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Identification of an important potential confound in CSF AD studies: aliquot volume

Abstract

Background: Cerebrospinal fluid (CSF) amyloid beta 1-42 (Aβ1-42), total tau (T-tau) and phosphorylated tau181 (P-tau) are finding increasing utility as biomarkers of Alzheimer's disease (AD). The purpose of this study was to determine whether measured CSF biomarker concentrations were affected by aliquot storage volume and whether addition of detergent-containing buffer mitigates any observed effects.

Methods: AD and control CSF was distributed into polypropylene tubes in aliquots of different volumes (50–1500 µL). Aβ1-42, T-tau and P-tau were measured with and without addition of Tween 20 (0.05%).

Results: Measured concentrations of Aβ1-42 increased two-fold with aliquot storage volume. A volume increase of 10 µL caused an Aβ1-42 increase of 0.95 pg/mL [95% confidence interval (CI) 0.36–1.50, p=0.02] in controls, and 0.60 pg/mL (CI 0.23–0.98 pg/mL, p=0.003) in AD samples. Following addition of Tween 20, the positive relationship between Aβ1-42 and aliquot volume disappeared. T-tau and P-tau were not significantly affected.

Conclusions: CSF aliquot storage volume has a significant impact on the measured concentration of Aβ1-42. The introduction of a buffer detergent at the initial aliquoting stage may be an effective solution to this problem.

Keywords: Alzheimer's disease; amyloid β; cerebrospinal fluid; tau; volume.

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Introduction

Cerebrospinal fluid (CSF) amyloid beta 1-42 (Aβ1-42), total tau (T-tau) and phosphorylated tau181 (P-tau) can be used to help diagnose Alzheimer's disease (AD) pathology in individuals with cognitive impairment [1]. These biomarkers are widely measured for clinical, research and drug development purposes. However there is considerable variability in collection procedures and laboratory handling methods, leading to inconsistency in the apparent concentrations of these analytes between individuals and between different laboratories [2].

Known confounding factors in the measurement of Aβ1-42 and T-tau concentrations include delays in sample analysis [3], diurnal variation [4], CSF contamination with blood or breakdown of the blood brain barrier [5, 6] and choice of storage tube material [7]. Of the various analytes, Aβ1-42 seems to be most influenced by such external factors, due at least in part to the hydrophobic nature of Aβ and its propensity to be adsorbed to the walls of collection containers, as well as to aggregate with itself and other proteins [5]. The use of polypropylene (PP) tubes mitigates the problem of adsorption to a large extent, but does not guarantee satisfactory pre-analytic behaviour as most PP tubes are made of copolymers to which Aβ adsorbs to different degrees depending on the tube manufacturer [7].

We hypothesised that apparent Aβ1-42, T-tau and P-tau concentrations could also be altered by varying the aliquot volume in which they are stored, and conjectured that increasing the ratio of CSF volume to surface area of PP storage container would result in decreased analyte adsorption. Furthermore, we explored whether we could mitigate any such effects by pre-treating with

a buffer-containing detergent that would lead to reduced tube surface adsorption and more complete measurement of analyte concentrations.

Materials and methods

Sample pools

CSF aliquots of different volumes, collected and stored in PP tubes at −80°C according to standardised operating procedures [1], were tested for Aβ1-42, T-tau, and P-tau. Experiments were conducted in five rounds. In each round, two pools of CSF were used: the first, from a cohort of de-identified subjects with CSF biomarker profiles consistent with AD. These were identified according to cut-offs that, taken together, are 90% sensitive and specific for AD [8]: Aβ1-42 <530 ng/L, T-tau >350 ng/L, P-tau >60 ng/L. An exception had to be made for the AD pool used in Rounds 4 and 5 due to insufficient quantities accessible. This pool was composed of CSF that met the criteria for Aβ1-42 and T-tau, but not P-tau. The second pool was composed of de-identified non-AD control (CTRL) CSF (all three biomarker concentrations within normal range). Pooled CSF was used due to the large CSF volume requirement of the study design. Different CSF pools were used over the rounds: Round 1 was unique and contained only neat CSF, Rounds 2 and 3 were identical except for the addition of a detergent buffer [Tween 20 (0.05%)] to the CSF of Round 3. Rounds 4 and 5 used identical pools, and included AD and control, neat and Tween, individual and pooled CSF types all in the same plate in order to verify comparability of results.

To confirm that pooled CSF would behave in the same way as that of individual patients, a series of aliquots of differing volumes was created from the CSF of single subjects (IND) and tested alongside the pooled samples.

Sample treatment

Pools were created from individual samples of CSF taken by lumbar puncture, which were poured into a PP 100 mL beaker. The CSF was mixed thoroughly for 30 min with a magnetic stirrer and then poured into a PP 50 mL falcon tube. This tube was centrifuged at 3000 *g* for 10 min. CSF from the centrifuge tube was then split into two equal halves, again using 50 mL falcons, and 0.05% tween was added to one. Samples were aliquoted into a range of volumes (50 µL, 75 µL, 100 µL, 125 µL, 250 µL, 500 µL, 1000 µL, 1500 µL) and frozen at −80°C. This process took 1.5 h. Every sample in this study was used on its second thaw, except the Round 2 individual subject pool which was used on its first thaw. Round 1 samples were incubated at −80°C for 2 months before testing, whilst Rounds 2–5 were incubated at −80°C for a week. All samples were stored in Sarstedt (Nümbrecht, Germany), 2 mL PP, skirted, screw top tubes (cat. 72.694.406).

Aliquots used in the Aβ1-42 and T-tau plates were thawed together at room temperature for approximately 1 h, and dispensed into disposable PP plates for dilution (1:8 and 1:2, respectively). P-tau aliquots were thawed by the same process, but not diluted. All solutions were mixed by continuous vortexing for 5 s.

Schedule and CSF analysis

Each round of experiments was conducted separately, and run over 2 days, with Aβ1-42 and T-tau plates tested in parallel on the first day. P-tau was commenced on the first day, incubated overnight, and concluded on the second. Aβ1-42 and T-tau were assayed on a Meso Scale Discovery 6000 platform, using MSD Human Aβ1-42 and MSD Human Total Tau kits, respectively (Meso Scale Discovery, Gaithersburg, MD, USA). P-tau was run with and INNOTEST® PHOSPHO-TAU(181P) ELISA kit (Innogenetics, Ghent, Belgium), and measured using a BMG Labtech FLUOstar Omega multi-mode microplate reader. In Rounds 1, 2 and 3, samples from the AD and CTRL pools were run in triplicate on each plate, whilst those from Rounds 4 and 5 were run in duplicate due to volume restrictions. The manufacturer's protocol was followed exactly for each assay, with the exception of vortex time in the P-tau assay where 5 s was preferred to 10 in the interest of plate set-up speed and consistency across all assays.

Statistical analysis

Linear regression was used to examine the relationship between analyte concentration values and sample volume. The median of the measured analyte concentration values was the dependent variable and sample volume was the independent variable of interest. Pooled samples were analysed separately and, to increase power, together over multiple rounds (Supplementary data, Table 1, which accompanies the article at http://www.degruyter.com/view/j/cclm.2013.51.issue-12/issue-files/cclm.2013.51.issue-12.xml), incorporating Round as a covariate. Individual samples were used as a point of comparison, but not analysed statistically as each was only run in a single round. All analyses were conducted in Stata Version 12.1. Graphs were created using SPSS version 21.

Surface area calculation

To calculate the relative surface area (SA) per volume in the storage tube used, measurements provided by the manufacturers (Sarstedt, Nümbrecht, Germany) were used to calculate the internal surface areas at each volume. This was done by adding the lateral SA of a hollow cylinder to that of a cone. All calculations were made using the relevant calculators on www.aqua-calc.com [9].

Results

Aβ1-42

For both control and AD CSF, combining Rounds 1, 2 and 4 an approximately two-fold increase in measured neat Aβ1-42 concentration was observed between the largest and smallest aliquot volumes (Figure 1A). In regression analysis, for controls, an increase of 10 µL aliquot volume was associated with a significant increase in Aβ1-42

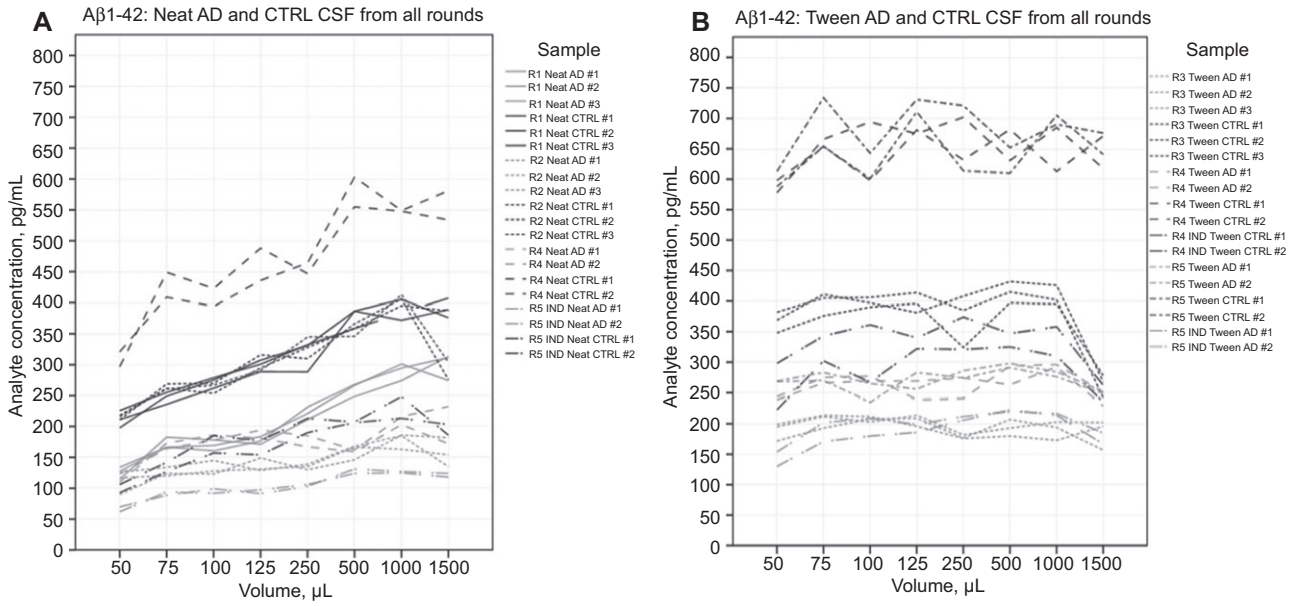

Figure 1 Shown are the results of Aβ1-42 over five rounds.
(A) Results of neat pooled and individual CSF Aβ1-42, Rounds 1–5, AD and control groups. Sample R1 neat CTRL #2 failed to react at 1000 µL (signal below blank) and has been excluded. (B) Results of 0.05% Tween-treated pooled and individual CSF Aβ1-42, Rounds 1–5, AD and control groups.

concentration (0.95 pg/mL [95% confidence interval (CI) 0.36–1.50, p=0.02]. A similar, stronger effect was seen in AD CSF: 0.60 pg/mL (CI 0.23–0.98 pg/mL, p=0.003). In Rounds 3, 4 and 5 (Figure 1B), 0.05% Tween 20 was added to all aliquots, following which there was no longer a significant relationship between aliquot volume and measured Aβ1-42 concentration in either the control CSF (Supplementary data, Table 1).

T-tau

In Rounds 1, 2 and 4 (Figure 2A) there was no association between measured T-tau concentration and aliquot volume for the control pools. An increase of 10 µL aliquot volume was associated with a non-significant increase in control T-tau concentration of 0.17 pg/mL (CI −0.53–0.87 pg/mL, p=0.06). For pooled AD samples an increase of 10 µL aliquot volume was associated with a non-significant increase in measured concentration of 0.05 pg/mL (CI −1.78–1.88 pg/mL, p=0.96). For the individual samples (Figure 2D) there was no significant association between volume and measured concentration in the individuals tested. The addition of Tween 20 (Figure 2B) made no difference to the relationship between T-tau and volume.

P-tau

The results for P-tau can be seen in Figure 3. In Rounds 1, 2 and 4 (Figure 3A) there was no significant association between neat sample P-tau concentration and aliquot volume in AD CSF (0.03 pg/mL (CI −0.01–0.06 pg/mL, p=0.10). In pooled control CSF samples (0.03 pg/mL (CI 0.00–0.05 pg/mL, p=0.03), the relationship was significant when all three rounds were combined (Supplementary Data, Table 1). With the addition of Tween 20 (Figure 3B) the association between P-tau and volume disappeared.

Tube surface area

Figure 4A shows the estimated surface area of each aliquot relative to volume, and compares this to the average results of Aβ1-42 at each volume. The average values were calculated from the combined Aβ1-42 data of relevant (i.e., neat or Tween) individual and pooled CSF from every round of experimentation. Lower volumes are associated with higher relative surface area and lower measured concentrations of Aβ1-42. This is further demonstrated by a strong inverse correlation (R^2=0.912) between concentration of neat Aβ1-42 and relative surface area (Figure 4B). No correlation (R^2=0.024) was found in Tween Aβ1-42 samples (Figure 4C).

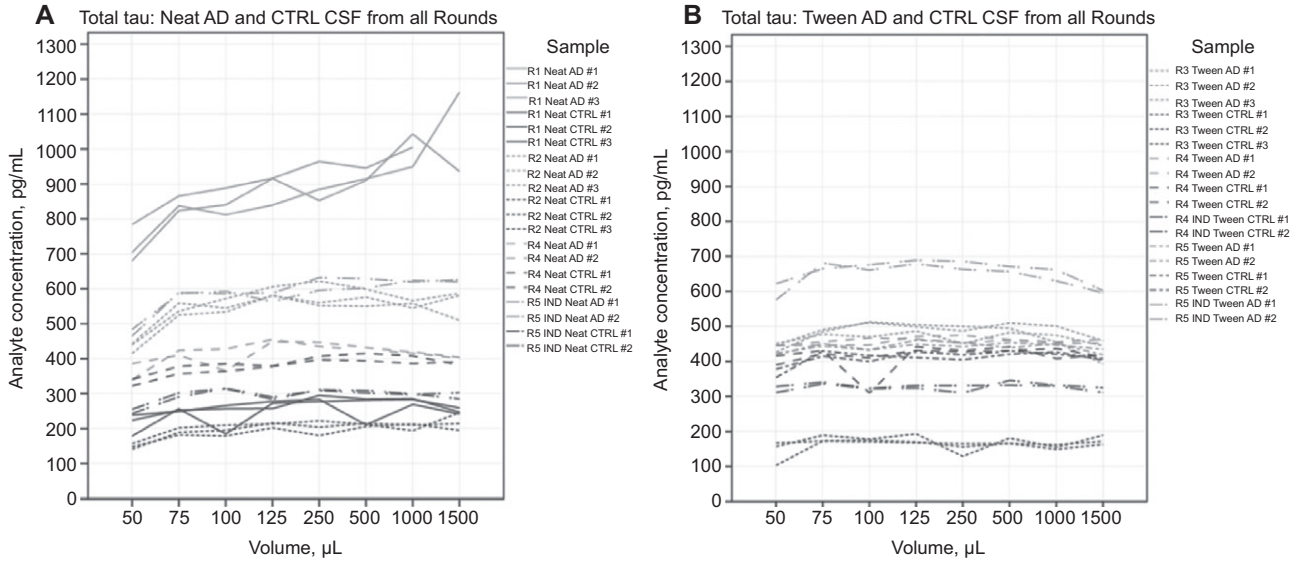

Figure 2 Shown are the results of T-tau over five rounds.
(A) Results of neat pooled and individual CSF Total tau, Rounds 1–5, AD and control groups. Samples R1 neat CTRL #2 and R4 neat AD #1 failed to react at 1500 µL and 500 µL, respectively (signal below blank). These data points have been excluded. (B) Results of Tween-treated pooled and individual CSF Total tau, Rounds 1–5, AD and control groups.

Discussion

This study demonstrates that the storage volume of CSF samples has significant impact on the measured concentration of Aβ1-42. The introduction of a buffer detergent to CSF samples at the initial aliquoting stage may be an effective solution to this problem. T-tau and P-tau concentrations generally appeared stable. These findings are consistent with the hypothesis that the hydrophobic properties of Aβ1-42 lead to adsorption of this protein to the walls of the storage vessel. In this section, we consider the impact of surface area, and other factors that may have influenced results.

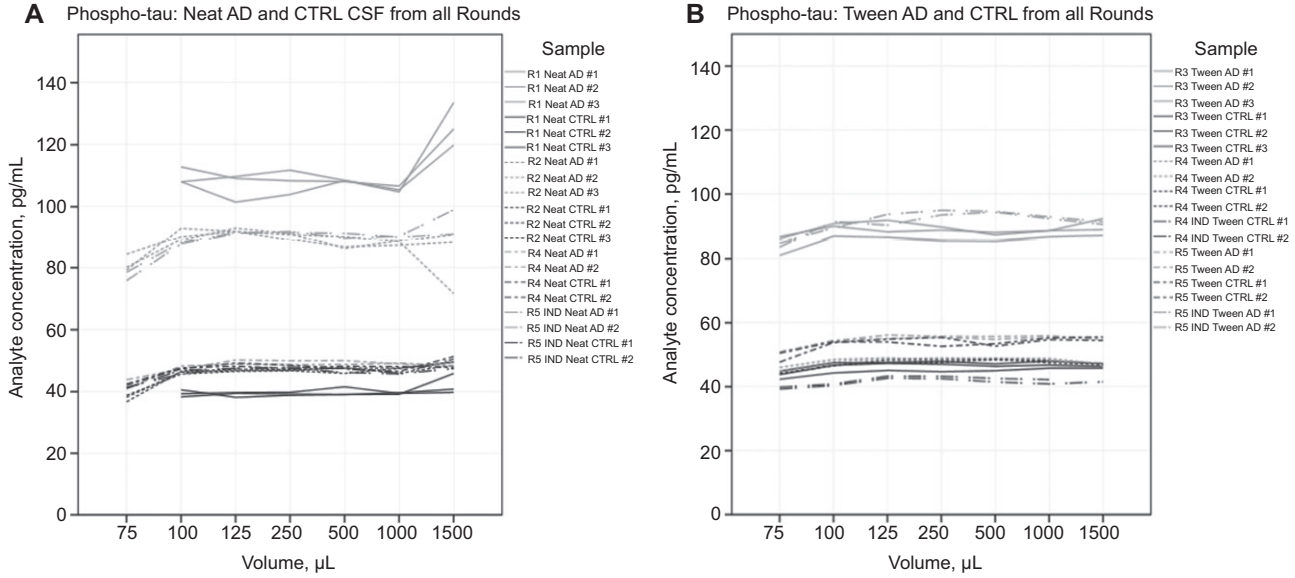

Figure 3 Shown are the results of P-tau over five rounds.
(A) Results of neat pooled and individual CSF Phospho-tau, Rounds 1–5, AD and control groups. (B): Results of Tween-treated pooled and individual CSF Phospho-tau, Rounds 1–5, AD and control groups.

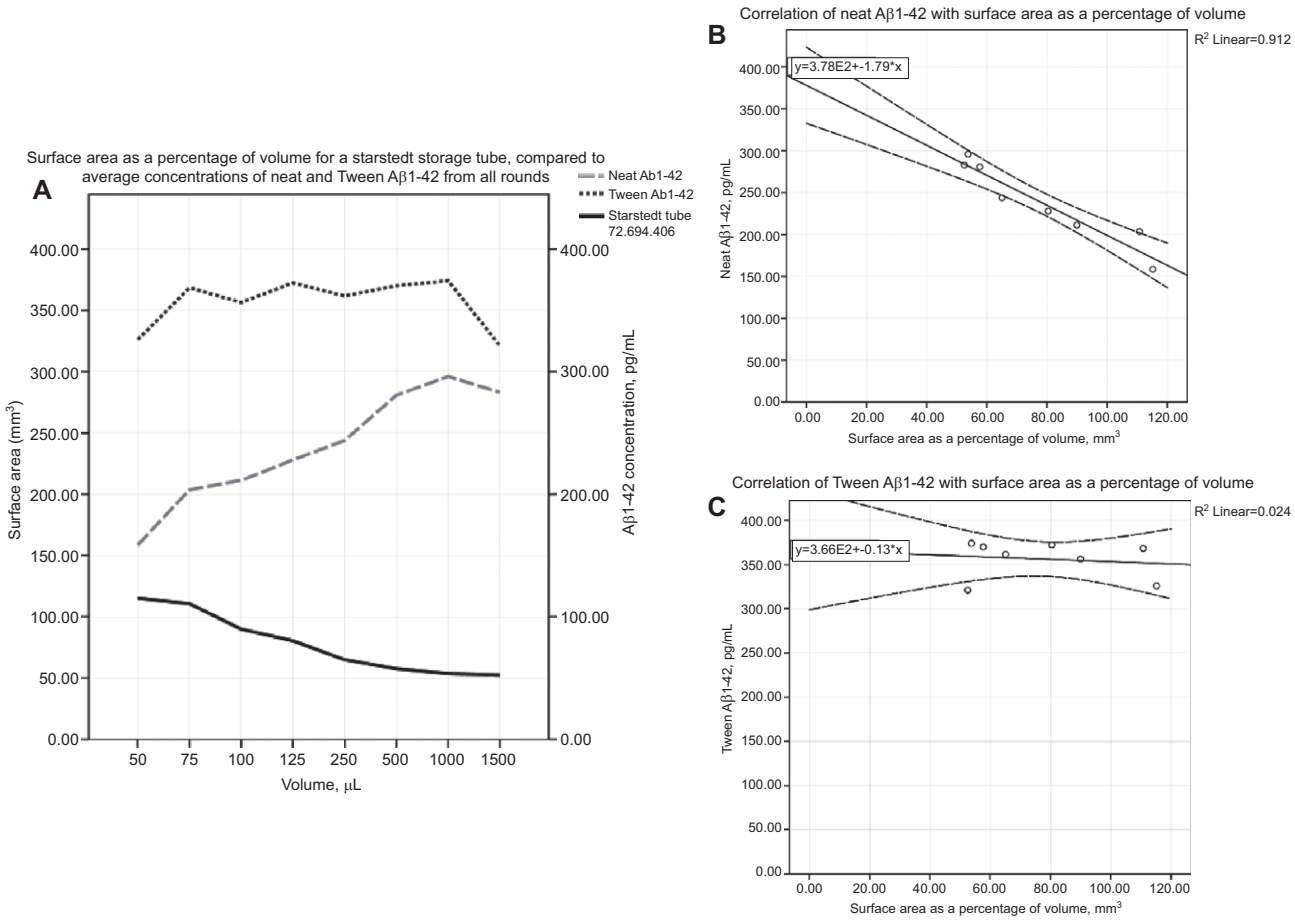

Figure 4 (A) Shown on the left y-axis are the calculated percentage values for surface area, relative to volume, for CSF in a 2 mL Sartstedt tube (72.694.406). Shown on the right y-axis are the average values, at each volume, for neat and Tween Aβ1-42 combining individual and pooled CSF data from all rounds. (B) Correlation analysis of average values for neat Aβ1-42 (combining individual and pooled CSF from all rounds) against surface area as a percentage of volume. Results show a strong trend for Aβ1-42 concentration to decline with increased surface area. Full line=best fit. Dashed line=95% confidence interval. (C) Correlation analysis of average values for neat Aβ1-42 (combining individual and pooled CSF from all rounds) against surface area as a percentage of volume. Results show no correlation between Aβ1-42 concentration and surface area. Full line=best fit. Dashed line=95% confidence interval.

Surface area

There was an inverse relationship between the measured concentration of Aβ1-42 and the surface area per volume ratio of storage tube tested (Figure 4). This suggests that surface adsorption is a key factor in the observed tendency for Aβ1-42 concentration to increase with storage volume.

In the pooled AD CSF of Round 1 there was an association between volume and measured T-tau concentration, but this could not be reproduced in subsequent assays. The control and individual pools for T-tau consistently demonstrated no significant effect between volume and concentration, and this was also the case for all P-tau pools taken individually. However, when analysed in combination the neat control P-tau pool did show significant concentration increase with volume, and therefore may also be affected by surface area dependent adsorption. It must be noted that the relative increase is of much smaller magnitude than observed in Aβ1-42 (expressed by the coefficient in Supplementary Data, Table 1) and is clinically irrelevant.

The frequent deviation in the concentrations of all three biomarkers which were stored in a 1500 µL volume is inexplicable and disturbing. It is difficult to rationalise this as an effect of volume in regard to surface area, especially as Tween 20 samples were also affected. Instead, it may be that when volume approximates a tube's maximum capacity, the remaining space is insufficient to allow thorough mixing by the method of vortexing used. Given the magnitude of deviation across multiple samples, this tendency deserves to be explored further.

Figure 4 shows that, although neat Aβ1-42 concentration and surface area are strongly correlated, they do not perfectly correspond. Additionally, Figures 1–3 show that the magnitude of concentration increase (or stability in the case of the tau isoforms) was not always the same. Therefore it seems that whilst surface area appears to play a key role in the effect, other factors may be involved.

Tween 20

The effect of Tween 20 provides further evidence for the hypothesis that protein is lost due to surface adsorption or aggregation. Tween 20 Aβ1-42 samples had considerably higher concentrations of Aβ1-42 than the neat samples. This suggests that a greater proportion of Aβ1-42 molecules were free in the solution of the storage tube, and is consistent with the findings of other studies [5, 10, 11]. If Tween 20 were to be added routinely to samples then Aβ1-42 cut points for clinical practice and clinical trials would require to be adjusted accordingly. Tween 20 tau sample results very closely reflected the results of neat samples, suggesting negligible quantities of tau are lost to surface adsorption, and that Tween 20 does not appreciably alter detection. This could suggest that the significant P-tau result may not be attributable to surface area derived adsorption.

Temperature

An alternative explanation of, or contributor to, the observed association between volume and concentration is the effect of differing thaw times. It is known that the number of freeze/thaw cycles have an effect on the protein concentration of a sample [3]. Every sample in this study was used on its second thaw or less. All aliquots were thawed together at room temperature for approximately 1 h, but large volume samples thaw more slowly (–80–approx. 21°C took approximately 60 min for a 1500 µL aliquot) and so spent less time at room temperature than the low volume samples. It may be that the additional time spent at room temperature for lower volume samples allowed more proteins (particularly Aβ1-42) to denature, become proteolysed, aggregate, or adhere. Kaiser et al. have shown that measured Aβ1-42 in CSF becomes elevated over time at room temperature. In their experiment, average Aβ1-42 concentration of samples frozen immediately was 790.8±329.2 pg/nL (CV=41.6%) compared to 848.5±287.1 pg/nL (CV=33.8%) in samples frozen after 24 h [12]. Tau species were not found to be significantly affected (T-tau=304.3±178.0 pg/nL versus 308.0±169.2 pg/nL; P-tau=59.4±29.9 pg/nL) [13]. However, even in Aβ1-42, difference was not significant after a period of 2 h (only becoming so after 24 h), and so unlikely to have affected our volume experiment. Additionally, the increase in concentration over time in Kaiser et al.'s study has been conjectured to be the result of cell lysis, as cells were not removed by centrifugation [5]. Bjerke et al. also reported no difference between thawing samples at room temperature, +4°C, and +20°C in a water bath [5]. However, to our knowledge no study has examined the impact of thawing different volumes, and it remains possible that this is a factor.

Further work

Greater adsorption of Aβ1-42 to vessel wall in proportion with volume to surface ratio is both credible and compatible with the results observed. The similar trend observed in the T-tau AD pool of Round 1, but not in other rounds, is not easily explained: this might suggest that T-tau is susceptible to differences in storage volume in certain circumstances (i.e., in pooling), but could also simply be a chance finding. Reflecting on the volume effect identified when P-tau rounds were combined, it is worth considering that the T-tau assay detects six isoforms of the protein, and it may be that some are more vulnerable than others to the factors previously discussed. Further studies are required to examine the extent of this phenomenon.

Conclusions

Aβ1-42, T-tau and P-tau are now widely used as diagnostic markers for Alzheimer's pathology. It is, therefore, concerning that a two-fold difference can exist between the measured Aβ1-42 concentrations of the 50 µL and 1500 µL volumes in both AD and control CSF. This could easily result in misclassification of individuals in both clinical and research settings, and is a source of variance that, to our knowledge, has not previously been considered or investigated. As we move towards 'analytical harmonisation' of CSF between centres [13], we propose that aliquot volume should also be standardised. Furthermore, as the addition of a readily available buffer detergent appears to neutralise the effect of Aβ1-42 adsorption (and potentially that of other biomarkers), the addition of Tween 20 to aliquots immediately before

sample storage should also be explored as a practical solution to the problem.

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Conflict of interest statement

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