameter to predict MCI-to-AD conversion. Three studies emphasized the value of A $\beta$ <sub>40</sub> levels or the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio* to discriminate AD from other neurodegenerative processes such as frontotemporal lobar degeneration (FTLD), vascular dementia (VaD), dementia with Lewy bodies (DLB) and cerebral amyloid angiopathy (CAA) [23-25]. All of these studies were performed on pre-classified groups of patients affected by these various pathologies. More recently, Slaets et al. validated the discriminative values of A $\beta$ <sub>40</sub> levels and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio* in autopsy-confirmed AD and non-AD patients [26].

In clinical practice, CSF biomarkers are carried out in patients with atypical or mixed presentation of dementia and have a major impact on the complex diagnosis that is made. Ambiguous situations are observed, such as isolated elevation of P-Tau or reduction of A $\beta$ <sub>42</sub> level alone. All previous data suggested that the measure of A $\beta$ <sub>40</sub> peptide levels and the calculation of A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio* could be helpful to further classify the patients displaying ambiguous profiles in daily practice. Therefore, the aim of this study was to establish whether

the addition of  $A\beta_{42}/A\beta_{40}$  ratio to other CSF biomarkers without prior stratification of patients could increase the level of evidence of AD or non-AD dementia in such situations (isolated elevation of P-Tau or isolated reduction of  $A\beta_{42}$  level).

## **Material and methods**

### ***Patients***

Between January 2009 and February 2011, CSF was collected from 386 patients through outpatient memory clinics all over the region Lorraine, with cognitive disorders assessment. All patients underwent thorough clinical investigations, including medical history, physical, neurological and neuropsychological examinations, screening laboratory tests and magnetic resonance imaging (MRI) or computerized tomography (CT) and single photon emission computerized tomography (SPECT) with technetium ( $(99m)Tc$ -HMPAO or ethyl cysteinate dimer ( $99m$  ECD)). CSF biomarkers were carried out according to European Federation of Neurological Societies guidelines (diagnosis doubt, atypical clinical presentations or early-onset dementia) or for amnesic mild cognitive impairment (MCI) according to research criteria for the diagnosis of AD published in 2007 [27].

### ***Clinical diagnostic criteria***

The diagnosis of probable or possible AD was made according to the NINCDS-ADRDA criteria (1984) [2] completed by individual imaging investigations. The diagnosis of MCI was made according to the Petersen criteria (MCI) (2004) [28]. The combination of AD and cerebrovascular disease (AD+CVD) was diagnosed when patients fulfilled criteria of probable AD in addition with vascular lesions on brain MRI and/or CT. The diagnosis of other dementias was made according to the following criteria: the NINDS-AIREN criteria for

vascular disease (1993) [29], the Neary criteria for FTLD (1998) [30] and the Mc Keith criteria for Lewy Bodies LBD (2005) [31]. Practically, in the 122 patients included in our study, evaluation of CSF biomarkers was performed because of diagnosis ambiguity. In these cases, final diagnosis was also established based on conclusions of multidisciplinary consultation meetings.

### ***Biological samples***

CSF was collected in the clinical setting by lumbar puncture (LP) of the L3-L4 or L4-L5 intervertebral space after local anaesthesia in non-fasting patients, as part of the investigation of patient's cognitive dysfunction. In the standard procedure, CSF was collected in a 5 mL Sarstedt polypropylene tubes (ref 65.806). All analyses were performed at the Biochemistry Laboratory of the University Hospital of Nancy (Central Laboratory) and all samples were transported and stored in standardized conditions according to the manufacturer's instructions. If samples could be delivered to the Central laboratory in less than 24 h after LP, they were immediately transported at 4°C. There, they were quickly centrifuged at 4,000 x g for 10 min at 4°C; 400 µL aliquots were removed and stored in 1.5 mL Sarstedt polypropylene microtubes (ref 72-692-105) at -80°C until assay. If samples could not be delivered to the Central laboratory in less than 24 h, they were centrifuged similarly in the local laboratory; 400 µL aliquots were removed and stored in 1.5 mL Sarstedt polypropylene microtubes (ref 72-692-105) at -20°C until transferred to the Central laboratory, always at -20°C. At the Central laboratory, samples were immediately stored at -80°C until assay. Finally all samples were stored at -80 °C in less than 24 h and underwent only one freeze-thaw cycle prior to the assay as described in the last international recommendations to standardize preanalytical confounding factors in AD CSF biomarkers [32].

### ***Biochemical analyses***

All analyses were performed under the same conditions in a unique Central laboratory (Biochemistry Laboratory of the Central University Hospital of Nancy, France). A $\beta$ <sub>42</sub>, Tau and P-Tau concentrations were determined using commercially available sandwich ELISA procedures (Innotest®, Innogenetics NV, Ghent, Belgium) according to the manufacturer's instructions. CSF A $\beta$ <sub>40</sub> level was quantified by ELISA using A $\beta$ (35-40) (1A10) antibody (Human Amyloid  $\beta$ (1-40) (N) Assay kit-IBL-Japan distributed by Innogenetics NV, Belgium) according to the manufacturer's instructions. Biomarker concentrations were proportional to optical density at 450 nm. A CSF pool sampled in the same polypropylene tubes and stored at -80°C was used as internal quality control in each experiment. Standards, samples and controls were run in duplicate. If the variation coefficient between both values was > 10%, the sample was re-assayed.

A $\beta$ <sub>42</sub> peptide, A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio* and P-Tau protein values of 500, 0.05 and 60 pg/mL, respectively, have been proposed as pathological thresholds. Several authors have proposed that P-Tau is more specific for AD than for other dementia [3, 33], even in autopsy-confirmed dementia series [34, 35]. We chose to conclude using primarily CSF A $\beta$ <sub>42</sub> peptide and P-Tau routinely measured according to the following criteria (Figure 1):

- A $\beta$ <sub>42</sub>  $\geq$  500 pg/mL and P-Tau < 60 pg/mL were considered as CSF profile with a low level of probability of AD,
- A $\beta$ <sub>42</sub> < 500 pg/mL and P-Tau  $\geq$  60 pg/mL were considered as CSF profile with a high level of probability of AD,
- A $\beta$ <sub>42</sub>  $\geq$  500 pg/mL and P-Tau  $\geq$  60 pg/mL or A $\beta$ <sub>42</sub> < 500 pg/mL and P-Tau < 60 pg/mL were considered as CSF profiles with an intermediate level of probability of AD and were included in the study.

For this last group, biological data were completed by CSF A $\beta$ <sub>40</sub> quantification and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio* calculation. The association of P-Tau and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> *ratio* led to a new biological conclusion (Figure 1):

- P-Tau < 60 pg/mL and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio  $\geq$  0.05 were considered as CSF profile with a low level of probability of AD,
- P-Tau  $\geq$  60 pg/mL and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio < 0.05 were considered as CSF profile with a high level of probability of AD,
- P-Tau < 60 pg/mL and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio < 0.05 and P-Tau  $\geq$  60 pg/mL and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio  $\geq$  0.05 were considered as CSF profiles with an intermediate level of probability of AD.

### ***Statistical analysis***

Demographic characteristics, clinical and biological data from patients were assessed using descriptive statistical methods: quantitative data are expressed as mean  $\pm$  SD, qualitative data by numbers and percentages. Bivariate analysis was performed to compare CSF profiles to the initial population. Comparison of 2 groups was performed by Fisher exact test for categorical variables and non-parametric test for continuous variables with 5% level of significance. Spearman correlation analyses were performed relating CSF biological parameters. Analyses were conducted with SAS software, version 9.3 (SAS Institute, Cary, NC).

## Results

### *Patient classification according to the initial CSF A $\beta$ <sub>42</sub> and P-Tau analysis*

Among the 386 CSF samples collected during the period, 10 samples were excluded because of pre-analytical non-conformity (Tau levels < 100 pg/mL or inappropriate type of tube in the majority of cases), thus 376 CSF samples were used in this study. Biological results showed:

- 150 CSF profiles with a high level of probability of AD (A $\beta$ <sub>42</sub> < 500 pg/mL and P-Tau  $\geq$  60 pg/mL),
- 102 CSF profiles with a low level of probability of AD (A $\beta$ <sub>42</sub>  $\geq$  500 pg/mL and P-Tau < 60 pg/mL), 2 typical CJD profiles (Tau > 1250 ng/mL without abnormalities in A $\beta$ <sub>42</sub> and P-Tau levels)
- 122 ambiguous CSF profiles with an intermediate level of probability of AD according to the criteria defined in Figure 1.

The patient group with ambiguous CSF profiles represented 31.6% of the initial population and included patients with combinations of A $\beta$ <sub>42</sub> < 500 pg/mL and P-Tau < 60 pg/mL (63%) or A $\beta$ <sub>42</sub>  $\geq$  500 pg/mL and P-Tau  $\geq$  60 pg/mL (37%). Demographic data and mean biomarker levels in the CSF for the 4 groups are shown in Table 1. The 122 patients with ambiguous biological profile and the whole initial population did not differ in age, gender and CSF biomarker levels.

### *A $\beta$ <sub>40</sub> quantitation and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio calculation in patients with non-ambiguous CSF profile (high or low level of probability of AD)*

To validate the use of  $A\beta_{42}/A\beta_{40}$  ratio for cases with ambiguous CSF biological profile only, we first measured CSF  $A\beta_{40}$  concentrations in a control group of 26 patients with a high level of CSF probability biomarkers of AD and by 27 patients with a low level of CSF probability biomarkers of AD. The median CSF value of  $A\beta_{40}$  was 10347 [4532 – 20116] pg/mL

$A\beta_{42}/A\beta_{40}$  ratio cut-off for distinguishing between AD and non-AD patients was 0.05 according to the recommendations of Innogenetics laboratory (personal communication). The median value obtained in the control group was 0.089 [0.049 – 0.193] for patients with a low probability level of AD and 0.034 [0.015 – 0.057] for patients with a high probability level of AD.

***$A\beta_{40}$  quantification and  $A\beta_{42}/A\beta_{40}$  ratio calculation in patients with an intermediate profile***

To determine whether  $A\beta_{42}/A\beta_{40}$  ratio could increase the level of probability of the AD pathophysiological process in cases of ambiguous biological profiles, we completed the biomarkers analysis with CSF  $A\beta_{40}$  quantitation and  $A\beta_{42}/A\beta_{40}$  ratio calculation for the 122 patients. The median CSF level of  $A\beta_{40}$  was 10289 [2467 – 24749] pg/mL and similar to the mean value obtained in patients with non ambiguous CSF biological profiles (Figure 2). Interestingly, we observed a significant difference ( $p = 0.0484$ ) in extreme values between both groups since  $A\beta_{40}$  levels ranged between 2467 and 24749 pg/mL in the group with ambiguous CSF biomarkers, whereas it ranged between 4532 and 20116 pg/mL in the control group (Figure 2).

For patients with ambiguous CSF biomarkers, the median value of  $A\beta_{42}/A\beta_{40}$  ratio was 0.05 (0.023 – 0.11). Among these 122 patients, 72 (59%) had an  $A\beta_{42}/A\beta_{40}$  ratio less than 0.05 suggesting an amyloidopathy-like pathology, and 50 (41%) had a normal ratio superior to 0.05.

The association of P-Tau and  $A\beta_{42}/A\beta_{40}$  *ratio* led to a new biological conclusion for patients with ambiguous biological profiles (Table 2). Thirty-five patients (29%) displayed a high level of CSF biomarkers probability of AD ( $p\text{Tau} \geq 60$  pg/mL and  $A\beta_{42}/A\beta_{40} < 0.05$ ) and 37 (30%) exhibited a low level of CSF biomarkers probability of AD ( $p\text{Tau} < 60$  pg/mL and  $A\beta_{42}/A\beta_{40} \geq 0.05$ ). Fifty patients remained with an intermediate level of CSF biomarkers probability of AD. Thirty-seven patients (30%) had an amyloidopathy-like profile (only a decrease in  $A\beta_{42}/A\beta_{40}$  *ratio*), while thirteen patients displayed only an elevation in CSF P-Tau concentrations. Therefore, the addition of  $A\beta_{42}/A\beta_{40}$  *ratio* improved the proportion of interpretable biological profiles from 69% to 87% in the initial population

#### ***Correlation between $A\beta$ peptides, Tau and P-Tau***

Correlations between each pair of biomarkers were plotted and Spearman factors were calculated.  $A\beta_{42}$  and  $A\beta_{40}$  were strongly correlated ( $r = 0.829$ ). Since the population of the study was composed of patients with an intermediate low level of CSF biomarkers probability of AD, a positive correlation ( $r = 0.691$ ) was found between  $A\beta_{42}$  and P-Tau. On the contrary, when considering  $A\beta_{40}$  within the biological data, a negative correlation was observed between  $A\beta_{42}/A\beta_{40}$  *ratio* and P-Tau ( $r = -0.49$ ,  $p < 0.0001$ ) as well as for CSF profiles associated with evidence of AD pathophysiological process (decrease in amyloid levels and increase in P-Tau concentrations) and for CSF profiles associated with evidence of non-AD like dementia (high levels of amyloid and low levels of P-Tau).

#### ***Comparison of biological data to final diagnosis***

The  $A\beta_{42}/A\beta_{40}$  *ratio*-completed biological data were then compared with each diagnosis group. We found that 86% of AD CSF profiles were correctly associated with AD, mixed dementia or MCI diagnosis. Amnesic MCI and LBD were described as putatively

associated with decreases in  $A\beta_{42}$  peptide level and/or in  $A\beta_{42}/A\beta_{40}$  *ratio*. Thirteen percent of the amyloidopathy-like profiles were associated with MCI or LBD diagnosis, while 73% of unaltered CSF profiles were associated with LBD or other pathologies (Table 3).

## Discussion

We show here that one-third of the CSF biological profiles of patients presenting atypical dementia were ambiguous. We demonstrate that the addition of  $A\beta_{40}$  peptide concentration and  $A\beta_{42}/A\beta_{40}$  *ratio* does not change the conclusion when usual biomarkers ( $A\beta_{42}$  and P-Tau) are concordant. In contrast, ambiguous results were improved when the  $A\beta_{42}/A\beta_{40}$  *ratio* was included, and increased the level of evidence of AD pathophysiological process or of dementia unrelated to AD.

The recently revised criteria for the diagnosis of AD recommend the use of biomarkers (CSF biomarkers and/or amyloid PET imaging and/or fluorodeoxyglucose (F18) PET scan) to assess the probability level of AD pathophysiological process [3]. We showed in clinical practice that CSF biomarkers measured in patients with atypical dementia did not always increase the level of diagnosis certainty because results can be ambiguous when absolute values of  $A\beta_{42}$  and P-Tau are used as readouts to optimally discriminate AD from non-AD as described in autopsy-confirmed dementia [34, 35]. In this specific population, we showed that the inclusion of  $A\beta_{42}/A\beta_{40}$  *ratio* in the criteria increased the level of evidence of AD pathophysiological process or dementia unlikely due to AD.

This *ratio* was initially proposed by Lecwuck et al. (2004) as able to improve the percentage of correctly classified patients, combined with Tau protein levels [35]. Later, the same group reported constitutively high- or low-CSF concentrations of total  $A\beta$  peptide in subjects, and suggested that inter-individual variability might lead to incorrect interpretation

[19]. Here it was very interesting to note that the addition of  $A\beta_{40}$  peptide concentration and  $A\beta_{42}/A\beta_{40}$  ratio did not change the conclusion when the usual biomarkers ( $A\beta_{42}$  and P-Tau) were initially concordant. In these cases, both the absolute value of  $A\beta_{42}$  and the  $A\beta_{42}/A\beta_{40}$  ratio equally discriminated patients with low or high level of probability of AD pathophysiological process. These data allowed us to conclude that the additional use of CSF  $A\beta_{40}$  in routine practice could be necessary only when  $A\beta_{42}$  and P-Tau were ambiguous but not when the initial biological profile was conclusive. Furthermore, a real difference was detected in the distribution of  $A\beta_{40}$  values from patients with ambiguous biological profile and those from patients with conclusive biological profile since CSF  $A\beta_{40}$  concentrations were significantly more dispersed in the group of patients with ambiguous biological profiles. This supports the hypothesis concerning varying brain amyloid load and the importance in normalizing CSF  $A\beta_{42}$  concentrations to  $A\beta_{40}$  rather than using absolute values of  $A\beta_{42}$ .

When CSF biological profile was ambiguous, we showed that additional analysis of  $A\beta_{40}$  peptide level and calculation of  $A\beta_{42}/A\beta_{40}$  ratio led to a new and more informative biological conclusion for 87% patients among those requiring biomarkers because of diagnosis uncertainty. In a recent study, Slaets et al. (2013) showed in a largely autopsy-confirmed population that adding CSF  $A\beta_{42}/A\beta_{40}$  ratio to the CSF AD biomarkers might have an added value for discriminating AD from non-AD demented patients in case of intermediate P-Tau levels [26]. To complete these data, our study allowed the assessment of the performance of CSF  $A\beta_{42}/A\beta_{40}$  ratio in daily clinical practice. In this context, CSF biomarkers were carried out in case of problematic diagnosis and were used to confirm or reverse the existence of AD pathophysiological process. We were then able to show the clinical relevance of  $A\beta_{42}/A\beta_{40}$  ratio in decreasing the proportion of intermediate biomarker results in undiagnosed subjects that would allow the physician to reach a relevant biological conclusion.

After the inclusion of  $A\beta_{42}/A\beta_{40}$  ratio to the 122 ambiguous CSF profiles, we obtained 29% of high level of evidence of AD pathophysiological process and 30% of low level of evidence of AD pathophysiological process. In 30% of the cases, we confirmed the isolated presence of amyloidopathy ( $ratio < 0.05$  associated with P-Tau  $< 60$  pg/mL). This type of result could be relevant for the physician because  $A\beta$  metabolism is altered early in AD neurodegenerative processes [36, 37]. Indeed, longitudinal studies of CSF biomarkers showed that the levels of  $A\beta$  peptide are already decreased at least 5 to 10 years before conversion to AD dementia, whereas Tau and P-Tau are markers of the advanced stage of AD [38]. Furthermore at the prodromal stage of AD, the  $A\beta_{42}/A\beta_{40}$  ratio demonstrated better diagnosis performance to predict AD conversion compared to the absolute value of  $A\beta_{42}$  [20]. Low levels of CSF  $A\beta$  peptide had also been described in clinically diagnosed cases with DLB, but not as much as in AD [38-42]. Vanderstichele et al. (2006) observed that CSF  $A\beta_{42}$  levels were not significantly different between DLB (285 to 495 pg/mL) and AD (338 to 532 pg/mL), and were significantly lower than that in the control group (551 to 830 pg/mL). Finally, they concluded that similar low levels of CSF  $A\beta$  peptide were found in AD and DLB, and that P-Tau discriminated both dementias with a cut-off value of 61 pg/mL [43]. We therefore propose that low CSF  $A\beta_{42}/A\beta_{40}$  ratio can provide an additional diagnosis element for isolated amyloidopathy and non-mixed DLB. Though this biological profile cannot be considered as a typical argument for DBL diagnosis, it could represent supplementary information for patients with hallucinations and/or extra-pyramidal syndrome and/or DAT-SCAN abnormalities.

Finally, after the addition of the  $A\beta_{42}/A\beta_{40}$  ratio, only 13 patients (11% of patients with ambiguous biological profile and only 3% of the population requiring CSF biomarkers to support dementia diagnosis) had a high CSF P-Tau level without amyloid abnormality. In these cases, AD cannot be excluded because CSF P-Tau remains the most robust marker for

discriminating AD from non-AD-dementia [33, 34] and it would be appropriate to follow these patients not only through CSF biomarker levels but also using PET amyloid imaging.

In addition to increasing diagnosis accuracy, the use of  $A\beta_{42}/A\beta_{40}$  *ratio* could contribute to the global standardization of CSF AD biomarkers. Today, variability, especially at pre-analytical steps, limits the usefulness of CSF biomarkers. According to the literature,  $A\beta_{42}$  peptide measurement would be more highly affected by this variability [5]. Perret-Liaudet *et al.* (2012) demonstrated that the type of collection/sampling tubes was a major pre-analytical source of variability, particularly for  $A\beta$  peptide, and represents a major limitation to define worldwide-established cut-off values [12]. It would be interesting to assess whether  $A\beta_{42}/A\beta_{40}$  *ratio* would be affected in the same way by these parameters, as is the case for the absolute value of  $A\beta_{42}$  peptide.

The lack of standardization data for  $A\beta_{40}$  constitutes a limitation to the extrapolation of our own results even if we tried to apply standard procedures towards sample collection, transport, preparation and storage in accordance with manufacturer's instructions, published data [12, 13] and constraints associated with clinical practice. Moreover, the autopsy-based confirmation of diagnoses would be ideal to complete this study and provide a more accurate cut-off for  $A\beta_{42}/A\beta_{40}$  *ratio* in the future.

In conclusion, our results confirm the value of CSF  $A\beta_{42}/A\beta_{40}$  *ratio* for improving interpretation of AD biomarkers in undiagnosed patients with ambiguous biological profile without modifying biological conclusion when usual biomarkers were conclusive. The CSF  $A\beta_{42}/A\beta_{40}$  *ratio* could be used specifically in this population to improve the level of certainty for the practitioner. However, further data concerning pre-analytical and analytical standardization of CSF  $A\beta_{40}$  is necessary to extend the use of this complementary biomarker in clinical practice.

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**Table 1. Demographic and biological data in each group and in the initial population**

Data are expressed as mean  $\pm$  SD. The range of values is indicated in brackets. All groups were compared to the initial population. Statistics were not performed on the small group of patients with CJD. Values were statistically different if  $p < 0.05$ .

	CSF profile with high probability of AD	CSF profile with intermediate level of probability of AD	CSF profile with low level of probability of AD	CSF Creutzfeldt-Jakob profile	Initial population
<b>Population</b>	150 (40%)	<b>122 (32%)</b>	102 (27%)	2 (1%)	<b>376 (100%)</b>
<b>Mean age <math>\pm</math> standard deviation (years)</b>	74.1 $\pm$ 8.9 [39-96] $p < 0.05^*$	<b>71 <math>\pm</math> 11.4</b> <b>[38-90]</b> <b>NS*</b>	68.1 $\pm$ 10.9 [24-87] $p < 0.05^*$	65.5 $\pm$ 4.9 [62-69] $p < 0.05^*$	<b>71.4 <math>\pm</math> 10,6</b> <b>[24-96]</b>
<b>Sex ratio M/F</b>	68/82 (0.83)	<b>62/60</b> <b>(1.03)</b>	58/44 (1.32)	2/0	<b>190/186</b> <b>(1.02)</b>
<b><i>A<math>\beta</math>42</i> (pg/mL)</b>	346 (141-496) $p < 0.001$	<b>469</b> <b>(163-1239)</b> <b>NS</b>	670 (503-1150) $p < 0.001$	(520-523)	<b>462</b> <b>(141-1239)</b>
<b><i>Tau</i> (pg/mL)</b>	517 (229-1250) $p < 0.001$	<b>315</b> <b>(102-1250)</b> <b>NS</b>	224 (102-528) $p < 0.001$	>1250	<b>360</b> <b>(102-1250)</b>
<b><i>P-Tau</i> (pg/mL)</b>	83 (61-358) $p < 0.001$	<b>56</b> <b>(13-193)</b> <b>NS</b>	43 (19-60) $p < 0.001$	(41-33)	<b>62</b> <b>(13-358)</b>

**Table 2. Distribution of A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio and P-tau and related conclusions**

	<b>A<math>\beta</math><sub>42</sub>/A<math>\beta</math><sub>40</sub> ratio &lt; 0.05</b>	<b>A<math>\beta</math><sub>42</sub>/A<math>\beta</math><sub>40</sub> ratio <math>\geq</math> 0.05</b>
<b>pTau <math>\leq</math> 60 pg/mL</b>	37 (30%) (Amyloidopathy)	37 (30%) (Normal)
<b>pTau &gt; 60 pg/mL</b>	35 (29%) (AD)	13 (11%) (Inconclusive)

**Table 3. Comparison of biological data to final diagnosis**

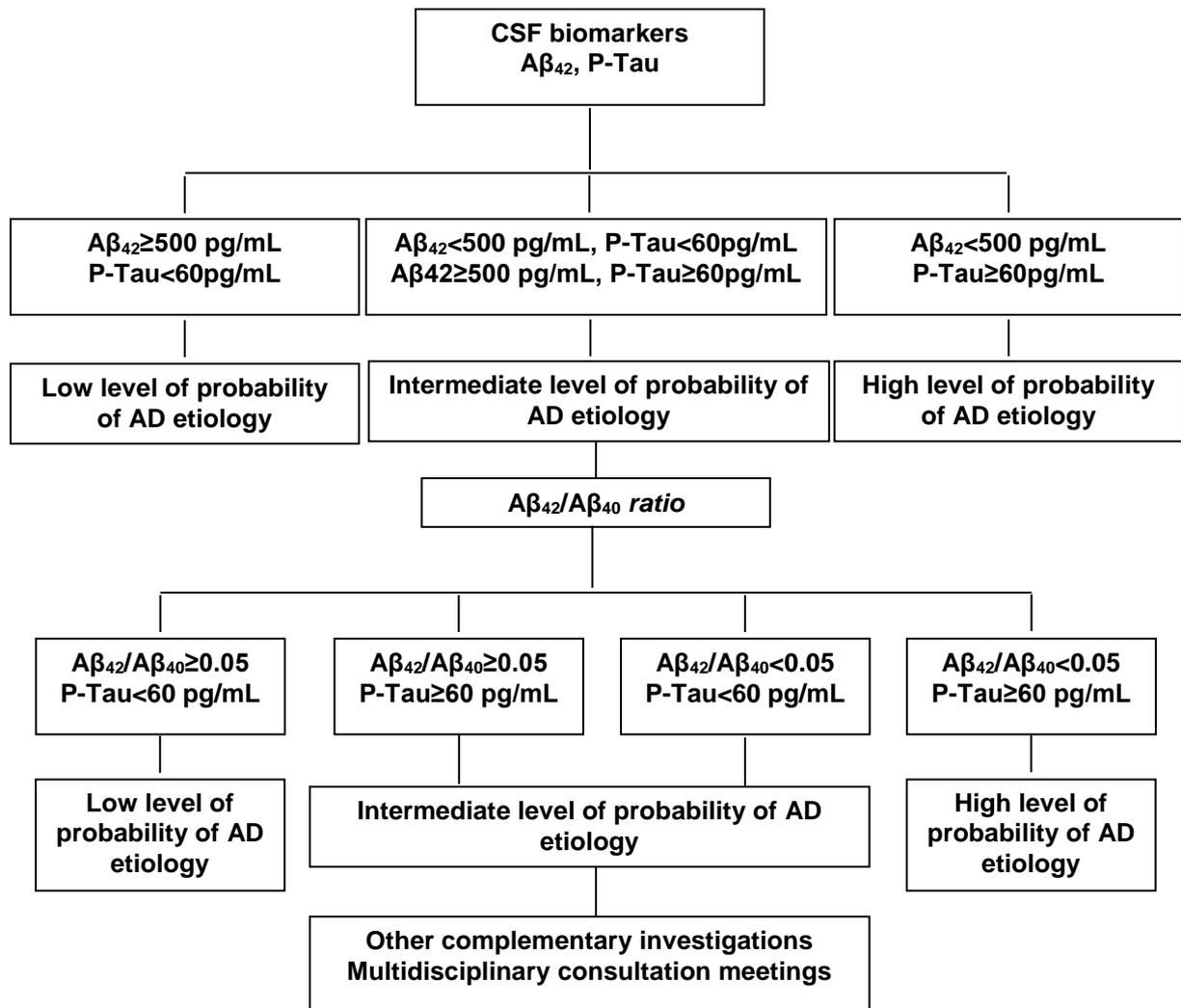
Data are presented as percentage of the total number of patients in each group

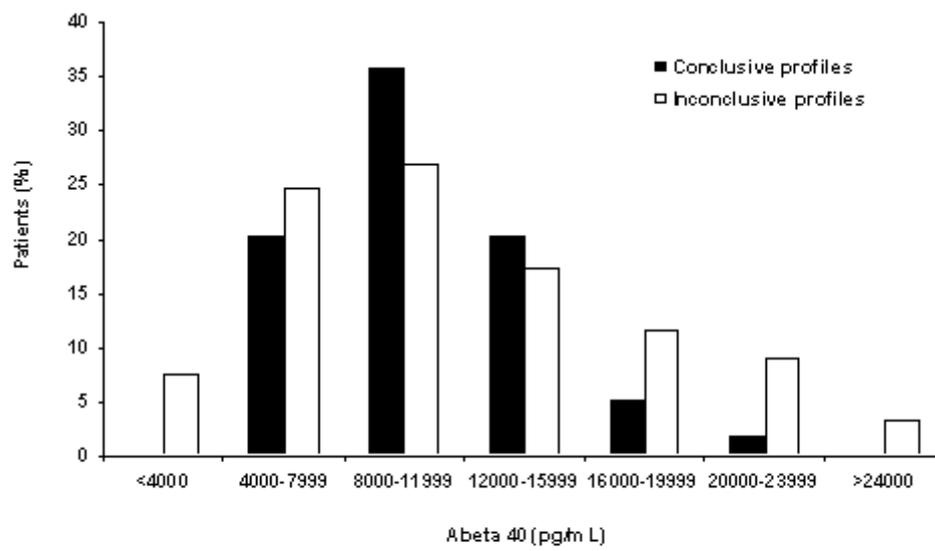
	<b>AD CSF profile (n = 35)</b>	<b>Unaltered final CSF profile (n = 37)</b>	<b>Amyloidopathy-like final profile (n = 37)</b>	<b>Inconclusive final profile (n = 13)</b>	<b>Total (n = 122)</b>
AD and mixed dementia	26 (74%)	4 (11%)	7 (19%)	2 (15%)	39 (32%)
MCI	5 (14%)	2 (5%)	2 (5%)	1 (8%)	10 (8%)
LBD	0	3 (8%)	3 (8%)	0	6 (5%)
Other pathologies	1 (3%)	24 (65%)	21 (57%)	8 (62%)	54 (44%)
Pending diagnosis	3 (9%)	4 (11%)	4 (11%)	2 (15%)	13 (11%)

## Figure legends

Figure 1: Decision tree for patient classification

Figure 2: Distribution of A $\beta$ 40 peptide in patients with inconclusive biological profiles (open bars) and in patients with conclusive biological profiles (closed bars)





**Figure1**