

## Preanalytical Variables and Alzheimer Disease Biomarker Concentrations in Cerebrospinal Fluid

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Cerebrospinal fluid (CSF)<sup>2</sup>  $\beta$ -amyloid ( $A\beta_{1-42}$ ) is one of the key biological forms of  $\beta$ -amyloid protein in brain tissue that inversely reflects brain amyloid burden with reduced concentrations in Alzheimer disease (AD) (1). Reductions in CSF  $A\beta_{1-42}$  have been shown to occur years before symptom onset (2) and to have good positive predictive value for progression from mild cognitive impairment to clinical AD (3). CSF concentrations of total tau (T-tau), a marker for the intensity of neuronal degeneration, and hyperphosphorylated tau (P-tau181P), thought to be a marker of neurofibrillary tangle pathology, are both increased in AD (4). The combination of low CSF  $A\beta_{1-42}$  with increased ratios of T-tau and P-tau181P to  $A\beta_{1-42}$  has been used to support the diagnosis of AD. One study has suggested that the T-tau: $A\beta_{1-42}$  ratio is the most robust biomarker combination (5). These biomarkers have been incorporated into the revised diagnostic criteria of AD (6) and also are well established as part of inclusion and exclusion criteria for clinical trials.

A low CSF  $A\beta_{1-42}$  does not always reflect brain amyloid deposition and it can also be seen in other non-AD causes of dementia such as Lewy body disease or vascular dementia (7). More importantly has come the realization that altered concentrations of CSF  $A\beta_{1-42}$  and other markers may be a consequence of both preanalytical and analytical biases that make it difficult to properly interpret the test result. In particular, lack of harmonization of the preanalytical sample handling procedures owing to variations in CSF collection, storage, or processing has hampered the comparison of CSF  $A\beta_{1-42}$ , T-tau, and P-tau181P concentrations between different laboratories and studies. An article in this issue of *Clinical Chemistry* by Le Bastard et al. (8) is therefore very timely, first to raise awareness of preanalytical considerations, but also to pro-

vide further insights on sources of variation that have implications for sample collection and handling.

Le Bastard et al. concluded that it is possible to collect CSF in a single large volume or several smaller volumes from their observation that the total volume of CSF collected does not affect the concentrations of tau and amyloid proteins (8). Fractionated sampling of lumbar CSF has been postulated to be important, given that brain-derived proteins usually show a rostrocaudal concentration gradient, with higher concentrations in ventricular CSF compared to lumbar CSF, thereby implying that different volumes or fractionated sampling could generate differences in CSF biomarker concentrations. However, no differences in  $A\beta_{1-42}$  and T-tau concentrations between lumbar CSF fractions were found, consistent with previous studies (9) and also true for P-tau181P. Comparison of centrifuged and noncentrifuged samples from the same fractions revealed that centrifugation had no effect on CSF biomarker concentrations in macroscopically non-blood-contaminated samples (8). The practical implication is that centrifugation is not required for CSF biomarker analyses, and this likely is true, as the authors discuss, even when CSF is contaminated with blood (8). Le Bastard et al. also subjected CSF samples to different protocols of freezing temperatures and delay before freezing. Freezing at  $-80^{\circ}\text{C}$  in comparison to initial freezing in liquid nitrogen resulted in borderline significantly lower  $A\beta_{1-42}$  concentrations, probably because freezing in liquid nitrogen quickly reduces degradation of proteins by protease activity, thereby preventing loss of  $A\beta_{1-42}$ . CSF T-tau and P-tau181P concentrations, but not  $A\beta_{1-42}$  concentrations, were found to differ significantly between samples frozen at  $-80^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ . The practical implication is that freezing of CSF samples at  $-80^{\circ}\text{C}$  as soon as possible after collection is recommended, both for long-term as well as short-term storage, owing to the effects of freezing at  $-20^{\circ}\text{C}$  on CSF biomarker concentrations. Freezing in liquid nitrogen, however, is not recommended, being impractical for most routine laboratory settings. Le Bastard et al. recommend minimizing any delay in freezing but do not suggest an explicit time frame. As they allude, this is an important practical consideration given that transport of samples to a reference laboratory usually takes at least 24 h. It would be ideal if  $A\beta_{1-42}$ , T-tau, and P-tau181P could be transported at room temperature or cooled, rather than on dry ice,

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Received March 3, 2015; accepted March 5, 2015.

Previously published online at DOI: 10.1373/clinchem.2015.240069

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<sup>2</sup> Nonstandard abbreviations: CSF, cerebrospinal fluid;  $\beta$ -amyloid,  $A\beta_{1-42}$ ; AD, Alzheimer disease; T-tau, total tau; P-tau181P, neuronal degeneration and hyperphosphorylated tau; ABSI, Alzheimer's Biomarkers Standardization Initiative.

which incurs significant costs. To study the influence of freeze–thaw cycles, samples were thawed up to 4 times and (re)frozen at  $-80^{\circ}\text{C}$ . A maximal decrease of 16% in  $\text{A}\beta_{1-42}$  concentration was noted after the fourth freeze–thaw cycle, a decrease which was consistently different from all other freeze–thaw cycles. T-tau showed an increase (nonsignificant) after 2 freeze–thaw cycles, after which there was a downward trend, resulting in a significant difference between freeze–thaw cycles 2 and 4 ( $-7\%$ ). One freeze–thaw cycle just before analysis is standard procedure, but Le Bastard et al. concluded that 1 or 2 additional freeze–thaw cycles could be allowed. Temperature of freezing, delay until freezing, and freeze–thaw cycles therefore were found to influence CSF biomarker concentrations, stressing the need for harmonized operating procedures for preanalytical sample handling. The differences observed in this study, however, were relatively small and the authors concluded that the impact of variations in preanalytical sample handling on the clinical value of these CSF biomarkers remains to be fully determined.

The report by Le Bastard et al. complements existing recommendations from the Alzheimer's Biomarkers Standardization Initiative (ABSI) with respect to preanalytical stringency for CSF biomarkers for AD, although it does not fully discuss other key preanalytical issues which were addressed by the ABSI (10). Interestingly, all the CSF samples in their study were taken at a standardized time (between 8 and 11 AM), with donors in a fasted condition, and collected into polypropylene cryovials. The ABSI stated that because there is currently no clear evidence to support a diurnal variation and because it seems to be likely that there is no diurnal variation with AD biomarkers, there is no need to recommend any particular time of day for lumbar puncture (10). The ABSI also states that there is no evidence that the individual biomarkers of a patient are influenced by food intake or glucose values and that fasting is not required for analysis of  $\text{A}\beta_{1-42}$ , T-tau, and P-tau181P biomarker concentrations (10). Both these recommendations, however, post-date the time that Le Bastard et al. conducted their study, and the stringency applied by the authors appropriately reflects their efforts to remove all potential sources of variation. The rationale for polypropylene collection tubes is that the use of tubes composed of glass or polystyrene has been shown to result in lower  $\text{A}\beta_{1-42}$ , although with less effect on T-tau or P-tau181P (11).

Le Bastard et al. determined CSF biomarker concentrations with commercially available single-analyte INNOTEST assays, thus removing between-assay variability as a consideration. However, variability between kit lots and between laboratories is another source of variation for biomarker measurements that must be considered. CVs between laboratories in the range of 20% to 30% have been seen for CSF biomarker variability in the

Alzheimer's Association QC program, but for T-tau and P-tau181P, between-kit lot effects were observed to be much less than between-laboratory effects (12). As a postanalytical consideration, overall assay variability has also contributed to an inability to assign universal biomarker cutoff values for clinical use.

The authors point out as a limitation of their study that physiological variability was limited through fractionated sampling in a relatively small number of patients that might not necessarily reflect daily clinical practice (8). The effect of the preanalytical variation might also depend on underlying brain pathology (AD vs non-AD dementias vs controls); thus replication in a larger and more heterogeneous population including a control group would be desirable (8).

The article by Le Bastard et al. raises awareness that the test result is not just a function of the analytical procedure alone, but that the overall “brain-to-brain” laboratory test cycle (13) needs to be taken into consideration, with a particular focus on the preanalytical phase, in which most sources of “error” or at least variation occur (14). It is remarkable that novel biomarkers such as CSF  $\text{A}\beta_{1-42}$ , T-tau, and P-tau181P can become incorporated into state-of-the-art guidelines (6) without full comprehension of all the facets of preanalytical variation that may confound interpretation. This is even more important in the case of AD, as the scientific community strives toward a better understanding of the underlying pathophysiology and toward a more robust evidence base for clinically viable diagnostic and prognostic markers that may ultimately leverage clinically important decision-making and influence outcomes. A harmonized approach for investigating preanalytical variables for novel biomarkers would be desirable, although there has been some progress to develop quality indicators in this important area (14).

The goals of using biomarkers for dementia are to enable presymptomatic diagnosis and to monitor disease progression and response to therapeutic interventions, often still in an investigative setting. As in other situations, interpretation should always be in full clinical context. Thus postanalytical as well as preanalytical and analytical factors need to be taken into consideration. Ultimately, the responsibility falls to authors of guidelines and individual laboratories to stipulate what level of preanalytical stringency is required, balancing clinical needs with practical expediency. Improved comprehension of the role of preanalytical variables in AD biomarkers based on carefully conducted studies, like the study of Le Bastard et al., is a helpful step forward.

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**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or

*analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

**Authors' Disclosures or Potential Conflicts of Interest:** *No authors declared any potential conflicts of interest.*

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