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Objectives: To assess the potential influence of the ratio between the storage tube surface area and the volume of cerebrospinal fluid (CSF) (surface/volume) on the quantifications of four Alzheimer's disease (AD) biomarkers on the Lumipulse G600II automated platform.
Methods: CSF samples of 10 consecutive patients were stored in 2 ml polypropylene tubes containing four dif-ferent CSF volumes: 1.5 ml, 1 ml, 0.5 ml and 0.25 ml. Concentration of CSF Aβ1-42, Aβ1–40, t-Tau and p-Tau were measured in all aliquots using the LUMIPULSE G600II automated platform from Fujirebio.
Results: Levels of CSF Aβ1-42 and Aβ1-40 were lower in samples stored with lower volumes (higher surface/volume ratios). This decrease was partly compensated by using the ratio Aβ1-42/Aβ1-40. Quantification of t-Tau and p-Tau were not influenced by this pre-analytical condition.
Conclusion: The surface/volume ratio can potentially influence the results of amyloid AD biomarkers. It appears essential to take into account the surface/volume ratio of the storage tubes when quantifying CSF biomarkers in clinical routine.

1. Introduction

Cerebrospinal fluid (CSF) biomarkers, in particular two amyloid peptides (Aβ1-42 and Aβ1-40), and total Tau (t-Tau) and 181-phosho-Tau (p-Tau), play a major role in the diagnosis of Alzheimer disease (AD). Numerous studies have shown a characteristic CSF biomarker pattern in AD that consists of a decrease of Aβ1-42 and the ratio Aβ1-42/Aβ-40 and an increase of t-Tau and p-Tau [1–3]. Experimental data have shown that pre-analytical conditions, such as the nature of the collection tube or the storage conditions among others, strongly influence the biomarker values and eventually can lead to misdiagnosis of AD [4–9]. Investigational studies have also established that the volume of CSF in storage tubes has an impact on the values of Aβ1-42 peptides [10] and that the consideration of the ratio Aβ1-42/Aβ1-40 could minimize this bias [11,12]. Thus, intense efforts have ultimately been developed to standardize pre-analytical treatment of human CSF samples in order to allow a wider clinical implementation of AD biomarkers for diagnosis in clinical practice and clinical trials [6,9,13–17]. The recent implementation of fully-automated platforms for the measurement of AD biomarkers has allowed reducing within-laboratory variability [18,19]. This increase in precision has also allowed a better characterization of the effect of some confounding pre-analytical factors that may not have been previously detected using conventional ELISA techniques. In the present study, we investigated the potential influence that the ratio between the storage tube surface area and the CSF volume (surface/volume) may have on the simultaneous quantification of four AD biomarkers (Aβ1-42, Aβ1-40, t-Tau and p-Tau) in the Lumipulse G600II automated platform.

2. Methods

CSF samples were obtained from 10 consecutive patients that

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Table 1
Patients' characteristics and biomarker measures.

ID	Sex	Clinical Diagnosis	MMSE	Aβ1-42* (pg/ml)	Aβ1-40* (pg/ml)	Aβ1-42/Aβ1-40*	tTau* (pg/ml)	pTau* (pg/ml)
1	Female	AD dementia	14	2665	18,249	0.146	517	68
2	Male	PPA	25	1141	11,601	0.098	660	44
3	Female	aMCI	29	2701	16,181	0.167	285	40
4	Female	aMCI	28	3409	21,164	0.161	629	60
5	Female	aMCI	26	806	8704	0.093	330	60
6	Female	aMCI	27	587	13,771	0.043	402	60
7	Male	naMCI	29	2497	17,149	0.146	408	50
8	Male	aMCI	25	1289	8264	0.156	204	29
9	Female	PSP	25	1590	10,802	0.147	319	31
10	Female	PPA	27	517	10,742	0.048	1011	190

For confidentiality, ages are not shown. Median and interquartile range for age were 71 and 8.5 years, respectively.
MMSE: Mini-Mental Status Examination; AD: Alzheimer's disease; PPA: Primary progressive aphasia; aMCI: amnestic mild cognitive impairment; naMCI: non-amnestic mild cognitive impairment; PSP: Progressive supranuclear palsy syndrome.
* Measures correspond to quantification in aliquots containing 1.5 ml of CSF.

underwent lumbar puncture for the analysis of CSF AD biomarkers at the Sant Pau Memory Unit in July 2018. All participants gave their written consent to participate in our CSF biomarker program. All CSF samples were collected in 10 ml polypropylene tubes (Sarstedt, Ref. 62.610.018), under standardized conditions [20,21]. Immediately after centrifugation at 2000*g* for 10 min at 4 °C, CSF volumes of 1.5 ml, 1 ml, 0.5 ml and 0.25 ml of each sample were aliquoted in 2 ml polypropylene tubes (Sarstedt, Ref. 72.694.007). Collection tubes and storage tubes were not pre-treated before their use. Total processing time (from lumbar puncture to storage) was less than 2 h in all cases. All aliquots were stored at −80 °C until analyses, and total storage time was less than four weeks in all cases.

On the day of the analysis, samples were thawed at room temperature and the tubes were vortexed to avoid freeze-thaw gradients. Aβ1-42, Aβ1-40, t-Tau and p-Tau were quantified in this order directly in the storage tubes using the LUMIPULSE G600II automated platform. All samples were processed using the same batch of kits and reagents, and all aliquots obtained from the same patient were processed in the same run of analysis to avoid run-to-run variability. Key performance characteristics of the assays are summarized in Supplementary Table 1.

Biomarker measures were transformed to percentages relative to their measure in the aliquot containing 1.5 ml (reference sample, 100%). We applied linear models to quantify the effect of the surface/volume ratio (factor) on the relative levels of biomarkers (dependent variable). The absolute levels of biomarkers were introduced as covariates. Level of significance was set at $\alpha = 0.05$ for all analyses.

3. Results

Table 1 shows the characteristics of all study participants and the biomarker results quantified in the reference sample (aliquots containing 1.5 ml of CSF, corresponding to the lowest surface/volume ratio). Levels of biomarker quantifications ranged from 517 to 3490 pg/ml for Aβ1-42, 8264 to 21,164 pg/ml for Aβ1-40, 204 to 1011 pg/ml for t-Tau and 29 to 190 pg/ml for p-Tau.

Fig. 1 illustrates biomarker measures in each polypropylene tube containing 0.25 ml, 0.5 ml, 1 ml and 1.5 ml, respectively. Results are expressed as percentage relative to the reference sample (1.5 ml). Table 2 shows the equivalence between CSF volumes and the surface/volume ratios in the tube that we tested. Our data show that the surface/volume ratio significantly influenced Aβ1-42 quantification (Fig. 1A), with levels decreasing progressively in lower volumes (higher surface/volume ratios); thus the Aβ1-42 levels in 1 ml, 0.5 ml and 0.25 ml aliquots of CSF were 7% (95%CI 2–12%, $p < .05$), 15%

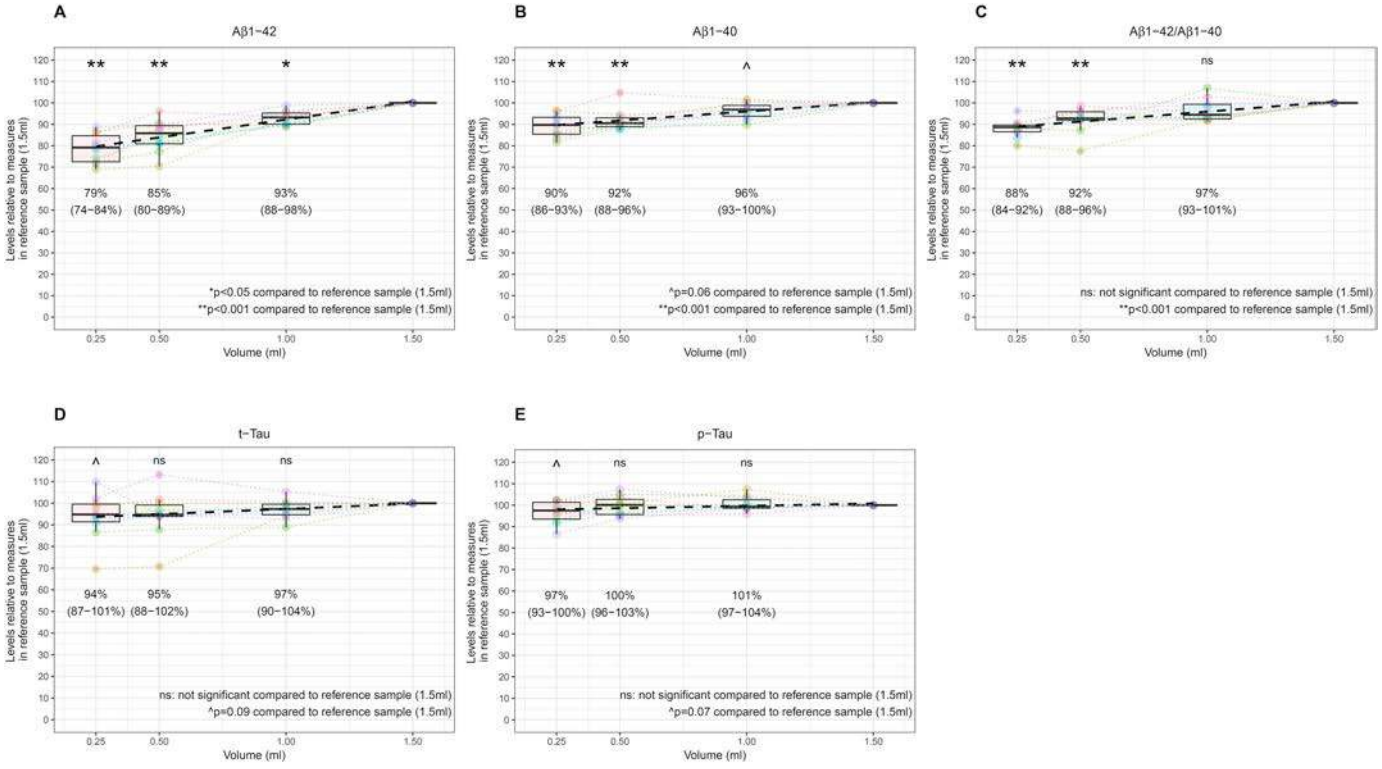

Fig. 1. Relative measures of Aβ1–42 (1A), Aβ1–40 (1B), Aβ1–42/Aβ1–40 (1C), t-Tau (1D) and p-Tau (1E) in tubes containing 0.25 ml, 0.5 ml, 1 ml and 1.5 ml. Values are expressed as mean percentage (and 95% confidence intervals) relative to their measures in the aliquot containing 1.5 ml. Each CSF sample is represented by a different coloured line. Black dashed-lines indicate linear regressions of relative levels for each biomarker over storage CSF volume.

Table 2
Equivalence between CSF volumes and the ratios between the surface area and CSF volume (surface/volume) in the tube tested in this study.

CSF volume (ml)	Total tube internal surface (mm^2)	Total surface/CSF Volume (mm^{-1})	Tube internal surface in contact with CSF (mm^2)	Surface in contact/CSF Volume (mm^{-1})
0.25	1123	4.50	141	0.56
0.5	1123	2.25	260	0.52
1	1123	1.12	498	0.51
1.5	1123	0.75	735	0.49

Note: These calculations are based on the following measures kindly provided by the tube manufacturing company: tube internal diameter = 8.41 mm, cylindrical section height = 40 mm, conical section height = 5 mm.

(95%CI 11–20%, p < .001) and 21% (95%CI 16–26%, p < .001) lower than those in the reference sample (1.5 ml), respectively. A similar pattern, although to a lesser extent, was observed for Aβ1-40 (Fig. 1 B). Levels of Aβ1-40 measured in tubes with 1 ml, 0.5 ml and 0.25 ml of CSF were 4% (95%CI 0–7%, p = .06), 8% (95%CI 4–12%, p < .001) and 10% (95%CI 7–14%, p < .001) lower than those in the reference sample, respectively. The use of the Aβ1-42/Aβ1-40 ratio could partly compensate these discrepancies (Fig. 1 C). The ratios measured in tubes with 0.5 ml and 0.25 ml of CSF were 8% (95%CI 4–12%, p < .001) and 12% (95%CI 8–16%, p < .001) lower than in those in the reference sample, respectively. The ratios measured in tubes with 1 ml were not significantly different than those in the reference sample (95%CI −1–7%, p = .11).

Next, we tested whether a similar effect was detectable when measuring tau proteins. As displayed in Fig. 1D and E, t-Tau and p-Tau were poorly influenced by the surface/volume ratio. The levels of t-Tau and p-Tau measured in tubes with 1 ml, 0.5 ml and 0.25 ml of CSF were not significantly different than those in the reference sample (only a trend was observed in the 0.25 ml sample compared to reference sample).

4. Discussion

Our work illustrates that lower surface/volume ratios are associated with a decrease in the quantification of Aβ1-42 and Aβ1-40 levels on the Lumipulse technology. Although applying the Aβ1-42/Aβ1-40 ratio could partly compensate this decrease, differences between different surface/volume conditions could still be detected. In contrast, the effect of the surface/volume tested was negligible for t-Tau and p-Tau.

Other previous studies have investigated the effect of CSF volume (or surface/volume) on AD biomarkers and reported similar results [10,22,23]. Toombs et al. found that an increase of 10 µl aliquot volume was associated with a significant increase of Aβ1–42 of 0.95 pg/ml (95%CI 0.36–1.50) [10] and 1.1 pg/ml [22]. Vanderstichele et al. found a 13.6% reduction in Aβ1-42 levels in aliquots of 0.5 ml compared to those containing 1.5 ml of CSF [23]. In our study, we estimate a similar reduction of 15% (95%CI 11–20%) for the same volume comparison (0.5 ml vs. 1.5 ml). This means that, for a sample with Aβ1-42 levels of 1000 pg/ml measured in the 1.5 ml aliquot, each 10 µl of decrease in the aliquot volume would be associated to an approximate reduction of 1.5 pg/ml (1.1–2.0 pg/ml) in Aβ1-42 levels. Minor discrepancies between studies could be partly explained by differences in the pre-analytical conditions, in particular the nature of the tubes used to collect and treat the CSF, which is known to affect the adsorption phenomenon of Aβ1-42, and the number of freeze-thaw cycles. Another source of variation might rely on differences in the immunoassays used in each study.

Vanderstichele et al. [23] found that the use of the Aβ1-42/Aβ1-40 ratio compensated the effect of the CSF volume on amyloid biomarkers, while Toombs et al. [22] reported that the ratio partially reduced the effect of the CSF volume although still being significant. Our data indicate that although the Aβ1-42/Aβ1-40 ratio could minimize the effect, it remained still detectable. Other factors, such as long-term stability, have been observed to affect differently CSF levels of Aβ1-42 and Aβ1-40 [24]. It has been described that pre-treating the surface of recipients with detergents may reduce the adsorption phenomenon [23], although we did not test this condition in the present study. It is possible that the hydrophobicity of the Aβ1-42 peptide compared to the Aβ1-40 peptide and the consequent adsorption to the storage tube surface explain their different behavior. Differences in the characteristics of the assays for the two isoforms could also explain part of these discrepancies.

The impact that changes in storage volume might have in the diagnosis of AD has previously been studied [4]. In our study, regarding Aβ1-42 and the Aβ1-42/Aβ1-40 ratio, 1 out of 10 samples would change its status from the normal to the abnormal range according to our local cutoffs due to differences in the storage volume. There would not be a significant status change regarding t-Tau or p-Tau. However, we acknowledge that we included a small number of CSF samples and that clinical diagnoses were not balanced or taken into account in the inclusion criteria, and therefore this proportions need to be taken cautiously. A larger study, specifically designed to address this issue, would provide a more accurate estimation of the clinical impact of changes in storage volume.

Considering the strong influence of pre-analytical conditions on biomarkers evaluation [6,9,13–15,17,25], we ensured the harmonization of all these parameters in our study. Thus, for a same patient's CSF, the storage volume (and therefore the surface/volume ratio) was the only variable that changed through experiments. We used a standard pre-analytical protocol, collected biological samples in a tube previously recommended by other consortia to limit adsorption phenomenon [4,5], and measured samples directly in the storage tubes to minimize the impact of repeated pipetting and transferring samples to different tubes [7]. In addition, the use of the same batch of Lumipulse reagents to quantify each biomarker and the fact that all samples from one patient were measured in the same run ensured a minimal inter-assay variation. It is noteworthy that absolute values of biomarkers in our study are not directly comparable from those obtained with other immunoassays and under different operating procedures. Our study has also some limitations. We only tested one type of tube, and we did not test other pre-analytical factors that could potentially compensate the decrease of amyloid levels in higher surface/volume conditions, such as the addition of detergent or the quantification in fresh (non-frozen) samples. Additionally, we did not test the potential inter-batch effect of storage tubes on amyloid levels.

Given the important role of CSF amyloid peptides for the biological diagnosis of AD, our results emphasize the necessity to take into account the CSF storage volume and surface/volume ratio when measuring AD biomarkers and when comparing results across studies. They also highlight the relevance of keeping the same surface/volume conditions in each laboratory to be able to use specific internal cutoffs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.12.021.

Acknowledgements

We would like to thank all the participants of this study and all the members of the clinical teams involved in their assessment.

This work was supported by CIBERNED and Instituto de Salud Carlos III (PI14/01561), jointly funded by Fondo Europeo de Desarrollo Regional (FEDER), Unión Europea, “Una manera de hacer Europa”. This work was also supported in part by Generalitat de Catalunya: 2014SGR-0235 and SLT006/17/125.

Fujirebio Europe NV provided kits and reagents to perform the study.

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