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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 $Aβ_{42}$ 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. $Aβ_{42}$, $Aβ_{40}$, and $Aβ_{38}$ 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 $Aβ_{42}$:$Aβ_{40}$ and $Aβ_{42}$:$Aβ_{38}$ ratios. However, the effect size of surface treatment was reduced in Aβ peptide ratios versus $Aβ_{42}$ alone. For $Aβ_{42}$:$Aβ_{40}$, the effect was reduced by approximately 50% (volume) and 75% (transfer) as compared to $Aβ_{42}$ 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 ($Aβ_{42}$), 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], $Aβ_{42}$ 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 $A\beta_{42}$ concentrations susceptible to variation in the preanalytical 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 $A\beta_{42}$ 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 $A\beta_{42}$ 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 mild cognitive impairment–AD conversion predictive power when $A\beta_{42}$ is considered in ratio to $A\beta_{40}$ [1,15,16,31,35,36]. $A\beta_{40}$ has been shown to be the most abundant Aβ peptide in the adult human brain [37] and does not generally have strong aggregative tendencies under physiological conditions or in vitro. Owing to this, CSF $A\beta_{40}$ has been proposed as a proxy for total Aβ production [33,38]. Another relatively abundant peptide, $A\beta_{38}$, has also demonstrated utility in ratio with $A\beta_{42}$ [35]. This is welcome news as, although biomarkers have facilitated earlier and more accurate diagnosis, improved patient group enrichment in current clinical trial design (i.e. more accurate, earlier disease phase biomarkers) is urgently required [39].

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 $A\beta_{42}$:$A\beta_{40}$, $A\beta_{42}$:$A\beta_{38}$, and $A\beta_{40}$:$A\beta_{38}$. $A\beta_{40}$:$A\beta_{38}$ 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 pathobiology. 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-laying position. Ten milliliters of CSF was collected at ambient room temperature into a 10-mL PP tube (Sarstedt, Nümbrecht, 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-I (1×) (Life Technologies; cat. 10565018) 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-mM 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 Geltrex 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 StemBANCC. 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 Aβx-38/x-40/x-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 four 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 Aβx-38/x-40/x-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 lme(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|" 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, "e" 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 $A\beta_{42}$ (4.3% and 9.9%), $A\beta_{40}$ (4.5% and 9.5%), and $A\beta_{38}$ (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 $A\beta_{42}/A\beta_{40}/A\beta_{38}$ concentration was observed to be significantly lower (all $P < .001$) in samples of

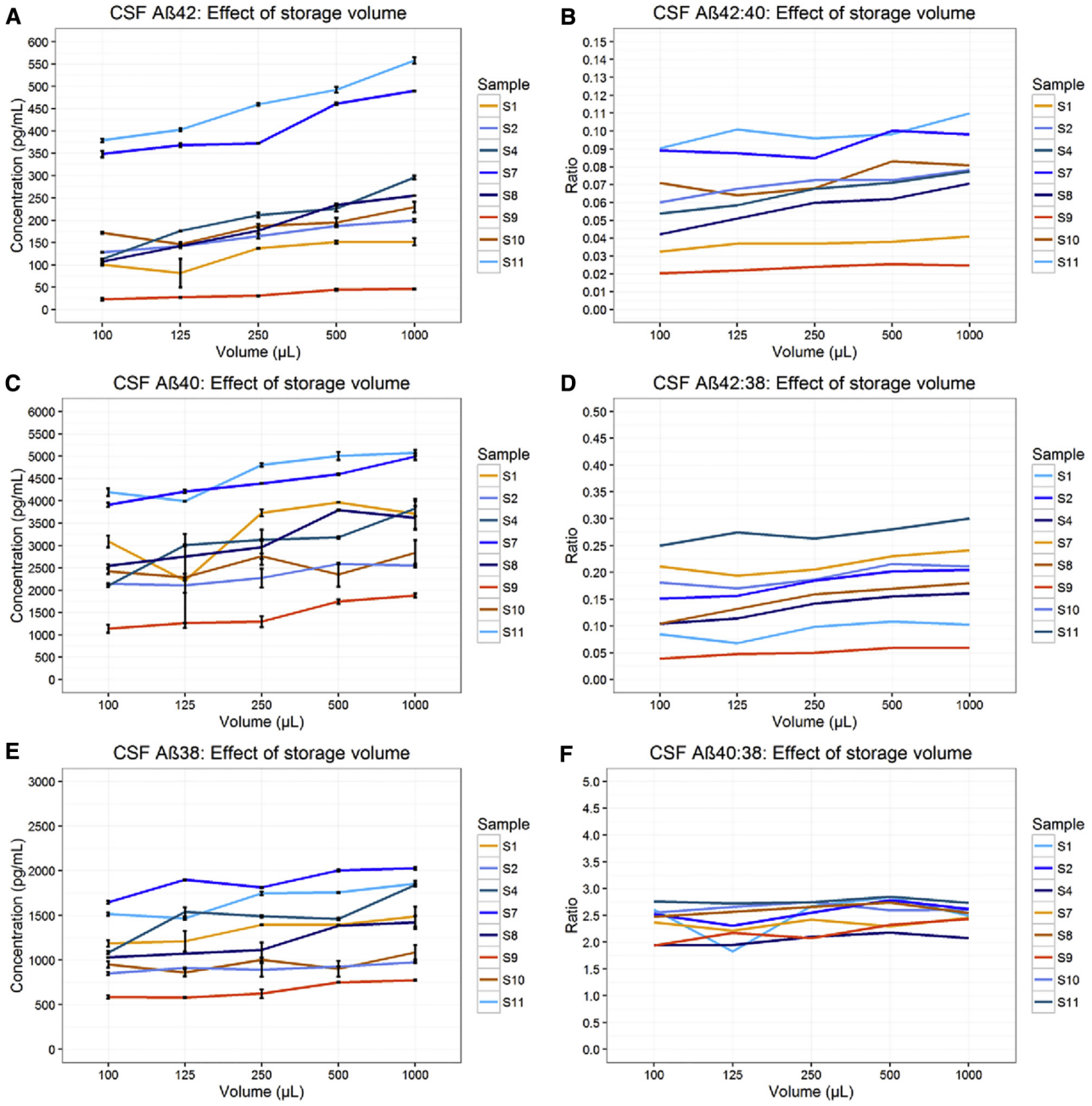

Fig. 1. Effect of storage volume on CSF Aβ. Results in CSF show the concentration of $A\beta_{42}$, $A\beta_{40}$, and $A\beta_{38}$ decreased with decreased storage volume (A, C, E). Concentration of $A\beta_{42}$ decreased proportionally more than $A\beta_{40}$ (B) and $A\beta_{38}$ (D) with lower storage volume, resulting in a decrease in the $A\beta_{42}$:$A\beta_{40}$ and $A\beta_{42}$:$A\beta_{38}$ ratios. (F) $A\beta_{40}$ showed a nonsignificant tendency to decrease more than $A\beta_{38}$, ($P = .05$). Data for sample S8 at 125 µL were excluded due to insufficient volume in the assay well; the line interpolates through this point. Error bars represent standard error of the mean. Abbreviations: CSF, cerebrospinal fluid; Aβ, amyloid β.

smaller storage volumes in both CSF and CM (Figs. 1 and 2). Results from these data predict a concentration change of $A\beta_{42}$: 1.1 pg/mL (0.6%), $A\beta_{40}$: 9.2 pg/mL (0.3%), and $A\beta_{38}$: 3.1 pg/mL (0.2%), for every 10-µL change in CSF storage volume, and a concentration change of $A\beta_{42}$: 0.5 pg/mL (0.3%), $A\beta_{40}$: 2.5 pg/mL (0.2%), and $A\beta_{38}$: 0.4 pg/mL (0.1%), for every 10 µL change in CM storage volume (Table 1). Results for CSF $A\beta_{42}$ are highly consistent with those previously reported for control CSF (a change of 0.95 pg/mL per 10 µL) [6].

Concordantly, ratios of $A\beta_{42}$:$A\beta_{40}$ and $A\beta_{42}$:$A\beta_{38}$ changed significantly with storage volume. In CSF $A\beta_{42}$:$A\beta_{40}$, change was 0.2% of an initial ratio value per 10 µL ($P < .001$), and in CSF $A\beta_{42}$:$A\beta_{38}$, change was 0.3% per 10 µL ($P < .001$) (Table 1). In CM, change in the $A\beta_{42}$:$A\beta_{40}$ and $A\beta_{42}$:$A\beta_{38}$ ratios were 0.1% and 0.2%

Table 1
Summary of results

Type	Peptide	Study	% Change per unit	P ($\alpha = 0.05$)	95% confidence interval	
CSF	$A\beta_{38}$	Vol	0.245	<.001	0.168	0.322
CSF	$A\beta_{40}$	Vol	0.318	<.001	0.202	0.435
CSF	$A\beta_{42}$	Vol	0.555	<.001	0.403	0.707
CSF	$A\beta_{42}$:$A\beta_{40}$	Vol	0.237	<.001	0.160	0.313
CSF	$A\beta_{42}$:$A\beta_{38}$	Vol	0.310	<.001	0.205	0.415
CSF	$A\beta_{40}$:$A\beta_{38}$	Vol	0.073	.054	−0.001	0.148
CSF	$A\beta_{38}$	Tra	15.756	<.001	14.818	16.695
CSF	$A\beta_{40}$	Tra	17.497	<.001	16.734	18.260
CSF	$A\beta_{42}$	Tra	22.359	<.001	21.177	23.541
CSF	$A\beta_{42}$:$A\beta_{40}$	Tra	4.862	<.001	3.990	5.734
CSF	$A\beta_{42}$:$A\beta_{38}$	Tra	6.603	<.001	5.409	7.796
CSF	$A\beta_{40}$:$A\beta_{38}$	Tra	1.741	<.001	1.111	2.372
CM	$A\beta_{38}$	Vol	0.105	<.001	0.053	0.156
CM	$A\beta_{40}$	Vol	0.162	<.001	0.106	0.217
CM	$A\beta_{42}$	Vol	0.331	<.001	0.235	0.427
CM	$A\beta_{42}$:$A\beta_{40}$	Vol	0.169	<.001	0.104	0.234
CM	$A\beta_{42}$:$A\beta_{38}$	Vol	0.226	<.001	0.160	0.293
CM	$A\beta_{40}$:$A\beta_{38}$	Vol	0.057	.028	0.007	0.108
CM	$A\beta_{38}$	Tra	2.411	<.001	1.676	3.205
CM	$A\beta_{40}$	Tra	4.789	<.001	4.103	5.711
CM	$A\beta_{42}$	Tra	7.786	<.001	6.993	9.219
CM	$A\beta_{42}$:$A\beta_{40}$	Tra	3.148	<.001	2.315	4.082
CM	$A\beta_{42}$:$A\beta_{38}$	Tra	5.508	<.001	4.532	6.799
CM	$A\beta_{40}$:$A\beta_{38}$	Tra	2.436	<.001	1.599	3.334

Abbreviations: CM, culture media; Aβ, amyloid β; CSF, cerebrospinal fluid.

NOTE. Volume unit is 10 μL, and transfer is 1 transfer. The table is divided into data generated for CSF and CM for storage volume (Vol) and serial transfer (Tra) treatments, respectively. Data are given for $A\beta_{42}$, $A\beta_{40}$, $A\beta_{38}$, and the ratio of these peptides transformed by natural logarithm (ln). Values given in "% Change per unit" are exponentiated coefficients of the mixed model as percent of Aβ concentration (pg/mL) or ratio change per unit of treatment. For the volume treatment, the unit of change is 1 μL, that is, the amount of Aβ lost per 1 μL of decreased storage volume. For serial transfer treatment, the unit of change is one transfer, that is, the amount of Aβ lost per transfer of sample to another tube.

of the ratio per 10 μL ($P < .001$), respectively (Table 1). The magnitude of change per unit volume was reduced in both CSF and CM ratios versus $A\beta_{42}$ alone. The ratio of $A\beta_{40}$:$A\beta_{38}$ showed a trend toward decreased $A\beta_{40}$, which bordered on significance in CSF ($P = .054$) and CM ($P = .028$) (Table 1).

3.3. Effect of serial tube transfer on Aβ peptide ratio

Detectable $A\beta_{42}$/$A\beta_{40}$/$A\beta_{38}$ concentration was observed to decrease significantly (all $P < .001$) over the transfer series in both CSF and CM (Figs. 3 and 4). 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 $A\beta_{42}$, an effect not observed in CM. The mean difference in $A\beta_{42}$ 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 $A\beta_{42}$ lost (as compared to $A\beta_{38} = 8.8\%$ and $A\beta_{40} = 24.1\%$). 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 $A\beta_{42}$ loss at first transfer may have been due to adsorption to the pipette tip, we conducted a pilot experiment to measure $A\beta_{42}$ peptide concentration change in response to a varying number of aspirations using the same tip. The number of fluid pumps had no effect on $A\beta_{42}$ 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 $A\beta_{42}$ cannot therefore be attributed to adsorption to the pipette tip.

To account for nonlinearity, data were transformed by ln and reanalyzed. After ln transformation, $A\beta_{42}$ decrease over serial tube transfers remained exaggerated in relation to $A\beta_{40}$ and $A\beta_{38}$ in both CSF and CM (Table 1). In CSF, the $A\beta_{42}$:$A\beta_{40}$ ratio decreased by 4.9% per transfer, and the $A\beta_{42}$:$A\beta_{38}$ decreased by 6.6% per transfer (Table 1). In CM, these were 3.1% and 5.5%, respectively. The decrease per transfer of $A\beta_{40}$:$A\beta_{38}$ was 1.7% in CSF and 2.4% in CM; in both fluids, the decrease of $A\beta_{40}$ relative to $A\beta_{38}$ 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 $A\beta_{42}$/$A\beta_{40}$/$A\beta_{38}$ 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 $A\beta_{42}$/$A\beta_{40}$/$A\beta_{38}$ 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 $A\beta_{42}$:$A\beta_{40}$ and $A\beta_{42}$:$A\beta_{38}$ demonstrated that decrease in $A\beta_{42}$ concentration per treatment unit was consistently

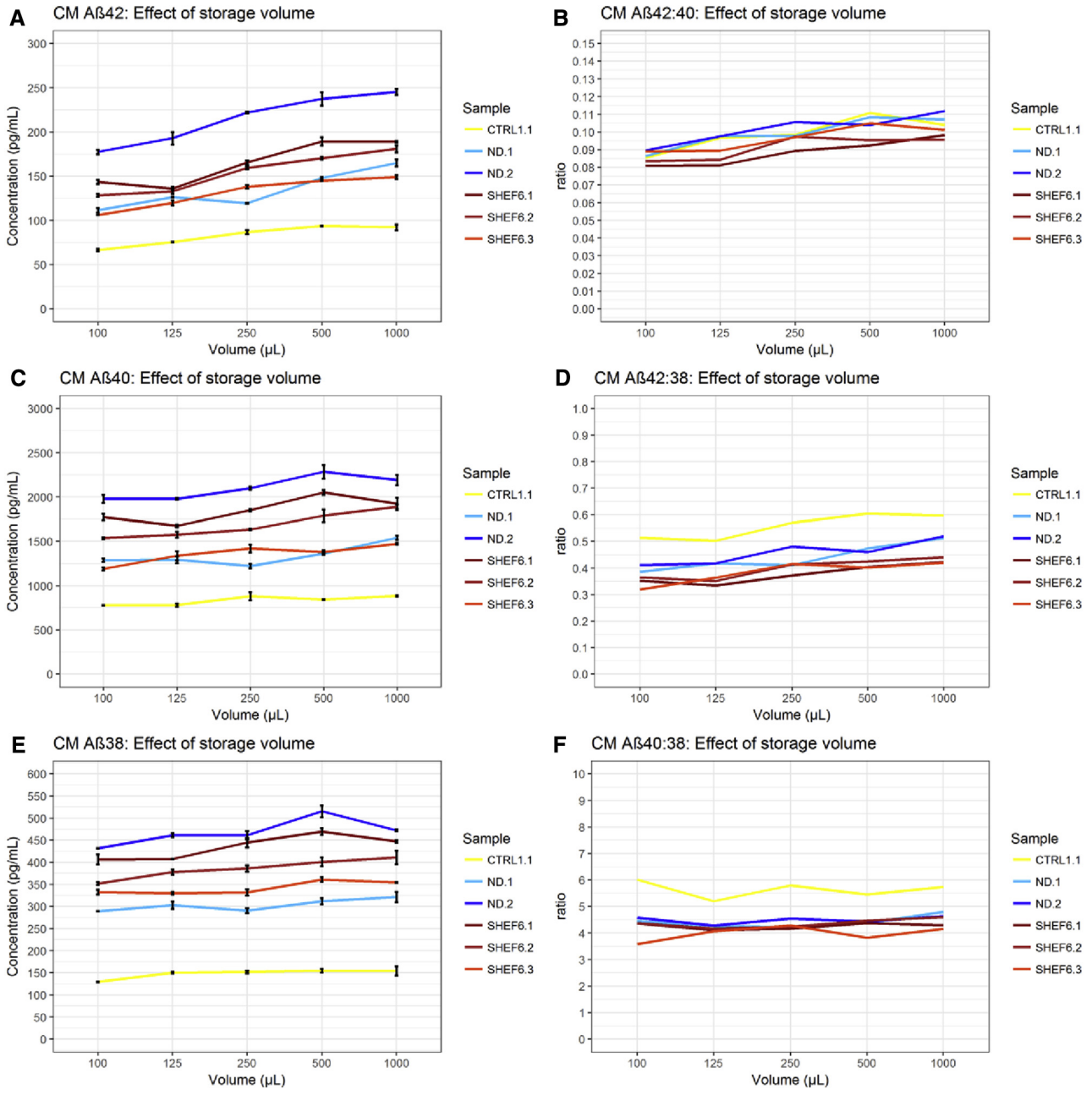

Fig. 2. Effect of storage volume on cell media Aβ. Results in CM show the concentration of $A\beta_{42}$, $A\beta_{40}$, and $A\beta_{38}$ decreased with decreased storage volume (A, C, E). Concentration of $A\beta_{42}$ decreased proportionally more than $A\beta_{40}$ (B) and $A\beta_{38}$ (D) with lower storage volume, resulting in a decrease in the $A\beta_{42}$:$A\beta_{40}$ and $A\beta_{42}$:$A\beta_{38}$ ratios. (F) $A\beta_{40}$ showed a tendency to decrease more than $A\beta_{38}$, although this was only weakly significant ($P = .03$). Abbreviations: CM, culture media; Aβ, amyloid β.

greater than that observed in $A\beta_{40}$ and $A\beta_{38}$, in both CSF and CM. In addition, $A\beta_{40}$:$A\beta_{38}$ ratios indicated that $A\beta_{40}$ may demonstrate a tendency toward greater concentration loss per treatment than $A\beta_{38}$.

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 $A\beta_{1–42}$ (−13.6%), $A\beta_{1–40}$ (−15.5%), and $A\beta_{1–38}$ (−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 $A\beta_{42}$:$A\beta_{40}$ ratio was not significantly altered by the difference in volume, whereas $A\beta_{42}$:$A\beta_{38}$ was altered by 3.4%. This is in contrast to our model that predicts larger, significant, changes in $A\beta_{42}$:$A\beta_{40}$ (23.7%) and $A\beta_{42}$:$A\beta_{38}$ (30.9%). It is worth noting that the volume effect is closely related to tube dimension [6,21], and our results

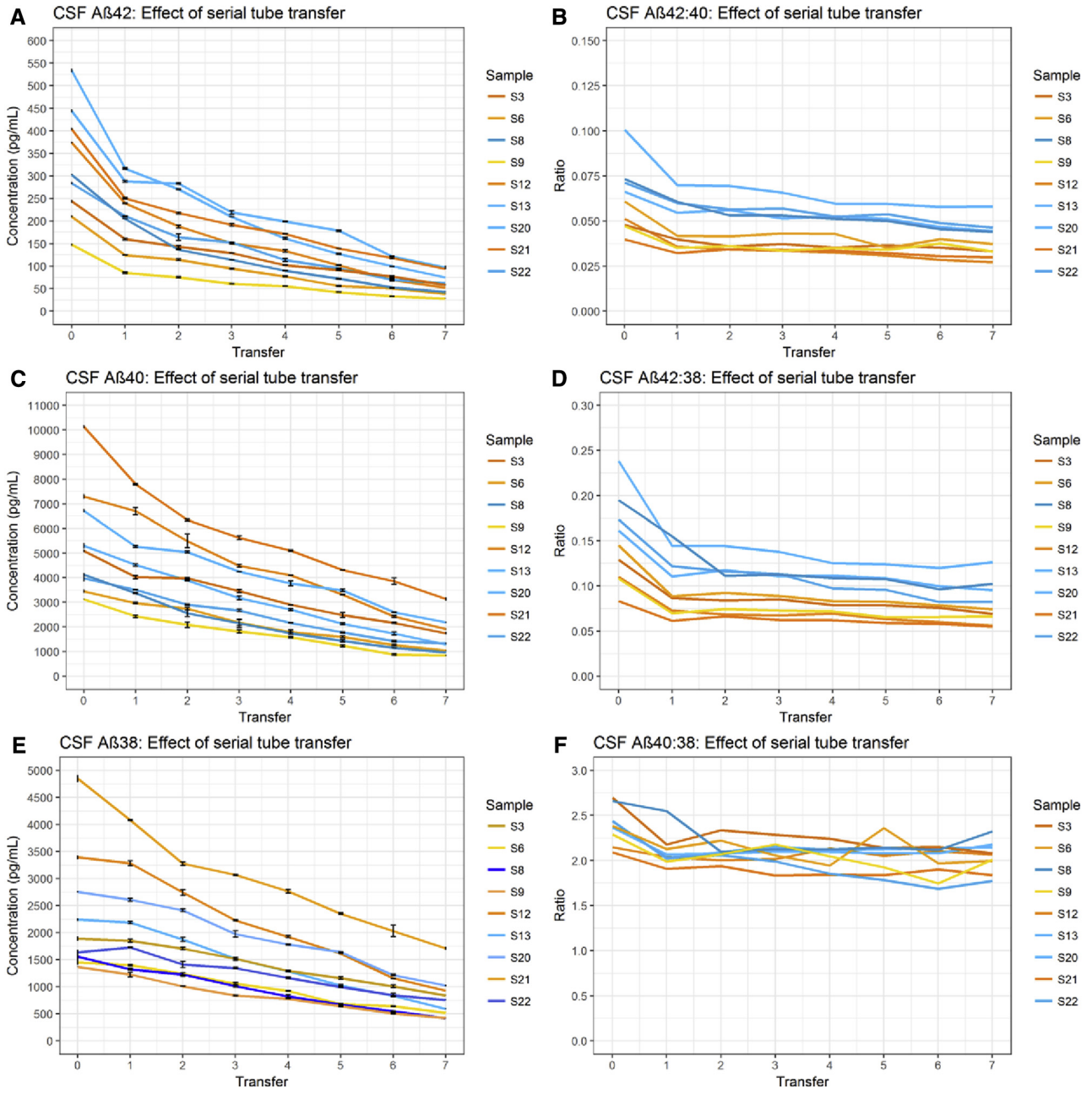

Fig. 3. Effect of serial tube transfer on CSF Aβ. Results in CSF show the concentration of $A\beta_{42}$, $A\beta_{40}$, and $A\beta_{38}$ decreased with consecutive transfer of sample to new storage tubes (A, C, E). Concentration of $A\beta_{42}$ decreased proportionally more than $A\beta_{40}$ (B) and $A\beta_{38}$ (D) with each transfer, resulting in a decrease in the $A\beta_{42}$:$A\beta_{40}$ and $A\beta_{42}$:$A\beta_{38}$ ratios. (F) In turn, the rate of $A\beta_{40}$ decrease with each transfer was greater than $A\beta_{38}$. Error bars represent standard error of the mean. Abbreviations: Aβ, amyloid β; CSF, cerebrospinal fluid.

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 $A\beta_{42}$ (11.0%), $A\beta_{40}$ (7.3%), and $A\beta_{38}$ (2.7%) in CSF collected into PP tubes. In addition, they report a concentration decrease of $A\beta_{42}$ (42.5%), $A\beta_{40}$ (27.8%), and $A\beta_{38}$ (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 $A\beta_{42}$ (39.0%) and $A\beta_{40}$ (24.1%), but smaller decrease of $A\beta_{38}$ (8.8%).

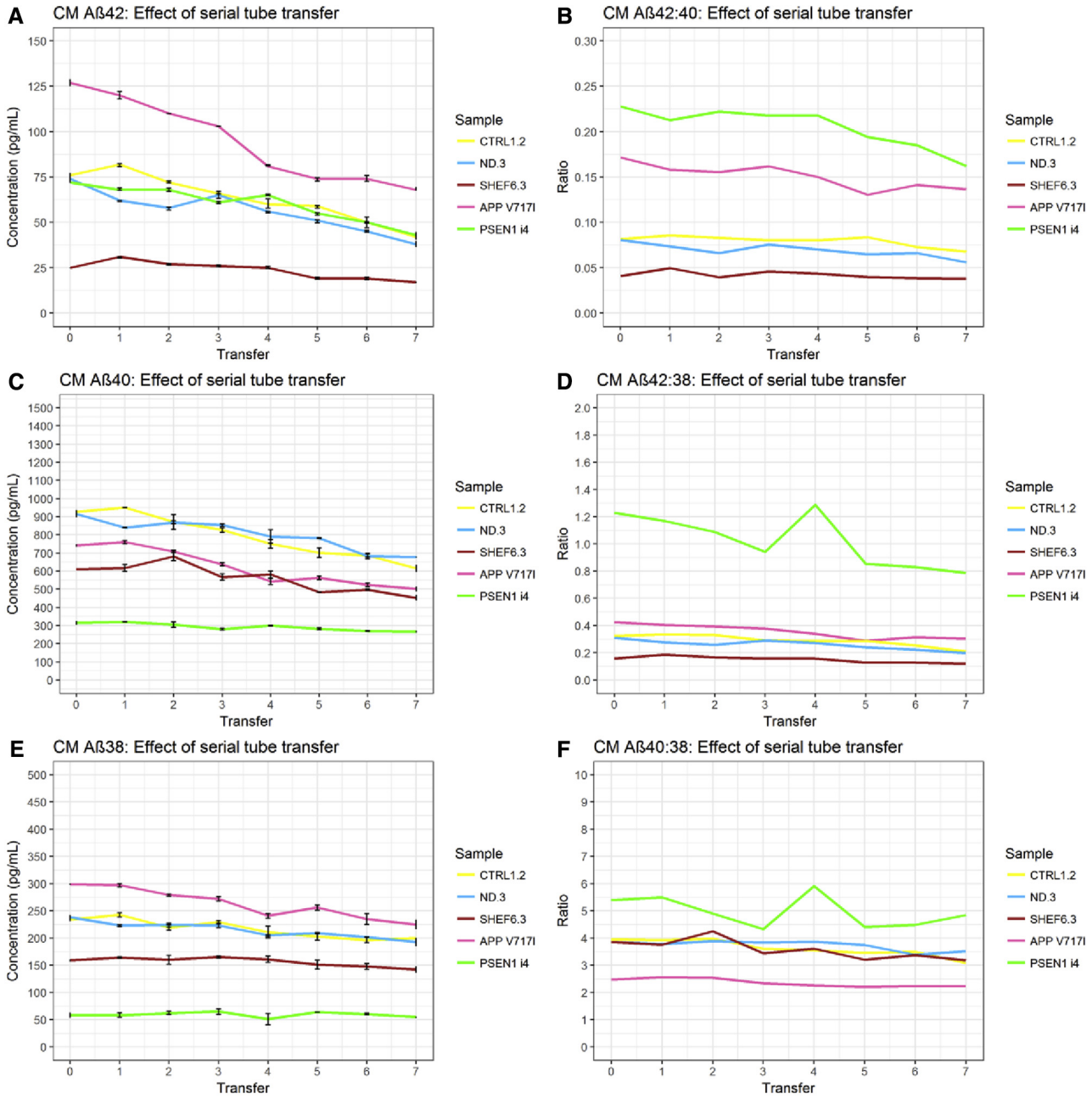

Fig. 4. Effect of serial tube transfer on cell media Aβ. Results in CM show the concentration of $A\beta_{42}$, $A\beta_{40}$, and $A\beta_{38}$ decreased with consecutive transfer of sample to new storage tubes (A, C, E). Concentration of $A\beta_{42}$ decreased proportionally more than $A\beta_{40}$ (B) and $A\beta_{38}$ (D) with each transfer, resulting in a decrease in the $A\beta_{42}$:$A\beta_{40}$ and $A\beta_{42}$:$A\beta_{38}$ ratios. (F) In turn, the rate of $A\beta_{40}$ decrease with each transfer was greater than $A\beta_{38}$. Abbreviations: CM, culture media; Aβ, amyloid β.

Willemse et al. [21] reported a 5% decrease (up to 10% in small volume samples) in $A\beta_{42}$ and $A\beta_{40}$ per transfer over four transfers between tubes and that $A\beta_{42}$:$A\beta_{40}$ therefore remained constant over transfer treatment. An effect size of 5%–10% is at odds with the $A\beta_{42}$ (22.3%) and $A\beta_{40}$ (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 Aβ 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

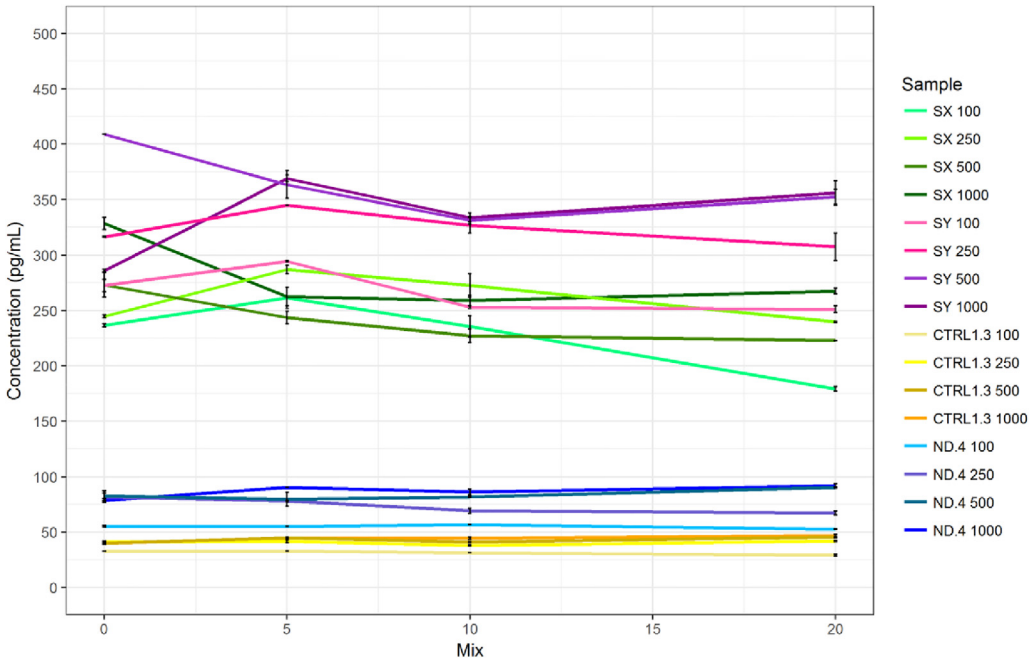

Fig. 5. CSF (SX and SY) and CM (CTRL and ND) $A\beta_{42}$: Effect of mixing by pipette across different storage volumes. Results show the concentration of $A\beta_{42}$ were not significantly affected by different levels of exposure to the pipette tip over a series of different volumes in either CSF or CM. Greater variability between measurements was observed in CSF measurements than those of CM. Error bars represent standard error of the mean. Abbreviations: CM, culture media; Aβ, amyloid β; CSF, cerebrospinal fluid.

observed at the first transfer step. Given this, we hypothesize the existence of a subpopulation of $A\beta_{42}$ 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 $A\beta_{42}$ fibrils [46,47]. These properties may contribute to differences observed in $A\beta_{42}$ versus $A\beta_{40}$ 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 $A\beta_{42}$:$A\beta_{40}$, the disparity between initial and treatment subsequent result was reduced by approximately 50% (volume) and 75% (transfer) as compared to $A\beta_{42}$ 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 $A\beta_{42}$ following differential exposure to polypropylene surfaces was significantly greater than $A\beta_{40}$ and $A\beta_{38}$ in both human CSF and neuronal cell CM. In addition, there was a tendency toward a pattern of greater loss of $A\beta_{40}$ relative to $A\beta_{38}$. 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 Aβ ratios in concert with individual peptides may reduce misinterpretation of Aβ assay results.

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The authors have declared that no conflict of interest exists.

RESEARCH IN CONTEXT

1. Systematic review: Amyloid β 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 Aβ fragments, which may have disease relevance or biomarker utility, and also for identification of factors affecting Aβ measurement from *in vitro* model fluids.

References

[1] Blennow K, Zetterberg H, Fagan AM. Fluid biomarkers in Alzheimer disease. Cold Spring Harb Perspect Med 2012;2.
[2] Dubois B, Feldman HH, Jacova C, Hampel H, Molinuevo JL, Blennow K, et al. Advancing research diagnostic criteria for Alzheimer's disease: The IWG-2 criteria. Lancet Neurol 2014;13:614–29.
[3] Perl DP. Neuropathology of Alzheimer's disease. Mount Sinai J Med 2010;77:32–42.
[4] Mandelkow EM, Mandelkow E. Biochemistry and cell biology of tau protein in neurofibrillart degeneration. Cold Spring Harb Perspect Med 2012;2:a006247.
[5] Schoonenboom NSM, Mulder C, Vanderstichele H, Van Elk E-J, Kok A, Van Kamp GJ, et al. Effects of processing and storage conditions on amyloid beta (1-42) and tau concentrations in cerebrospinal fluid: Implications for use in clinical practice. Clin Chem 2005; 51:189–95.
[6] Toombs J, Paterson RW, Lunn MP, Nicholas JM, Fox NC, Chapman MD, et al. Identification of an important potential confound in CSF AD studies: Aliquot volume. Clin Chem Lab Med 2013; 51:2311–7.
[7] Wang Z-XX, Tan L, Liu J, Yu J-TT. The essential role of soluble Aβ oligomers in Alzheimer's disease. Mol Neurobiol 2015:1905–24.
[8] Lucey BP, Gonzales C, Das U, Li J, Siemers ER, Slemmon JR, et al. An integrated multi-study analysis of intra-subject variability in cerebrospinal fluid amyloid-β concentrations collected by lumbar puncture and indwelling lumbar catheter. Alzheimers Res Ther 2015;7:53.
[9] Cicognola C, Chiasserini D, Parnetti L. Preanalytical confounding factors in the analysis of cerebrospinal fluid biomarkers for Alzheimer's disease: The issue of diurnal variation. Front Neurol 2015;6:143.
[10] Kaiser E, Schönknecht P, Thomann PA, Hunt A, Schröder J. Influence of delayed CSF storage on concentrations of phospho-tau protein (181), total tau protein and beta-amyloid (1-42). Neurosci Lett 2007; 417:193–5.
[11] Le Bastard N, Aerts L, Sleegers K, Martin JJ, Van Broeckhoven C, De Deyn PP, et al. Longitudinal stability of cerebrospinal fluid biomarker levels: Fulfilled requirement for pharmacodynamic markers in Alzheimer's disease. J Alzheimer's Dis 2013;33:807–22.
[12] Sancesario GMG, Esposito Z, Nuccetelli M, Bernardini S, Sorge R, Martorana A, et al. Aβ1-42 Detection in CSF of Alzheimer's disease is influenced by temperature: Indication of reversible Aβ1-42 aggregation? Exp Neurol 2010;223:371–6.
[13] Murphy BM, Swarts S, Mueller BM, van der Geer P, Manning MC, Fitchmun MI. Protein instability following transport or storage on dry ice. Nat Methods 2013;10:278–9.
[14] Slemmon JR, Meredith J, Guss V, Andreasson U, Andreasen N, Zetterberg H, et al. Measurement of Abeta1-42 in cerebrospinal fluid is influenced by matrix effects. J Neurochem 2012;120:325–33.
[15] Slemmon JR, Shapiro A, Mercken M, Streffer J, Romano G, Andreasen N, et al. Impact of cerebrospinal fluid matrix on the detection of Alzheimer's disease with Abeta42 and influence of disease on the total-Abeta42/Abeta40 ratio. J Neurochem 2015;135:1049–58.
[16] Toombs J, Paterson RW, Schott JM, Zetterberg H. Amyloid-beta 42 adsorption following serial tube transfer. Alzheimers Res Ther 2014; 6:5.
[17] Perret-Liaudet A, Pelpel M, Tholance Y, Dumont B, Vanderstichele H, Zorzi W, et al. Risk of Alzheimer's disease biological misdiagnosis linked to cerebrospinal collection tubes. J Alzheimer's Dis 2012; 31:13–20.
[18] Lewczuk P, Lelental N, Spitzer P, Maler JM, Kornhuber J. Amyloid-beta 42/40 cerebrospinal fluid concentration ratio in the diagnostics of Alzheimer's disease: Validation of two novel assays. J Alzheimer's Dis 2014;43:183–91.
[19] Murray AN, Palhano FL, Bieschke J, Kelly JW. Surface adsorption considerations when working with amyloid fibrils in multiwell plates and Eppendorf tubes. Protein Sci 2013;22:1531–41.
[20] Lewczuk P, Beck G, Esselmann H, Bruckmoser R, Zimmermann R, Fiszer M, et al. Effect of sample collection tubes on cerebrospinal fluid concentrations of tau proteins and amyloid B peptides. Clin Chem 2006;52:331–4.
[21] Willemse E, van Uffelen K, Brix B, Engelborghs S, Vanderstichele H, Teunissen C. How to handle adsorption of cerebrospinal fluid amyloid-beta (1-42) in laboratory practice? Identifying problematic handlings

and resolving the issue by use of the Aβ42/Aβ40 ratio. Alzheimer's Dement 2017;13:885–92.
[22] Toombs J, Foiani MS, Paterson RW, Heslegrave A, Wray S, Schott JM, et al. Effect of spinal manometers on cerebrospinal fluid amyloid-β concentration. J Alzheimer's Dis 2017;56:885–91.
[23] Reijn TSM, Rikkert MO, Van Geel WJA, De Jong D, Verbeek MM. Diagnostic accuracy of ELISA and xMAP technology for analysis of amyloid beta42 and tau proteins. Clin Chem 2007;53:859–65.
[24] Fagan AM, Shaw LM, Xiong C, Vanderstichele H, Mintun MA, Trojanowski JQ, et al. Comparison of analytical platforms for cerebrospinal fluid measures of beta-amyloid 1-42, total tau, and p-tau181 for identifying Alzheimer disease amyloid plaque pathology. Arch Neurol 2011;68:1137–44.
[25] Ellis TA, Li J, Leblond D, Waring JF. The relationship between different assays for detection and quantification of amyloid beta 42 in human cerebrospinal fluid. Int J Alzheimers Dis 2012;2012:984746.
[26] Vos SJB, Visser PJ, Verhey F, Aalten P, Knol D, Ramakers I, et al. Variability of CSF Alzheimer's disease biomarkers: Implications for clinical practice. PLoS One 2014;9:e100784.
[27] Mattsson N, Andreasson U, Persson S, Carrillo MC, Collins S, Chalbot S, et al. CSF biomarker variability in the Alzheimer's Association Quality Control Program. Alzheimer's Dement 2013;9:251–61.
[28] Bjerke M, Portelius E, Minthon L, Wallin A, Anckarsäter H, Anckarsäter R, et al. Confounding factors influencing amyloid Beta concentration in cerebrospinal fluid. Int J Alzheimers Dis 2010;2010:1–12.
[29] del Campo M, Mollenhauer B, Bertolotto A, Engelborghs S, Hampel H, Simonsen AH, et al. Recommendations to standardize preanalytical confounding factors in Alzheimer's and Parkinson's disease cerebrospinal fluid biomarkers: an update. Biomark Med 2012;6:419–30.
[30] Le Bastard N, De Deyn PP, Engelborghs S. Importance and impact of preanalytical variables on Alzheimer disease biomarker concentrations in cerebrospinal fluid. Clin Chem 2015;61:734–43.
[31] Vanderstichele H, Bibl M, Engelborghs S, Le Bastard N, Lewczuk P, Molinuevo JL, et al. Standardization of preanalytical aspects of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: A consensus paper from the Alzheimer's Biomarkers Standardization Initiative. Alzheimer's Dement 2012;8:65–73.
[32] Vanderstichele HMJ, Janelidze S, Demeyer L, Coart E, Stoops E, Herbst V, et al. Optimized standard operating procedures for the analysis of cerebrospinal fluid Ab42 and the ratios of Ab isoforms using low protein binding tubes. J Alzheimer's Dis 2016;53:1121–32.
[33] Wiltfang J, Esselmann H, Bibl M, Hüll M, Hampel H, Kessler H, et al. Amyloid β peptide ratio 42/40 but not Aβ42 correlates with phospho-Tau in patients with low- and high-CSF Aβ40 load. J Neurochem 2007;101:1053–9.
[34] Mo J-A, Lim J-H, Sul A-R, Lee M, Youn YC, Kim H-J. Cerebrospinal fluid β-Amyloid1–42 levels in the differential diagnosis of Alzheimer's disease—systematic review and meta-analysis. PLoS One 2015;10:1–16.
[35] Janelidze S, Zetterberg H, Mattsson N, Palmqvist S, Vanderstichele H, Lindberg O, et al. CSF Aβ42/Aβ40 and Aβ42/Aβ38 ratios: better diagnostic markers of Alzheimer disease. Ann Clin Transl Neurol 2016;3:154–65.
[36] Dorey A, Perret-Liaudet A, Tholance Y, Fourier A, Quadrio I. Cerebrospinal fluid Aβ40 improves the interpretation of Aβ42 concentration for diagnosing Alzheimer's disease. Front Neurol 2015;6:247.
[37] Schieb H, Kratzin H, Jahn O, Möbius W, Rabe S, Staufenbiel M, et al. Beta-amyloid peptide variants in brains and cerebrospinal fluid from amyloid precursor protein (APP) transgenic mice: comparison with human Alzheimer amyloid. J Biol Chem 2011;286:33747–58.
[38] Hansson O, Zetterberg H, Buchhave P, Andreasson U, Londos E, Minthon L, et al. Prediction of Alzheimer's disease using the CSF Abeta42/Abeta40 ratio in patients with mild cognitive impairment. Dement Geriatr Cogn Disord 2007;23:316–20.
[39] Fiandaca MS, Mapstone ME, Cheema AK, Federoff HJ. The critical need for defining preclinical biomarkers in Alzheimer's disease. Alzheimer's Dement 2014;10:S196–212.
[40] Moore S, Evans LDB, Andersson T, Portelius E, Smith J, Dias TB, et al. APP metabolism regulates tau proteostasis in human cerebral cortex neurons. Cell Rep 2015;11:689–696.
[41] Shi Y, Kirwan P, Smith J, Robinson HPC, Livesey FJ. Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. Nat Neurosci 2012;15:477–486, S1.
[42] Sposito T, Preza E, Mahoney CJ, Setó-Salvia N, Ryan NS, Morris HR, et al. Developmental regulation of tau splicing is disrupted in stem cell-derived neurons from frontotemporal dementia patients with the 10 + 16 splice-site mutation in MAPT. Hum Mol Genet 2015;24:5260–5269.
[43] Ludtmann MHR, Arber C, Bartolome F, De Vicente M, Preza E, Carro E, et al. Mutations in valosin-containing protein (VCP) decrease ADP/ATP translocation across the mitochondrial membrane and impair energy metabolism in human neurons. J Biol Chem 2017;292:8907–8917.
[44] ISO 5725-2:1994(en), Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method [Internet], 1994. Available at: https://www.iso.org/obp/ui/#iso:std:11834:en. Accessed February 11, 2017.
[45] Natascha W, Wente W, Müller P. Application Note 180 – Eppendorf LoBind®: Evaluation of protein recovery in Eppendorf Protein LoBind Tubes and Plates [Internet]. Hamberg, Germany: Eppendorf AG; 2010. p. 1–5. Available at: https://www.google.co.uk/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=0ahUKEwjClOWPrIDaAhVqCMAKHR95CJ8QFgg1MAE&url=https%3A%2F%2Fonline-shop.eppendorf.us%2FUS-en%2Feshopdownload%2Fdownloadbykey%2F118990_Application-Note_186&usg=AOvVaw2PpGq2UXAzCc1nrwJrZfbm. Accessed June 7, 2017.
[46] Colvin MT, Silvers R, Ni QZ, Can TV, Sergeyev I, Rosay M, et al. Atomic resolution structure of monomorphic Ab42 amyloid fibrils. J Am Chem Soc 2016;138:9663–9674.
[47] Wälti MA, Ravotti F, Arai H, Glabe CG, Wall JS, Böckmann A, et al. Atomic-resolution structure of a disease-relevant Aβ(1-42) amyloid fibril. Proc Natl Acad Sci U S A 2016;113:E4976–84.
[48] Meisl G, Yang X, Hellstrand E, Frohm B, Kirkegaard JB, Cohen SIA, et al. Differences in nucleation behavior underlie the contrasting aggregation kinetics of the Aβ40 and Aβ42 peptides. Proc Natl Acad Sci U S A 2014;111:9384–9.
[49] Esbjörner EK, Chan F, Rees E, Erdelyi M, Luheshi LM, Bertoncini CW, et al. Direct observations of amyloid beta self-assembly in live cells provide insights into differences in the kinetics of Abeta(1-40) and Abeta(1-42) aggregation. Chem Biol 2014;21:732–42.
[50] Moores B, Drolle E, Attwood SJ, Simons J, Leonenko Z. Effect of surfaces on amyloid fibril formation. PLoS One 2011;6:e25954.
[51] Rocha S, Krastev R, Thünemann A, Pereira M, Möhwald H, Brezesinski G. Adsorption of amyloid beta-peptide at polymer surfaces: a neutron reflectivity study. Chemphyschem 2005;6:2527–34.