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Effect of Patient-Specific Preanalytic Variables on CSF A β _{1–42} Concentrations Measured on an Automated Chemiluminescent Platform

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Abstract

Background: Cerebrospinal fluid (CSF) biomarkers are increasingly used to confirm the accuracy of a clinical diagnosis of mild cognitive impairment or dementia due to Alzheimer disease (AD). Recent evidence suggests that fully automated assays reduce the impact of some preanalytical factors on the variability of these measures. This study evaluated the effect of several preanalytical variables common in clinical settings on the variability of CSF β -amyloid 1–42 (A β _{1–42}) concentrations.

Methods: A β _{1–42} concentrations were measured using the LUMIPULSE G1200 from both freshly collected and frozen CSF samples. Preanalytic variables examined were: (1) patient fasting prior to CSF collection, (2) blood contamination of specimens, and (3) aliquoting specimens sequentially over the course of collection (i.e., CSF gradients).

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SUPPLEMENTAL MATERIAL

Supplemental material is available at *The Journal of Applied Laboratory Medicine* online.

Results: Patient fasting did not significantly affect CSF A β_{1-42} levels. While assessing gradient effects, A β_{1-42} concentrations remained stable within the first 5 1-mL aliquots. However, there is evidence of a gradient effect toward higher concentrations over successive aliquots. A β_{1-42} levels were stable when fresh CSF samples were spiked with up to 2.5% of blood. However, in frozen CSF samples, even 0.25% blood contamination significantly decreased A β_{1-42} concentrations.

Conclusions: The preanalytical variables examined here do not have significant effects on A β_{1-42} concentrations if fresh samples are processed within 2 h. However, a gradient effect can be observed on A β_{1-42} concentrations after the first 5 mL of collection and blood contamination has a significant impact on A β_{1-42} concentrations once specimens have been frozen.

INTRODUCTION

Alzheimer disease (AD), the most prevalent form of age-related dementia, is distinguished by neuronal degeneration, leading to progressive loss of cognitive functions (1, 2). A diagnosis of AD, based on clinical criteria, is uncertain in the clinical stage of mild cognitive impairment (MCI) or mild dementia, making definitive diagnosis challenging (3). Accuracy of clinical diagnostic criteria early in disease course is also limited by overlapping clinical profiles among individuals with other causes of cognitive decline. For example, studies have found that 12–23% of patients diagnosed with mild AD do not meet neuropathological criteria (4).

Since pathological changes in the brain are known to be reflected in CSF, biomarkers have increasingly been utilized as a tool to aid in diagnosis of MCI and dementia due to AD (5). The revised research criteria for MCI and dementia due to AD, as well as the A/T/N framework for AD, emphasize the importance of using biomarkers to improve accuracy of diagnosis in research settings, with the ultimate goal of application to routine clinical use (6–8). These recommendations are based on evidence that CSF biomarkers can detect underlying neuropathology when mild cognitive symptoms are present (8). Altered concentrations of CSF core protein biomarkers [total tau (T-tau), phosphorylated tau (p-Tau), and amyloidbeta 42 (A β_{1-42})] have demonstrated utility in characterizing biochemical alterations associated with AD (9). More specifically, fibrillar A β_{1-42} is the main peptide responsible for the generation of amyloid plaques, one of 2 pathological markers of AD. In humans, brain amyloid pathology is associated with low levels of A β_{1-42} in CSF (10). However, AD CSF biomarker concentrations can show considerable variation due to the impact of preanalytic variables such as tube type, temperature, centrifugation, blood contamination, sample gradient, etc. (8, 11). Preanalytic variability is partially negated by the advancement of automated assays; however, additional studies are needed to determine the impact of preanalytic variables to address the applicability of these assays in clinical settings.

Recent international studies have emphasized concentration inconsistency of core AD biomarkers and cut-off values between centers, using assay kits that are not fully automated, with the most significant variability seen in A β_{1-42} concentrations (12). Therefore, automated assays have been developed to improve standardization, so reproducibility and interpretation of biomarker results can be used in both research and clinical settings. Recent

reports suggest automated assays reduce variability in A β _{1–42} concentrations (13). This study aims to evaluate the impact of real-world preanalytical variables (fasting, inadvertent blood contamination, CSF gradients) particularly prone to vary in clinical settings at an individual level as opposed to systemic variables (e.g., tube type, centrifugation, etc.) that can be standardized. This study also sought to address recent reports of significantly reduced analytical variability, using frozen specimens, in one of the new chemiluminescent enzyme immunoassay platforms, the LUMIPULSE G1200 (13).

METHODS

Research Participants

CSF was obtained from 35 patients examined in the Johns Hopkins Center for CSF disorders who provided consent for biospecimen banking for research. This included patients with a range of neurologic disorders [e.g., normal pressure hydrocephalus (NPH) and AD] and patients with no history of a cognitive disorder who were referred for lumbar puncture (LP) for assessment of headache and whose opening pressures and CSF constituents were normal. Patients diagnosed with idiopathic NPH met standard guidelines (14). Patients diagnosed with AD satisfied NIA–AA criteria based on clinical, cognitive, and neuroimaging assessments (15). The Institutional Review Board of Johns Hopkins School of Medicine approved this study.

Lumbar Puncture Procedures

LP procedures were performed under sterile conditions. Patients had a spinal needle placed between the L3/L4 or L4/L5 intervertebral space. LPs were done using a Quincke needle (22 G) as is standard of care at the center.

Sample Testing

Each sample was analyzed within 2 h of collection after additional processing steps from appropriate protocols. A β _{1–42} was quantified directly from the collection tube or as specified by appropriate protocol using the LUMIPULSE G β -Amyloid 1–42 assay. CSF samples were tested using fully automated chemiluminescent enzyme immunoassay (CLEIA), LUMIPULSE G1200 (Fujirebio Diagnostics). Operators were trained and tested over 3 days to ensure competency with the analyzer and sample handling procedures. Quality control testing was performed beginning each test day to ensure control levels (low, medium, high) were within target ranges (see Supplemental Table 1 in the online Data Supplement). Each patient CSF sample was used to test a single preanalytic variable. All samples were coded; no information could be identified to an individual patient.

Impact of Fasting Prior to Lumbar Puncture

Patients evaluated for impact of food intake on CSF measures were admitted to the inpatient unit. Each patient underwent an extended lumbar drainage trial following clinical standard of care. Patients had a flexible catheter placed into the lumbar spine, draining CSF into a drainage chamber for 3 days. The time, caloric counts, and type of food consumed were recorded (Supplemental Table 2). On the third morning of CSF drainage, the initial CSF sample was drawn just before the patient consumed breakfast. Patients agreed to fast for a

minimum of 6 h before initial sample collection. Prior to collection of CSF blood glucose measurements were obtained by finger stick using the Bayer Contour Next Test Strip every 1 h $\times$ 10 min $\pm$ 3 h. To account for dead space in the lumbar catheter, 7 mL of CSF was discarded. Subsequently, 3 mL of CSF was collected, of which 1 mL was collected into a polystyrene tube provided in the Carefusion Safe-T plus Adult LP tray for CSF glucose measurements and 2 mL was collected in 2-mL Sarstedt 72.703.600 polypropylene tubes for LUMIPULSE G β -amyloid 1–42 measurements. Samples were transported on wet ice, centrifuged [2000g ($\pm$ 200g) 10 min ($\pm$ 30 s) at 5 $\pm$ 3 °C], and tested on the LUMIPULSE G1200.

Impact of Sample Gradient

LPs to assess impact of sample gradients were conducted in an outpatient setting. Twenty 1 mL CSF aliquots were collected from each patient sequentially by gravity drip method over 5–10 min into Sarstedt 72.703.600 tubes. Samples were transported on wet ice, centrifuged [2000g ($\pm$ 200 g) 10 min ($\pm$ 30 s) at 5 $\pm$ 3 °C], and analyzed.

Impact of Deliberate Blood Contamination

To examine the potential impact of inadvertent blood contamination on fresh CSF, 3 8 mL CSF samples were collected from each patient in an outpatient setting into Sarstedt 62.610.018 polypropylene collection tubes. Whole-blood samples were collected from the same patients using EDTA vacutainer tubes directly after LP. It was determined in a separate experiment that EDTA did not interfere with A β _{1–42} concentrations (Supplemental Table 3). Samples were transported and stored at room temperature until centrifugation [2000g ($\pm$ 200g) 10 min ($\pm$ 30 s) at 5 $\pm$ 3 °C], then split into 3 sets. Test and control aliquots were aliquoted into new Sarstedt 62.610.018 tubes. The third, sixth, and ninth unspiked CSF aliquots were controls and other aliquots were deliberately spiked with whole blood from the same patient (0.1, 0.5, 1, 2.5, 5, 10%). Samples were vortexed for 10 s and analyzed (Supplemental Table 4).

To examine potential impact of inadvertent blood contamination on frozen CSF a second set of CSF samples (2 mL each) were collected and deliberately spiked with varying amounts of whole blood (0.25, 0.5, 2.5, 5%) (Supplemental Fig. 1). Samples were stored in Corning MCT-150-C-S collection tubes in –80 °C freezers for 4 years, then thawed, vortexed for 3 s, and analyzed.

Statistical Analysis

Each experiment performed can be conceptualized as a repeated-measures analysis of variance (ANOVA) with the test condition as the factor of interest. We, therefore, implemented 1-way ANOVA with person-level random effects as the primary analysis. To ensure appropriateness of this analysis for the data, analyses were staged as follows:

First, fixed effects 2-way ANOVA with factors of test condition and person ID was performed. Residuals from these models were examined using *q*-normal plots to evaluate the assumption of normally distributed errors and identify extreme values. In cases where distribution was considerably skewed, a logarithm transformation was applied to the

response variable; this sufficed in all cases to yield reasonably normally distributed errors. Fresh blood spiking yielded 1–2 extreme outliers. Estimates of the person-level fixed effect were examined using q -normal plots to assess reasonableness of a normal random-effects assumption.

Mixed-effects 1-way ANOVA with a test condition factor and person-wise random effects were then performed. A normal distribution assumption was judged reasonable for most analyses; for 1 experiment where this was not the case, inferences were made using a bootstrap with 1000 replications. For the fresh blood spiking experiment, sensitivity analyses removing outliers were performed to assure analytic findings were not overly influenced by these values.

In all cases, the global Wald test of the null hypothesis of no variation among test conditions was performed. These addressed the sensitivity of biomarker analyses to variations in test conditions evaluated.

RESULTS

Study Participants

Ten patients were included in assessment of the impact of fasting: 2 cognitively normal (CN), 1 AD, 2 non-AD dementia (NAD), 5 NPH. A total of 6 patients participated in the assessment of the impact of sample gradient: 1 CN, 5 AD, and 18 patients participated in examination of blood contamination: 2 CN, 3 AD, 8 NAD, and 5 NPH. Demographic and clinical information for each patient is in Supplemental Tables 5–8.

Impact of Fasting

The impact of patient fasting on CSF $A\beta_{1-42}$, CSF glucose and blood glucose levels were evaluated at 4 time points (6 h fasting, 1 h postbreakfast, 2 h postbreakfast, and 3 h postbreakfast). As seen in Fig. 1 and Table 1, no significant changes in CSF $A\beta_{1-42}$ levels were observed postmeal (PM) time points relative to fasting state (6 h fasting: 1337 ± 107 ; 1 h PM: 1373 ± 122 pg/mL, 2 h PM: 1359 ± 119 pg/mL; 3 h PM: 1362 ± 121 pg/mL). This result is consistent with studies investigating plasma $A\beta_{1-42}$ levels with and without fasting (16). Of note, blood glucose concentrations varied significantly over time. There was a peak at 1 h, followed by a progressive return to near-baseline values ($P < 0.001$). CSF glucose exhibited a dip at 1 h (-0.041 units) followed by a progressive increase thereafter ($P = 0.016$). There was no statistically significant variation in CSF $A\beta_{1-42}$ concentration over fasting/breakfast conditions ($P = 0.886$). Both CSF analyses were considerably more precise than the blood glucose analysis, with nearly all variation in measurements (94–99%) attributable to differences between people. In blood, 34% of overall variation was attributable to glucose measurement error (Table 1 and Supplemental Fig. 2).

Impact of Deliberate Blood Contamination

The impact of blood contamination on $A\beta_{1-42}$ concentration in fresh and frozen CSF samples was examined. In fresh CSF, concentrations varied significantly across test conditions ($P < 0.001$), but this appeared to be driven primarily by spiking concentrations

above 2.5%. In 7 fresh CSF samples spiked with levels of whole blood up to 10%, CSF $A\beta_{1-42}$ levels were stable at 0.1, 0.5, 1.0, and 2.5% blood contamination with a mean percentage difference and standard deviation of $0 \pm 2\%$. (Fig. 2, Table 1). In frozen CSF, $A\beta_{1-42}$ concentrations were highly sensitive to spiking with even low concentrations of blood. In frozen CSF samples from 11 patients, blood contamination as little as 0.25% significantly affected $A\beta_{1-42}$ concentrations (Fig. 3, Table 1). When 2 outliers (5 and 10% from patient 1) from the fresh blood spiking experiment were removed, the extent of difference in mean for these test conditions versus without spiking, while still significant, was reduced considerably.

Impact of Sample Gradient

$A\beta_{1-42}$ concentration (pg/mL) was measured in 20 serially collected 1-mL CSF aliquots. Three replicates were performed for each aliquot. There was a significant trend in concentrations over aliquots, with lowest concentrations found over the first 5 mL and highest concentrations in the later aliquots ($P = 0.027$) (Table 1). Linear regression was performed characterizing $A\beta_{1-42}$ concentrations as a straight-line relationship with aliquot number: the coefficient was 0.810 (indicating an increase in concentration, on average, of 0.81% per successive aliquot) with $P = 0.001$ and 95% CI = (0.344, 1.28). A residual plot indicated this model fit well on average, but revealed evidence of considerable serial correlation (Fig. 4). In the generalized estimating equations analysis (with distinct aliquot terms), evidence for between-aliquot variation was stronger than in the mixed-effects model ($P = 0.0012$). In the AR1 fit, the estimated serial correlation in $A\beta_{1-42}$ concentration between successive aliquots was 0.336 (Table 1). Over successive aliquots, the trend coefficient was very similar to the mixed-effects analysis ($b = 0.794$) and the statistical significance remained strong ($P = 0.005$; 95% CI = 0.236, 1.35). The estimated serial correlation between successive aliquots was 0.254 (Fig. 4).

There is evidence both of systematic “drift” toward higher concentrations over successive aliquots and of considerable, positive autocorrelation in concentrations over successive aliquots after accounting for systematic differences or trends.

DISCUSSION

The present results indicate that several preanalytic variables do not have a significant impact on a fully automated assay for CSF $A\beta_{1-42}$. Food intake did not affect CSF $A\beta_{1-42}$ levels, even though blood glucose measurements varied greatly. Additionally, sample gradient up to the first 5 mL of CSF did not appear to be an influencing factor for the LUMIPULSE G β -Amyloid 1–42 assay, but after the first 5 mL does show a trend toward increasing $A\beta_{1-42}$ concentrations. Last, the data suggests that fresh blood contamination up to 2.5% does not affect CSF $A\beta_{1-42}$ levels. However, even minimal blood contamination in frozen samples (e.g., 0.25%) significantly reduces CSF $A\beta_{1-42}$ levels. Collectively, these findings provide valuable guidelines for the assessment of CSF AD biomarkers in clinical settings.

Impact of Fasting

In routine clinical practice, fasting is not recommended for diagnostic LPs, as other routine CSF measures are not affected by food. Moreover, it is not uncommon for clinical research sites to request that patients fast at least 6 h prior to the LP to reduce variability (6, 17). This can deter recruitment if a patient is to fast overnight. Although there have been studies that show the stability of A β levels in plasma (16), our study is the first to measure fresh CSF A β_{1-42} concentrations in patients after an overnight fast followed by a typical breakfast. Concentration of A β_{1-42} was found to be stable, regardless of the fasting state, while blood glucose measurements varied greatly. Thus, the present findings suggest that fasting before LP is not required to generate stable CSF A β_{1-42} results.

Impact of Deliberate Blood Contamination

Often CSF is contaminated by blood during an LP procedure (16). A traumatic tap with a significant amount of blood can be expected in approximately 14–20% of (18). Blood contamination can lead to an increase in protein degradation after 6 h of incubation (16,19). At present, there is no consensus about the precise definition of a traumatic tap. However, it is recommended that CSF be discarded if it is visibly blood-contaminated (16, 20). In this study, CSF A β_{1-42} concentrations were evaluated in both frozen and fresh CSF spiked with blood. Janelidze et al. observed dose-dependent and time-dependent decreases in A β_{1-42} levels after blood contamination in freshly collected CSF stored at room temperature for up to one (21). In our study, blood contamination did not negatively affect the integrity of A β_{1-42} concentrations in freshly collected samples spiked with up to 2.5% fresh blood if samples were processed and analyzed within 2 h of collection, even though samples were visibly grossly contaminated (Fig. 5). Processing and analysis within 2 h can be accomplished in most clinical labs that process CSF routinely.

Interestingly, when frozen CSF samples spiked with blood (0.25%) were evaluated, significant decreases in A β_{1-42} concentrations were observed even at low concentrations. This suggests blood borne proteases may induce protein degradation after 2 h of collection or during the freeze-thaw process and A β_{1-42} releases from amyloid-binding proteins (8, 11, 19) Janelidze et al., studied blood contamination effects at 0.1, 1, and 10% after being stored for 2 weeks at –20 °C and used a roller mixer before analysis. In the case of visible blood contamination, they did not notice a significant change in A β_{1-42} concentrations up to 10% at measurement. Their study differs from ours since our samples were frozen for 4 years after collection as is common in longitudinal biomarker studies since long-term storage does not significantly impact A β_{1-42} concentrations (22, 23), thawed at room temperature and vortexed for 3 s before being measured.

Since traumatic taps are common in clinical practice, this study supports previous findings that samples should be transported to the laboratory directly after collection and blood contaminated CSF should not be analyzed if samples have undergone one freeze-thaw cycle to avoid misinterpretation of A β_{1-42} protein expression levels.

Impact of Sample Gradient

Recently, studies have revealed concern regarding potential sample gradient effects on CSF. Specifically, studies have suggested that removing too small a volume of CSF might only reflect the $A\beta_{1-42}$ levels in the lumbar thecal sac, while removing a large volume may increase the concentration of brain-derived proteins, like $A\beta_{1-42}$ (16). Additional studies suggest a standard CSF volume should be collected at LP (16). However, there is no standard consensus on what amount should be collected. This lack of consensus can be attributed to the variety of protein and peptides in CSF with different physicochemical properties (24). We did not observe any difference in $A\beta_{1-42}$ levels within the first 5 mL of collection. However, after 5 mL of CSF collection, a gradient effect toward higher concentrations over successive aliquots emerged, demonstrating serial CSF sampling can alter physiology, confounding results. While other studies have not observed this gradient effect, there are pertinent differences. First, most have examined CSF after freezing or tube transfer rather than testing fresh CSF, potentially impacting $A\beta_{1-42}$ concentration due to additional handling (11, 25). Additionally, many studies have tested CSF in varying volumes of aliquots within the same collection or large aliquots of 10 mL after discarding the initial 2–3 mL (11, 16). In contrast, when CSF was collected in 1-mL aliquots, as is done in routine clinical practice, our study detected no significant gradient effects up to 5 mL with respect to fresh CSF $A\beta_{1-42}$ concentrations negating the need to discard precious CSF.

LIMITATIONS

The present study is not without limitations. It is possible that Type II error may be present in our analyses; thus, in future studies a larger sample cohort will prove beneficial in addressing this topic. A more robust patient cohort including various forms of mixed dementia would also be useful to address whether the patient outlier result, in the fresh blood contamination study is specific to the patient's condition (Fig. 2). In the present study, samples were spiked and analyzed within $2 \text{ h} \pm 10 \text{ min}$ from collection time. Investigating longer incubation times after CSF spiking would be helpful in fully understanding the impact of blood contamination in CSF and analyzing $<0.1\%$ blood contamination effects.

Furthermore, we did not examine the impact of CSF handling factors such as collection and storage tube type, extended storage conditions, sample volume and mixing, freeze–thaw effects, and transport conditions in the present study as that is the subject of a separate analysis. We also did not analyze $A\beta_{1-40}$ t-tau, and p-tau analytes. However, future studies have been initiated to investigate the potential impact of these preanalytical factors and their impact on these additional core proteins will be reported later.

CONCLUSIONS

The ability to analyze AD biomarkers in routine clinical practice requires establishment of preanalytical protocols and handling across the CSF biomarker landscape to ensure uniformity within the field. If CSF biomarkers are to be adopted into clinical routine, it is essential to have uniform preanalytical protocols for CSF handling. The utilization of universal preanalytical protocols will permit accurate comparison of CSF biomarkers and reduce potential diagnostic variability found in biomarker analysis between laboratories and

multicenter trials. We demonstrate for the first time that fasting is not critical, highlight the differential effects of blood contamination on fresh vs frozen CSF samples, and that gradients up to the first 5 mL do not materially change A β _{1–42} concentrations, but demonstrate a gradient effect toward higher concentrations with continued serial sampling. Our findings regarding impact of fasting, blood contamination, and CSF gradients are essential in refining the clinical protocol for future application of CSF biomarkers in clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations:

CSF	cerebrospinal fluid
LP	lumbar puncture
A β _{1–42}	β -amyloid 1–42
AD	Alzheimer disease
NPH	normal pressure hydrocephalus

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IMPACT STATEMENT

While the utility of CSF biomarkers in the diagnosis of Alzheimer disease has been shown in multiple research studies, the transition to clinic has been fraught with concerns about the influence of preanalytic variables on the assays used to measure biomarkers, in particular A β 42. While analytic variability has been reduced by automated electrochemiluminescent assays and standardized protocols and tubes have minimized some preanalytic variables, the influence of food, variable blood contamination, and CSF gradients has not been systematically studied. This study specifies how these variables influence A β 42 concentrations and provides interpretation of factors likely to be encountered in clinic settings.

Fig. 1.

Impact of fasting on CSF Aβ₁₋₄₂ levels. Aβ₁₋₄₂ concentration (pg/mL) in fresh CSF measured in fasted patients (>6h), and 1, 2, and 3h postbreakfast. Three replicates were performed for each time point. Error bars represent SEM.

Fig. 2.

Impact of blood contamination on CSF Aβ₁₋₄₂ levels in fresh specimens. Aβ₁₋₄₂ concentration (pg/mL) in fresh CSF spiked with whole blood (0.1, 0.5, 1, 2.5, 5, 10%). Three replicates were analyzed for each time point. Error bars represent SEM.

Fig. 3.

Impact of blood contamination on CSF Aβ₁₋₄₂ levels in frozen specimens. Aβ₁₋₄₂ concentration (pg/mL) in frozen CSF samples ($n=11$) spiked with whole blood (0.25, 0.5, 2.5, 5%). Mean storage time for frozen specimens, 48 months. Two replicates were performed for each time point. Error bars represent SEM.

Fig. 4.

Impact of gradient effects on CSF $A\beta_{1-42}$ levels in fresh specimens. CSF $A\beta_{1-42}$ values adjusted for patient-specific fixed effects vs aliquots with the fitted trend line from the regression overlaid. Adjusting for patient-specific effects, these were subtracted from the CSF $A\beta_{1-42}$ values.

Fig. 5. Visualization of fresh CSF spiked with varying concentrations of fresh blood. Tubes of fresh CSF spiked with whole blood. From left to right: neat, 0.1, 0.5, 1, 2.5, 5, and 10%.

Table 1.

Analysis of preanalytic effects on $A\beta_{1-42}$ levels.

Experiment	Condition	Difference vs reference ^a	condition	P value	95% CI	Global test P value	Fraction of variance due to person
Fasting-CSF $A\beta_{1-42}$ (log values)	1 h post	-4.2		0.737	-28.7, 20.3	0.886	0.993
	2 h post	-9.5		0.447	-34.0, 15.0		
	3 h post	-2.0		0.873	-26.5, 22.5		
Spiking—frozen CSF $A\beta_{1-42}$	0.25%	-223.6		0.020	-411.4, -35.8	<0.001	0.555
	0.50%	-381.7		<0.001	-569.5, -193.9		
	2.50%	-784.5		<0.001	-922.4, -546.7		
	5.00%	-785.6		<0.001	-973.4, -597.8		
	0.10%	0.286		0.993	-67.5, 68.1	<0.001	0.982
Spiking—fresh CSF $A\beta_{1-42}$	0.50%	-1.71		0.960	-69.5, 66.1		
	1.00%	-2.86		0.934	-70.7, 64.9		
	2.50%	-13.0		0.707	-80.8, 54.8		
	5.00%	-85.4		0.014	-153.2, -17.6		
	10.00%	-95.6		0.007	-160.3, -24.8		
	2	4.8		0.572	-11.95, 21.62	0.027	0.997
	3	5.5		0.521	-11.28, 22.28		
	4	2.0		0.815	-14.78, 18.78		
	5	3.7		0.669	-13.12, 20.45		
	6	12.7		0.139	-4.12, 29.45		
Gradient—aliquots	7	-2.8		0.741	-19.62, 13.95		
	8	-2.7		0.755	-19.45, 14.12		
	9	8.3		0.330	-8.45, 25.12		
	10	15.8		0.064	-95, 32.62		
	11	6.8		0.425	-9.95, 23.62		
	12	11.0		0.199	-5.78, 27.78		
	13	20.8		0.015	4.05, 37.62		
	14	6.7		0.436	-10.12, 23.45		
	15	4.5		0.599	-12.28, 21.28		
	16	14.8		0.083	-1.95, 31.62		

Experiment	Condition	Difference vs reference ^a	condition	P value	95% CI	Global test P value	Fraction of variance due to person
Fasting—blood glucose	17	12.8		0.134	−3.95, 29.62		
	18	29.8		0.000	13.05, 46.62		
	19	11.2		0.192	−5.62, 27.95		
	20	12.2		0.155	−4.62, 28.95		
Fasting—CSF glucose (log values)	1 h post	45.8		<0.001	22.5, 69.1	<0.001	0.661
	2 h post	11.2		0.345	−12.1, 34.5		
	3 h post	4.9		0.680	−18.4, 28.2		
	1 h post	−0.041		0.089	−0.09, 0.01	0.016	0.941
	2 h post	0.002		0.918	−0.05, 0.05		
	3 h post	0.037		0.130	−0.01, 0.08		

^aFor all fasting experiments: reference = 9 h fasting. For both spiking experiments, reference = none. For the gradient experiment, reference was the first aliquot.