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RESEARCH ARTICLE

Pre-analytical protocol for measuring Alzheimer's disease biomarkers in fresh CSF

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Abstract

Introduction: We aimed to establish a standardized, routine-use pre-analytical protocol for measuring Alzheimer's disease (AD) biomarkers in cerebrospinal fluid (CSF).

Methods: The effect of pre-analytical factors (sample collection/handling/storage/transportation) on biomarker levels was assessed using freshly collected CSF. Tube type/sterilization was assessed using previously frozen samples. A low-bind false-bottom tube (FBT, Sarstedt) was used for all experiments, except tube types/sterilization experiments. Biomarkers were measured using Elecsys CSF assays.

Results: Amyloid beta $(A\beta)_{1\text{-}42}$ levels varied by tube type, using a low-bind FBT reduced variation. $A\beta_{1\text{-}42}$ levels were higher with no mixing versus roller/inversion mixing. $A\beta_{1\text{-}42}$ levels were lower with horizontal versus upright transportation; this was resolved by maximal tube filling and storage at 2°C to 8°C. $A\beta_{1\text{-}40}$ levels were less strongly affected. Phospho-tau and total-tau levels were largely unaffected.

Discussion: We propose an easy-to-use, standardized, routine-use pre-analytical protocol, using low-bind FBTs, for measuring AD CSF biomarkers in clinical practice.

KEYWORDS
Alzheimer's disease, amyloid beta, biomarker, cerebrospinal fluid, diagnosis, pre-analytical, tau

1 | BACKGROUND

Accurate and timely diagnosis of Alzheimer's disease (AD) is vital to improve patient care and support clinical trials of novel treatments,[1] and will be especially important with the introduction of disease-modifying therapies, particularly amyloid-targeting immunotherapies. AD is usually diagnosed by exclusion, based on clinical evaluation, neuropsychological testing, and brain imaging.[2] However, clinical diagnosis

of AD is suboptimal, with variable accuracy rates depending on the clinical/neuropathological criteria used.[1,3,4] Furthermore, mixed pathologies are common in clinical Alzheimer's-type dementia.[5]

Cerebrospinal fluid (CSF) biomarkers for amyloid beta (Aβ) peptides A$\beta_{1\text{-}42}$ and A$\beta_{1\text{-}40}$, phospho-tau (181P; P-tau), and total-tau (T-tau) may improve the accuracy of early AD diagnosis and evidence to support their incorporation into routine clinical practice is accumulating. Biomarker ratios P-tau/A$\beta_{1\text{-}42}$ and T-tau/A$\beta_{1\text{-}42}$ have demonstrated high concordance with amyloid status according to positron-emission tomography and predict future clinical progression to AD.[4] Criteria for appropriate use of the lumbar puncture (LP) procedure and CSF testing in AD diagnosis have therefore been proposed[6] and incorporated into research diagnostic guidelines for AD.[1,4,7–10]

AD CSF biomarker measurements may be influenced by pre-analytical factors (eg, sample handling/storage), which can vary between clinical sites.[11–15] A$\beta_{1\text{-}42}$ levels are particularly affected by pre-analytical factors, as the peptide is prone to aggregation and surface binding; thus it is important to use polypropylene tubes for collection, as A$\beta_{1\text{-}42}$ will adhere to other types of tubes.[13,16] A new 2.5 mL low-bind false-bottom tube (FBT; Sarstedt) was recently developed to address this issue based on the present study. When assessing the impact of pre-analytical factors, selecting the correct reference standard is important and should ideally comprise non-processed CSF samples freshly collected (never frozen) in low-bind tubes. However, this approach has only been applied systematically in one study.[15] Furthermore, biomarker concentrations should be measured using highly reliable methods, for example, the Elecsys CSF immunoassays (Roche Diagnostics), so that subtle effects of pre-analytical factors can be evaluated. Studies utilizing frozen CSF samples and/or less reliable biomarker assays in their methodology are unlikely to detect all effects of pre-analytical variables.

Several criteria must be achieved before implementing AD CSF biomarkers in routine clinical practice: (1) development/validation of reliable assays for measuring biomarkers; (2) determination of criteria to guide appropriate use of these biomarkers; and (3) establishment of a pre-analytical protocol for measuring biomarkers and standardizing testing between laboratories. As mentioned above, the first two criteria have been achieved. Thus, until now, there has been an unmet need to develop a standardized pre-analytical protocol for measuring AD CSF biomarkers. We evaluated the impact of pre-analytical factors on the measurement of AD CSF biomarkers, including tube type, sample handling procedures, and storage/transportation conditions. We aimed to establish a simplified, easy-to-use CSF handling protocol, intended for implementation in clinical practice, to minimize variation in CSF biomarker measurement (in particular, loss of A$\beta_{1\text{-}42}$) and facilitate comparison of biomarker data between laboratories by using fresh CSF (increasing the possibility of using CSF analysis in locations that do not have immediate access to a laboratory or the ability to freeze samples at −80°C before transporting samples for analysis).

HIGHLIGHTS

- Biomarkers were measured in fresh, non-processed cerebrospinal fluid (CSF) collected in low-bind tubes
- CSF amyloid beta (Aβ)$_{1-42}$ levels were mildly to moderately affected by pre-analytical factors
- Pre-analytical factors had similar/slightly less pronounced effects on CSF Aβ_{1-40}
- CSF P-tau and T-tau levels were largely unaffected by pre-analytical factors
- We propose an easy-to-use, standardized, routine-use pre-analytical protocol

RESEARCH IN CONTEXT

- Systematic review: Cerebrospinal fluid (CSF) biomarkers amyloid beta (Aβ)$_{1-40}$ and Aβ_{1-42}, phospho-tau, and total-tau improve the accuracy of early Alzheimer's disease (AD) diagnosis. However, pre-analytical factors can influence CSF biomarker measurements, which may result in variability and complicate the implementation of these diagnostic tools in routine clinical practice.
- Interpretation: Following a comprehensive analysis of the impact of pre-analytical factors (including tube type, sample handling procedures, and storage/transportation conditions) on the measurement of AD CSF biomarkers, we propose a novel, simplified, standardized, routine-use pre-analytical protocol for handling fresh CSF samples prior to measuring AD biomarkers.
- Future directions: Evaluation of a standardized pre-analytical protocol for handling frozen CSF samples is ongoing, and data on mid-term storage of frozen samples is still required. Further research is also needed to evaluate how the new protocol will improve generalizability of biomarker results across clinical centers and testing laboratories.

JOURNAL CLASSIFICATION

- 280.030 Diagnostic Techniques and Procedures
- 280.080 Laboratory Techniques and Procedures
- 600.130 Clinical Laboratory Techniques
- 670.100 Neurodegenerative
- 670.210 Neurodegenerative, Tauopathies

2 | METHODS

2.1 | Study design

Most experiments used freshly collected (never frozen) CSF samples from patients undergoing diagnostic LP for suspected normal-pressure hydrocephalus in clinical practice at Skåne University Hospital in Malmö, Sweden (N = 35 patients). Inclusion criteria included ability to give informed consent and successful collection of ≥40 mL CSF; exclusion criteria were age <18 years and need for an interpreter. Studying patients with suspected normal-pressure hydrocephalus enabled collection of large CSF sample volumes for comparison of multiple pre-analytical factors. Fresh CSF samples were used to better simulate routine clinical practice and were collected directly into a novel low-bind FBT, suitable for use with several platforms. CSF was collected using an add-on lumbar fluid manometer (LFM), except when assessing the effect of using drip collection versus LFM introduction; whereas LFM is not used widely for the analysis of CSF biomarkers, add-on LFM is routinely used at the Malmö site to increase dripping speed when collecting large volumes of CSF. Samples were analyzed within 4 to 6 hours after collection. No further handling steps were performed beyond the steps varied in each pre-analytical factor experiment outlined in Section 2.3.

For experiments in previously frozen CSF (analyzed in-house at Roche), samples purchased from vendors were thawed for ≈30 minutes and roller-mixed for 20 minutes, and then pipetted into tubes. Some samples were refrozen/rethawed before analysis. No clinical data are known with respect to the previously frozen CSF samples.

CSF concentrations of $A\beta_{1\text{-}42}$, $A\beta_{1\text{-}40}$, P-tau, and T-tau were measured either with the Conformité Européen approved Elecsys CSF assays (frozen CSF samples) or using Elecsys β-Amyloid(1-42) CSF, Elecsys β-Amyloid(1-40) CSF, Elecsys Phospho-Tau (181P) CSF, and Elecsys Total-Tau CSF immunoassays (fresh CSF; all markers currently under development) on the cobas e 601 analyzer (Roche Diagnostics).

All samples (regardless of preparation method) were randomized within run before measurement. Statisticians were not blinded to the collection method of each sample, but all samples were analyzed identically, irrespective of the pre-analytical protocol, and the main results were validated by an independent statistical programmer. Comparisons between pre-analytical factors were performed within each individual.

The study protocol was approved by the regional ethics committee in Lund, Sweden, and the study was conducted in accordance with the principles of the Declaration of Helsinki and International Conference on Harmonization guidelines for Good Clinical Practice. All patients provided written informed consent.

2.2 | Study assessments: previously frozen CSF samples

The effect of tube type/manufacturer was assessed using different tube-filling volumes and the following tubes: Sarstedt FBT 2.5, and Sarstedt 2.0 and 0.5 mL; Nunc 1.8 mL; Eppendorf LoBind 1.5 mL and Snap-Cap 1.5 mL; and Nalgene 0.5 mL. The effect of using low-bind versus non–low-bind Sarstedt 2.5 mL FBTs was assessed using tube-filling volumes of 0.75, 1.5, and 2.5 mL. The effect of tube sterilization status (sterilization vs no sterilization) was assessed using the Sarstedt FBT and tube-filling volumes of 1 and 2.5 mL. Biomarker concentrations were measured after 24 hours of storage at room temperature (RT).

2.3 | Study assessments: freshly collected CSF samples

To evaluate if LFM had a methodological effect, gravity-drip collection versus LFM introduction was assessed using eight 1 mL aliquots of freshly collected CSF samples; aliquots 1 to 4 were collected using a gravity-drip method, and the LFM was introduced for the next four. Biomarker levels in each aliquot were compared with the average level measured in the first 4 mL of CSF collected via gravity-drip method (aliquots 1 to 4).

Effect of LP gradient was tested as part of the tube-filling volume experiment (see below). Data were analyzed by order of collection during LP, and the percentage change in measured biomarker level per mL of CSF collected was approximated based on the mean recoveries from the first 20 mL of CSF collected. The effect of using different mixing procedures (no mixing, roller-mixing, vortexing) was assessed using tube-filling volumes of 0.75 and 1.75 mL. The effect of sample storage conditions was assessed at different time-points for samples stored at RT (up to 8 days) and 2°C to 8°C (up to 15 days).

Sample contamination with blood (0%, 0.1%, 1%) was assessed using samples stored at RT (fresh and 6 days) and 2°C to 8°C (fresh and 14 days). CSF samples were spiked with patients' own whole blood according to these dilution procedures: 0.1%, 15 μL prediluted blood (1:10 dilution; 0.5 mL blood and 4.5 mL phosphate-buffered saline, roller-mixed for 15 minutes) was added to each 1.5 mL CSF aliquot and roller-mixed at RT for 15 minutes; 1%, 15 μL undiluted blood was added to each 1.5 mL CSF aliquot and roller-mixed at RT for 15 minutes. To assess the effect of centrifugation versus no centrifugation, the first 2 mL of CSF was collected and split into two aliquots—one centrifuged, one not. Blood contamination status (cell counts, visual hemolysis) was recorded, and biomarker measurements following centrifugation versus no centrifugation were compared.

Transportation/storage conditions were assessed using samples stored at room temperature and 2°C to 8°C with tube-filling volumes of 1.5 and 3 mL: upright transportation and direct measurement (4 to 6 hours after sample collection); upright transportation and measurement after 40 hours of storage in upright position; upright transportation and measurement after 40 hours of inverting; upright transportation and measurement after 40 hours of roller-mixing; horizontal transportation and direct measurement; horizontal transportation and measurement after 24 hours of horizontal storage; horizontal transportation and measurement after 40 hours of horizontal storage.

Effect of using different tube-filling volumes of 2.25 to 3 mL (increments: 0.25 mL) was assessed in samples transported in an

upright and horizontal position and stored at RT and 2°C to 8°C.

2.4 | Statistical analyses

To allow presentation of results across different concentrations and comparison between patients, CSF biomarker recoveries were calculated for each patient. Recovery (%) was defined as the ratio between the measured biomarker concentration and mean concentration measured in all aliquots of the reference condition specified for each experiment (see figure legends). For the centrifugation analysis, Spearman-Kendall correlations for biomarker concentrations were calculated in centrifuged versus non-centrifuged samples. Statistical analyses were performed using statistical programming language R (v3.2.2).

3 | RESULTS

3.1 | CSF samples

Previously frozen CSF sample pools, CSF samples from several individual donors, and freshly collected CSF samples from 35 patients (mean age, 75.5 years; 51% female) with suspected normal-pressure hydrocephalus were included in the analyses. Median (median absolute deviation) $A\beta_{1\text{-}42}$ concentration for these patients was 801.8 (278.5) pg/mL; additional characteristics and CSF biomarker levels/ratios are presented in Table S1.

3.2 | Impact of pre-analytical factors on measurement of $A\beta_{1\text{-}42}$

3.2.1 | Tube type/manufacturer, low-bind versus non–low-bind tube type, and tube sterilization

$A\beta_{1\text{-}42}$ recovery varied substantially by tube type/manufacturer, partly dependent on the tube-filling volume used (Figure 1A). Sarstedt FBT showed the highest robustness against absolute differences in fill volume, with no difference in recovery between 2.5 versus 1.66 mL fill volumes; Nunc and Eppendorf snap-cap were less robust. Sarstedt 2.0 and Eppendorf LoBind had higher variability in recoveries, and the fill volume effects were not consistent. In general, smaller fill volume led to lower recoveries, and smaller tubes (0.5 mL) had consistently lower recoveries (90% to 95%). Collection of CSF directly into a low-bind tube suggested a potentially reduced variation in $A\beta_{1\text{-}42}$ recovery, compared with using a non-low-bind tube (Figure 1B); however, only one aliquot per volume was available for testing the non-low-bind tube. Mean $A\beta_{1\text{-}42}$ recoveries were within ±5% of the reference condition (sterilized tube, 2.5 mL tube-filling volume) when using sterilized and unsterilized low-bind FBTs, indicating no clear impact of tube sterilization (Figure 1C and D). Collection technique and LP gradient are presented in the Supplementary Materials and Figure S1.

3.2.2 | Mixing procedure and storage conditions

Mean $A\beta_{1\text{-}42}$ recoveries were higher when no mixing was used, compared with roller-mixing or vortexing, for each tube-filling volume tested, although loss of recovery was minimal for the 1.75 mL tube-filling volume using vortexing (Figure 2A). The impact of tube-filling volume on $A\beta_{1\text{-}42}$ recovery varied according to mixing procedure used; vortexing showed strong volume dependency, with lower volumes resulting in lower $A\beta_{1\text{-}42}$ recovery (Figure 2A).

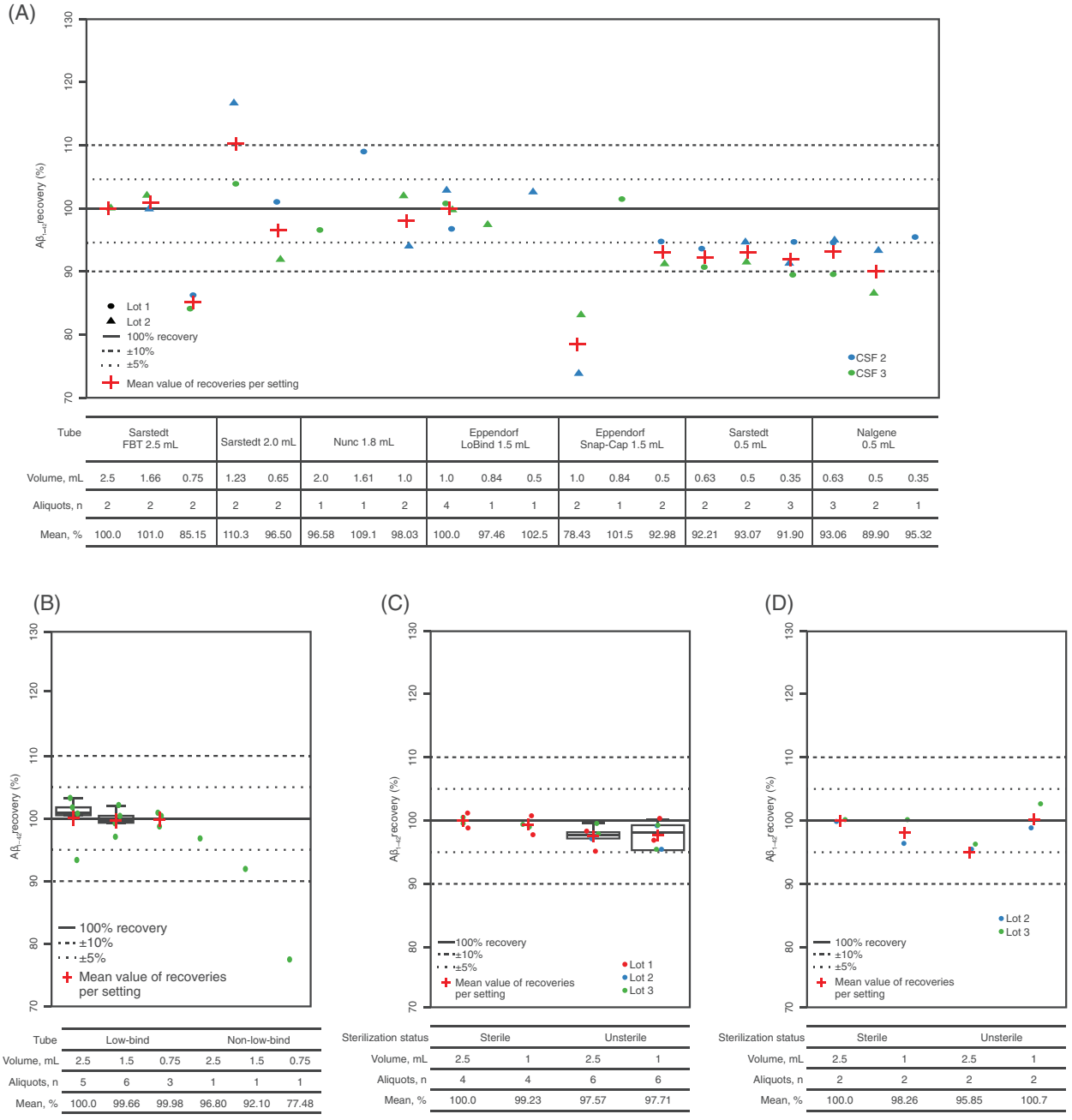
$A\beta_{1\text{-}42}$ recovery was stable for up to 49 hours at RT and up to 15 days at 2°C to 8°C. Mean $A\beta_{1\text{-}42}$ recoveries for each aliquot were within ±5% of measurements in the reference condition (fresh samples measured within 4 to 6 hours of collection), except for the aliquot stored for 6 days at RT (Figure 2B).

3.2.3 | Blood contamination and centrifugation

No substantial effect on $A\beta_{1\text{-}42}$ recovery was observed when up to 1% blood contamination, which exceeds levels associated with visible hemolysis (ie, very reddish appearance), was added to CSF samples stored for up to 6 days at RT and up to 14 days at 2°C to 8°C. Mean $A\beta_{1\text{-}42}$ recoveries across all tested patients for each aliquot were within ±10% of measurements in the reference condition (fresh, uncontaminated samples; Figure 3A and B). There was also no substantial effect on $A\beta_{1\text{-}42}$ concentrations with centrifugation versus no centrifugation, when using the first 2 mL of CSF collected (Figure 3C). However, it is noteworthy that blood contamination was rarely observed in the first 2 mL of CSF collected.

3.2.4 | Transportation/storage conditions and tube-filling volume

When using a tube-filling volume of 1.5 mL and storing samples at RT, $A\beta_{1\text{-}42}$ recovery was substantially lower for samples transported horizontally versus in an upright position (Figure 4A). For example, horizontal transportation of 1.5 mL CSF samples resulted in a 15.5% reduction in mean $A\beta_{1\text{-}42}$ recovery, which remained after storage for 40 hours at RT, compared with the corresponding reference condition (upright transportation, measurement within 4 to 6 hours of sample collection; Figure 4A). This reduction in recovery was resolved by increasing the tube-filling volume from 1.5 to 3.0 mL and storing at 2°C to 8°C. When using a tube-filling volume of 3.0 mL and storing at 2°C to 8°C, mean values for $A\beta_{1\text{-}42}$ recovery for all transportation/store conditions were within ±5% of the reference condition (Figure 4B). The effect of horizontal versus upright transportation on recovery values for $A\beta_{1\text{-}42}$ remained small when the tube-filling volume was reduced to 2.25 mL (Figure 4C and D); however, we recommend a conservative tube-filling volume of at least 2.5 mL in the final pre-analytical protocol (Section 3.4).



Tube	Sarstedt FBT 2.5 mL			Sarstedt 2.0 mL		Nunc 1.8 mL			Eppendorf LoBind 1.5 mL			Eppendorf Snap-Cap 1.5 mL			Sarstedt 0.5 mL			Nalgene 0.5 mL		
Volume, mL	2.5	1.66	0.75	1.23	0.65	2.0	1.61	1.0	1.0	0.84	0.5	1.0	0.84	0.5	0.63	0.5	0.35	0.63	0.5	0.35
Aliquots, n	2	2	2	2	2	1	1	2	4	1	1	2	1	2	2	2	3	3	2	1
Mean, %	100.0	101.0	85.15	110.3	96.50	96.58	109.1	98.03	100.0	97.46	102.5	78.43	101.5	92.98	92.21	93.07	91.90	93.06	89.90	95.32

Tube	Low-bind			Non-low-bind		
Volume, mL	2.5	1.5	0.75	2.5	1.5	0.75
Aliquots, n	5	6	3	1	1	1
Mean, %	100.0	99.66	99.98	96.80	92.10	77.48

Sterilization status	Sterile		Unsterile	
Volume, mL	2.5	1	2.5	1
Aliquots, n	4	4	6	6
Mean, %	100.0	99.23	97.57	97.71

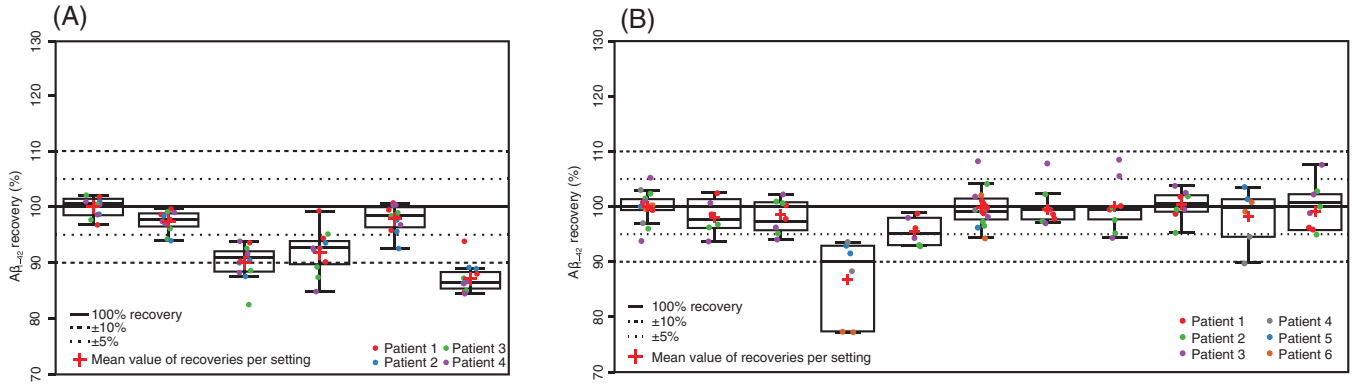
Sterilization status	Sterile		Unsterile	
Volume, mL	2.5	1	2.5	1
Aliquots, n	2	2	2	2
Mean, %	100.0	98.26	95.85	100.7

FIGURE 1 Recovery of CSF A$\beta_{1\text{-}42}$ according to (A) tube type/manufacturer, (B) use of low-bind versus non–low-bind Sarstedt 2.5 mL FBTs, and sterilization status (sterilization vs no sterilization) in samples analyzed (C) within 4-6 hours of collection and (D) after storage at RT for 24 hours. Recovery (%) is defined as the ratio between the measured biomarker concentration and the mean concentration measured in the reference condition: (A) Sarstedt FBT 2.5 mL tube, 2.5 mL tube-filling volume; (B) low-bind Sarstedt FBT, 2.5 mL tube-filling volume; (C) and (D) sterilized tube, 2.5 mL tube-filling volume. Abbreviations: A$\beta_{1\text{-}42}$, amyloid beta$_{1\text{-}42}$; CSF, cerebrospinal fluid; FBT, false-bottom tube; RT, room temperature. Each Lot refers to a different batch of tube lot

3.3 | Impact of pre-analytical factors on measurement of A$\beta_{1\text{-}40}$, P-tau, T-tau, A$\beta_{1\text{-}42}$/A$\beta_{1\text{-}40}$, P-tau/A$\beta_{1\text{-}42}$, and T-tau/A$\beta_{1\text{-}42}$

Effects of pre-analytical factors on measurement of A$\beta_{1\text{-}40}$, P-tau, T-tau, A$\beta_{1\text{-}42}$/A$\beta_{1\text{-}40}$, P-tau/A$\beta_{1\text{-}42}$, and T-tau/A$\beta_{1\text{-}42}$ are presented in Figures S2-12.

In general, the impact of pre-analytical factors on A$\beta_{1\text{-}40}$ recovery was similar or less than that observed with A$\beta_{1\text{-}42}$. A$\beta_{1\text{-}40}$ recovery was affected by tube type/manufacturer, although not always in the same direction as A$\beta_{1\text{-}42}$, and the impact was generally less for A$\beta_{1\text{-}40}$. Effects of collection technique and mixing procedure on A$\beta_{1\text{-}40}$ recovery were similar to A$\beta_{1\text{-}42}$, but to a lesser extent. Results for the effect of LP gradient, sample storage conditions, blood contamination, and



Mixing	No mixing		Roller mixing		Vortexing	
Volume, mL	1.75	0.75	1.75	0.75	1.75	0.75
Aliquots, n	12	12	12	12	12	12
Mean, %	100.0	97.34	89.98	91.94	97.82	87.18
SD	1.73	1.82	3.13	3.72	2.48	2.55
CV, %	1.93	1.75	3.14	3.72	1.92	2.42

Temperature	RT					2-8°C					
Duration	Fresh	25h	49h	6d	8d	Fresh	25h	49h	8d	14d	15d
Aliquots, n	15	6	8	6	6	15	9	9	8	6	9
Mean, %	100.0	98.13	98.43	86.83*	95.52	99.81	99.99	100.0	100.4	98.21	99.28
CV, %	2.50	2.77	3.18	1.73	1.60	3.04	3.00	3.35	2.56	2.15	2.97

FIGURE 2 Recovery of CSF A$\beta_{1\text{-}42}$ according to (A) mixing procedure (no mixing, roller-mixing, vortexing) and (B) sample storage conditions (temperature and duration). Recovery (%) is defined as the ratio between the measured biomarker concentration and the mean concentration measured in the reference condition: (A) no mixing, 1.75 mL tube-filling volume; and (B) measurement within 4-6 hours of sample collection in fresh samples stored at RT. NOTE: Data from different patients were used for each experiment; patient numbers do not correspond to the same patient across figures. *The lower recovery observed at day 6 versus day 8 in the samples stored at RT is likely due to a degree of variability in these measurements. Abbreviations: A$\beta_{1\text{-}42}$, amyloid beta $_{1\text{-}42}$; CSF, cerebrospinal fluid; CV, coefficient of variation; RT, room temperature; SD, standard deviation

centrifugation on A$\beta_{1\text{-}42}$ and A$\beta_{1\text{-}40}$ recovery were similar. Reductions in A$\beta_{1\text{-}40}$ recoveries following horizontal transportation of 1.5 mL CSF samples stored at RT, compared with upright transportation/direct measurement, were smaller in magnitude than reductions observed for A$\beta_{1\text{-}42}$.

P-tau and T-tau measurements were largely unaffected by the pre-analytical factors tested. Recoveries for both biomarkers were stable when samples were stored up to 8 days at RT and up to 15 days at 2°C to 8°C.

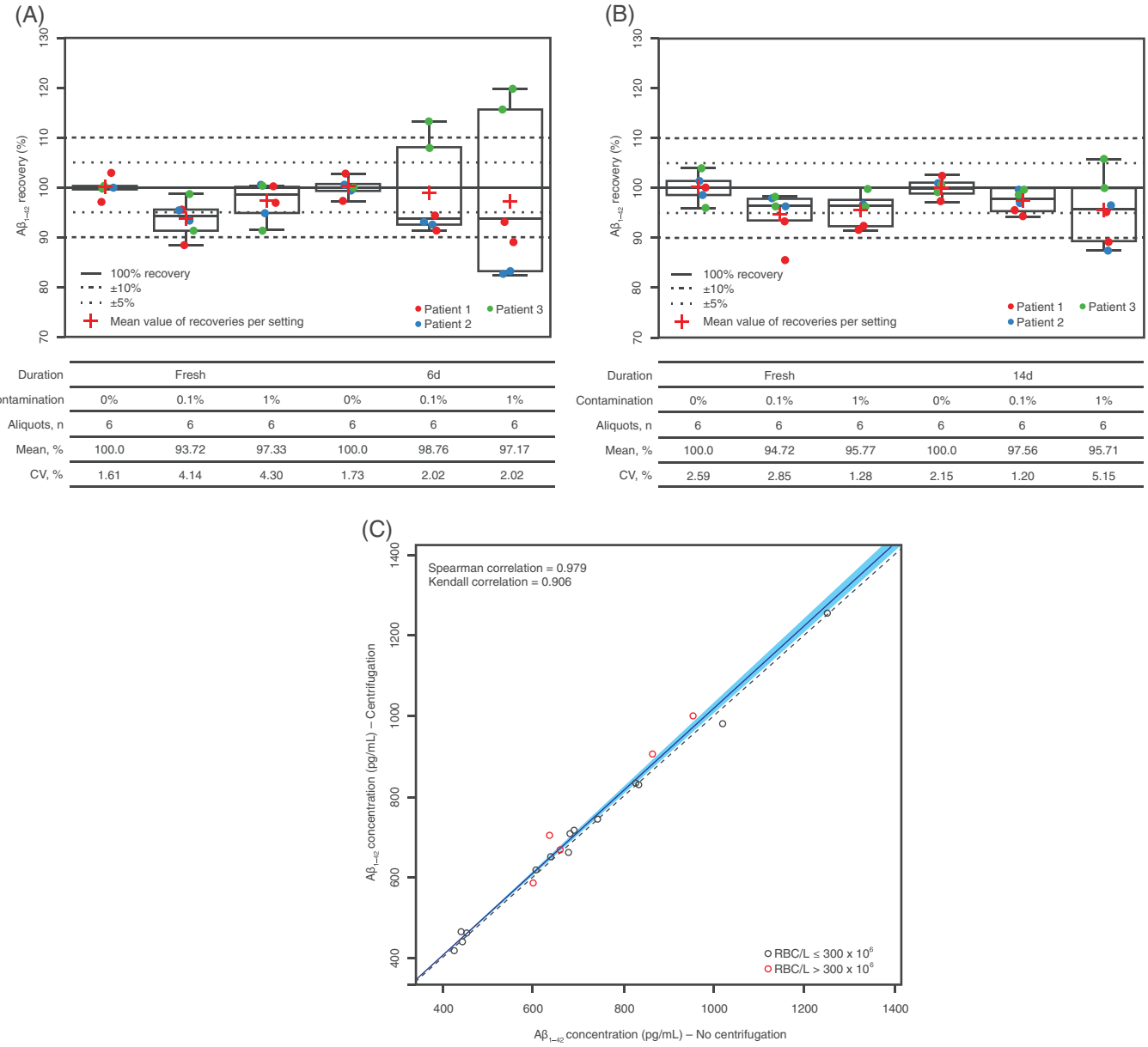
Results for the A$\beta_{1\text{-}42}$/A$\beta_{1\text{-}40}$, P-tau/A$\beta_{1\text{-}42}$, and T-tau/A$\beta_{1\text{-}42}$ ratios were reflective of the individual constituent biomarkers. As the impact of pre-analytical factors on A$\beta_{1\text{-}40}$ was generally similar or less than that observed with A$\beta_{1\text{-}42}$, the A$\beta_{1\text{-}42}$/A$\beta_{1\text{-}40}$ ratio accordingly showed a weaker effect than A$\beta_{1\text{-}42}$ alone. Because the individual tau biomarkers were largely unaffected by pre-analytical factors, the tau ratios typically showed the reverse of the effect of pre-analytical factors on A$\beta_{1\text{-}42}$ alone.

3.4 | Standardized, routine-use pre-analytical protocol for handling AD biomarkers in fresh CSF samples

Based on these comprehensive analyses, we propose a novel, standardized pre-analytical protocol for handling fresh CSF samples before measuring AD biomarkers in routine clinical practice (Figure 5). The first 2 mL of CSF collected should not be used for AD biomarker analysis, and ≥2.5 mL of CSF should be collected into a low-bind FBT; no further handling steps should be performed. Samples can be transported/stored for up to 15 days at 2°C to 8°C, and should be measured immediately at the testing site.

4 | DISCUSSION

Since AD CSF biomarkers were identified as potential tools for diagnosing AD and monitoring treatment effects in clinical trials, significant progress has been made in their development and standardization.[17] Fully automated immunoassays have been developed for reliable/accurate measurement of A$\beta_{1\text{-}42}$, A$\beta_{1\text{-}40}$, P-tau, and T-tau concentrations in CSF,[18–20] and a reference measurement procedure for A$\beta_{1\text{-}42}$ has been approved by the Joint Committee for Traceability in Laboratory Medicine (JCTLM).[18,21,22] The observation of significant intra-/inter-laboratory variation in CSF biomarker measurements led to the creation of a global quality control program (Alzheimer's Association QC Program[23]), which aims to standardize CSF biomarker measurements across laboratories to facilitate direct comparisons of results, and promote greater understanding of pre-analytical/analytical factors that may influence CSF biomarker variability.[23,24] The fully automated Elecsys β-Amyloid(1-42) CSF immunoassay, the first assay for CSF A$\beta_{1\text{-}42}$ to be standardized to the JCTLM-approved reference measurement procedure, has been shown by the Alzheimer's Association QC Program to dramatically reduce real-world measurement variability.[18] Until now, the remaining step was to establish a simplified and validated pre-analytical protocol for the measurement of AD CSF biomarkers, which was the focus of the present study.



Duration	Fresh			6d		
Contamination	0%	0.1%	1%	0%	0.1%	1%
Aliquots, n	6	6	6	6	6	6
Mean, %	100.0	93.72	97.33	100.0	98.76	97.17
CV, %	1.61	4.14	4.30	1.73	2.02	2.02

Duration	Fresh			14d		
Contamination	0%	0.1%	1%	0%	0.1%	1%
Aliquots, n	6	6	6	6	6	6
Mean, %	100.0	94.72	95.77	100.0	97.56	95.71
CV, %	2.59	2.85	1.28	2.15	1.20	5.15

FIGURE 3 Recovery of CSF A$\beta_{1\text{-}42}$ according to blood contamination in samples stored at (A) RT and (B) 2°C-8°C, and (C) centrifugation status (centrifugation vs no centrifugation). Recovery (%) is defined as the ratio between the measured biomarker concentration and the mean concentration measured in the reference condition: (A) and (B) measurement within 4-6 hours of sample collection in fresh samples, 0% contamination. NOTE: Data from different patients were used for each experiment; patient numbers do not correspond to the same patient across figures. Abbreviations: A$\beta_{1\text{-}42}$, amyloid beta$_{1\text{-}42}$; CSF, cerebrospinal fluid; CV, coefficient of variation; RBC, red blood cell; RT, room temperature

We assessed the impact of a range of pre-analytical factors on the measurement of CSF biomarkers A$\beta_{1\text{-}42}$, A$\beta_{1\text{-}40}$, P-tau, T-tau, and their derivative ratios (A$\beta_{1\text{-}42}$/A$\beta_{1\text{-}40}$, P-tau/A$\beta_{1\text{-}42}$, T-tau/A$\beta_{1\text{-}42}$). Based on our findings, we propose a novel, easy-to-use, standardized, routine-use pre-analytical protocol for handling AD biomarkers in fresh CSF samples. This protocol is intended primarily for use in clinical practice; trials of CSF biomarkers may require specific conditions, depending on the parameter under investigation, which may necessitate different protocols. Our protocol offers several advantages: only one tube is required for collection and measurement; simplified handling procedure; no transfer steps required and thus no loss of analyte and less variability; and sample transportation does not require additional actions. The last point is particularly important because LP procedures and dementia evaluations are also performed outside the hospital setting and without access to in-house facilities for CSF biomarker analysis. Our protocol advises against the use of assisting tools to collect CSF, other than an LP needle; if assisting tools are required, the first 2 mL of CSF after applying the instrument should not be used to measure AD biomarkers. Although Nunc tubes showed the highest recoveries overall, the new Sarstedt low-bind FBT was less sensitive to the

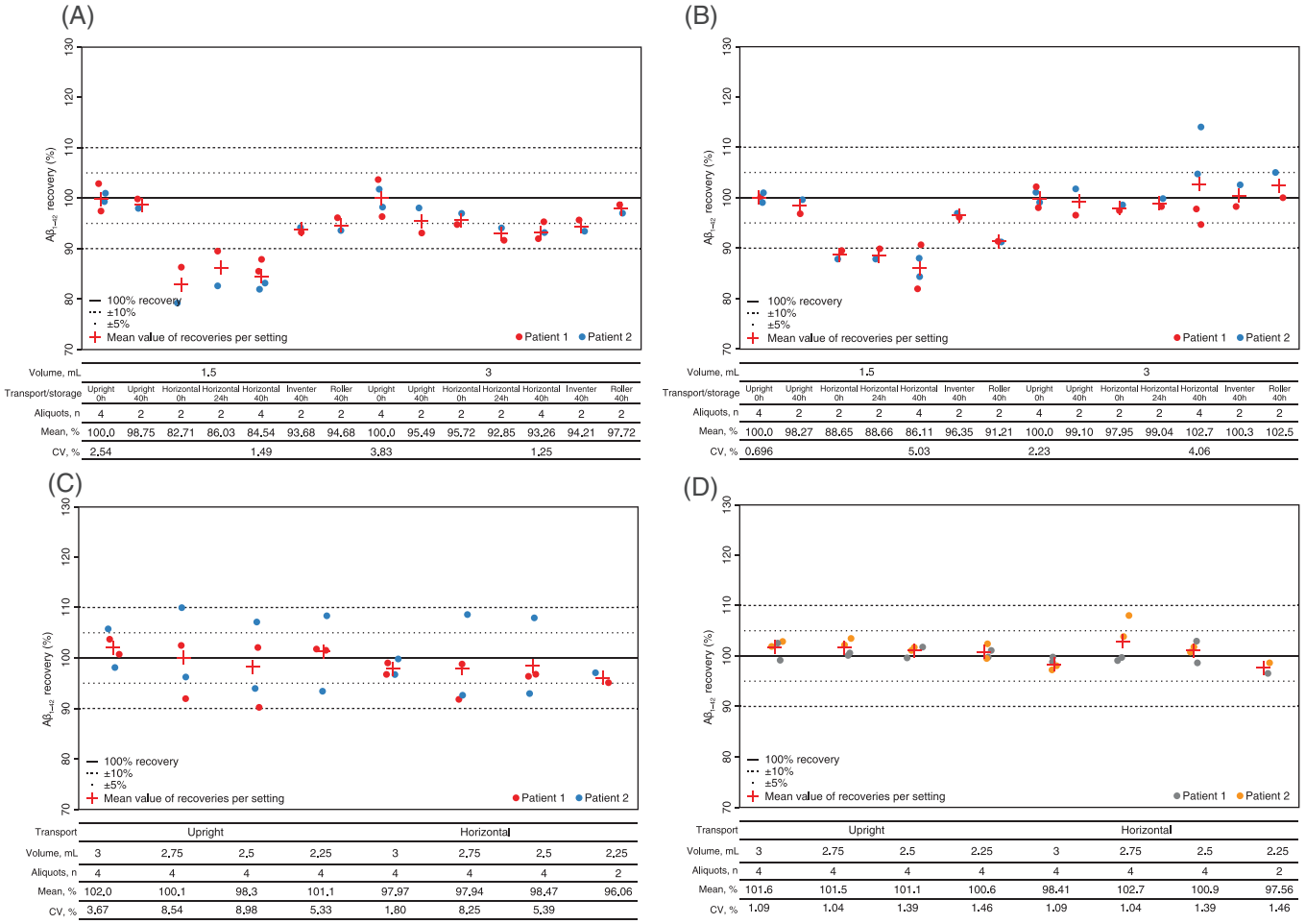


FIGURE 4 Recovery of CSF Aβ_{1-42} according to transportation condition in samples with tube-filling volumes of 1.5 and 3 mL stored at (A) RT and (B) 2°C-8°C, and tube-filling volume in samples transported in an upright and horizontal position and stored at (C) room temperature and (D) 2°C-8°C. Recovery (%) is defined as the ratio between the measured biomarker concentration and the mean concentration measured in the reference condition: (A) and (B) upright transportation and measurement within 4-6 hours of sample collection (upright 0 hours) for each tube-filling volume (1.5 and 3 mL); (C) and (D) mean recovery for 3 mL tube-filling volume across both transportation conditions (upright and horizontal). NOTES: Data from different patients were used for each experiment; patient numbers do not correspond to the same patient across figures. Transportation/storage conditions: upright 0 hours, upright transportation and measurement within 4-6 hours of sample collection; upright 40 hours, upright transportation and measurement after 40 hours of storage in upright position; horizontal 0 hours, horizontal transportation and measurement within 4-6 hours of sample collection; horizontal 24 hours, horizontal transportation and measurement after 24 hours of storage in horizontal position; horizontal 40 hours, horizontal transportation and measurement after 40 hours of storage in horizontal position; inverter 40 hours, upright transportation and measurement after 40 hours of inverting; roller 40 hours, upright transportation and measurement after 40 hours of roller-mixing. Abbreviations: Aβ_{1-42}, amyloid beta$_{1-42}$; CSF, cerebrospinal fluid; CV, coefficient of variation; RT, room temperature

absolute change in fill volume and had a lower variability of recoveries, suggesting lower aliquot-to-aliquot variability. Therefore, the protocol incorporates the new Sarstedt low-bind FBT, which was used by 10 clinicians at one site for CSF sample collection during the study.

There was no substantial impact on measured AD biomarker levels when up to 1% blood contamination was added to CSF samples, which exceeds levels associated with visible hemolysis. However, a recent study showed that higher levels of blood contamination up to 10% can result in dramatically reduced CSF Aβ_{1-42} levels.[15] Because the level of blood contamination is difficult to visibly estimate, we recommend avoiding use of CSF samples with visible hemolysis for the analysis of AD biomarkers, to ensure reliable results. The same study[15] also showed that CSF Aβ_{1-42} and Aβ_{1-40} levels are not affected by mixing of samples or centrifugation of non-blood-contaminated samples, and are stable for up to 72 hours at RT and up to 1 week at 4°C.[15] However, the researchers did not observe significant reduction in CSF Aβ_{1-42} levels when comparing the first portion of collected CSF (1-5 mL) versus the last portion (15 to 20 mL), suggesting that any portion of the first 1 to 20 mL can be used.[15] Our study builds on this previous research through a more comprehensive analysis of the effect of pre-analytical factors on AD CSF biomarker levels, including additional pre-analytical factors (eg, transportation conditions) and CSF biomarkers/ratios

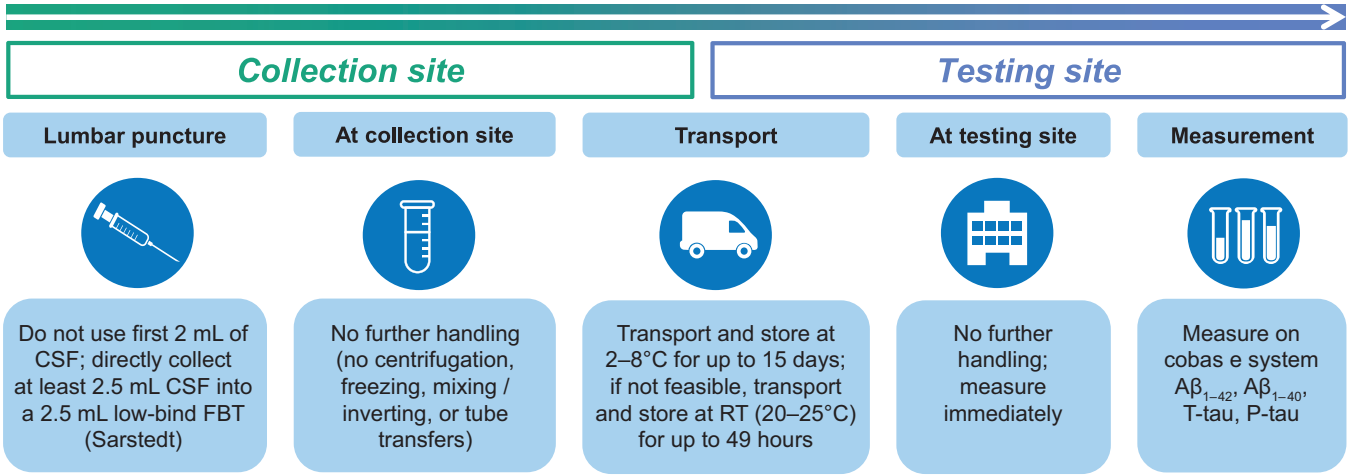


FIGURE 5 A novel, standardized, routine-use pre-analytical protocol for handling fresh CSF samples prior to measuring Alzheimer's disease biomarkers. Abbreviations: $A\beta_{1\text{-}40}$, amyloid-$\beta_{1\text{-}40}$; $A\beta_{1\text{-}42}$, amyloid-$\beta_{1\text{-}42}$; CSF, cerebrospinal fluid; FBT, false-bottom tube; P-tau, phospho-tau (181P); RT, room temperature; T-tau, total tau

(P-tau, T-tau, $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$, P-tau/$A\beta_{1\text{-}42}$, T-tau/$A\beta_{1\text{-}42}$). Although previous studies have proposed using the $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio[25,26] due to it being more robust against pre-analytical factors than single analytes, our new protocol removes the pre-analytical variation related to sample handling; hence, measuring $A\beta_{1\text{-}42}$ alone could avoid the potential problems of using the $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio (ie, measuring two analytes, both of which have some analytical variation) while remaining robust against pre-analytical factors. Use of low-bind tubes for CSF sample collection is also supported by previous research.[13]

Disease-modifying therapies in development for AD are likely to be most effective in early disease.[14,27] Although blood biomarkers are currently under investigation and have become an important factor, CSF biomarkers are still a key tool for accurate/early diagnosis of AD, and can detect neuropathological changes in patients with mild cognitive impairment or before symptom onset.[28–30] Therefore, the ability to accurately and reliably measure CSF biomarkers is likely to be increasingly important in the diagnosis of AD and the evaluation of treatment effectiveness with the anticipated introduction of disease-modifying immunotherapies targeting $A\beta$ in routine clinical practice. Our standardized, routine-use pre-analytical protocol for CSF AD biomarkers could reduce the impact of sample-handling variability between sites, and thus improve accuracy of biomarker measurements, enable inter-study/laboratory comparisons, support CSF biomarker use in clinical practice, and facilitate the introduction of global assay cutoffs.[14] It could also allow longitudinal comparisons within a single individual who has undergone different collection/measurement procedures. Our protocol is for use with fresh CSF samples; evaluation of a standardized protocol for frozen CSF samples has been initiated,[15] but data on mid-term storage of frozen samples are warranted. In addition, the CSF samples in our study were taken from patients with unknown diagnoses; pre-analytical factors may affect T-tau and P-tau differently in AD patients due to hyper-phosphorylation of tau. However, both T-tau and P-tau appear much more robust against pre-analytical variation than amyloid peptides, including in samples from AD patients.[31] Further research is needed to evaluate how the new protocol will improve generalizability of biomarker results across clinical centers and testing laboratories.

5 | CONCLUSION

Following a comprehensive analysis of the impact of various pre-analytical factors, we propose a novel, simplified, standardized, routine-use pre-analytical protocol for analysis of AD biomarkers in fresh CSF samples, which uses newly developed low-bind tube technology and minimizes the need for post-collection sample processing (no mixing, inverting, centrifugation, or tube transfer). This protocol is recommended for implementation in clinical routine to reduce the impact of pre-analytical factors, and thus reduce assay measurement variability and enable comparison of AD CSF biomarker results between studies and laboratories.

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CONFLICTS OF INTEREST

Oskar Hansson acquired research support (for the institution) from Roche, GE Healthcare, Biogen, AVID Radiopharmaceuticals, and Euroimmun; in the past 2 years, he has received consultancy/speaker

fees (paid to the institution) from Biogen and Roche. Sandra Rutz is an employee of Roche Diagnostics. Henrik Zetterberg has served at scientific advisory boards for Wave, Samumed, CogRx, and Roche Diagnostics; has given lectures in symposia sponsored by Biogen and Alzecure; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. Ekaterina Bauer, Susanne Burget, Teresa Hähl, Ekaterina Manuilova, and Simone Wahl are employees of Roche Diagnostics. Mehmet Can Mert is an agent representing Roche Diagnostics.

Kaj Blennow has served as a consultant or at advisory boards for Alector, Biogen, CogRx, Lilly, MagQu, Novartis, and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. Erik Stomrud has no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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