Letter to the Editor

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The impact of Tween 20 on repeatability of amyloid β and tau measurements in cerebrospinal fluid

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To the Editor,

Cerebrospinal fluid (CSF) concentrations of amyloid β peptides 38, 40 and 42 (Aβ38, Aβ40, Aβ42), total tau (T-tau) and phosphorylated tau_{181} (P-tau) are increasingly used as biomarkers to support a clinical diagnosis of Alzheimer's disease (AD), and to track disease progression in observational research studies and clinical trials [1]. It is therefore of great importance to understand the analytical repeatability (i.e. the variability of repeated measures of the same sample assayed by the same operator) of currently available enzyme-linked immunosorbent assays (ELISAs). In the interest of improving diagnostic reliability within and between sites there is a need to identify strategies to reduce analytical variance. Presently the U.S. Food and Drugs Administration (FDA) criteria for bioanalytical assay precision demands an average percent coefficient of variation (%CV) of <15% between measurements for a quantitative immunoassay to be regarded as having acceptable quality for clinical use [2]. Better analytical precision will be required to reliably monitor the in vivo biomarker responses to disease modifying drugs, and detect clinically relevant biomarker changes in patients over time, based on reports from longitudinal studies (e.g. P-tau 2.20 pg/mL decrease per year, Aβ42 decrease 11.9 pg/mL per year in AD-AD patients) [3].

The aim of this study was first to measure the analytical repeatability of ELISA-based assays for amyloid peptides and tau; and second to test the hypothesis that adding 0.05% Tween 20, a non-ionic surfactant that has previously been found to mitigate variation introduced by protein-tube surface interactions in CSF Aβ42 measurements [4, 5], would also decrease the variability between measurements.

Six de-identified CSF samples, each of 11 mLs, were used in this study: three were from individual subjects, and three pooled samples formed by mixing 2.2 mL CSF from five individual subject samples (Figure 1A and B). All samples were collected according to the standard operating procedure of the Sahlgrenska Academy at the University of Gothenburg (supplementary table in [6]), and with ethical approvals from the London Queen Square Ethics Committee (individual samples) and University of Gothenburg (pooled samples). Each of the six 11 mL CSF samples was split into two 5.5 mL samples (Figure 1C) and 2.75 μL (0.05%) Tween 20 was added to one of these (Figure 1D), following which both 5.5 mL samples were aliquoted at 500 μL volume, into 2 mL, polypropylene, DNase/RNase free tubes (Sarstedt, Nümbrecht, Germany, cat. 72.694.406) (Figure 1E). These aliquots were then stored at −80 °C. Aβ38, 40 and 42 were measured simultaneously in the same assay plate (MSD Aβ Peptide Panel 1 V-plex, 6E10 antibody). T-tau was measured separately using MSD T-tau V-plex assay. Both

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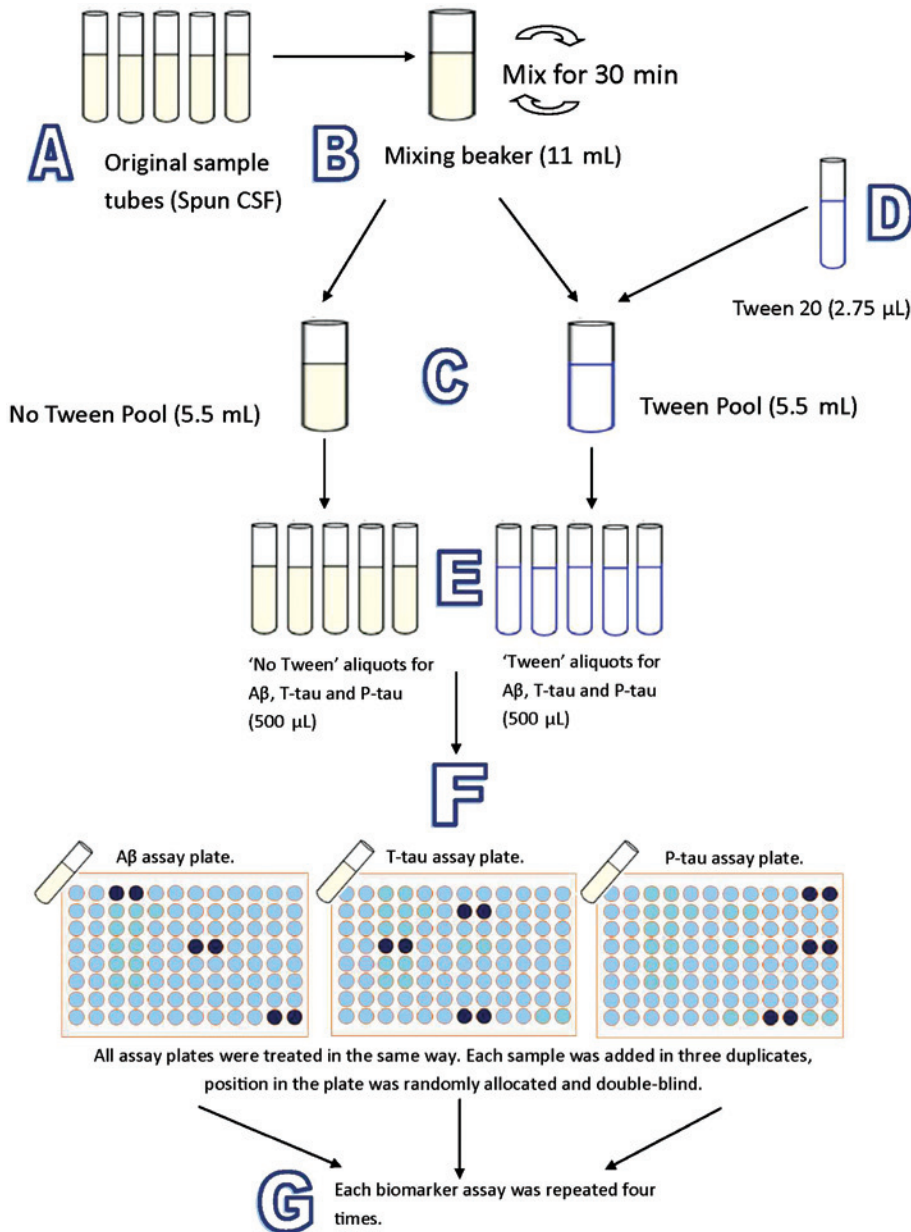

Figure 1: Experiment procedure.
Detailed are the various steps of the experiment.

of these assays were run on a Meso Scale Discovery 6000 platform. P-tau was measured separately by INNOTEST Phospho-tau (181P) ELISA and run using a BMG Labtech FLUOstar Omega multi-mode microplate reader (Figure 1F). In terms of sample layout within plate, each of the (three) biomarker assays were treated the same. Each sample was added to the plate in three pairs (i.e. six wells total – 1,2 | 3,4| 5,6), in order to collect intra-assay variation data for each sample (Figure 1F). It is common laboratory practice to use two wells for each sample (known as running a sample 'in duplicate'), and distributing samples in this way was intended to simulate this multiple times. Additionally, the identity of each sample tube was masked, and renamed in a different order by a colleague, thus the experiment was conducted under double-blind conditions. The experiment was repeated four times (i.e. four plates were run for Aβ, T-tau and P-tau) (Figure 1G), in order to collect data on inter-assay variation. Each repeat took place on a different day, and was conducted by the same operator.

To analyse measurement variance, %CVs were calculated according to ISO standards [7], and the results from aliquots with Tween added ('Tween') and those without ('No Tween') were compared using paired t-tests in R [8]. To investigate the contribution of covariates, linear

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Table 1: T-test and mixed model analysis.

T-test analysis

Sample	Aβ38		Aβ40		Aβ42		T-tau		P-tau	
	Intra (%CV)	Inter (%CV)	Intra (%CV)	Inter (%CV)	Intra (%CV)	Inter (%CV)	Intra (%CV)	Inter (%CV)	Intra (%CV)	Inter (%CV)
733	2.00	7.30	3.50	4.70	2.20	6.20	6.40	8.80	4.40	9.70
733T	2.50	5.70	3.70	5.40	2.10	2.70	4.00	7.80	1.90	7.70
724	3.00	5.40	3.50	3.50	1.90	3.50	5.40	8.20	1.70	5.50
724T	2.40	5.60	3.80	4.30	2.40	2.90	5.10	7.90	2.60	7.90
806	2.00	5.90	2.80	5.80	2.50	8.20	6.10	6.80	2.00	5.40
806T	2.70	5.30	3.50	3.80	2.50	3.50	2.50	9.20	1.40	4.00
Pool1	3.60	5.60	3.60	4.30	2.90	4.40	3.10	6.30	1.20	6.40
Pool1T	2.00	5.30	4.10	4.60	2.20	3.80	2.70	5.30	2.20	5.40
Pool2	3.20	5.50	2.10	4.70	2.10	6.40	3.90	8.90	2.40	5.10
Pool2T	2.60	4.90	3.70	4.80	2.50	3.30	12.70	12.70	2.20	5.50
Pool3	1.90	5.30	2.80	3.90	2.00	4.70	2.90	6.50	1.60	4.30
Pool3T	1.90	6.40	2.40	4.60	1.70	4.60	4.00	8.40	1.80	5.00
Average %CV Tween	2.35	5.53	3.53	4.58	2.23	3.47	5.17	8.55	2.02	5.92
Average %CV No Tween	2.62	5.83	3.05	4.48	2.27	5.57	4.63	7.58	2.22	6.07
Two-tailed t-test (df=5) p=	0.476	0.453	0.134	0.827	0.862	**0.043**	0.778	0.293	0.719	0.830

Mixed model analysis

CSF Subject type	Individual	Pool	Individual	Pool	Individual	Pool	Individual	Pool	Individual	Pool
Tween residual variance relative to No Tween	−7%	+4%	+0.3%	+19%	**−45%**	−15%	+14%	+11%	−6%	−10%
p=	0.698	0.820	0.986	0.318	**0.001**	0.364	0.461	0.591	0.700	0.558

T-test: The t-test analysis shows the mean intra- and inter-assay %CV by sample for each biomarker. This mean was calculated from 12 %CVs derived from each sample duplicate pair (n=3 within each plate) across all plates (n=4). A two-tailed, paired t-test compared Tween and No Tween sample versions for each biomarker. Inter-plate measurements of Aβ42 showed significant difference dependent on Tween status (p=0.04) with Tween samples having lower %CVs. Mixed model: Results of a linear mixed model analysis showing the effect of Tween 20 on measurement variation relative to samples without Tween for individual or pooled subject CSF for each biomarker. Results were calculated using a linear mixed effects model on data transformed by the natural logarithm (ln). Addition of Tween 20 to samples tended to lower the residual variance of Aβ42. However, this was only significant in individual subject CSF. Bold formatting marks values of statistical significance, and serves no other purpose.

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mixed model analyses were conducted using the nlme [9] package in R. To allow for increasing variance as concentration increased, the dependent variance in all analyses was concentration on a log scale (pg/mL). The interaction between Tween status and intra-assay sample repeat were the independent variables. A random intercept for sample was included, as was a random effect of Tween to allow for variability in the effect of Tween between samples.

The degree of variability between measurements for all samples and for all biomarkers, whether intra- or inter-assay, was <10% (Table 1), meaning that concentration measurements can be considered highly repeatable regardless of Tween 20 status. For measurements of Aβ42 in the same sample across different assays, %CVs of 'Tween' samples were significantly lower than in 'No Tween' samples ($p=0.04$) (Table 1 t-test). Further exploration with the use of linear mixed model analysis revealed that this result was driven by individual subject samples, whilst variance in pooled samples also decreased but did not reach significance (Table 1 Mixed model). No significant differences were found in any of the other biomarkers. Finally, concentration of Aβ peptides increased significantly with Tween (Aβ38=42% increase, $p\leq 0.0001$; Aβ40=43% increase, $p\leq 0.0001$; Aβ42=69% increase, $p\leq 0.0001$). No significant change was observed in the detectable concentration of P-tau ($p=0.9$), with a trend towards an increase in T-tau ($p=0.9$).

These results are consistent with data previously reported [4, 5], and showed that measurement variation was very low in all biomarkers throughout this study. Furthermore, results showed that repeat measurement variation of Aβ42 in individual subject CSF was improved by the addition of 0.05% Tween 20. If this finding generalises to datasets with greater levels of analytical variance, e.g. multi-site initiatives, it could be of clinical relevance. Following recent setbacks in AD drug development [10], it is now widely considered that a longitudinal and collaborative approach to research and clinical trial work in neurodegenerative disease is required if meaningful therapeutic progress is to be achieved. Reliable measurements are essential to such a strategy, and the potential to improve this for Aβ42 by adding 0.05% Tween 20 is worth further consideration. Despite the number of repeats, a limitation of this study was the small number of samples tested. Results should be treated as preliminary, and provide a reference effect size to inform future work.

This study shows that ELISA based measurement of neurodegenerative biomarkers in CSF treated with and without 0.05% Tween 20 can be highly repeatable for individual and pooled patient samples given strict standardisation of procedure.

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