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CSF A β ₄₂/A β ₄₀ as means to limiting tube- and storage-dependent pre-analytical variability in clinical setting

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Running title: A β ₄₂/A β ₄₀ ratio *in pre-analytical steps*

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Abstract

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

Objective. The objectives of this study were to compare, in clinical setting the impact of collection tubes and the repetition of freeze/thaw cycles on A β ₄₂ and A β ₄₀ concentrations and consequently determine if the A β ₄₂/A β ₄₀ ratio could resolve the diagnosis difficulties related to these pre-analytical parameters.

Methods. CSF from 35 patients was collected in different PP and stored at -80°C after sampling. For CSF collected in the reference tube, three successive freeze-thaw cycles were done. A β ₄₂ and A β ₄₀ concentrations were determined in each condition in order to calculate the A β ₄₂/A β ₄₀ ratio.

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

Conclusion. The use of A β ₄₂/A β ₄₀ ratio rather than A β ₄₂ alone could contribute towards pre-analytical standardization, thus allowing the general use of CSF AD biomarkers in routine practice.

Key words: Alzheimer's disease, A β ₄₀ peptide, A β ₄₂/A β ₄₀ ratio, A β ₄₂ peptide, cerebrospinal fluid, pre-analytical standardization, tube, freeze-thaw cycles

Introduction

Alzheimer's disease (AD) diagnosis criteria were revised in 2011 to assimilate the advances in the understanding of AD (1). Although clinical symptoms still constitute the core of the diagnosis, new criteria now include biomarkers related to the AD pathophysiological processes such as cerebrospinal fluid (CSF) and imaging biomarkers. In the CSF, AD is biologically characterized by a reduction in CSF A β 42 peptide levels which is correlated with the density of neuritic plaques (2) and by an increase in CSF Tau and phosphorylated Tau (P-tau) levels associated with neurodegeneration and neocortical neurofibrillary pathology (3). The combination of these three parameters have shown high levels of sensitivity and specificity as well as diagnostic accuracy in discriminating AD *versus* controls (4).

In 2004, Lewczuk *et al.* (5) was the first to propose A β 42/A β 40 ratio rather than the absolute value of A β 42 peptide to improve the percentage of appropriately diagnosed patients. Since this publication, several studies have largely demonstrated the interest of using A β 42/A β 40 ratios for better interpretative CSF analysis and increase the level of evidence of AD process (6-13). A β 40 peptide is the most abundant A β peptide in the brain and its CSF level might therefore be considered to most closely reflect the total A β load in the brain. CSF A β 40 was characterized by a great inter-individual variability (14) and was long considered as unchanged in AD. Today, findings are less clear-cut. Indeed, Bousiges *et al.* (15) confirmed that CSF A β 40 level was not significantly different in AD than in the control group, whereas it was decreased in patients with body Lewy dementia (BLD). Thus, A β 42/A β 40 allowed better discrimination of AD from BLD than A β 42 alone (15). On the contrary, others studies showed lower CSF A β 40 levels in AD patients than in controls (13, 16). More recently, Dorey *et al.* demonstrated that absolute value of CSF A β 40 levels allowed the correct classification of AD patients with non-pathological A β 42 levels and control patients with pathological A β 42 levels (11).

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

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

Material and methods

Patients

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

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 MCI according to research criteria for the diagnosis of AD published in 2007 (25). Each subject had signed a written informed consent for CSF assessment, analysis and storage and protocol was approved by local ethical committee.

Samples

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

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

Freeze-thaw cycles procedure

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

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

Biochemical analyses

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

Statistical analysis

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

Results

Demographical characteristics

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

Effect of tube type on A β peptide concentrations and A β 42/A β 40 ratio

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

Furthermore, individually, all the correlations between the ratios were significant for T1 vs TR, T1 vs T2 and T2 vs TR ($r_s = 0.957$ $p = 0.0001$; $r_s = 0.913$ $p = 0.0001$; $r_s = 0.899$ $p = 0.0001$ respectively). Bland-Altman plots showed that the number of observations located outside the corresponding interval (differences within mean ± 1.96 SD) is 2 (5.7%) for T1 vs TR; 3 (8.6%) for T1 vs T2 and 2 (5.7%) for T2 vs TR. Overall, it should be noted that 2

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

Effect of free-thaw cycles on A β peptide concentrations and A β 42/A β 40 ratios

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

On the contrary, after the third freeze-thaw cycle, A β 42 levels were similar to those measured after one cycle and were significantly increased comparatively to the levels obtained after 2 cycles (+ 21.6% (11.1 to 40.7), $p < 0.001$). Similarly, A β 40 levels were statistically higher after the third cycle than after both first and second cycles (+ 17.8% (6.9 to 35.5), $p < 0.001$ and + 21.6% (10.8 to 52.4), $p < 0.001$). Consequently A β 42/A β 40 ratio was similar after 2 and 3 freeze-thaw cycles (-6.6% (-9.1 to 11.5), NS). Freeze-thaw cycles-induced variations in A β

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Additional contribution

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

Table 1. CSF A β 42, A β 40 and A β 42/A β 40 median values according to the PP tube type.

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

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

Table 2. Influence of CSF protein concentration on tube-associated variation after one freeze/thaw cycle. Rs: Spearman's rank correlation coefficient. NS: Not significant, n=35.

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

Table 3. Influence of A β levels on tube-associated variation after one freeze/thaw cycle.

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

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

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

Figure legends

Figure 1: Variations of CSF A β 42, A β 40 and A β 42/A β 40 values according to the PP tube type. Box-plots of the dispersion of A β 42 (A), A β 40 (B) and A β 42/A β 40 ratio (C). Values were statistically different if ** $p < 0.01$, *** $p < 0.001$ or not significantly different (NS), $n = 35$.

Figure 2: Individual variations of CSF A β 42, A β 40 and A β 42/A β 40 values according to the PP tube type. Bland-Altman plots showing the number of observations located outside the corresponding interval (differences within mean ± 1.96 SD), $n = 35$.

Figure 3: Variations of CSF A β 42, A β 40 and A β 42/A β 40 values according the number of freeze/thaw cycles. Box-plots present the dispersion of A β 42 (A), A β 40 (B) and A β 42/A β 40 ratio (C). Values were statistically different if *** $p < 0.001$ or not significantly different (NS), $n = 24$.

Figure 1

Figure 2

Figure 3