Title: The Alzheimer's Association international guidelines for handling of cerebrospinal fluid for routine clinical measurements of amyloid β and tau

Abstract

The core cerebrospinal fluid (CSF) Alzheimer's disease (AD) biomarkers amyloid beta (Aβ42 and Aβ40), total tau, and phosphorylated tau, have been extensively clinically validated, with very high diagnostic performance for AD, including the early phases of the disease. However, between-center differences in pre-analytical procedures may contribute to variability in measurements across laboratories. To resolve this issue, a workgroup was led by the Alzheimer's Association with experts from both academia and industry. The aim of the group was to develop a simplified and standardized pre-analytical protocol for CSF collection and handling before analysis for routine clinical use, and ultimately to ensure high diagnostic performance and minimize patient misclassification rates. Widespread application of the protocol would help minimize variability in measurements, which would facilitate the implementation of unified cut-off levels across laboratories, and foster the use of CSF biomarkers in AD diagnostics for the benefit of the patients.

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Alzheimer’s disease (AD) is a devastating and progressive neurodegenerative disease with symptoms that exist along a continuum composed of three stages: a lengthy preclinical phase without symptoms but with measurable changes in the brain (through imaging techniques), cerebrospinal fluid (CSF) and blood, and two subsequent phases, mild cognitive impairment (MCI) and dementia, in which symptoms vary in magnitude [1–6].

Diagnosing AD at its earliest stages is challenging. However, biomarkers, biological indicators that can be measured in the brain, CSF, and blood, and evaluated as potential indicators of the disease [7], are promising tools that provide the potential for early detection and increased clinician confidence in AD diagnosis [8]. AD biomarkers can be obtained from brain imaging methods such as magnetic resonance imaging (MRI) or positron emission tomography (PET), as well as CSF or blood tests. Despite the utility of brain imaging methods, they can only identify the presence of a certain type of pathology once levels pass a certain threshold. In contrast, biomarkers from CSF are considered a more direct readout of protein levels and are therefore a more sensitive assay [9].

The four core AD CSF biomarkers include two isoforms of the protein β-amyloid (Aβ), Aβ(1-42) and Aβ(1-40), as well as total tau (tTau) and phosphorylated tau (pTau) [7]. Aβ(1-42) is the primary constituent of the amyloid plaques that are found in AD [10–12], while hyperphosphorylated tau is a primary constituent of neurofibrillary tangles [13–15]; together, plaques and tangles form the pathological hallmarks of the disease.

A large body of literature has validated that each of these core CSF biomarkers are reflective of AD brain pathology. For example, low levels of CSF Aβ(1-42) are correlated with brain Aβ levels [13,16–20], while elevated levels of CSF pTau are found in AD patients [21], and correlate with the presence of cortical neurofibrillary tangles in the majority of studies [22–24]. In contrast, increased CSF levels of tTau are not specific to AD [21], but instead reflect neuronal injury/degeneration more generally and are thus also increased in conditions such as traumatic brain injury [25], stroke [26] and Creutzfeldt-Jakob disease [27] that do not show elevated pTau levels. The 2018 National Institute on Aging (NIA)-Alzheimer’s Association Research Framework, created to provide guidance on how AD imaging and CSF biomarkers should be in used in research, groups them into three categories that are collectively known as the AT(N) scheme [28]. The Aβ (A) designation includes CSF Aβ(1-42) or the Aβ(1-42)/ Aβ(1-40) ratio; the tau (T) designation includes CSF pTau; and the neurodegeneration or neuronal injury (N) designation includes CSF neurofilament light (NfL) or tTau.

There are several commercially available assays that measure these core CSF biomarkers and many are approved for use in the diagnosis of AD in the European Union [29–35]. In the United States, however, PET imaging remains the only Food and Drug Administration-approved method for biomarker measurement in the disease [36]. Despite widespread use in Europe, studies of these core CSF biomarkers have found significant inter-laboratory and batch-to-batch variability [7, 37]. There are three possible sources of variability to be considered when interpreting CSF biomarker results or when defining CSF biomarker cutoffs: (1) pre-analytical (i.e. due to differences in the collection, handling, and storage of CSF) [38]; (2) analytical (i.e. due to differences in technician skill or how the assays are used in the lab, and lot-to-lot variability of reference materials, kits and/or kit components [37,39]; and (3) biological/patient-related (i.e. confounding factors linked to patients, such as patient age).
There have been a number of efforts to reduce analytic variability and harmonize results obtained from different vendors. The International Federation of Clinical Chemistry and Laboratory Medicine Working Group for CSF proteins (WG-CSF), working closely with the Alzheimer’s Association Global Biomarker Standardization Consortium (GBSC), developed certified reference materials (CRMs) 40. For use as value assignment of CSF Aβ(1-42) in the CRMs, they developed four liquid chromatography mass spectrometry (LC-MS) methods for absolute quantification of Aβ(1-42), of which two were fully validated analytically and approved by the Joint Committee for Traceability in Laboratory Medicine as Reference Measurement Procedures (C11RMP9 and C12RMP1, respectively) [7,41]. Initiatives to obtain standardization of Aβ(1-40) and total-Tau are ongoing.

In addition, a number of quality control programs have been initiated to monitor the immunoassay variability for Aβ (Aβ42, Aβ40, Aβ38) and tau proteins (total tau, phosphorylated tau) that occurs between batches and across laboratories [9,35,42–46]. The largest effort, launched in 2009 by the Alzheimer’s Association, was an international quality control program involving more than 85 laboratories across 20 countries [9]. By the recent introduction of automated measurement of biomarkers and improved in-process quality control for critical assay components, today’s commercially available immunoassays developed on random access analyzers offer higher precision, better lot-to-lot consistency, and lower between-laboratory variation such that analytic variability is much less of a problem than before.

Variability surrounding patient selection has also been sufficiently addressed due to the recently published appropriate use criteria (AUC) for lumbar puncture and CSF testing for AD diagnosis, established by a multidisciplinary workgroup convened by the Alzheimer’s Association [47].

However, a large source of variability remains with respect to pre-analytics. Following the integration of certified reference materials for Aβ1-42 in commercial assays, pre-analytics are considered the last piece of the variability puzzle that remains to be solved. This pre-analytic variability still precludes the establishment of general biomarker cut-off values and the adoption of a standard CSF biomarker protocol, makes proper diagnosis and treatment more difficult, and presents challenges for clinical trial enrollment [37].

Unfortunately, across the landscape, the pre-analytical protocols currently in use vary considerably between studies and centers, and such heterogeneity influences the absolute levels of biomarkers measured. Given that different biomarkers, and indeed even isoforms of the same biomarker, exhibit distinct profiles of stability and aggregation, and thus differ in their susceptibility to pre-analytical variables, it is important to systematically evaluate best practices for the collection, storage, and processing of individual CSF samples before analysis of a specific protein biomarker [7]. In a systematic review of pre-analytical variables that affect CSF biomarkers in AD, Aβ1-42 was found to be especially susceptible relative to pTau and tTau [48]. For example, variables such as sample storage volume and tube surface adsorption appear to dramatically affect Aβ1-42 levels [49–53].

Although some groups have led development initiatives for clearer pre-analytical protocols [45, 48,54], these protocols have not been adopted, nor have they been widely implemented (e.g. across both academic and industry settings), despite its proven success [55]. In the present communication, we report a unified, impartial approach by a workgroup, led by the Alzheimer’s Association and composed of key opinion leaders in academia and industry, to develop a standardized pre-analytic protocol. The workgroup examined the impact of a number of pre-analytical factors on measured CSF biomarker concentrations: sample collection
methodology; blood contamination; tube type and filling volume; tube transfer steps and pipetting; mixing; transportation; and sample stability. The primary goal of this effort is to ensure that stakeholders in industry and academia will adopt and implement the recommended protocol in clinical routine. The adoption of a global standard pre-analytic protocol is critical to establishing universal cut-off values for the measurement CSF AD biomarker levels, especially Aβ1-42. Without such standardization, variability will both continue to weaken the confidence of clinicians when evaluating measured levels of biomarkers as diagnostic criteria for AD and negatively impact clinical trials.

2. Recommendations
The CSF Pre-Analitics Consortium was formed with the goal of developing a simple, easy to implement, data-driven and consensus-based pre-analytical protocol. The consensus protocol was based on the available literature focusing on Aβ1-42 as this marker is particularly susceptible to variations in pre-analytical handling [48]. Our research identified several areas where data was insufficient, especially in regards to experiments where a proper standard of truth (i.e., fresh CSF obtained directly in protein low-binding (LoB) tubes, which minimize binding of Aβ to the test tube walls, and analyzed after LP without any pre-analytical steps in-between collection and analyses within hours after LP) was lacking. We consequently performed new experiments that have now been published elsewhere in three different publications [56, Hansson et al (in press), Darrow et al (in press)]. Several pre-analytical factors were systematically evaluated by the CSF pre-analitics consortium, including tube type, storage procedures, optimal filling volume, transportation conditions, mixing procedures, the need for centrifugation and tube type. Of these factors, tube type, tube filling volume, transfer of CSF from one tube to another, transportation conditions, and mixing procedure had the biggest impact on the measured concentrations of the core AD biomarkers (Table 1).

Table 1. Main findings of recent studies on the effects of pre-analytical factors on CSF AD biomarkers.

<table>
<thead>
<tr>
<th>Main findings</th>
<th>Main references</th>
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<tr>
<td>Fasting has no effect on CSF Aβ concentrations</td>
<td>Darrow et al (in press)</td>
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<tr>
<td>When collecting CSF using the drip method, the concentrations of Aβ are stable when collecting up to at least 20 ml of CSF (after removing the first 1-2 ml CSF).</td>
<td>Janelidze et al [56]; Hansson et al (in press)</td>
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<td>The use of LoB tubes mitigates loss of Aβ. However, even when using LoB tubes all new tube types must be evaluated to determine if extended contact between sample and tube (including the cap of the tube) affects the Aβ 42 levels.</td>
<td>Vanderstichele et al [49]; Hansson et al (in press)</td>
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<tr>
<td>A low CSF volume vs tube surface area might reduce Aβ concentrations.</td>
<td>Vanderstichele et al [49]; Hansson et al (in press)</td>
</tr>
<tr>
<td>Transfer of CSF from one tube to another results in loss of Aβ.</td>
<td>Vanderstichele et al [49]</td>
</tr>
<tr>
<td>Aβ concentrations in fresh CSF are stable at RT for up to 2 days and at 4°C for up to 14 days.</td>
<td>Janelidze et al [56]; Hansson et al (in press);</td>
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<tr>
<td>Mixing of fresh CSF is not necessary before analyses and can reduce Aβ levels. However, if a frozen CSF samples is used it</td>
<td>Janelidze et al [56]; Hansson et al (in press)</td>
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should be mixed using a roller mixer after thawing to reduce variation.

| Centrifugation of CSF samples is not necessary (if no visible blood contamination). | Janelidze et al [56]; Hansson et al (in press) |
| Blood contamination visible to the naked eye (>0.1-1.0%) might reduce Aβ concentrations in fresh CSF, but this effect is mitigated by centrifugation and/or storage at 4°C or -20°C. | Janelidze et al [56]. |
| Transportation can have effects on Aβ concentrations, especially if the tube is i) not transported in upright position, ii) the CSF volume vs tube volume is low, and iii) the temperature is > 2-8 °C. | Hansson et al (in press) |
| CSF Aβ42/40 ratio generally more resistant variations in pre-analytical procedures than Aβ42 alone. | Vanderstichele et al [49]; Hansson et al (in press) |
| Freezing of CSF at -20°C or -80°C for at least up to 2 weeks does not change Aβ concentrations. | Janelidze et al [56] |

Based on current evidence, we recommend the following protocol when analyzing fresh CSF samples (Figure 1). Fasting is not necessary when collecting CSF. We recommend using an ordinary lumbar puncture needle. The first 1-2 ml of collected CSF should not be used for AD biomarker diagnostics. The next CSF should be collected using the drip method, directly into a LoB tube and should come from the first 20 ml tapped from the patient. Although some clinicians prefer the syringe pull method due to speed of collection, we strongly recommend the drip method for the portion of CSF to be used for AD biomarker measurements, which is 1.0-3.0 mL depending on which validated tube is used (see below). This step will take less than two minutes and thus does not significantly slow down the collection procedure, and reduces the risk that Aβ binds to the plastic of any syringe used. The remaining CSF may be collected using the syringe pull method if more sample material is needed for additional research purposes.

The same LoB tube should be used for collection and transport of CSF to avoid any transfer of CSF from one tube to another. The LoB tube must be validated, and the established biomarker cutoffs for Aβ42, Aβ42/40, P-tau and T-tau only apply when using a particular validated tube with a certain filling volume. For example, a sterile, false-bottom, low bind polypropylene tube from Sarstedt (Cat # 63.614.699) must be used for the Elecsys AD biomarker assays and this tube should be filled with 2.5mL CSF. For the Lumipulse platform 10 ml (Cat# 62.610.018) and 1.5 ml Sarstedt tubes (Cat# 72.703.600) have been validated for direct testing using the fresh CSF collection procedures described here. If a new LoB tube would be used then that new tube must be compared to the already validated tube. Experiments that need to be done include showing that fresh CSF can be stored up to 48h at RT, up to 14 days at 2-8°C and at -20°C or -80°C in the new tube, resulting in similar biomarker levels (approximately 5%) compared to analyses of fresh CSF stored in the already validated tube. Further, an experiment showing effects of transport is also crucial and again the results should be (approximately 5%) compared to the validated tube. The same experiments are needed if the recommended filling volume of a validated tube would be changed.

Further, we recommend not mixing or centrifuging the tube as there is no clear benefit to this step for core AD biomarker analysis. If there is a visible blood contamination, the CSF should not be used unless contamination can be removed by centrifugation, and CSF free of visible blood contamination should be used instead for AD biomarker analyses. However,
Centrifugation should normally not be done because each transfer of CSF to a new tube might result in lower Aβ values. Please note that the recommendations in this paper focus on β-amyloid and tau, and centrifugation guidance may vary with other biomarkers.

The validated tubes with a predetermined filling volume CSF should be transported (with cold blocks) and stored at 2-8°C for up to 14 days before analyses, because of lower susceptibility to blood contamination, violations in filling volume and transport effects at 2-8°C compared to RT. If this is not possible, samples can be stored at RT for up to 48 h before analyses, but we still recommend transport with cold blocks, especially if longer transportation is needed (i.e. outside the hospital).

![Figure 1](Image)

**Figure 1.** Recommended pre-analytical protocol for collection, storage, sample handling, and measurement of fresh CSF

As an alternative to storing and transporting fresh CSF, the validated tube filled with a predefined volume CSF can be frozen -20°C or -80°C during storage and transport for up to two weeks (Figure 2). Otherwise the protocol is identical to the fresh CSF protocol, except that rolling mixing before analyses might reduce variability [56]. Future studies need to determine if longer storage of frozen CSF is adequate.

![Figure 2](Image)

**Figure 2.** Recommended pre-analytical protocol for collection, storage, sample handling, and measurement of frozen CSF.
3. Summary

This report presents unified pre-analytical protocols for the analysis of fresh and short-term frozen CSF samples. It was designed to be independent of analytical technique/assay format and is intended for use in routine clinical diagnosis and screening/enrollment in trials. The protocols were designed to be easy to use and to reduce the variability of CSF AD biomarker results.

The recommended protocols are not intended for long-term storage of samples in clinical studies (i.e. collection of multiple aliquots, and long-term storage). Data for long-term storage will be presented at a later time. Second, this protocol was developed only for Aβ1-42, Aβ1-40, pTau, and tTau. Confounding factors and sampling techniques that affect additional biomarkers, if validated, will need to be assessed and collection protocols developed separately.

The protocols are based on the drip technique for collection. The extra time to collect this CSF volume by the drip technique is negligible. If syringe pull is standard in some centers, this technique can be applied after the protocol for additional sampling.

Finally, we want to emphasize that established biomarker cut offs for a certain method can only be used in clinical practice when the current protocols are used together with a validated tubed filled with a predefined volume of CSF. It is important to note that forthcoming studies referencing this unified protocol will present pre-analytical study results from experiments conducted using additional LoB tubes consistent with the data presented herein.

Although there are many benefits of a unified pre-analytical protocol, a number of challenges and unanswered questions remain. The most significant challenge will be getting the standardized protocol implemented by different centers and users. Changing applied procedures is a major hurdle. In addition, the current protocol does not immediately imply that we can use the same cutoffs for each marker when different methods are used for analyses. That will require further work and also depends on other factors such as standardization of the assays.

4. References


