Importance and Impact of Preanalytical Variables on Alzheimer Disease Biomarker Concentrations in Cerebrospinal Fluid

Nathalie Le Bastard,1,2 Peter Paul De Deyn,1,3,4 and Sebastiaan Engelborghs1,3*

BACKGROUND: Analyses of cerebrospinal fluid (CSF) biomarkers (β-amyloid protein, total tau protein, and hyperphosphorylated tau protein) are part of the diagnostic criteria of Alzheimer disease. Different preanalytical sample procedures contribute to variability of CSF biomarker concentrations, hampering between-laboratory comparisons. The aim of this study was to explore the influence of fractionated sampling, centrifugation, freezing temperature, freezing delay, and freeze–thaw cycles on CSF biomarker analyses.

METHODS: We studied fractionated sampling in sequential aliquots of lumbar CSF. Centrifuged and noncentrifuged samples from the same fraction were compared. CSF samples were subjected to different protocols (liquid nitrogen, −80 °C, and −20 °C; 24 h at 2–8 °C; and 24 and 48 h at room temperature). To study the influence of freeze–thaw cycles, samples were thawed up to 4 times and refrozen at −80 °C. CSF was collected in polypropylene tubes. We measured CSF biomarker concentrations with commercially available single-analyte Innotest assays.

RESULTS: CSF biomarker concentrations from non–blood-contaminated samples are not influenced by centrifugation or fractionated sampling. Freezing temperature and delayed storage can affect biomarker concentrations; freezing of CSF samples at −80 °C as soon as possible after collection is recommended. Consecutive freezing and thawing of CSF samples up to 3 times had little effect.

CONCLUSIONS: Temperature of freezing, delay until freezing, and freeze–thaw cycles significantly influence CSF biomarker concentrations, stressing the need for standard operating procedures for preanalytical sample handling. The differences observed in this study are, however, relatively small, and the impact on the clinical value of these CSF biomarkers needs to be determined.

© 2015 American Association for Clinical Chemistry
power. Most studies that have evaluated effects of freezing and thawing agree that these manipulations result in decreased concentrations of \( \beta \)-amyloid; however, there is disagreement on the number of freeze–thaw cycles acceptable to maintain \( \beta \)-amyloid concentrations at a constant level (10–13, 16–18).

Differences in preanalytical sampling procedures across studies are summarized in Fig. 1. To facilitate consensus on preanalytical standard operating procedures (SOPs) for CSF sampling with regard to biomarker analyses in an evidence-based manner, we investigated the influence of fractionated sampling, centrifugation, freezing temperature, freezing delay, and repeated freeze–thaw cycles on CSF \( \beta \)-amyloid, T-tau, and P-tau181P concentrations in 1 experimental setup.

**Methods**

**CSF SAMPLING AND HANDLING**

We obtained CSF from 38 patients admitted to the Memory Clinic of Hospital Network Antwerp between January and August 2010. All LPs were performed in the context of a diagnostic workup of presumed cognitive deterioration. Demographic and clinical data of the population included are described in Supplemental Data, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue5, and summarized in online Supplemental Table 1. All patients and/or their relatives gave informed consent. This study was approved by local medical ethics committees (University of Antwerp and Memory Clinic of Hospital Network Antwerp).

LPs were performed in the morning (between 0800 and 1100), with the patient in a sitting position, at the L3/L4 or L4/L5 interspaces with a 20-gauge, 3.5-inch Quincke point spinal needle (Becton Dickinson). All patients were in a fasting state (since the evening before) at the time of LP. Routine investigation included cell count and determination of total protein and glucose concentrations. The different CSF handling procedures are explained in detail below and depicted in Fig. 2. We used a structured study design to evaluate the effect of fractionated sampling (protocol 1: gradient effect), centrifugation (protocol 2: sample homogeneity), freezing temperatures and delayed sample storage (protocol 3), and freeze–thawing (protocol 4).
For protocols 1 and 2, CSF from prospectively sampled patients \((n = 20)\) was collected into 7 consecutive polypropylene cryovials (Nalgene® cat. nos. 5000–1020, maximum volume 1.5 mL, and 5000–0050, maximum volume 4.5 mL): 5 fractions (C1–C5) of 1.5 mL and 2 fractions (C6–C7) of 4.5 mL. The final cryovial (C7) was gently mixed before pipetting 1.5 mL CSF into a new cryovial (noncentrifuged C7.1). The same tubes were used for storage.

To investigate the influence of the fractionated sampling on CSF biomarker concentrations (protocol 1), we used the C3 and the noncentrifuged C7.1 fractions. The C3 fraction was chosen over the C1/C2 fractions because of possible blood contamination in the first milliliter of CSF. The C3 fraction was also pipetted into another vial (before freezing) to eliminate possible effects due to tube adsorption on only the C7.1 fraction. C, cerebrospinal fluid fraction; RT, room temperature; liq, liquid; f/t, freeze-thaw cycle. The boxes explain the goals of the different protocols.

For protocols 3 and 4, the C6 fraction from prospectively sampled patients (protocol 1), we used the C3 and the noncentrifuged C7.1 fractions. The C3 fraction was chosen over the C1/C2 fractions because of possible blood contamination in the first milliliter of CSF. The C3 fraction was also pipetted into another vial (before freezing) to eliminate possible effects due to tube adsorption on only the C7.1 fraction. Fractions C3 and C7.1 contained CSF taken 7.5 mL apart from each other. The remaining CSF in the large cryovial C7 was then centrifuged for 10 min at 1200g (Eppendorf centrifuge 5702, rotor A-4–38), and the supernatant was pipetted into a new cryovial (C7.1*).

To investigate the influence of centrifugation in atraumatic CSF (erythrocyte count <500/mm³) (protocol 2), we measured biomarker concentrations in the noncentrifuged and centrifuged C7.1 fractions. For protocols 1 and 2, only CSF samples with erythrocyte count <500/mm³, leukocyte count <10/mm³, and total CSF protein concentration between 12 and 60 mg/dL were included, to analyze samples representative of healthy CSF. All CSF samples for protocols 1 and 2 were frozen in liquid N₂ at the same time within 2 h (range 20–120 min) after sampling at the hospital and transported to our Biobank facilities for storage at −80 °C until analysis.

For protocols 3 \((n = 22)\) and 4 \((n = 20)\), the C6 fraction from prospectively sampled patients with a total volume of 4.5 mL was centrifuged for 10 min at 1200g and divided into 10 fractions of 350 µL that were transported at room temperature to our Biobank facilities.
To investigate the influence of freezing temperature and freezing delay (protocol 3), 6 CSF aliquots were frozen in liquid N₂ before storage at −80 °C (snap freezing), −80 °C (slow freezing, ultralow temperature), or 20 °C (slow freezing, low temperature); or were incubated at 2–8 °C for 24 h, room temperature for 24 h, or room temperature for 48 h. Samples incubated at 2–8 °C or room temperature were all transferred to −80 °C after the proposed time period.

To investigate the influence of repeated freeze–thaw cycles (protocol 4), we used the 4 remaining aliquots of the C6 fraction. We measured CSF biomarker concentrations after a maximum of 4 freeze–thaw cycles. At each thawing cycle, CSF samples were kept at room temperature for 2.5 h and refrozen at −80 °C for at least 1 day. Samples were not vortex-mixed during the thawing procedure and, to exclude the effect of tube absorption, CSF samples were also not transferred into another cryovial after thawing.

CSF BIOMARKER ANALYSIS
We measured CSF concentrations of Aβ₁₋₄₂, T-tau, and P-tau₁₈₁P with commercially available single-analyte ELISA kits (Innotest® β-Amyloid₁₋₄₂, Innotest hTau-Ag, and Innotest Phospho-Tau₁₈₁P, Fujirebio Europe) in April and October 2010. For protocol 3, the mean delay between sampling and analysis was 47 days (range 14–127). For protocol 4, the mean delay between sampling and analysis was 124 days (range 40–166).

All samples were tested according to the test instructions provided by the manufacturer. The measurement ranges of the test kits are described in the package inserts (Aβ₁₋₄₂, 125–2000 pg/mL; T-tau, 75–1200 pg/mL; P-tau₁₈₁P, 15.6–500 pg/mL). If the T-tau concentrations obtained were above the highest calibrator concentration of 1200 pg/mL, samples were retested by extension of the calibrator concentration range through inclusion of 2400 pg/mL as the highest calibrator concentration in the standard curve. Out-of-range biomarker values refer to values outside these calibration ranges. All samples from 1 patient within 1 protocol were analyzed during the same ELISA run to exclude test variability as a potential cause of variation between the different aliquots obtained from 1 patient.

STATISTICAL ANALYSES
CSF biomarker concentrations were log₁₀-transformed before data analysis. Data analysis was performed with mixed models, controlling for use of different kit lots. We calculated relative median differences between different treatments together with the associated 95% CIs. A hypothesis test was considered significant if its associated P value was <0.050. Analyses were performed with and without the samples with out-of-range biomarker values. Only data including the out-of-range samples are presented in all figures and tables. If the results without the out-of-range samples were meaningfully different, that is stated in the text. Statistical analyses were performed with SAS version 9.2.

Results
FRACTIONATED SAMPLING AND CENTRIFUGATION (PROTOCOLS 1 AND 2)
Median erythrocyte and leukocyte counts were 1/mm³ (range 0–245/mm³) and 0/mm³ (range 0–4/mm³), respectively. Median protein and glucose concentrations were 34 mg/dL (range 22–55 mg/dL) and 56 mg/dL (range 45–79 mg/dL), respectively. The relative median differences in concentrations of the 3 markers were not found to be significant for fractionated sampling and centrifugation (Table 1). The maximal difference between CSF fractions C3 and C7.1 was approximately 5% for Aβ₁₋₄₂ and P-tau₁₈₁P. For T-tau, uncertainty was larger (14%) owing to 1 outlier. After excluding this outlier, the maximal difference between C3 and C7.1 fractions was approximately 9%. The maximal difference between CSF fractions C7.1 and C7.1* was approximately 7% for all 3 biomarkers.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ₁₋₄₂, pg/mL</td>
<td>421 (269–855)</td>
<td>421 (223–907)</td>
<td>426 (246–875)</td>
</tr>
<tr>
<td>T-tau, pg/mL</td>
<td>411 (71–2183)</td>
<td>368 (63–1224)</td>
<td>386 (63–1466)</td>
</tr>
<tr>
<td>P-tau₁₈₁P, pg/mL</td>
<td>61 (13–239)</td>
<td>59 (13–299)</td>
<td>58 (13–318)</td>
</tr>
</tbody>
</table>

None of the comparisons were statistically significant.
Median protein and glucose concentrations were 35 mg/dL (range 16–110 mg/dL) and 55 mg/dL (range 41–79 mg/dL), respectively. Samples that needed to be frozen on the same day (liquid N\textsubscript{2}, −80 °C, −20 °C) were frozen within 4 h after sampling (median 122 min; range 75–230 min).

Snap freezing of samples in liquid N\textsubscript{2} led to (borderline) significantly higher A\textsubscript{β1–42} concentrations in comparison to freezing at −20 °C (\textit{P} = 0.048), whereas the difference between freezing at −20 °C and −80 °C was nonsignificant (\textit{P} = 0.135) (Table 2). Freezing at −20 °C led to significantly lower T-tau and P-tau\textsubscript{181P} concentrations in comparison to freezing at −80 °C (\textit{P} = 0.012 and 0.005, respectively). Freezing at −20 °C led to significantly lower T-tau and P-tau\textsubscript{181P} concentrations in comparison to freezing at −80 °C (\textit{P} = 0.012 and 0.005, respectively). Freezing at −20 °C led to significantly lower T-tau and P-tau\textsubscript{181P} concentrations in comparison to freezing at −80 °C (\textit{P} = 0.012 and 0.005, respectively). Freezing at −20 °C led to significantly lower T-tau and P-tau\textsubscript{181P} concentrations in comparison to freezing at −80 °C (\textit{P} = 0.012 and 0.005, respectively).

There were no significant differences between freezing at −80 °C and a delay in freezing. The relative median differences between the different conditions are visualized in Fig. 3.

**FREEZE–THAW CYCLES (PROTOCOL 4)**

Median protein and glucose concentrations were 35 mg/dL (range 16–110 mg/dL) and 55 mg/dL (range 41–79 mg/dL), respectively. Samples were frozen within 4 h after sampling (median 115 min; range 75–230 min).

Lower A\textsubscript{β1–42} concentrations were found in samples that underwent 4 freeze–thaw cycles in comparison with all other samples (1 vs 4, \textit{P} = 0.022; 2 vs 4, \textit{P} < 0.001; 3 vs 4, \textit{P} = 0.002) (Fig. 4). The T-tau concentrations were also lower after 4 freeze–thaw cycles in comparison with samples that underwent 2 freeze–thaw cycles (\textit{P} = 0.016), but were not significantly different from samples with 1 or 3 freeze–thaw cycles. Freezing and thawing of CSF had no effect on P-tau\textsubscript{181P} concentrations.

### Discussion

Poor standardization of preanalytical sample handling procedures has hampered the comparison of CSF A\textsubscript{β1–42}, T-tau, and P-tau\textsubscript{181P} concentrations between different laboratories or studies. In this study, we examined the effects of fractionated sampling, centrifugation, freezing temperature, freezing delay, and freeze–thaw cycles. CSF biomarker concentrations from non–blood-contaminated samples were not found to be significantly influenced by centrifugation or fractionated sampling.

### Table 2. CSF biomarker concentrations for protocol 3 (freezing temperature and delay) and 4 (freeze–thaw cycles).\footnote{Data are medians (range). Significant differences are indicated in bold. See also Figure 2.}

<table>
<thead>
<tr>
<th>Protocol</th>
<th>A\textsubscript{β1–42}, pg/mL</th>
<th>T-tau, pg/mL</th>
<th>P-tau\textsubscript{181P}, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>−80 °C\textsuperscript{b} (n = 22)</td>
<td>300 (165–579)</td>
<td>359 (46–1875)</td>
<td>53 (14–172)</td>
</tr>
<tr>
<td>−20 °C</td>
<td>313 (166–636)</td>
<td>348 (52–1550)</td>
<td>52 (10–165)</td>
</tr>
<tr>
<td>Liquid N\textsubscript{2}</td>
<td>335 (172–594)</td>
<td>396 (63–1604)</td>
<td>0.048</td>
</tr>
<tr>
<td>24 h at 2–8 °C</td>
<td>307 (145–576)</td>
<td>386 (52–1890)</td>
<td>0.296</td>
</tr>
<tr>
<td>24 h at room temperature</td>
<td>324 (178–612)</td>
<td>403 (48–1871)</td>
<td>0.732</td>
</tr>
<tr>
<td>48 h at room temperature</td>
<td>320 (195–558)</td>
<td>384 (50–1919)</td>
<td>0.804</td>
</tr>
</tbody>
</table>

| Protocol 4: freeze–thaw cycles (n = 20) |
|-------------------------------|-------------|--------------------------------|
| 1 freeze–thaw cycle\textsuperscript{b} | 428 (190–837) | 417 (89–2063) |
| 2 freeze–thaw cycles | 443 (229–839) | 422 (90–2228) |
| 3 freeze–thaw cycles | 411 (229–939) | 413 (86–1928) |
| 4 freeze–thaw cycles | 398 (178–833) | 406 (80–2070) |

\[a\] Data are medians (range). Significant differences are indicated in bold. See also Figure 2.

\[b\] Standard procedure.
Different freezing temperatures and delays in the freezing process introduced differences compared with freezing at −80 °C, which is considered to be the standard procedure. The consecutive freezing and thawing of CSF samples for up to 3 times demonstrated little effect on biomarker concentrations.

**Fig. 3.** Relative median differences (%) between the different freezing conditions and introductions of a delay before freezing, compared with freezing at −80 °C.

Results are presented as the relative median difference with 95% CI. For example, the relative median difference for Aβ_{1–42} comparing samples that remained at room temperature (RT) for 48 h and samples that were frozen immediately at −80 °C is approximately 8%, indicating that the Aβ_{1–42} concentrations are 8% higher in samples that were kept at room temperature for 48 h.

**Fig. 4.** Relative median differences (%) between the different freeze–thaw cycles.

Results are presented as the relative median difference with 95% CI. For example, the relative median difference for Aβ_{1–42} comparing samples that underwent 1 and 4 freeze–thaw cycles is approximately 8%, indicating that the Aβ_{1–42} concentrations were 8% lower in the samples that underwent 4 freeze–thaw cycles in comparison to samples that underwent only 1 freeze–thaw cycle. 1/2, 1 vs 2; 1/3, 1 vs 3; 1/4, 1 vs 4; 2/3, 2 vs 3; 2/4, 2 vs 4; 3/4, 3 vs 4.

**FRACTIONATED SAMPLING**

The volume of CSF withdrawn varies across centers, patients (evacuating LP in case of normal-pressure hydrocephalus), or study type (research, routine, clinical trial), and CSF can be collected in consecutive polypropylene vials or in a large polypropylene tube before aliquoting.
Brain-derived proteins usually show a rostro-caudal concentration gradient, with higher concentrations in ventricular CSF compared with lumbar CSF (5, 8), implying that different volumes or fractionated sampling could generate differences in CSF biomarker concentrations. However, differences in Aβ1–42 and T-tau between lumbar CSF fractions were not found (6). Our data on lumbar CSF fractions agree with previous studies and showed that this is also true for P-tau181P, although a difference of only 7.5 mL CSF was investigated (between 2 fractions), and the estimated lumbosacral CSF volume of healthy individuals, though highly variable, is much more than 7.5 mL (19).

**CENTRIFUGATION**

In the case of blood-contaminated CSF, freezing of samples without prior centrifugation might lead to hemolysis, which is known to cause aberrant results in the determination of several other substances present in erythrocytes (e.g., neuron-specific enolase (20) or synuclein (21)). Blood-contaminated CSF was defined as >500 erythrocytes/mm³, and the detection limit of visual inspection of CSF for blood contamination is approximately 0.05% (vol/vol) blood (22). As a consequence of blood–brain barrier deficits, abundant plasma proteins could influence the outcome of CSF tests, although it was shown in the past that the ex vivo addition of a number of plasma proteins followed by a direct measurement in the assay or overnight incubation of the sample did not affect Aβ1–42 concentrations, except for conjugated bilirubin and fibrinogen (17).

Therefore, we wondered whether centrifugation of CSF is mandatory for non–blood-contaminated samples. Our results indicate that centrifugation has no effect on CSF biomarker concentrations in macroscopically non–blood-contaminated samples. For Aβ1–42, 2 studies that did not freeze–thaw their CSF samples before analysis yielded contradictory results; one showed increasing Aβ1–42 concentrations in centrifuged samples independent of the centrifugation temperature (4 °C or room temperature) (6), whereas the other showed stable Aβ1–42 concentrations independent of the time lapse (1, 4, 48, or 72 h) between collection and centrifugation (11). Another study on Aβ1–42 that did freeze the centrifuged and noncentrifuged samples before analysis showed no changes in Aβ1–42, independent of the technology platform (12). All studies investigating the effect of centrifugation on T-tau and P-tau181P showed no differences (10–12). Moreover, no significant changes in Aβ1–42 arose from the addition of 5000 lysed erythrocytes/mm³ (6). The concentrations of Aβ1–42 and T-tau were also determined in 1 severely blood-contaminated sample (28.800 erythrocytes/mm³) that had been divided, with 1 aliquot being centrifuged before freezing and 1 aliquot not centrifuged, which did not lead to a considerable difference (11). The present data, combined with the majority of the data from the literature, allows us to conclude that centrifugation (before or after freezing) does not affect CSF biomarker concentrations, probably not even when CSF is contaminated with blood. Although Aβ1–42 can also be found in plasma, originating from different pools such as platelets, muscle, and liver, its concentration is much lower than in CSF (23) and is therefore unlikely to significantly alter the concentrations measured in blood-contaminated CSF. Maximal differences of 9% and 7% for fractionated sampling and centrifugation, respectively, were smaller than the reported interassay CV for the assays used (10, 17). In addition, centrifugation of the non–blood-contaminated samples did not lead to a significantly different intraassay CV for Aβ1–42 (4.6% vs 3.8%), T-tau (4.1% vs 4.8%), or P-tau181P (2.2% vs 1.4%). However, the possible impact of hemoglobin concentrations and/or the number of red blood cells on assay performance has not been studied in detail.

**FREEZING TEMPERATURE**

Freezing of samples can be done at different temperatures: −20 °C, −80 °C, and −196 °C (liquid N₂) are most commonly used. From long-term stability studies, it is already known that Aβ1–42 concentrations in samples frozen at −80 °C are stable for at least 2 years (6, 18), as are concentrations of T-tau and P-tau181P when stored at −20 °C for at least for 2 and 4 years, respectively (12, 18). A storage artifact is seen for cystatin C analysis after freezing at −20 °C, but not at −80 °C (24). Long-term stability of CSF samples in liquid N₂, or direct freezing of a sample in liquid N₂ compared with other freezing conditions, has never been investigated extensively. Freezing at −80 °C in comparison to (initial) freezing in liquid N₂ resulted in borderline significantly lower Aβ1–42 concentrations, whereas the comparison of −80 °C with −20 °C was not found to be significant. We can speculate that freezing in liquid N₂ quickly reduces degradation of proteins by protease activity and stabilizes all proteins in the CSF, thereby preventing loss of full-length Aβ1–42. However, freezing in liquid N₂ immediately after CSF collection should then have led to higher Aβ1–42 concentrations in comparison to freezing in liquid N₂ after 2 h, which was shown not to be the case (14), and freezing at −80 °C should have also resulted in higher concentrations than freezing at −20 °C. Indeed, T-tau and P-tau181P concentrations differed significantly between −80 °C and −20 °C frozen samples, but not those of Aβ1–42. Although a significant difference was found for Aβ1–42 after freezing in liquid N₂ in comparison to freezing at −80 °C, these results should be interpreted with caution, because of the relatively large CI for Aβ1–42 in comparison to T-tau and P-tau181P, indicative of a larger variation during measure-
ment for $A\beta_{1-42}$. One other study evaluating the effect of different freezing temperatures for $A\beta_{1-42}$ found no difference (6). In conclusion, freezing in liquid $N_2$ yields the highest concentrations for all 3 markers but is not the most practical solution, and freezing of CSF samples at $-20\,^\circ C$ pending biomarker analysis, which would be preferred from a practical point of view, is discouraged. The fact that long-term studies show stable concentrations for all 3 biomarkers means that differences between liquid $N_2$, $-80\,^\circ C$, and $-20\,^\circ C$ are most likely attributable to the initial freezing conditions. This is also seen for tubes that immediately adsorb $A\beta_{1-42}$ (25).

FREZING DELAY

CSF samples are frequently shipped to reference laboratories at room temperature, with cooling, or after freezing (Fig. 1). Shipment of samples by means of regular mail takes at least 24 h. Therefore, $A\beta_{1-42}$, T-tau, and $P$-tau$_{181P}$ should preferably be stable at room temperature for several days (also because shipment of CSF samples on dry ice is very costly). In comparison with immediate (within 4 h) freezing of samples at $-80\,^\circ C$, the $A\beta_{1-42}$ concentration gradually increased by almost 15% during 48 h of storage at room temperature. Bjerke et al. (6) stated that storage at room temperature for $>24$ h did not significantly affect $A\beta_{1-42}$ concentrations in comparison to both fresh samples and storage at $-80\,^\circ C$ until analysis. Another study found relatively stable CSF biomarker concentrations even after 4 days, although unsystematic variation increased over time (18). In contrast to this, $A\beta_{1-42}$ has been found to decrease during the first 48 h (11) or, more in line with the present study, even to increase within 24 h (14). An increase could be explained by deoligomerization of $A\beta_{1-42}$ or the release of $A\beta_{1-42}$ from amyloid-binding proteins, such as (pre)albumin or (apo)lipoproteins. The largest proportion of $A\beta$ in plasma and CSF is bound to proteins (26, 27). A higher ratio of free to bound $A\beta$ could substantially alter $A\beta_{1-42}$ concentrations. Incubation of the assay at different temperatures influences the outcome of the $A\beta_{1-42}$ assay, with higher $A\beta_{1-42}$ concentrations at higher temperatures (17), and storage of samples at higher temperatures does seem to have the same effect on $A\beta_{1-42}$ (24 h at 2–8 °C or room temperature, $P = 0.020$), in contrast to what has previously been published on a smaller population (11). In addition, Sancesario et al. (15) also demonstrated (reversible) increased $A\beta_{1-42}$ in samples that were kept at 37 °C before freezing, an effect that was seen for samples from AD patients, not controls. T-tau was found to be stable at room temperature (14, 16, 28), for up to 22 days in 1 particular study, but tended to decrease in that same study after 12 days when stored at 37 °C degrees (11), which would indicate that stability of T-tau is also dependent on temperature. No effects of storage at room temperature on $P$-tau$_{181P}$ were found in previous studies (10, 14, 16). One study that examined possible differences between storage at 4 °C for 4, 24, and 72 h and immediate freezing at $-80\,^\circ C$ could not demonstrate an effect (10). In conclusion, we found significant differences in $A\beta_{1-42}$ and $P$-tau$_{181P}$ concentrations after delayed storage before freezing.

FREEZE–THAW CYCLES

Our results showed a maximal decrease of 16% in $A\beta_{1-42}$ concentration after the fourth freeze–thaw cycle, which is consistently different from all other freeze–thaw cycles. Previous studies also found decreasing $A\beta_{1-42}$ after the third (−20%) or fifth (−15%) freeze–thaw cycle (11, 12, 17), whereas no differences were found between fresh CSF samples and samples frozen and thawed once (11, 13, 17). T-tau showed a (nonsignificant) increase after 2 freeze–thaw cycles, after which a decreasing trend was imposed, resulting in a significant difference between freeze–thaw cycles 2 and 4 (−7%). T-tau is considered to be very stable, as several authors failed to find significant differences in T-tau concentrations between samples frozen and thawed once (11, 13) and in up to 6 freeze–thaw cycles (11, 12, 16), although Schoonenboom et al. (11) also observed an increase in T-tau followed by a decrease. $P$-tau$_{181P}$, on the other hand, proved to be very stable in the present study, and the small CIs point to a low variability in evolution of $P$-tau$_{181P}$ among the different individuals, in accord with the results of earlier studies (10, 16, 29). Zimmermann et al. (18) found no significant systematic changes of the biomarker concentrations, but did observe increasing unsystematic variation after 3 freeze–thaw cycles, especially for $A\beta_{1-42}$ and T-tau. In summary, $A\beta_{1-42}$ and possibly also T-tau are influenced by repeated freeze–thaw cycles.

LIMITATIONS OF THE STUDY

Physiological variability was limited through fractionated sampling in a relatively small number of patients, which is both a strength (variability of the results is largely due to variability of the preanalytical variables under study) and a limitation (the limited physiological variability might not necessarily reflect daily clinical practice). Moreover, the impact of the preanalytical variables might depend on the brain pathology (AD vs non-AD dementias vs controls). Therefore, the lack of a control group is another limitation of the study. A replication study in a larger and more heterogeneous population including a control group might strengthen the conclusions of this article.

Conclusions

The following recommendations can be proposed as a result of the present study.

Clinical Chemistry 61:5 (2015) 741
Fractionated sampling: It is possible to collect CSF in 1 large volume (for division into aliquots at a later phase) or several smaller volumes, since the total volume of CSF collected does not affect the concentrations of tau and amyloid proteins.

Centrifugation: If atraumatic samples are used, centrifugation is not required for CSF biomarker analyses.

Freezing temperature: Freezing at −80 °C can be a general recommendation for long-term as well as short-term storage, since freezing at −20 °C seems to influence CSF biomarker concentrations. Freezing in liquid N2 is not recommended.

Freezing delay: The delay in freezing should be minimized.

Freeze–thaw cycles: One freeze–thaw cycle just before analysis is standard procedure, but 1 or 2 additional freeze–thaw cycles are allowed.

Centrifugation: If atraumatic samples are used, centrifugation is not required for CSF biomarker analyses.

Freezing temperature: Freezing at −80 °C can be a general recommendation for long-term as well as short-term storage, since freezing at −20 °C seems to influence CSF biomarker concentrations. Freezing in liquid N2 is not recommended.

Freezing delay: The delay in freezing should be minimized.

Freeze–thaw cycles: One freeze–thaw cycle just before analysis is standard procedure, but 1 or 2 additional freeze–thaw cycles are allowed.

Authors’ Disclosures or Potential Conflicts of Interests: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interests:

Employment or Leadership: N. Le Bastard, Fujirebio Europe.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.

Research Funding: This work is part of the BIOMARKPD project within the EU Joint Programme for Neurodegenerative Disease Research (JPND). The work was supported by the Research Foundation Flanders (FWO), the Agency for Innovation by Science and Technology (IWT), the Belgian Science Policy Office Interuniversity Attraction Poles (IAP) program, and the Flemish government-initiated Methusalem excellence grant. Innogenex assays were provided by Fujirebio Europe (Ghent, Belgium). N. Le Bastard, Alzheimer’s Biomarkers Standardization Initiative (ABSI); P.P. De Deyn, the central Biobank facility of the Institute Born-Bunge/University Antwerp; S. Engelenborgs, the University of Antwerp Research Fund, the Alzheimer Research Foundation (SAO-FRA), ABSI, and the EU/EFFP InnovAXE Medicines Initiative Joint Undertaking (EMI grant no. 115372).

Expert Testimony: None declared.

Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: The authors thank Ellis Niemantsverdriet, MSc, Charisse Somers, MSc (BIODEM, UAntwerp), and Inge Bats (Institute Born-Bunge) for administrative assistance as well as Fujirebio Europe (Ghent, Belgium) for kindly providing the Innogenex assays.

References

23. Le Bastard N, Aerts L, Leurs J, Blomme W, De Deyn PP, Engelborgs S. No correlation between time-linked...


