CSF Biomarkers

Amyloid β peptides are differentially vulnerable to preanalytical surface exposure, an effect incompletely mitigated by the use of ratios

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Abstract

Introduction: We tested the hypothesis that the amyloid β (Aβ) peptide ratios are more stable than Aβ42 alone when biofluids are exposed to two preanalytical conditions known to modify measurable Aβ concentration.

Methods: Human cerebrospinal fluid (CSF) and culture media (CM) from human cortical neurons were exposed to a series of volumes and polypropylene surfaces. Aβ42, Aβ40, and Aβ38 peptide concentrations were measured using a multiplexed electrochemiluminescence immunoassay. Data were analyzed using mixed models in R.

Results: Decrease of measurable Aβ peptide concentrations was exaggerated in longer peptides, affecting the Aβ42:Aβ40 and Aβ42:Aβ38 ratios. However, the effect size of surface treatment was reduced in Aβ peptide ratios versus Aβ42 alone. For Aβ42:Aβ40, the effect was reduced by approximately 50% (volume) and 75% (transfer) as compared to Aβ42 alone.

Discussion: Use of Aβ ratios, in conjunction with concentrations, may mitigate confounding factors and assist the clinical diagnostic process for Alzheimer’s disease.

Keywords: Alzheimer’s disease; Amyloid β ratio; Preanalytical factors; Cerebrospinal fluid; Cell culture media; Surface adsorption

1. Background

The cerebrospinal fluid (CSF) concentrations of the 42 amino acid form of amyloid β (Aβ42), total tau protein (T-tau), and phosphorylated tau (P-tau) are core biomarkers of Alzheimer’s disease (AD) [1] and are incorporated in the clinical diagnostic process [2]. Largely constituting the neuropathological hallmarks of AD [3], these proteins are also integral to the validation and study of AD models in a research context.

While tau is soluble and concentrations remain relatively stable over a range of conditions [4–6], Aβ42 is well known to be highly labile and prone to aggregate, a property that underpins a range of dynamic structures and their contribution to the disease process [7]. These properties

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make Aβ42 concentrations susceptible to variation in the pre-analytical process. Factors potentially include CSF collection technique [8], diurnal collection time [9], interval between collection and freezing [10,11], temperature [12], pH [13], sample matrix composition [14,15], sample exposure to storage surfaces [6,16–22], and assay measurement variation [23–27]. Several articles have presented studies providing data from assessments of multiple factors [5,28–32].

Further to preanalytical factors, concentrations of CSF Aβ also vary between individuals [33]; thus, individuals with constitutively high or low quantities of Aβ42 relative to chosen diagnostic “cut points” may be vulnerable to misinterpretation of test results. Recent meta-analysis shows that when comparing AD versus nondemented controls and non-AD dementias, CSF Aβ42 has a pooled sensitivity of 0.80 (95% confidence interval = 0.78–0.82) and a pooled specificity of 0.76 (95% confidence interval = 0.74–0.78) [34]. Increasingly, reports suggest improved diagnostic and specificity of 0.76 (95% confidence interval 0.80–0.82) and a pooled 

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The aim of the present study was to extend previous work on the effect of surface exposure on Aβ peptide concentration by assessing the impact on ratios of Aβ42:Aβ40, Aβ32:Aβ38, and Aβ40:Aβ38. Aβ40:Aβ38 is not immediately relevant to the clinic, but better understanding of the relationship between production and interaction of different Aβ fragments may prove useful to future understanding of AD pathology. In addition to CSF, we also examined cell culture media (CM) from human glutamatergic cortical neurons derived from induced pluripotent stem cells (iPSCs). Neurons differentiated from the iPSCs of AD and non-AD donors can act as disease-relevant models with a fully human genetic background. Aβ secreted from these cells into the CM represents a key biomarker for AD-relevant neurobiology. In this context, ratios of Aβ are increasingly used to understand nuances of amyloidogenic pathways [40], and it is important to expand knowledge of preanalytical factors affecting Aβ measurement in this medium.

2. Methods

2.1. Preparation of CSF

This study used de-identified CSF from patients of unknown disease status. Ethical approval was received from the regional ethics board at the University of Gothenburg for the CSF pools used in this study. Samples were collected by lumbar puncture. All lumbar punctures were conducted before 13:00, between L3/L4 and L4/L5 in a sitting or side-lying position. Ten milliliters of CSF was collected at ambient room temperature into a 10-mL PP tube (Sarstedt, Nünbrecht, Germany; cat. 62.9924.284). In the case of visible blood contamination, the CSF was discarded and tap continued in a new tube once bleeding had stopped. Samples selected for inclusion had no erythrocyte contamination visible to the eye. Samples were centrifuged at 2200 relative centrifugal force for 10 minutes at 20°C, transferred to another 10-mL tube (Sarstedt; cat. 62.9924.284), and stored at −80°C. CSF samples were then thawed at 21°C for 1 hour to pool CSF to sufficient volume for experiment in Sarstedt 2-mL PP tubes (cat. 72.694.406), refrozen at −80°C, and thawed (21°C for 1 hour) for assay. CSF was transported on dry ice by international courier and received, frozen, within 24 hours of sending, and immediately stored at −80°C on reception.

2.2. Preparation of cell CM

The N2B27 cell CM used in this study was composed of a 1:1 solution of DMEM/F12 + GlutaMax-1 (1 ×) (Life Technologies; cat. 10565017) and Neurobasal Medium (1 ×) (Life Technologies; cat. 12348017) with the following supplements: 1 × N2 supplement (Life Technologies; cat. 17502-048), 1 × B27 supplement (Life Technologies; cat. 17504-044), 100-μM MEM nonessential amino acids (Life Technologies; cat. 11140-050), 100-μM 2-mercaptoethanol (Life Technologies; cat. 31350-010), 50-U/mL penicillin and 50-μg/mL streptomycin (Life Technologies; cat. 15070063), 5-μg/mL insulin (Sigma-Aldrich; cat. I9278), and 1-nM glutamine (Life Technologies; cat. no. 25030-024). Fresh N2B27 was made every 7 days and stored at 4°C.

2.3. Neuronal culture

Glutamatergic cortical neurons were generated from human iPSCs following a protocol previously described [41–43]. Briefly, iPSCs (cultured on Geltrix and fed with Essential 8) were induced toward a neuronal fate by treatment with N2B27 media supplemented with SMAD (a family of human protein homologues of the drosophila “mothers against decapentaplegic” [Mad]) and the proteins encoded by the C. elegens gene “small body size” [Sma]) inhibitors, SB431542 (10 μM) and Dorsomorphin (1 μM), for 12 days. Neuronal precursor colonies were expanded on laminin-coated plates and maintained in N2B27 media until cultures of mature cortical neurons were generated, at least 80 days after induction. Five different cell lines were used in this study: CTRL and ND were fibroblast-derived iPSC lines from nondegenerative controls, generated using retroviral transduction (obtained from the laboratory of Dr Tilo Kunath). SHEF6 was a human embryonic stem cell line obtained from the UK Stem Cell Bank. APP (derived
from an amyloid precursor protein V717I mutation patient) and PSEN (derived from a presenilin-1 T113-114ins intron 4 deletion mutation patient) were fibroblast-derived iPSC lines generated using retroviral transduction, obtained from Stem-BANCC. Cell CM were collected after 4-day incubation in VWR 15-mL PP tubes (cat: 21008-216), pooled and centrifuged at 2000 relative centrifugal force for 5 minutes at 21°C. Supernatant was aliquoted in Sarstedt 2-mL PP tubes (cat. 72.694.406), stored at −80°C, and thawed at 21°C immediately before assay.

2.4. Volume experiments

To investigate the effect of storage volume on the ratios of Aβ peptides, each CSF (n = 8) and CM (n = 6) sample was thawed and aliquoted into Sarstedt 2-mL PP tubes (cat. 72.694.406) in a volume series: 1000, 500, 250, 125, and 100 µL. Aliquots were refrozen at −80°C and later thawed at 21°C for 1 hour and assayed for Aβ38-40-42 using a Meso Scale Discovery V-PLEX Aβ peptide kit (6E10). Assays were performed on a Meso Scale Discovery SECTOR 6000, according to manufacturer protocol, which is freely available.

To examine the contribution of the pipette to any effect, a separate group of samples (CSF n = 2 and CM n = 2) were aliquoted into five different volumes (100, 250, 500, and 1000 µL) and, immediately before sample dilution during assay, each volume for each sample was mixed with a varying number of pumps (0, 5, 10, and 20) with a pipette tip (TipOne; Starlab, Milton Keynes; cat. S1113-1700). Tips used for samples given the 0 pump treatment were therefore not prewetted.

All samples were added to the assay plate in duplicate by multichannel in randomized, double-blind order. All samples and reagents of volume <5 mL were mixed by vortex (Vortex-Genie 2; Scientific Industries) at speed setting 10 for 5 seconds, and all samples and reagents of volume >5 mL were mixed on a roller for 5 minutes. Pipette tips (TipOne; Starlab, Milton Keynes; cat. S1112-1720, cat. S1113-1700, and cat. S1110-3700) were prewetted with three pumps when aspirating all solutions unless otherwise stated. The same pipette tip was used to create the volume series of each sample.

2.5. Serial transfer experiments

Each CSF (n = 9) and CM (n = 5) sample was thawed, aliquoted into Sarstedt 2-mL PP tubes at a volume of 1000 µL, refrozen at −80°C, and later thawed for assay. The sample was mixed by vortex and transferred from the storage aliquot (tube 0) to seven consecutive Sarstedt 2-mL PP tubes (tubes 1–7), leaving 100 µL of sample in each tube. This process took approximately 10 minutes to complete. Samples were then assayed immediately for Aβ38-40-42 as described for the volume experiment. All samples were added to the assay plate in duplicate by multichannel in randomized, double-blind order. Mixing practice and pipette tips used were as described in section 2.4. The same pipette tip was used to create the transfer series of each sample. Data from two CSF samples previously reported (S12 and S13 in this study, previously AD and CT [36]) were included in the analyzed data set. These samples were prepared according to the same protocol just described and were included to bolster the power of the data set.

2.6. Statistical analysis

The relationship between analyte measurement and sample treatment (volume or transfer step) was assessed by mixed model analysis. Data normality was assessed by histogram, qq-plot, and Shapiro-Wilk test, and linearity was assessed by a scatterplot of the residual variance. All analyses set α at 0.05 and confidence intervals at 95%. The formula used for the mixed model was ln(sample concentration ~ treatment + X, random = ~1|sample) + ε, where the dependent variable is “sample concentration”—the average of duplicate concentration (or ratio) values of a given Aβ peptide in pg/mL (numeric data), the fixed effect variable is “treatment”—the volume or transfer series as relevant (numeric data), X represents other fixed effects (such as disease status, cell type, assay plate, and sample pooling status) where these variables were relevant, and the random effect variable is “sample”—variation unaccounted for differences samples (factor data). “~1|sample” specifies an independent intercept for each sample. ε represents residual variation not accounted for by the stated parameters of the model.

While data from the volume study met the mixed model’s assumption of linearity, the data from the transfer study did not. To meet this requirement, a separate analysis was conducted wherein average concentration was transformed by the natural logarithm (ln) and used as the dependent variable. To calculate the proportional change per treatment unit, “ε” was exponentiated to the power of the model’s output coefficient. Graphs were composed using the ggplot2 package in R. Intra- and inter-assay percentage coefficients of variance were calculated according to ISO 5725-2 standards [44].

3. Results

3.1. Assay variation

Intra- and inter-assay variations were calculated from the concentrations of an internal control CSF sample included in assay plates in both volume and transfer studies. Levels of variation (intra- and inter-assay percentage coefficients of variance, respectively) for Aβ32 (4.3% and 9.9%), Aβ40 (4.5% and 9.5%), and Aβ38 (1.6% and 5.3%) were within what is generally considered acceptable range for research assays (<20%).

3.2. Effect of storage volume on Aβ peptide ratio

Detectable Aβ42/Aβ40/Aβ38 concentration was observed to be significantly lower (all P < .001) in samples of...
smaller storage volumes in both CSF and CM (Figs. 1 and 2). Results from these data predict a concentration change of Aβ42: 1.1 pg/mL (0.6%), Aβ40: 9.2 pg/mL (0.3%), and Aβ38: 3.1 pg/mL (0.2%), for every 10-μL change in CSF storage volume, and a concentration change of Aβ42: 0.5 pg/mL (0.3%), Aβ40: 2.5 pg/mL (0.2%), and Aβ38: 0.4 pg/mL (0.1%), for every 10 μL change in CM storage volume (Table 1). Results for CSF Aβ42 are highly consistent with those previously reported for control CSF (a change of 0.95 pg/mL per 10 μL) [6].

Concordantly, ratios of Aβ42:Aβ40 and Aβ42:Aβ38 changed significantly with storage volume. In CSF Aβ42:Aβ40, change was 0.2% of an initial ratio value per 10 μL (P < .001), and in CSF Aβ42:Aβ38, change was 0.3% per 10 μL (P < .001) (Table 1). In CM, change in the Aβ42:Aβ40 and Aβ42:Aβ38 ratios were 0.1% and 0.2%.
3.3. Effect of serial tube transfer on Aβ peptide ratio

Detectable Aβ_{42}/Aβ_{40}/Aβ_{38} concentration was observed to decrease significantly (all P < .001) over the transfer series in both CSF and CM (Table 1). Results from untransformed CSF data were highly consistent with observations made of control CSF in a previous study [16]. However, these data violated the model’s assumption of linearity. Concentration loss in CSF between transfer 0 and transfer 1 was particularly pronounced for Aβ_{42}, an effect not observed in CM. The mean difference in Aβ_{42} between transfer 0 and transfer 1 was 95.8 pg/mL (paired, two-tailed t-test P < .001), whereas the mean difference between transfer 1 and the final concentration at transfer 7 was 149.7 pg/mL (paired, two-tailed t-test P < .001). This highlights that, in CSF, the first transfer accounted for 39% of the total Aβ_{42} lost (as compared to Aβ_{38} = 8.8% and Aβ_{40} = 24.1%). The decrease in all Aβ peptides remained significant between transfers 1 and 7 after transfer 0 was removed. This effect was not observed in CM samples.

To test whether exaggerated Aβ_{42} loss at first transfer may have been due to adsorption to the pipette tip, we conducted a pilot experiment to measure Aβ_{42} peptide concentration change in response to a varying number of aspirations using the same tip. The number of fluid pumps had no effect on Aβ_{42} peptide concentration in either CSF or CM, indeed paired, two-tailed t-test showed no significant difference between 0 pumps and 5 pumps, although it was observed that measurement variability was greater in the 0 pump group (Fig. 5). The initial exaggerated decrease in Aβ_{42} cannot therefore be attributed to adsorption to the pipette tip.

To account for nonlinearity, data were transformed by ln and reanalyzed. After ln transformation, Aβ_{42} decrease over serial tube transfers remained exaggerated in relation to Aβ_{40} and Aβ_{38} in both CSF and CM (Table 1). In CSF, the Aβ_{42}:Aβ_{40} ratio decreased by 4.9% per transfer, and the Aβ_{42}:Aβ_{38} decreased by 6.6% per transfer (Table 1). In CM, these were 3.1% and 5.5%, respectively. The decrease per transfer of Aβ_{40}:Aβ_{38} was 1.7% in CSF and 2.4% in CM; in both fluids, the decrease of Aβ_{40} relative to Aβ_{38} was significant (P < .001).

4. Discussion

In this study, we explored the effect of storage volume and serial between-tube transfer on the concentration of Aβ_{42}/Aβ_{40}/Aβ_{38} in human CSF and the CM of human cortical neurons derived from iPSCs. We report a novel finding: First, Aβ peptides are differentially affected by changes in sample surface exposure and raise the implication that subpopulations of Aβ peptide structures may be differentially vulnerable to surface exposure. Second, we show that ratios are less sensitive to surface exposure than peptides considered alone, although the effect is still significant.

4.1. Aβ peptides are differentially vulnerable to surface exposure

CSF and CM Aβ_{42}/Aβ_{40}/Aβ_{38} concentrations decreased as a result of two different PP surface exposure treatments (storage volume and serial tube transfer), closely replicating observations we previously reported in CSF [6,16]. Results are consistent with irreversible peptide adsorption to the tube surface, although the experiments did not test for this mechanism directly. Importantly, this decrease did not occur at the same rate for each peptide. Ratios of Aβ_{42}:Aβ_{40} and Aβ_{42}:Aβ_{38} demonstrated that decrease in Aβ_{42} concentration per treatment unit was consistently
greater than that observed in Aβ40 and Aβ38, in both CSF and CM. In addition, Aβ40:Aβ38 ratios indicated that Aβ40 may demonstrate a tendency toward greater concentration loss per treatment than Aβ38.

To our knowledge experiments such as these have not previously been published on CM. However, a body of work has been growing on the impact of preanalytical surface adsorption in CSF. Vanderstichele et al. [32] observed significant decreases in Aβ1–42 (−13.6%), Aβ1–40 (−15.5%), and Aβ1–38 (−10.6%) between CSF stored at 1500 µL (Sarstedt; cat. 72.706) and 500 µL (Sarstedt; cat. 72.730.006) in PP tubes, but not in Eppendorf LoBind tubes. They found that the Aβ42:Aβ40 ratio was not significantly altered by the difference in volume, whereas Aβ42:Aβ38 was altered by 3.4%. This is in contrast to our model that predicts larger, significant, changes in Aβ42:Aβ40 (23.7%) and Aβ42:Aβ38 (30.9%). It is worth noting that the volume effect is closely related to tube dimension [6,21], and our results...
represent the difference between 1000 and 100 μL rather than 1500 and 500 μL, which have different relative surface area exposure to the conical portion of the tube. In addition, the tubes used by Vanderstichele et al. for each volume were not the same, and neither matched the tube we tested (Sarstedt; cat. 72.694.007), which may reduce the direct comparability of results. With regard to the preanalytical effects of tube transfer, the observations between this group and our own align more closely. Vanderstichele et al. [32] observed significantly lower concentrations of Aβ42 (11.0%), Aβ40 (7.3%), and Aβ38 (2.7%) in CSF collected into PP tubes. In addition, they report a concentration decrease of Aβ42 (42.5%), Aβ40 (27.8%), and Aβ38 (16.7%) after one transfer between PP tubes (Sarstedt; cat 62.554.502 and either 72.706 or 72.730.006). In comparison, at the first transfer, our results showed a similar decrease of Aβ42 (39.0%) and Aβ40 (24.1%), but smaller decrease of Aβ38 (8.8%).
Willemse et al. [21] reported a 5% decrease (up to 10% in small volume samples) in $\alpha_{42}$ and $\alpha_{40}$ per transfer over four transfers between tubes and that $\alpha_{42}$:$\alpha_{40}$ therefore remained constant over transfer treatment. An effect size of 5%–10% is at odds with the $\alpha_{42}$ (22.3%) and $\alpha_{40}$ (17.5%) decrease per transfer that we observed over equivalent transfers. The tubes used by Willemse et al. (Sarstedt; cat. 72.694.007) are identical to the tubes we studied (Sarstedt; cat. 72.694.406), except that cat. 72.694.406 is certified DNA and RNase free. It is not clear why our data should be divergent, other factors are seemingly involved, and a degree of interlaboratory variation should be taken into consideration until these factors are identified.

Repeated aspirations and ejections from the same tip did not significantly alter $\alpha$ concentration in either CSF or CM. Indeed even when the tip was not prewetted, no effect was seen, contrary to what others have described [21]. Therefore, this cannot account for the initial exaggerated decrease we observed.

![Fig. 4. Effect of serial tube transfer on cell media $\alpha$. Results in CM show the concentration of $\alpha_{42}$,$\alpha_{40}$, and $\alpha_{38}$ decreased with consecutive transfer of sample to new storage tubes (A, C, E). Concentration of $\alpha_{42}$ decreases proportionally more than $\alpha_{40}$ (B) and $\alpha_{38}$ (D) with each transfer, resulting in a decrease in the $\alpha_{42}$:$\alpha_{40}$ and $\alpha_{42}$:$\alpha_{38}$ ratios. (F) In turn, the rate of $\alpha_{40}$ decrease with each transfer was greater than $\alpha_{38}$. Abbreviations: CM, culture media; $\alpha$, amyloid $\alpha$.](image-url)
observed at the first transfer step. Given this, we hypothesize the existence of a subpopulation of Aβ42 that is more readily adsorbed to PP and rapidly depleted from solution. This interpretation would fit the work of Vanderstichele et al. [32], but not Willemse et al. [21]. A similar, adsorption-attributed, initial effect on fluorescein-labeled bovine serum albumin (BSA), also found in the B27 fraction of the CM used in our study, has been reported [45]. It is possible that competition for surface binding sites by this and other proteins of the CM matrix might explain why the first transfer step effect was not observed in these samples, although we did not examine fluid protein content as a variable.

Although still not well understood, Aβ monomers, oligomers, and fibrils adopt a range of conformations in solution, and indeed current models highlight the importance of C-terminal sequence for multimeric stability and predict the presence of a laterally exposed hydrophobic “patch” unique to certain Aβ42 fibrils [46,47]. These properties may contribute to differences observed in Aβ42 versus Aβ40 nucleation rate constants [48,49] and the range of adsorption dynamics at different polymer surfaces [50,51]. Our results, which are preliminary, highlight the risk that potentially disease-relevant peptide subpopulations may be differentially vulnerable to loss during preanalytical processes.

4.2. Aβ ratios are less vulnerable to preanalytical surface exposure

Despite disparities in the size of transfer and volume effects between studies on PP tubes, our data and those of others [21,32] converge on the reduction of preanalytical surface exposure effect when a ratio of Aβ peptides is used. For Aβ42:Aβ40, the disparity between initial and treatment subsequent result was reduced by approximately 50% (volume) and 75% (transfer) as compared to Aβ42 alone (Table 1). It is difficult to give a hard estimate for the amount of concentration loss necessary to mislead interpretation of the ratio, and this will depend on how close the individual peptide values are to a chosen diagnostic threshold. However, it is reasonable to expect reduction in the range, or “gray zone”, of diagnostic uncertainty if preanalytically derived “noise” can be mitigated. This is also relevant to cell models in AD research, where Aβ measurement variability within and between cell lines presents a hindrance to experiment repeatability, which use of ratios may help reduce.

4.3. Limitations

This study was limited by the relatively low number of independent samples used.

4.4. Summary

Loss of Aβ42 following differential exposure to polypropylene surfaces was significantly greater than Aβ40 and Aβ38 in both human CSF and neuronal cell CM. In addition, there was a tendency toward a pattern of greater loss of Aβ40 relative to Aβ18. Despite differences between peptides, Aβ ratios were less strongly affected by storage volume and tube
transfer treatments than peptides considered individually. We conclude that the $A\beta_{42}:A\beta_{40}$ and $A\beta_{42}:A\beta_{38}$ ratios may predispose toward a risk of a false-negative diagnostic result for AD if samples are not treated in a standardized manner, though risk of misinterpretation may be less attendant than to $A\beta_{42}$ alone. Reporting $A\beta$ ratios in concert with individual peptides may reduce misinterpretation of $A\beta$ assay results.

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RESEARCH IN CONTEXT

1. Systematic review: Amyloid $\beta$ 42 ($A\beta_{42}:A\beta_{40}$) ratio has attracted interest as a biomarker for Alzheimer’s disease (AD) with a number of recent clinical studies demonstrating high sensitivity and specificity. Work on identifying preanalytical factors relevant to the ratio is ongoing. The authors have comprehensively cited this literature.

2. Interpretation: Our findings add to growing evidence supporting $A\beta_{42}:A\beta_{40}$ and $A\beta_{42}:A\beta_{38}$ ratios as reliable biomarkers for AD and expand the experimental perspective beyond cerebrospinal fluid (CSF) to cell media. Significantly we highlight that longer peptides adsorb to polypropylene surfaces, in a context relevant to clinical diagnostics, to a greater extent than shorter peptides.

3. Future directions: The manuscript forms a platform for further characterization of peptide-surface interaction for a wider range of $A\beta$ fragments, which may have disease relevance or biomarker utility, and also for identification of factors affecting $A\beta$ measurement from in vitro model fluids.

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