

## Cerebrospinal Fluid Biomarkers

# Amyloid $\beta$ 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  $\beta$  (A $\beta$ ) peptide ratios are more stable than A $\beta$ <sub>42</sub> alone when biofluids are exposed to two preanalytical conditions known to modify measurable A $\beta$  concentration.

**Methods:** Human cerebrospinal fluid (CSF) and culture media (CM) from human cortical neurons were exposed to a series of volumes and polypropylene surfaces. A $\beta$ <sub>42</sub>, A $\beta$ <sub>40</sub>, and A $\beta$ <sub>38</sub> peptide concentrations were measured using a multiplexed electrochemiluminescence immunoassay. Data were analyzed using mixed models in R.

**Results:** Decrease of measurable A $\beta$  peptide concentrations was exaggerated in longer peptides, affecting the A $\beta$ <sub>42</sub>:A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub>:A $\beta$ <sub>38</sub> ratios. However, the effect size of surface treatment was reduced in A $\beta$  peptide ratios versus A $\beta$ <sub>42</sub> alone. For A $\beta$ <sub>42</sub>:A $\beta$ <sub>40</sub>, the effect was reduced by approximately 50% (volume) and 75% (transfer) as compared to A $\beta$ <sub>42</sub> alone.

**Discussion:** Use of A $\beta$  ratios, in conjunction with concentrations, may mitigate confounding factors and assist the clinical diagnostic process for Alzheimer's disease.

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### Keywords:

Alzheimer's disease; Amyloid  $\beta$  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  $\beta$  (A $\beta$ <sub>42</sub>), 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], A $\beta$ <sub>42</sub> 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 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  $A\beta$  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  $A\beta$  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  $A\beta$  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  $A\beta$  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  $A\beta$  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.  $A\beta$  secreted from these cells into the CM represents a key biomarker for AD-relevant neurobiology. In this context, ratios of  $A\beta$  are increasingly used to understand nuances of amyloidogenic pathways [40], and it is important to expand knowledge of preanalytical factors affecting  $A\beta$  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-1 (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- $\mu$ M MEM nonessential amino acids (Life Technologies; cat. 11140-050), 100- $\mu$ M 2-mercaptoethanol (Life Technologies; cat. 31350-010), 50-U/mL penicillin and 50- $\mu$ g/mL streptomycin (Life Technologies; cat. 15070063), 5- $\mu$ g/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. elegans* gene ‘small body size’ [Sma]) inhibitors, SB431542 (10  $\mu$ M) and Dorsomorphin (1  $\mu$ M), 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 A $\beta$  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  $\mu$ L. Aliquots were refrozen at -80°C and later thawed at 21°C for 1 hour and assayed for A $\beta$ <sub>38/x-40/x-42</sub> using a Meso Scale Discovery V-PLEX A $\beta$  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  $\mu$ L) 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  $\mu$ L, 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  $\mu$ L of sample in each tube. This process took approximately 10 minutes to complete. Samples were then assayed immediately for A $\beta$ <sub>38/x-40/x-42</sub> 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  $\alpha$  at 0.05 and confidence intervals at 95%. The formula used for the mixed model was  $\text{lme}(\text{sample concentration} \sim \text{treatment} + X, \text{random} = \sim 1 | \text{sample}) + \epsilon$ , where the dependent variable is “sample concentration”—the average of duplicate concentration (or ratio) values of a given A $\beta$  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.  $\epsilon$  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 $\beta$ <sub>42</sub> (4.3% and 9.9%), A $\beta$ <sub>40</sub> (4.5% and 9.5%), and A $\beta$ <sub>38</sub> (1.6% and 5.3%) were within what is generally considered acceptable range for research assays (<20%).

#### 3.2. Effect of storage volume on A $\beta$ peptide ratio

Detectable A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub>/A $\beta$ <sub>38</sub> concentration was observed to be significantly lower (all  $P < .001$ ) in samples of

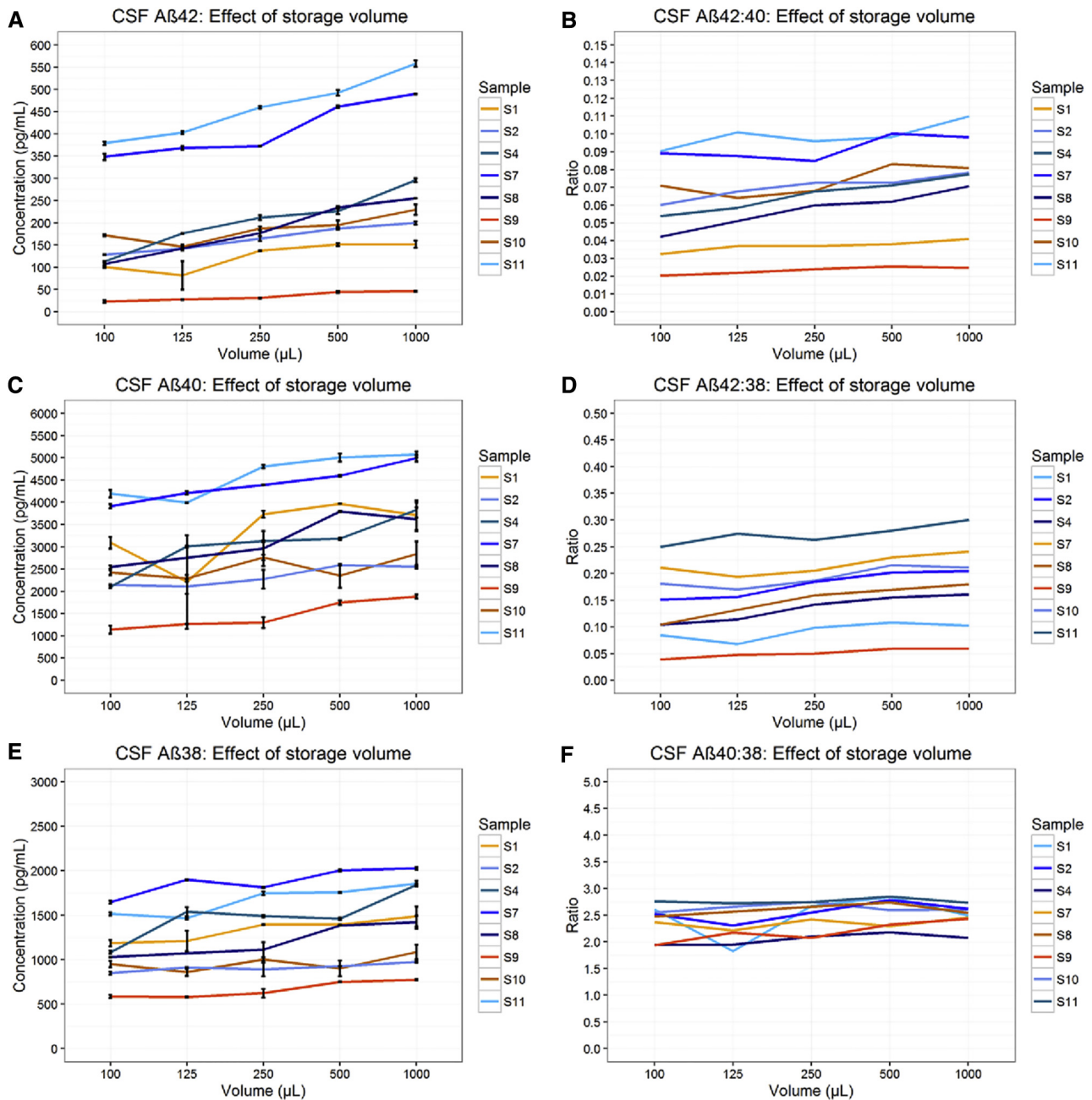


Fig. 1. Effect of storage volume on CSF Aβ. Results in CSF show the concentration of Aβ<sub>42</sub>, Aβ<sub>40</sub>, and Aβ<sub>38</sub> decreased with decreased storage volume (A, C, E). Concentration of Aβ<sub>42</sub> decreased proportionally more than Aβ<sub>40</sub> (B) and Aβ<sub>38</sub> (D) with lower storage volume, resulting in a decrease in the Aβ<sub>42</sub>:Aβ<sub>40</sub> and Aβ<sub>42</sub>:Aβ<sub>38</sub> ratios. (F) Aβ<sub>40</sub> showed a nonsignificant tendency to decrease more than Aβ<sub>38</sub>, (*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 Aβ<sub>42</sub>: 1.1 pg/mL (0.6%), Aβ<sub>40</sub>: 9.2 pg/mL (0.3%), and Aβ<sub>38</sub>: 3.1 pg/mL (0.2%), for every 10-μL change in CSF storage volume, and a concentration change of Aβ<sub>42</sub>: 0.5 pg/mL (0.3%), Aβ<sub>40</sub>: 2.5 pg/mL (0.2%), and Aβ<sub>38</sub>: 0.4 pg/mL (0.1%), for every 10 μL change in CM storage volume (Table 1). Results for CSF Aβ<sub>42</sub> are highly consistent

with those previously reported for control CSF (a change of 0.95 pg/mL per 10 μL) [6].

Concordantly, ratios of Aβ<sub>42</sub>:Aβ<sub>40</sub> and Aβ<sub>42</sub>:Aβ<sub>38</sub> changed significantly with storage volume. In CSF Aβ<sub>42</sub>:Aβ<sub>40</sub>, change was 0.2% of an initial ratio value per 10 μL (*P* < .001), and in CSF Aβ<sub>42</sub>:Aβ<sub>38</sub>, change was 0.3% per 10 μL (*P* < .001) (Table 1). In CM, change in the Aβ<sub>42</sub>:Aβ<sub>40</sub> and Aβ<sub>42</sub>:Aβ<sub>38</sub> ratios were 0.1% and 0.2%



Table 1  
Summary of results

Type	Peptide	Study	% Change per unit	P (α = 0.05)	95% confidence interval	
CSF	Aβ <sub>38</sub>	Vol	0.245	<.001	0.168	0.322
CSF	Aβ <sub>40</sub>	Vol	0.318	<.001	0.202	0.435
CSF	Aβ <sub>42</sub>	Vol	0.555	<.001	0.403	0.707
CSF	Aβ <sub>42</sub> :Aβ <sub>40</sub>	Vol	0.237	<.001	0.160	0.313
CSF	Aβ <sub>42</sub> :Aβ <sub>38</sub>	Vol	0.310	<.001	0.205	0.415
CSF	Aβ <sub>40</sub> :Aβ <sub>38</sub>	Vol	0.073	.054	-0.001	0.148
CSF	Aβ <sub>38</sub>	Tra	15.756	<.001	14.818	16.695
CSF	Aβ <sub>40</sub>	Tra	17.497	<.001	16.734	18.260
CSF	Aβ <sub>42</sub>	Tra	22.359	<.001	21.177	23.541
CSF	Aβ <sub>42</sub> :Aβ <sub>40</sub>	Tra	4.862	<.001	3.990	5.734
CSF	Aβ <sub>42</sub> :Aβ <sub>38</sub>	Tra	6.603	<.001	5.409	7.796
CSF	Aβ <sub>40</sub> :Aβ <sub>38</sub>	Tra	1.741	<.001	1.111	2.372
CM	Aβ <sub>38</sub>	Vol	0.105	<.001	0.053	0.156
CM	Aβ <sub>40</sub>	Vol	0.162	<.001	0.106	0.217
CM	Aβ <sub>42</sub>	Vol	0.331	<.001	0.235	0.427
CM	Aβ <sub>42</sub> :Aβ <sub>40</sub>	Vol	0.169	<.001	0.104	0.234
CM	Aβ <sub>42</sub> :Aβ <sub>38</sub>	Vol	0.226	<.001	0.160	0.293
CM	Aβ <sub>40</sub> :Aβ <sub>38</sub>	Vol	0.057	.028	0.007	0.108
CM	Aβ <sub>38</sub>	Tra	2.411	<.001	1.676	3.205
CM	Aβ <sub>40</sub>	Tra	4.789	<.001	4.103	5.711
CM	Aβ <sub>42</sub>	Tra	7.786	<.001	6.993	9.219
CM	Aβ <sub>42</sub> :Aβ <sub>40</sub>	Tra	3.148	<.001	2.315	4.082
CM	Aβ <sub>42</sub> :Aβ <sub>38</sub>	Tra	5.508	<.001	4.532	6.799
CM	Aβ <sub>40</sub> :Aβ <sub>38</sub>	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 Aβ<sub>42</sub>, Aβ<sub>40</sub>, Aβ<sub>38</sub>, 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 Aβ<sub>42</sub> alone. The ratio of Aβ<sub>40</sub>:Aβ<sub>38</sub> showed a trend toward decreased Aβ<sub>40</sub>, 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 Aβ<sub>42</sub>/Aβ<sub>40</sub>/Aβ<sub>38</sub> 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 Aβ<sub>42</sub>, an effect not observed in CM. The mean difference in Aβ<sub>42</sub> 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<sub>42</sub> lost (as compared to Aβ<sub>38</sub> = 8.8% and Aβ<sub>40</sub> = 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<sub>42</sub> loss at first transfer may have been due to adsorption to the pipette tip, we conducted a pilot experiment to measure Aβ<sub>42</sub> 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<sub>42</sub> 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<sub>42</sub> 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<sub>42</sub> decrease over serial tube transfers remained exaggerated in relation to Aβ<sub>40</sub> and Aβ<sub>38</sub> in both CSF and CM (Table 1). In CSF, the Aβ<sub>42</sub>:Aβ<sub>40</sub> ratio decreased by 4.9% per transfer, and the Aβ<sub>42</sub>:Aβ<sub>38</sub> decreased by 6.6% per transfer (Table 1). In CM, these were 3.1% and 5.5%, respectively. The decrease per transfer of Aβ<sub>40</sub>:Aβ<sub>38</sub> was 1.7% in CSF and 2.4% in CM; in both fluids, the decrease of Aβ<sub>40</sub> relative to Aβ<sub>38</sub> 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<sub>42</sub>/Aβ<sub>40</sub>/Aβ<sub>38</sub> 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<sub>42</sub>/Aβ<sub>40</sub>/Aβ<sub>38</sub> 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<sub>42</sub>:Aβ<sub>40</sub> and Aβ<sub>42</sub>:Aβ<sub>38</sub> demonstrated that decrease in Aβ<sub>42</sub> concentration per treatment unit was consistently

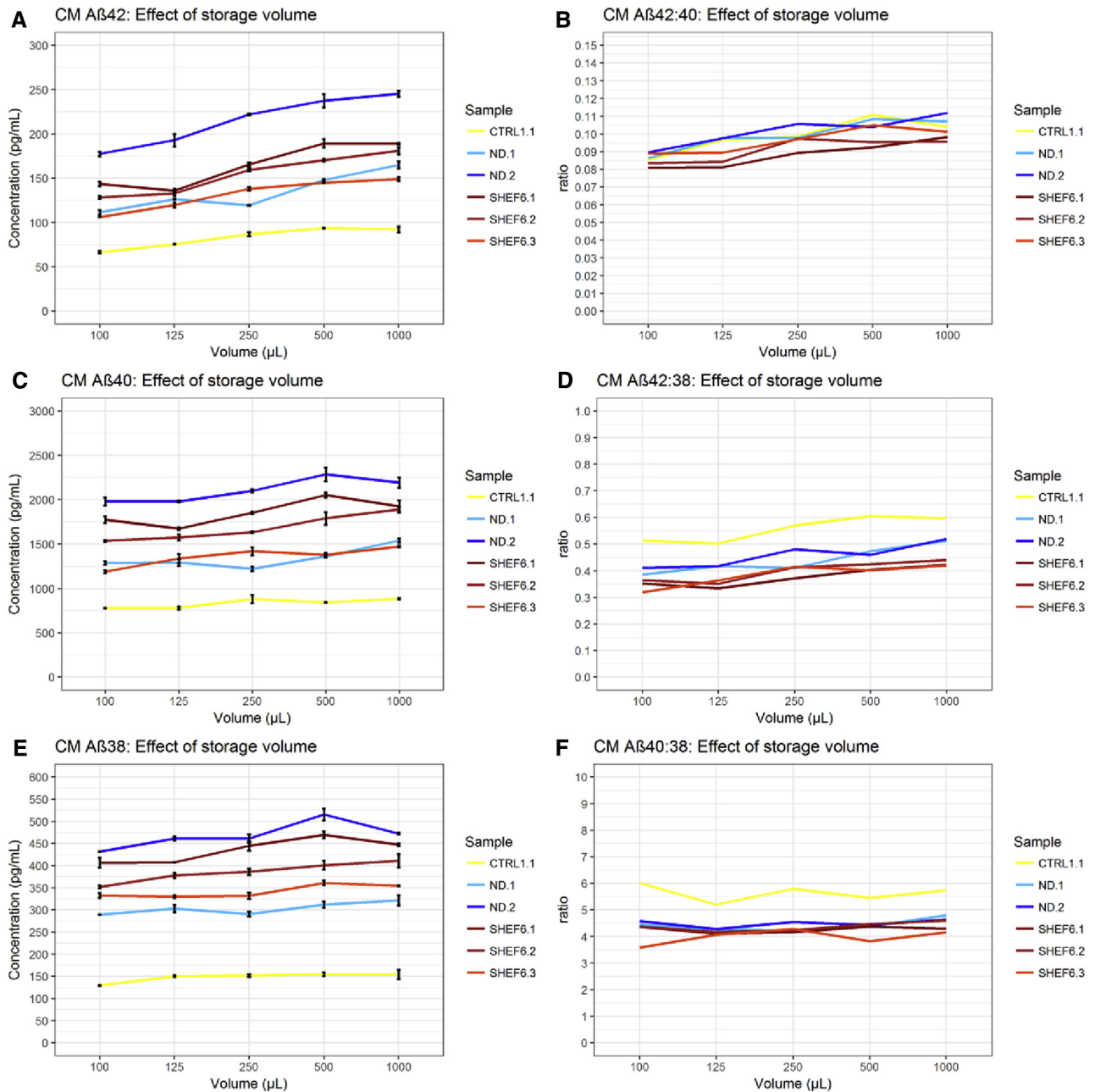


Fig. 2. Effect of storage volume on cell media Aβ. Results in CM show the concentration of Aβ<sub>42</sub>, Aβ<sub>40</sub>, and Aβ<sub>38</sub> decreased with decreased storage volume (A, C, E). Concentration of Aβ<sub>42</sub> decreased proportionally more than Aβ<sub>40</sub> (B) and Aβ<sub>38</sub>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

greater than that observed in Aβ<sub>40</sub> and Aβ<sub>38</sub>, in both CSF and CM. In addition, Aβ<sub>40</sub>:Aβ<sub>38</sub> ratios indicated that Aβ<sub>40</sub> may demonstrate a tendency toward greater concentration loss per treatment than Aβ<sub>38</sub>.

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 Aβ<sub>1-42</sub> (−13.6%), Aβ<sub>1-40</sub>

(−15.5%), and Aβ<sub>1-38</sub> (−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 Aβ<sub>42</sub>:Aβ<sub>40</sub> ratio was not significantly altered by the difference in volume, whereas Aβ<sub>42</sub>:Aβ<sub>38</sub> was altered by 3.4%. This is in contrast to our model that predicts larger, significant, changes in Aβ<sub>42</sub>:Aβ<sub>40</sub> (23.7%) and Aβ<sub>42</sub>:Aβ<sub>38</sub> (30.9%). It is worth noting that the volume effect is closely related to tube dimension [6,21], and our results

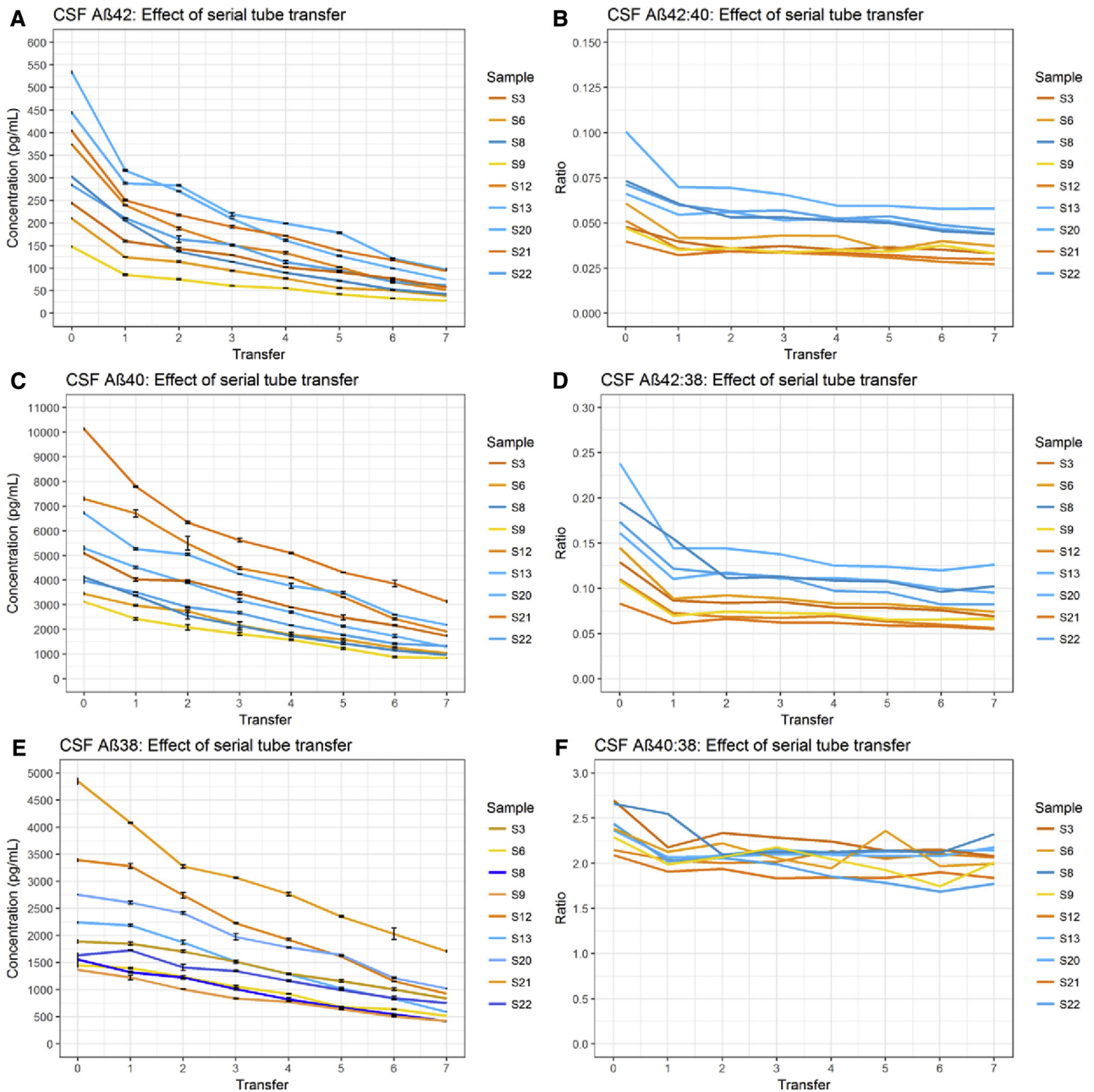


Fig. 3. Effect of serial tube transfer on CSF Aβ. Results in CSF show the concentration of Aβ<sub>42</sub>, Aβ<sub>40</sub>, and Aβ<sub>38</sub> decreased with consecutive transfer of sample to new storage tubes (A, C, E). Concentration of Aβ<sub>42</sub> decreased proportionally more than Aβ<sub>40</sub> (B) and Aβ<sub>38</sub> (D) with each transfer, resulting in a decrease in the Aβ<sub>42</sub>:Aβ<sub>40</sub> and Aβ<sub>42</sub>:Aβ<sub>38</sub> ratios. (F) In turn, the rate of Aβ<sub>40</sub> decrease with each transfer was greater than Aβ<sub>38</sub>. 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 Aβ<sub>42</sub> (11.0%), Aβ<sub>40</sub> (7.3%), and Aβ<sub>38</sub> (2.7%) in CSF collected into PP tubes. In addition, they report a concentration decrease of Aβ<sub>42</sub> (42.5%), Aβ<sub>40</sub> (27.8%), and Aβ<sub>38</sub> (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 Aβ<sub>42</sub> (39.0%) and Aβ<sub>40</sub> (24.1%), but smaller decrease of Aβ<sub>38</sub> (8.8%).

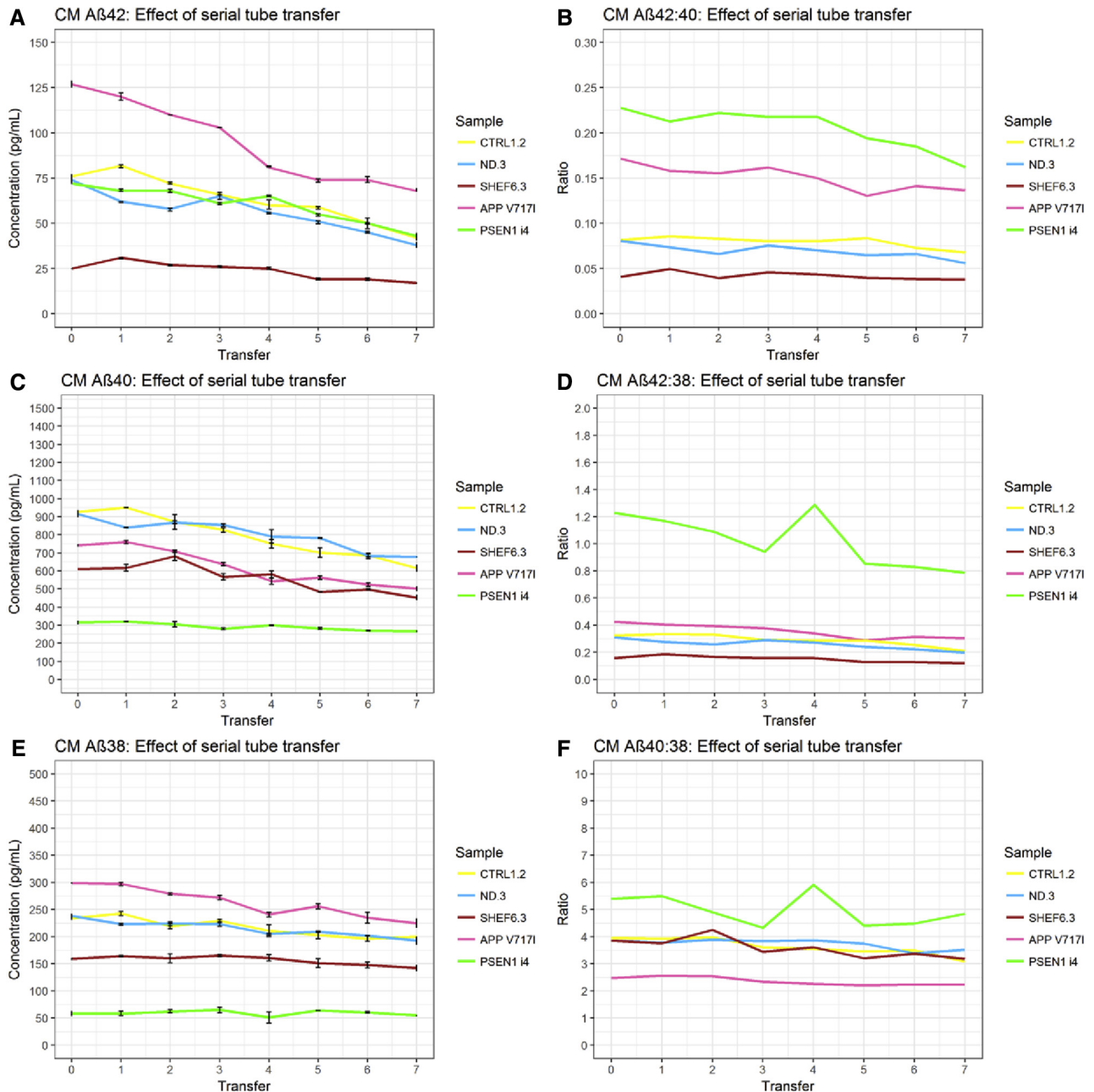


Fig. 4. Effect of serial tube transfer on cell media A $\beta$ . Results in CM show the concentration of A $\beta_{42}$ , A $\beta_{40}$ , and A $\beta_{38}$  decreased with consecutive transfer of sample to new storage tubes (A, C, E). Concentration of A $\beta_{42}$  decreased proportionally more than 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; A $\beta$ , amyloid  $\beta$ .

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 certi-

fied 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 A $\beta$  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



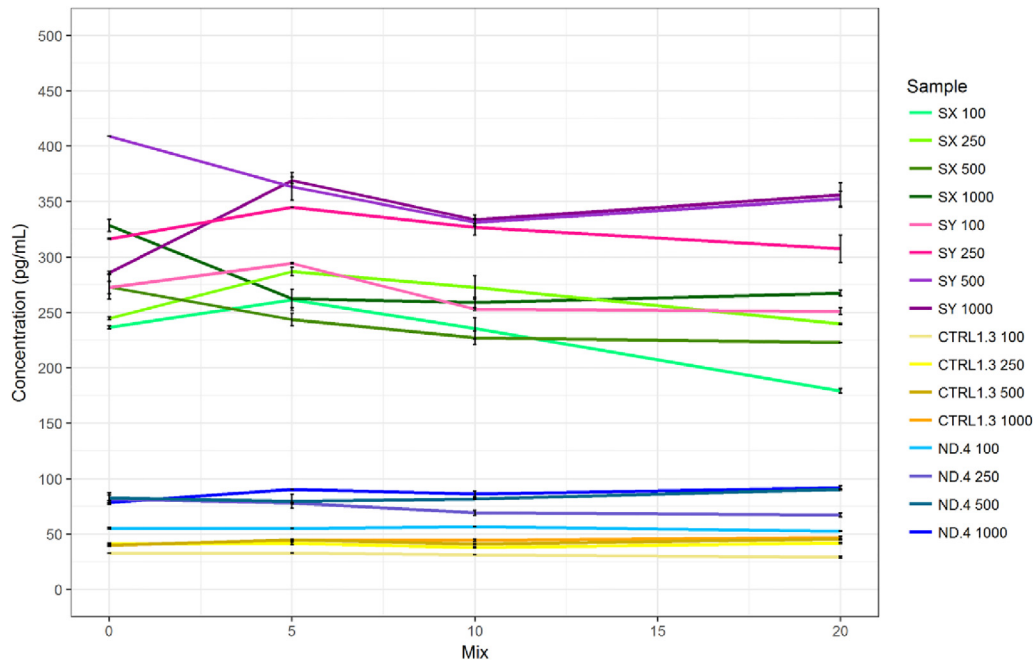


Fig. 5. CSF (SX and SY) and CM (CTRL and ND)  $A\beta_{42}$ : Effect of mixing by pipette across different storage volumes. Results show the concentration of  $A\beta_{42}$  were not significantly affected by different levels of exposure to the pipette tip over a series of different volumes in either 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;  $A\beta$ , amyloid  $\beta$ ; 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,  $A\beta$  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. $A\beta$ 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  $A\beta$  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  $A\beta$  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,  $A\beta$  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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The authors have declared that no conflict of interest exists.

### 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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