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## **Cerebrospinal Fluid $A\beta_{42}/A\beta_{40}$ as a Means to Limiting Tube- and Storage-Dependent Pre-Analytical Variability in Clinical Setting**

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1 **CSF A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> as means to limiting tube- and storage-dependent pre-analytical**  
2 **variability in clinical setting**

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13

14 **Running title:** A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio *in pre-analytical steps*

15

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26 Abstract

27 **Background.** Alzheimer cerebrospinal fluid (CSF) biomarkers have recently been included in  
28 the criteria for Alzheimer's disease (AD) diagnosis. Unfortunately, their wider use in routine  
29 and interpretation require more standardization, particularly for the pre-analytical steps. In  
30 particular, A $\beta$ <sub>42</sub> peptide measurement is strongly influenced by the type of collection tube and  
31 by repeated freeze/thaw cycles.

32 **Objective.** The objectives of this study were to compare, in clinical setting the impact of  
33 collection tubes and the repetition of freeze/thaw cycles on A $\beta$ <sub>42</sub> and A $\beta$ <sub>40</sub> concentrations  
34 and consequently determine if the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio could resolve the diagnosis difficulties  
35 related to these pre-analytical parameters.

36 **Methods.** CSF from 35 patients was collected in different PP and stored at -80°C after  
37 sampling. For CSF collected in the reference tube, three successive freeze-thaw cycles were  
38 done. A $\beta$ <sub>42</sub> and A $\beta$ <sub>40</sub> concentrations were determined in each condition in order to calculate  
39 the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio.

40 **Results.** Our results showed that CSF A $\beta$ <sub>42</sub> and A $\beta$ <sub>40</sub> values were significantly different  
41 according to the type of collection tube and the number of freeze/thaw cycles. Although the  
42 calculation of the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio eliminated the effect of PP tube-dependent variation, this  
43 was not the case for freeze-thaw cycle-associated variation.

44 **Conclusion.** The use of A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio rather than A $\beta$ <sub>42</sub> alone could contribute towards  
45 pre-analytical standardization, thus allowing the general use of CSF AD biomarkers in routine  
46 practice.

47

48 **Key words:** Alzheimer's disease, A $\beta$ <sub>40</sub> peptide, A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio, A $\beta$ <sub>42</sub> peptide,  
49 cerebrospinal fluid, pre-analytical standardization, tube, freeze-thaw cycles

50 **Introduction**

51 Alzheimer's disease (AD) diagnosis criteria were revised in 2011 to assimilate the advances in  
52 the understanding of AD (1). Although clinical symptoms still constitute the core of the  
53 diagnosis, new criteria now include biomarkers related to the AD pathophysiological  
54 processes such as cerebrospinal fluid (CSF) and imaging biomarkers. In the CSF, AD is  
55 biologically characterized by a reduction in CSF A $\beta$ 42 peptide levels which is correlated with  
56 the density of neuritic plaques (2) and by an increase in CSF Tau and phosphorylated Tau (P-  
57 tau) levels associated with neurodegeneration and neocortical neurofibrillary pathology (3).  
58 The combination of these three parameters have shown high levels of sensitivity and  
59 specificity as well as diagnostic accuracy in discriminating AD *versus* controls (4).  
60 In 2004, Lewczuk *et al.* (5) was the first to propose A $\beta$ 42/A $\beta$ 40 ratio rather than the absolute  
61 value of A $\beta$ 42 peptide to improve the percentage of appropriately diagnosed patients. Since  
62 this publication, several studies have largely demonstrated the interest of using A $\beta$ 42/A $\beta$ 40  
63 ratios for better interpretative CSF analysis and increase the level of evidence of AD process  
64 (6-13). A $\beta$ 40 peptide is the most abundant A $\beta$  peptide in the brain and its CSF level might  
65 therefore be considered to most closely reflect the total A $\beta$  load in the brain. CSF A $\beta$ 40 was  
66 characterized by a great inter-individual variability (14) and was long considered as  
67 unchanged in AD. Today, findings are less clear-cut. Indeed, Bousiges *et al.* (15) confirmed  
68 that CSF A $\beta$ 40 level was not significantly different in AD than in the control group, whereas  
69 it was decreased in patients with body Lewy dementia (BLD). Thus, A $\beta$ 42/A $\beta$ 40 allowed  
70 better discrimination of AD from BLD than A $\beta$ 42 alone (15). On the contrary, others studies  
71 showed lower CSF A $\beta$ 40 levels in AD patients than in controls (13, 16). More recently, Dorey  
72 *et al.* demonstrated that absolute value of CSF A $\beta$ 40 levels allowed the correct classification  
73 of AD patients with non-pathological A $\beta$ 42 levels and control patients with pathological  
74 A $\beta$ 42 levels (11).

75

76 Various papers have pointed out pre-analytical and analytical variability between laboratories  
77 for A $\beta$ 42 peptide (17-21). Tubes for collection, sample handling and sample storage  
78 conditions were especially noted as critical factors (22-23). In the clinical setting, despite  
79 numerous recommendations, the use of a non-recommended tube to collect CSF and the  
80 thawing of sample are two abnormalities frequently encountered. This is particularly the case  
81 when CSF AD biomarkers are analyzed for a large geographical area and samples are  
82 aliquoted and frozen by a local lab close to the applicant hospital before being transferred to a  
83 central laboratory. Transport conditions are unfortunately not always respected, leading to  
84 premature thawing of the samples. These pre-analytical confounding factors strongly  
85 influence A $\beta$ 42 peptide analysis, potentially leading to misinterpretation of biological  
86 profiles. A clear understanding of how these factors influence A $\beta$  levels would therefore  
87 represent a major step for a general widespread clinical application.

88

89 In this study, we sought to determine if the A $\beta$ 42/A $\beta$ 40 ratio, which might be less affected by  
90 the CSF collection tube and the repetition of freeze-thaw cycles, could be more largely used  
91 to limit variability due to these two major pre-analytical confounding parameters.

92

## 93 **Material and methods**

### 94 **Patients**

95 Thirty-five subjects needing CSF AD biomarkers (Tau, P-Tau, A $\beta$ 42 and A $\beta$ 40 peptides)  
96 were recruited in the Clinical and Research Memory Center of Nancy (France) *i.e.* patients  
97 with atypical clinical presentation or early-onset dementia (24).

98 .

99 CSF biomarkers were carried out according to European Federation of Neurological Societies  
100 guidelines (diagnosis doubt, atypical clinical presentations, or early-onset dementia) or for  
101 amnesic MCI according to research criteria for the diagnosis of AD published in 2007 (25).  
102 Each subject had signed a written informed consent for CSF assessment, analysis and storage  
103 and protocol was approved by local ethical committee.

104

### 105 **Samples**

106 CSF was collected by lumbar puncture of the L3-L4 or L4-L5 intervertebral space after local  
107 anaesthesia in non-fasting patients. Two mL CSF were collected in 3 different polypropylene  
108 (PP) tubes, always in the same order (1, R, 2). Ten mL Sarstedt PP tube (ref 62.610.201, PP +  
109 polyethylene copolymer) was chosen as the reference tube (tube R), most commonly as the  
110 standard tube in French laboratories. Five mL Gosselin PP tube (ref TP 10-03, PP), and 15  
111 mL Biosciences Discovery Labware Falcon tube (ref 352096, PP + Polyethylene copolymer)  
112 were respectively named tube 1 and tube 2.

113 All CSF were transported to the Biochemistry Laboratory of the University Hospital of  
114 Nancy, at 4 °C in less than 4 hours after the lumbar puncture. There, they were centrifuged at  
115 4,000 x g for 10 minutes at 4°C (FROILABO® centrifuge). Samples with blood  
116 contamination were excluded. CSF was sampled by 250 µL each in 500 µL PP storage tubes  
117 (ref 045513, Dutscher®) and stored at -80 °C until assay (THERMO SCIENTIFIC® freezer).

### 118 **Freeze-thaw cycles procedure**

119 The freeze-thaw cycles study was carried out with CSF collected in tube R but only 24  
120 samples contained enough CSF to prepare at least 2 samples (250 µL each). Cycles 1 and 2  
121 were done with 2 different 250 µL samples. Cycle 3 were done with the residual 120 µL CSF

122 after second thawing step. Time at room temperature during a freeze-thaw cycle was 45 min.  
123 A $\beta$ 42 and A $\beta$ 40 concentrations were determined at each step in order to calculate A $\beta$ 42/A $\beta$ 40  
124 ratio. For the freezing step, samples were stored at -80°C for at least 24h.

## 125 **Biochemical analyses**

126 A $\beta$ 42 and A $\beta$ 40 concentrations were determined using commercially available sandwich  
127 ELISA procedures (INNOTEST®, Fujirebio, Ghent, Belgium) according to the  
128 manufacturer's instructions. For each sample, A $\beta$ 42/A $\beta$ 40 ratio was calculated. A CSF pool  
129 stored at -80 °C in PP tubes was used as internal quality control for each assay. Standards,  
130 controls and samples were run in duplicate. Measures were done at 450 nm with a BIO-  
131 TEK® spectrophotometer (MWGt Lambda Scan 200, BIO-TEK®, USA). Data were analyzed  
132 with KC4 software v.3.3 (BIO-TEK®, USA). In cases of variation coefficients exceeding  
133 10%, the sample was re-assayed.

## 134 **Statistical analysis**

135

136 Variables were expressed, according to their distributions, as number, percent, mean  $\pm$   
137 Standard Deviation or median with Inter Quartile Range (IQR) and minimum maximum  
138 (range). Comparisons of CSF biological parameters for the 3 tubes and for the 3 repeated  
139 freeze-thaw cycles were performed by non-parametric test (Friedman test) with 5% level of  
140 significance. When the Friedman test concluded to a significant difference, a Dunn test was  
141 performed in order to identify the differences among the tubes or among the freeze-thaw  
142 cycles. To study the stability of the ratio between different tubes, we used the Spearman's  
143 rank correlation coefficient (Spearman's rho;  $r_s$ ) and the Bland-Altman plot to analyze  
144 correlation and agreement.

145

## 146 **Results**

### 147 **Demographical characteristics**

148 Thirty-five subjects were included in the study. Mean age was  $74 \pm 10$  years (range: 53-89  
149 years) and gender ratio (men/women) was 0.7.

150

### 151 **Effect of tube type on A $\beta$ peptide concentrations and A $\beta$ 42/A $\beta$ 40 ratio**

152 Each comparison was done between median values because A $\beta$  peptide concentrations and  
153 A $\beta$ 42/A $\beta$ 40 ratio were not normally distributed. A $\beta$ 42 and A $\beta$ 40 concentrations ranged from  
154 252.5 to 1506 pg/mL and from 2379 to 18052 pg/mL, respectively. Median and extreme A $\beta$   
155 peptide concentration and A $\beta$ 42/A $\beta$ 40 ratio are summarized in Table 1 for each tube. Box-  
156 plots of A $\beta$ 42 and A $\beta$ 40 concentrations and A $\beta$ 42/A $\beta$ 40 ratios are presented in Figure 1.  
157 Concentrations were quite variable and extended all over the range of measure. Detectable  
158 A $\beta$ 42 and A $\beta$ 40 concentrations of samples in different PP tubes were significantly different  
159 ( $p < 0.001$  for both peptides). Results from these data showed lower median values of A $\beta$ 42  
160 and A $\beta$ 40 peptides in tubes 1 -6.4% (-40.2 to 10.9),  $p < 0.01$  and -12.9% (-59.5 to -6.5),  
161  $p < 0.01$ , respectively) and 2 (-26.7 (-47 to 21.7)  $p < 0.001$  and -29.9% (-62.6 to 52.2),  $p < 0.001$ ,  
162 respectively) compared to tube R. However, no significant difference in the median of  
163 A $\beta$ 42/A $\beta$ 40 ratio was globally observed between the 3 tubes in the whole population  
164 (Friedman test,  $p = 0.549$ ) (Table 1, Figure 1).

165 Furthermore, individually, all the correlations between the ratios were significant for T1 vs  
166 TR, T1 vs T2 and T2 vs TR ( $r_s = 0,957$   $p = 0.0001$ ;  $r_s = 0,913$   $p = 0.0001$ ;  $r_s = 0,899$   $p = 0.0001$   
167 respectively). Bland-Altman plots showed that the number of observations located outside the  
168 corresponding interval (differences within mean  $\pm 1.96$  SD) is 2 (5.7%) for T1 vs TR; 3  
169 (8.6%) for T1 vs T2 and 2 (5.7%) for T2 vs TR. Overall, it should be noted that 2



170 observations located outside the intervals were the same for T1 vs T2 and T2 vs TR. Thus 30  
171 observations among the 35 (85.7%) are located within the 95% limits of agreement (Figure 2).  
172 Finally, median CSF protein and glucose concentrations were 0.47 g/L (range from 0.19 to  
173 0.81 g/L) and 3.55 mmol/L (range from 1.27 to 6.2 mmol/L), respectively. In this range of  
174 CSF protein concentrations and after one freeze/thaw cycle, variations in CSF A $\beta$ 42 or A $\beta$ 40  
175 according to the tube were not influenced by CSF protein level (Table 2). On the contrary,  
176 A $\beta$ 42 or A $\beta$ 40 mean values were weakly correlated with variations in peptide levels  
177 according the type of tube used (Table 3).

178

### 179 **Effect of free-thaw cycles on A $\beta$ peptide concentrations and A $\beta$ 42/A $\beta$ 40 ratios**

180 Twenty-four CSF were used for the freeze-thaw cycles study. A $\beta$  peptide concentrations and  
181 A $\beta$ 42/A $\beta$ 40 ratio were not normally distributed. Median and extreme A $\beta$  peptide  
182 concentration and A $\beta$ 42/A $\beta$ 40 ratios are summarized in Table 4. Box-plots of A $\beta$ 42 and A $\beta$ 40  
183 concentrations and A $\beta$ 42/A $\beta$ 40 ratios are presented in Figure 2. Median values of A $\beta$ 42 and  
184 A $\beta$ 40 concentrations were observed to be significantly different with the number of  
185 freeze/thaw cycles. Results from these data showed lower A $\beta$ 42 level after 2 freeze-thaw  
186 cycles compared to one (- 17.6% (-27.9 to -14),  $p < 0.001$ ) whereas A $\beta$ 40 level was not  
187 significantly changed -3.1% (-23.9 to 17.4), NS). Consequently, a significant difference for  
188 A $\beta$ 42/A $\beta$ 40 ratio (-15.8% (-18.2 to 1.4),  $p < 0.001$ ) was observed.

189 On the contrary, after the third freeze-thaw cycle, A $\beta$ 42 levels were similar to those measured  
190 after one cycle and were significantly increased comparatively to the levels obtained after 2  
191 cycles (+ 21.6% (11.1 to 40.7),  $p < 0.001$ ). Similarly, A $\beta$ 40 levels were statistically higher after  
192 the third cycle than after both first and second cycles (+ 17.8% (6.9 to 35.5),  $p < 0.001$  and +  
193 21.6% (10.8 to 52 .4),  $p < 0.001$ ). Consequently A $\beta$ 42/A $\beta$ 40 ratio was similar after 2 and 3  
194 free-thaw cycles (-6.6% (-9.1 to 11.5), NS). Freeze-thaw cycles-induced variations in A $\beta$

195 peptide concentrations and A $\beta$ 42/A $\beta$ 40 ratios led to biological misinterpretations in 4 patients  
196 among 24 (15%) since A42/A $\beta$ 40 ratio was lower or higher than reference threshold (0.07)  
197 according the freeze-thaw cycle.

198

199

## 200 **Discussion**

201 In this study, we showed that the use of the CSF A $\beta$ 42/A $\beta$ 40 ratio rather than A $\beta$ 42 alone  
202 could solve some pre-analytical difficulties which currently limit the standardization process  
203 and thus wider routine use of CSF biomarkers. We demonstrated that while A $\beta$ 42/A $\beta$ 40 ratio  
204 eliminated PP tube-dependent analytical variability, it did not completely resolve freeze-thaw  
205 cycle-associated variations.

206

207 Currently, PP is clearly recommended as the best material for CSF collection tubes, due to  
208 limited variability in A $\beta$ 42 values (26). However, heterogeneous results were also obtained  
209 with PP collection tubes from different suppliers (18). Indeed, by investigating the  
210 composition of 11 different PP tubes, Perret-Liaudet *et al.* revealed the presence of co-  
211 polymers such as polyethylene which are reported to largely influence A $\beta$ 42 adsorption (27)  
212 and led to more than 50% disparity in A $\beta$ 42 values depending on the tube used (18). This  
213 study led to the conclusion that A $\beta$ 42 cut-off needed to be established by the laboratory using  
214 a single type of sampling tube (28). Recommendations were made for the least adsorbent tube  
215 (Tube R in our study) to be used routinely in French labs (29). This tube is composed of PP  
216 and polyethylene. Here we chose to use a commonly used tube (tube 1, PP only) and another  
217 tube containing PP and polyethylene similar to tube R. As in Perret-Liaudet study (18), we  
218 observed that tube 2 was the most adsorbent for A $\beta$ 42 but also for A $\beta$ 40 suggesting that

219 information about tube composition provided by the supplier was insufficient to anticipate the  
220 adsorption potential of the tube.

221 A $\beta$ 40 is known to be less hydrophobic than A $\beta$ 2 peptide (30) suggesting a lesser tendency to  
222 interact with sampling tubes, but no study is available in the literature. We demonstrated here  
223 that variations in A $\beta$ 40 concentrations according to the tube type were equivalent to those  
224 observed with A $\beta$ 42 concentrations leading to A $\beta$ 42/A $\beta$ 40 ratios that were not globally  
225 significantly different, and independent of the tube type. Individual analysis showed that  
226 variations of the ratio according to the tube type were close to the mean except for 5 patients,  
227 mostly with ratio values very high relative to the threshold of 0.07 and without influence on  
228 the biological interpretation. These results support those of Lewczuk *et al.* (26) and  
229 Vanderstichele *et al.* (31) which concluded that A $\beta$ 42/A $\beta$ 40 ratio was comparable between PP  
230 and polystyrene, polycarbonate tubes (26) or PP and low protein binding tubes (31). Thus the  
231 use of A $\beta$ 42/A $\beta$ 40 ratio rather than A $\beta$ 42 alone could also represent a solution towards  
232 minimizing pre-analytical variability related to the various collection PP tube. In daily  
233 practice, CSF collection in a non-recommended tube is a frequent mistake that leads to  
234 rejection of the sample and exposing the patient a second time to lumbar puncture-associated  
235 adverse effects and discomfort. The absence of variation in A $\beta$ 42/A $\beta$ 40 ratio could be used as  
236 an alternative solution, rather than carrying out another CSF sampling.

237

238 Freeze/thaw cycles before A $\beta$ 42 peptide analysis represent another critical pre-analytical step,  
239 especially for CSF transferred from others hospitals since CSF AD biomarkers are analyzed in  
240 one laboratory for a given geographic area. Unfortunately, data from published studies are  
241 extremely discordant. Several studies shown that samples could be frozen and thawed up to  
242 two times without any effect on the AD biomarkers in CSF (20, 32), whereas other  
243 demonstrated a decrease in A $\beta$ 42 values after one (33) or two freeze/thaw cycles (34). Taken

244 together, these data led to imprecise recommendations suggesting the avoidance of repeated  
245 freeze/thawing of samples (28). Finally, the use of A $\beta$ 42/A $\beta$ 40 ratios rather than A $\beta$ 42 alone  
246 could also have been an interesting solution. Only one publication showed that A $\beta$ 40 level  
247 was stable for 2 freeze/thaw cycles (32). Otherwise Vanderstichele *et al.*, recently reported  
248 that an additional freeze-thaw cycle in a PP tube reduced both CSF A $\beta$ 42 and A $\beta$ 40 levels  
249 whereas the A $\beta$ 42/A $\beta$ 40 ratio was not significantly affected (31). Here, we observed a  
250 significant decrease in A $\beta$ 42 levels between the first and the second cycle while A $\beta$ 40 levels  
251 remained stable. Interestingly, both A $\beta$ 42 and A $\beta$ 40 were significantly increased between the  
252 second and the third freeze/thaw cycles. These results led to two major conclusions. Firstly,  
253 A $\beta$ 42/A $\beta$ 40 ratio did not prevent freeze-thaw cycle-associated variability in A $\beta$  peptide  
254 concentrations, which could lead to misdiagnosis. Indeed, 4 samples out of 24 included (15%)  
255 had ratio values above or below the pathological cut-off, depending on the considered  
256 freeze/thaw cycle. This result demonstrates the need to work with a defined number of  
257 freeze/thaw cycles and to establish a ratio cut-off according selected conditions. Secondly, the  
258 A $\beta$ 42 and A $\beta$ 40 level re-increase in CSF after 3 freeze/thaw cycles suggested that amyloid  
259 degradation was not the unique cause of variability. Aliquot volume was identified as a  
260 potential confounding factor in CSF A $\beta$ 42 peptide measurement (35-36). In our study, an  
261 extra variation factor occurred, since the total CSF volume was lower in cycle 3 than cycles 1  
262 and 2. This could contribute to the variability between freeze/thaw cycles. However, it is  
263 known that tube A $\beta$ 42 adsorption is rapid, and is a saturable phenomenon (18). Here, the  
264 volume was effectively lower in cycle 3 than cycle 1, but CSF was left in the initial storage  
265 tube during the entire experimental period. In studies on the influence of aliquot volume such  
266 as that of Toombs *et al.* (35), different storage volumes (50-1500  $\mu$ L) were tested in  
267 independent storage tubes. No data about the impact of the variation of volume of CSF in the  
268 initial storage tube are available in literature. However, the decrease in CSF volume in

269 storage tube could modify the equilibrium state between CSF and tube walls, and eventually  
270 lead to the release of A $\beta$  peptide from tube to CSF. Further investigation would be necessary  
271 to clarify this issue. Other processes could be taken into account such as conformational  
272 changes of A $\beta$  peptides, oligomerization, protein binding or modification in adsorption of  
273 amyloid on the tube wall which could depend on delay to freezing and changes in freezing or  
274 thawing temperatures (37). Moreover, a protein desorption processing could occur and it  
275 could be dependent on several parameters such as temperature, pressure (38), or eventually  
276 CSF volume variations. Finally, the impact of repeated freeze/thaw cycles on amyloid level  
277 variability seems to be a very complex process which requires further investigation to  
278 determine the respective involvement of amyloid species, storage conditions and nature of the  
279 storage tube.

280

281 The systematic use A $\beta$ 42/A $\beta$ 40 ratio could limit variability associated with some pre-  
282 analytical aspects, but results should be confirmed by further investigations with a larger  
283 cohort and by testing PP tubes from other suppliers. The effects of freezing seem to be  
284 dependent on the storage tube type and a cross-study exploring the link between PP tubes and  
285 repeated freeze/thaw cycle on amyloid pre-analytical variability could be relevant. Moreover,  
286 few data are available about amyloid level in fresh CSF. Blennow *et al.* (39) showed that  
287 A $\beta$ 42 concentrations were highly consistent between fresh and frozen CSF samples. It would  
288 be interesting to know if similar results would be obtained with A $\beta$ 40 and A $\beta$ 42/A $\beta$ 40 ratio.

289

290 There are several limitations to note in this study. Firstly, patients were not selected according  
291 to their diagnosis, whereas A $\beta$ 42 and A $\beta$ 40 levels seem to influence tube-associated  
292 variations. However, this choice was deliberate because this study was voluntarily conducted  
293 in a clinical setting before knowing final diagnosis and A $\beta$  values. Secondly, time delay

294 between LP and centrifugation was very broad (< 4h) and could affect the amyloid peptide  
295 adsorption process. Again, the aim of this study was to evaluate the CSF A $\beta$ 42/A $\beta$ 40 as  
296 means to limiting variability in clinical settings. Consequently, reducing this delay to less than  
297 4h would be very difficult. Today, AD biomarkers are not only research biomarkers, but also  
298 are largely integrated in diagnostic tools. It is thus very important to have information about  
299 pre-analytical confounding factors in daily practice. Moreover, A $\beta$  adsorption occurred  
300 quickly, measurable within 15min of storage and did not increase with longer incubation  
301 times, suggesting that this delay should have little influence (18).

302

303 In conclusion, our study demonstrates that the use of A $\beta$ 42/A $\beta$ 40 rather than A $\beta$ 42 could  
304 contribute to pre-analytical standardization which will allow generalizing the use of CSF AD  
305 biomarkers. If this ratio seems to be interesting to solve PP tube-dependent variability, it is  
306 not relevant or even inappropriate to limit freeze/thaw cycle associated variability. Further  
307 investigation must be carried out to better understand complex interactions between these two  
308 critical pre-analytical factors.

309

### 310 **Additional contribution**

311 The authors gratefully acknowledge all geriatricians and neurologists from CMRR. They also  
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313

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Tube	Variable	Median (IQR)	Min	Max	p
Tube R	A $\beta$ 42	607.9 (270.7)	438.8	1506.0	
	A $\beta$ 40	9983.0 (5352.2)	3356.2	18052.0	
	A $\beta$ 42/A $\beta$ 40 ratio	0.064 (0.059)	0.033	0.162	
Tube 1	A $\beta$ 42	569.1 (282.9)	296.0	1435.0	0.008
	A $\beta$ 40	8695.0 (5664.0)	2910.3	15151.0	0.001
	A $\beta$ 42/A $\beta$ 40 ratio	0.070 (0.054)	0.037	0.147	Friedmann NS
Tube 2	A $\beta$ 42	446.0 (222.4)	252.5	1407.7	0.0001
	A $\beta$ 40	6994.5 (5308.2)	2379.0	13416.0	0.0001
	A $\beta$ 42/A $\beta$ 40 ratio	0.069 (0.064)	0.022	0.151	Friedmann NS

466 **Table 1. CSF A $\beta$ 42, A $\beta$ 40 and A $\beta$ 42/A $\beta$ 40 median values according to the PP tube type.**

467 Data are expressed as median (IQR), minimal and maximal values. All groups were  
 468 compared to tube R. Median values were statistically different from tube R according to the  
 469 Dunn test after a significant Friedman test if  $p < 0.01$ . NS: Not significant, n=35.

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	$\Delta$ Tube R/tube1	$\Delta$ TubeT/tube 2
A $\beta$ 42	$r_s = -0.255$ $p = 0.137$	$r_s = 0.042$ $p = 0.801$
A $\beta$ 40	$r_s = -0.216$ $p = 0.201$	$r_s = -0.060$ $p = 0.728$

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479 **Table 2. Influence of CSF protein concentration on tube-associated variation after one**

480 **freeze/thaw cycle.** Rs: Spearman's rank correlation coefficient. NS: Not significant, n=35.

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	$\Delta$ Tube R/tube1	$\Delta$ TubeT/tube 2
A $\beta$ 42	$r_s = 0.157$	$r_s = 0.393$
	$p = 0.366$	$p = 0.020$
A $\beta$ 40	$r_s = -0.344$	$r_s = -0.28$
	$p = 0.043$	$p = 0.873$

495 **Table 3. Influence of A $\beta$  levels on tube-associated variation after one freeze/thaw cycle.**

496 Rs: Spearman's rank correlation coefficient. NS: Not significant, n=35.

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Cycle	Variable	Median (IQR)	Min	Max	p
Cycle 1	A $\beta$ 42	631.8 (478.6)	438.8	1506.0	
	A $\beta$ 40	11363.0 (5666.1)	3356.2	18052.0	
	A $\beta$ 42/A $\beta$ 40 ratio	0.057 (0.058)	0.033	0.155	
Cycle 2	A $\beta$ 42	520.9 (324.3)	346.3	1237.3	0.0001
	A $\beta$ 40	11009.0 (5571.0)	3938.9	19905.0	1
	A $\beta$ 42/A $\beta$ 40 ratio	0.048 (0.042)	0.026	0.101	0.0001
Cycle 3	A $\beta$ 42	633.3 (425.4)	417.4	1404.3	0.13
	A $\beta$ 40	13384.5 (7138.7)	4364.9	23321.0	0.0001
	A $\beta$ 42/A $\beta$ 40 ratio	0.045 (0.046)	0.025	0.104	0.0001

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514 **Table 4. CSF A $\beta$ 42, A $\beta$ 40 and A $\beta$ 42/A $\beta$ 40 median values according to number of**  
515 **freeze/thaw cycles.** Data are expressed as median (IQR), minimal and maximal values. All  
516 groups were compared to tube R. Median values were statistically different from tube R  
517 according to the Dunn test after a significant Friedman test if  $p < 0.01$ . NS: Not significant,  
518  $n=24$ .

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530 **Figure legends**

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532 **Figure 1: Variations of CSF A $\beta$ 42, A $\beta$ 40 and A $\beta$ 42/A $\beta$ 40 values according to the PP tube**

533 **type.** Box-plots of the dispersion of A $\beta$ 42 (A), A $\beta$ 40 (B) and A $\beta$ 42/A $\beta$ 40 ratio (C). Values

534 were statistically different if \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  or not significantly different (NS),  $n =$

535 35.

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537 **Figure 2: Individual variations of CSF A $\beta$ 42, A $\beta$ 40 and A $\beta$ 42/A $\beta$ 40 values according to**

538 **the PP tube type.** Bland-Altman plots showing the number of observations located outside

539 the corresponding interval (differences within  $\text{mean} \pm 1.96 \text{ SD}$ ),  $n = 35$ .

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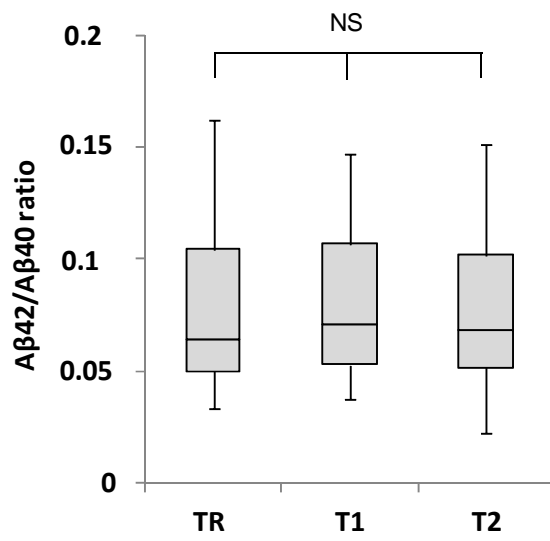
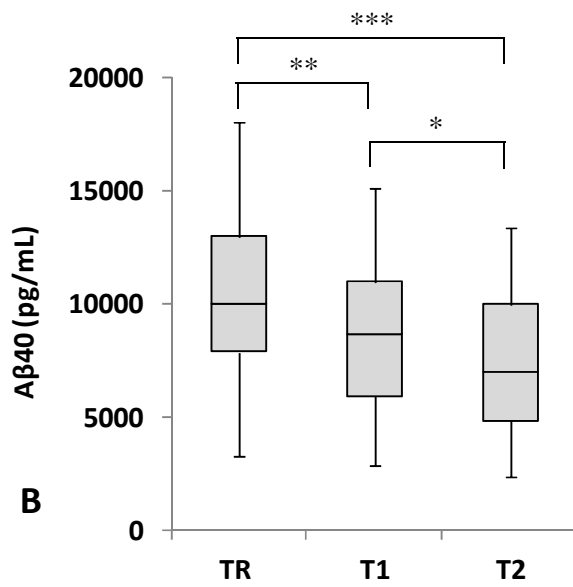
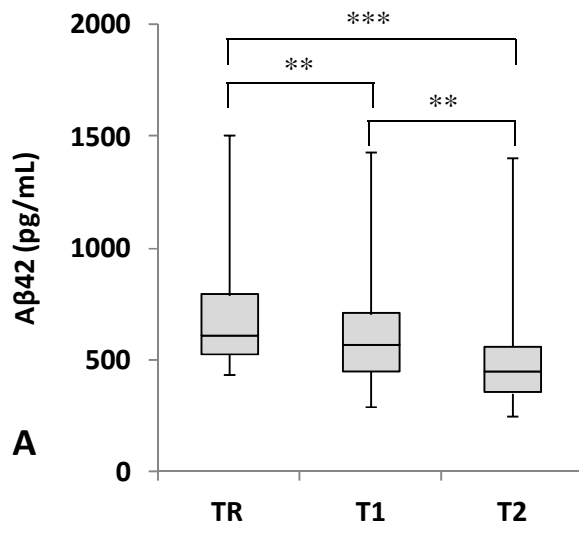
541 **Figure 3: Variations of CSF A $\beta$ 42, A $\beta$ 40 and A $\beta$ 42/A $\beta$ 40 values according the number of**

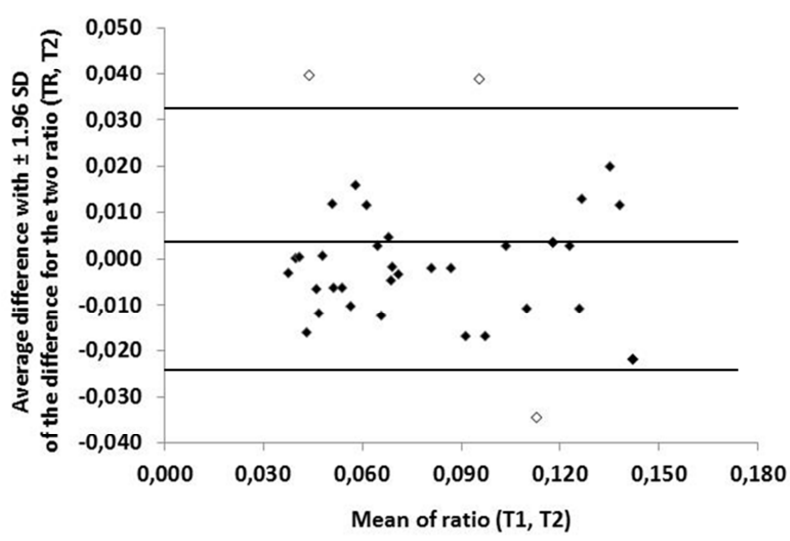
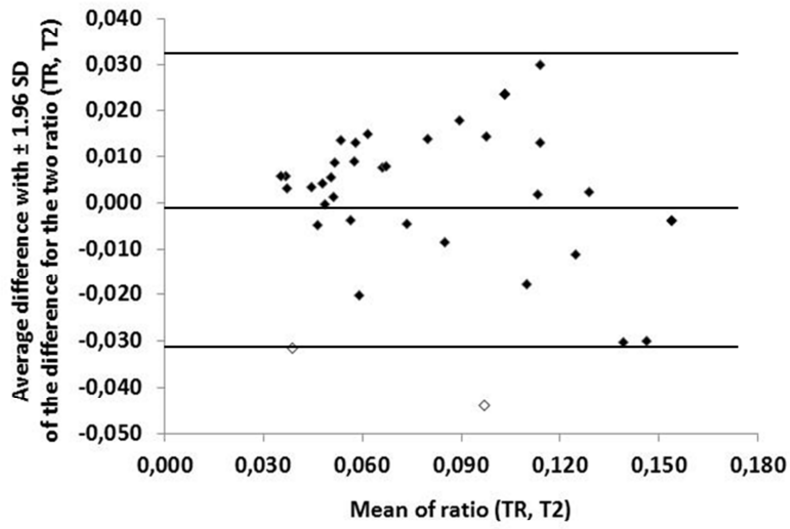
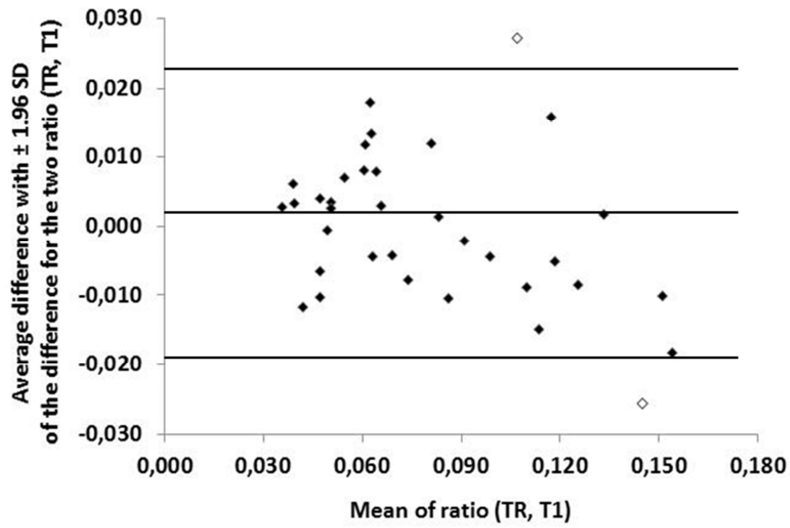
542 **freeze/thaw cycles.** Box-plots present the dispersion of A $\beta$ 42 (A), A $\beta$ 40 (B) and A $\beta$ 42/A $\beta$ 40

543 ratio (C). Values were statistically different if \*\*\*  $p < 0.001$  or not significantly different

544 (NS),  $n = 24$ .

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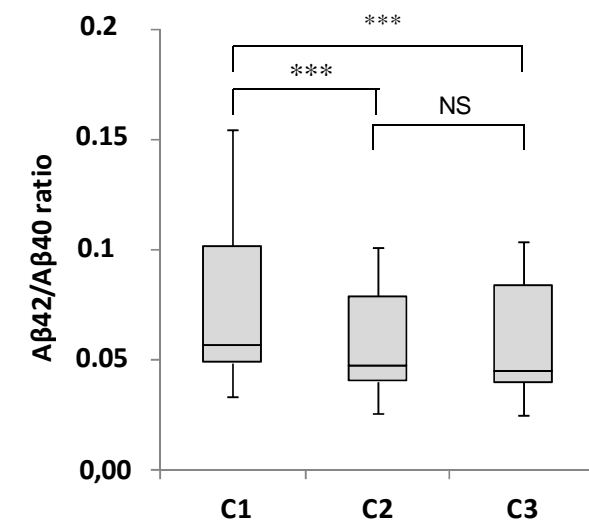
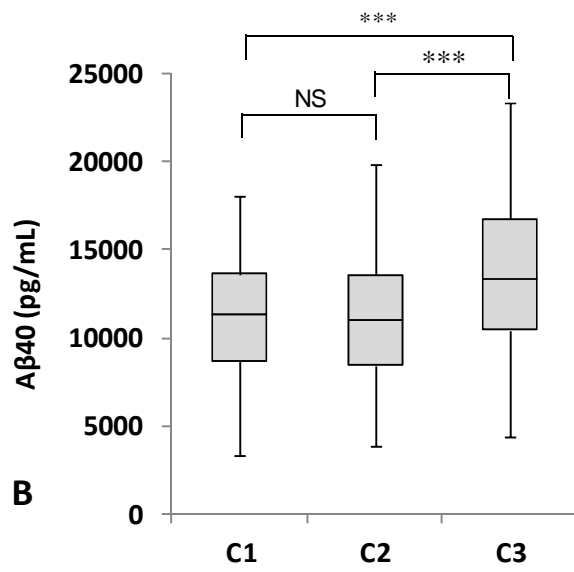
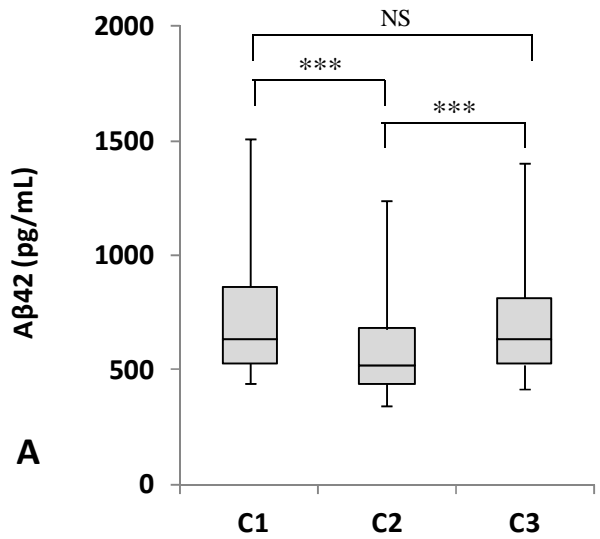




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Figure 2



**C**

Figure 3

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