Importance and impact of preanalytical variables on Alzheimer disease biomarker concentrations in cerebrospinal fluid

Importance and impact of pre-analytical variables on Alzheimer's disease biomarker concentrations in cerebrospinal fluid

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List of abbreviations:

$A\beta_{1-42}$	β-amyloid protein of 42 amino acids
T-tau	total tau protein
$P\text{-}tau_{181P}$	tau phosphorylated at threonine 181
CSF	cerebrospinal fluid
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Associations
LP	lumbar puncture
SOP	standard operating procedures
RT	room temperature
ELISA	enzyme-linked immunosorbent assay
OD	optical density
CI	confidence interval
CV	coefficient of variation

Abstract

Background: CSF biomarker analyses (β-amyloid ($A\beta_{1-42}$), total tau (T-tau) and hyperphosphorylated tau ($P\text{-}tau_{181P}$)) are part of the diagnostic criteria of Alzheimer's disease (AD). Different pre-analytical 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: Fractionated sampling was studied in sequential aliquots of lumbar CSF. Centrifuged and non-centrifuged samples from the same fraction were compared. CSF samples were subjected to different protocols (liquid nitrogen; -80°C; -20°C; 24h at 2-8°C; 24h and 48h at room temperature). To study the influence of freeze-thaw cycles, samples were thawed up to four times and (re)frozen at -80°C. CSF was collected in polypropylene tubes. CSF biomarker concentrations were determined 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 for up to three 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 pre-analytical 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.

1. Introduction

The cerebrospinal fluid (CSF) biomarkers β-amyloid ($A\beta_{1-42}$), total tau (T-tau) and hyperphosphorylated tau ($P\text{-}tau_{181P}$) have been integrated into the revised diagnostic criteria of Alzheimer's disease (1). AD CSF biomarker concentrations can show considerable variation. The sources of these variations can be found in patient selection, pre-analytical, analytical, or post-analytical aspects of interpretation of the biomarker data (2, 3).

Pre-analytical confounding factors include the lumbar puncture (LP) procedure, tubes for collection and storage of the sample, and sample handling and storage. Several studies have been undertaken to quantify their effects (reviewed by Vanderstichele et al. (2) and del Campo et al (4)). Introduction of confounding factors starts at sample collection. Rostro-caudal gradients for brain-derived CSF biomarkers are assumed (5). However, these gradients do not lead to significant differences in $A\beta_{1-42}$ and T-tau concentrations between lumbar CSF fractions (6), but controversy exists for the comparison of cisternal/ventricular and lumbar CSF (7-9). Concentrations have not been found to be influenced by centrifugation (10-12), except for one recent well-designed study that demonstrated increased $A\beta_{1-42}$ in centrifuged samples, independent of centrifugation temperature (6).

Studies aimed at unraveling the effect of storage conditions are less clear-cut (6, 10, 11, 13-16) owing to different methodological approaches or the lack of statistical power. Most studies that evaluated effects of freezing and thawing agree that these manipulations result in decreased concentrations of $A\beta_{1-42}$; however, there is disagreement on the number of freeze-thaw cycles acceptable to maintain the $A\beta_{1-42}$ concentrations at a constant level (10-13, 16-18)

Differences in pre-analytical sampling procedures across studies are summarized in **Figure1**. To facilitate consensus on pre-analytical standard operating procedures (SOP) 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 $A\beta_{1-42}$, T-tau and P-tau_{181P} concentrations in one experimental set-up.

2. Methods

2.1. CSF sampling and handling

CSF was obtained from 38 patients admitted to the Memory Clinic of Hospital Network Antwerp (ZNA) between January and August 2010. All LPs were performed in the context of a diagnostic work-up of presumed cognitive deterioration. Demographic and clinical data of the population included are described in the **Supplementary Data** and summarized in **Supplementary Table 1**. All patients and/or their relatives gave informed consent. This study was approved by the local medical ethics committees (UAntwerp, ZNA).

LPs were performed in the morning (between 8 and 11a.m.), with the patient in a sitting position, at the L3/L4 or L4/L5 interspaces using a 20 Gauge, 3.5 inch Quincke point spinal needle (Becton, Dickinson and Company). 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 **Figure2**. A well-structured study design was used to evaluate the effect of fractionated sampling (Protocol1~gradient effect), centrifugation (Protocol2~sample homogeneity), freezing

temperatures / delayed sample storage (Protocol3), and freeze-thawing (Protocol4). A full description of the test procedures will help the reader to review differences between published literature data.

For protocols 1 and 2, CSF from prospectively sampled patients (n=20) was collected into seven consecutive polypropylene cryovials (Nalgene® cat.no.5000-1020 (maximum volume: 1.5mL) and 5000-0050 (4.5mL)): five fractions (C1-C5) of 1.5mL and two fractions (C6-C7) of 4.5mL. The last cryovial (C7) was gently mixed before pipetting 1.5mL CSF into a new cryovial (non-centrifuged C7.1). The same tubes were used for storage.

To investigate the influence of the fractionated sampling on CSF biomarker concentrations, the C3 and the non-centrifuged C7.1 fractions were used (Protocol1). The C3 fraction was chosen over the C1/C2 fractions because of possible blood contamination in the first mL 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, by also using a second tube for the C3 fraction. Fractions C3 and C7.1 contained CSF taken 7.5mL apart from each other. The remaining CSF in the large cryovial C7 was then centrifuged for 10min at 1200xg (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), biomarker concentrations were determined in the non-centrifuged and centrifuged C7.1 fractions (Protocol2). For protocols 1 and 2, only CSF samples with an erythrocyte count <500/mm³, a leukocyte count <10/mm³ and a total CSF protein concentration between 12 and 60mg/dL were included in order to analyze samples, representative of normal CSF. All CSF samples for protocols 1 and 2 were frozen in liquid nitrogen (N_2) at the same time within two hours (range: 20-120min) 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 4.5mL was centrifuged for 10min at 1200xg (Eppendorf centrifuge 5702, rotor A-4-38) and aliquoted into 10 fractions of 350µL, that were transported at room temperature (RT) to our Biobank facilities.

To investigate the influence of freezing temperature and freezing delay, six CSF aliquots were either frozen in liquid N_2 before storage at -80°C (snap freezing); at -80°C (slow freezing, ultra-low temperature); at -20°C (slow freezing, low temperature); or incubated at 2-8°C for 24±2h; or incubated at RT for 24±2h; or incubated at RT for 48±2h (Protocol 3). Samples incubated at 2-8°C or RT were all transferred to -80°C after the proposed time period.

To investigate the influence of repeated freeze-thaw cycles, the four remaining aliquots of the C6 fraction were used (Protocol4). CSF biomarker concentrations were determined after a maximum of four freeze-thaw cycles. At each thawing cycle, CSF samples were kept at RT for 2.5h and refrozen at -80°C for at least one day. Samples were not vortexed during the thawing procedure and, to exclude the effect of tube absorption, CSF samples were also not transferred into another cryovial after thawing.

2.2. CSF biomarker analysis

CSF concentrations of $A\beta_{1-42}$, T-tau and P-tau_{181P} were determined with commercially available single-analyte ELISA kits (INNOTEST® β-$AMYLOID_{(1-42)}$, INNOTEST® hTAU-Ag, INNOTEST® $PHOSPHO\text{-}TAU_{(181P)}$; Fujirebio Europe) in April and October 2010. For protocol 3, the mean delay between sampling and analysis

was 47 days (min-max 14-127). For protocol 4, the mean delay between sampling and analysis was 124 days (min-max 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 ($A\beta_{1-42}$: 125-2000pg/mL, T-tau: 75-1200pg/mL, P-tau_{181P}: 15,6-500pg/mL). If the T-tau concentrations obtained were above the highest calibrator concentration of 1200pg/mL, samples were retested by extension of the calibrator concentration range through inclusion of 2400pg/mL as the highest calibrator concentration in the standard curve. Out-of-range biomarker values refer to values outside these calibrator ranges. All samples from one patient within one protocol were analyzed during the same ELISA run to exclude test variability as a potential cause of variation between the different aliquots obtained from one patient.

2.3. Statistical analyses

CSF biomarker concentrations were $\log_{10}$-transformed prior to data analysis. Data analysis was performed using mixed models, controlling for use of different kit lots. Relative median differences between different treatments were calculated together with the associated 95% confidence intervals (CI). A hypothesis test was considered significant if its associated *P*-value was less than 0.050. Analyses were performed with and without the samples with out-of-range biomarker values. Only data including the out-of-range samples were presented in all figures and tables. If the results without the out-of-range samples were meaningfully different, it is mentioned as such in the text. Statistical analyses were performed using SAS v9.2.

3. Results

3.1. Fractionated sampling and centrifugation (Protocol1 and 2)

Median erythrocyte and leukocyte count were 1/mm³ (range: 0-245/mm³) and 0/mm³ (range: 0-4/mm³), respectively. Median protein and glucose concentrations were 34mg/dL (range: 22-55mg/dL) and 56mg/dL (range: 45-79mg/dL), respectively. The relative median differences in concentrations of the three markers were not found significant for fractionated sampling and centrifugation (**Table1**). The maximal difference between CSF fractions C3 and C7.1 was about 5% for $A\beta_{1\text{-}42}$ and P-tau_{181P}. For T-tau, uncertainty was larger (14%) due to one outlier. After excluding this outlier, the maximal difference between C3 and C7.1 fractions was about 9%. The maximal difference between CSF fractions C7.1 and C7.1* was about 7% for all three biomarkers.

3.2. Freezing temperature and freezing delay (Protocol3)

Median protein and glucose concentrations were 35mg/dL (range: 16-110mg/dL) and 55mg/dL (range: 41-79mg/dL), respectively. Samples that needed to be frozen on the same day (liquid N_2, -80°C, -20°C) were frozen within four hours after sampling (median: 122min; range: 75-230min).

Snap-freezing of samples in liquid N_2 led to (borderline) significantly higher $A\beta_{1\text{-}42}$ concentrations in comparison to freezing at -80°C (*P*=0.048), whereas the difference between freezing at -20°C and -80°C was non-significant (*P*=0.135). Freezing at -20°C led to significantly lower T-tau and P-tau_{181P} concentrations in comparison to freezing at -80°C (*P*=0.012 and *P*=0.001, respectively). Excluding the out-of-range samples, the relative median difference in T-tau between -20°C and -80°C did not remain significant (3.37% 95%CI: -0.32% - 6.94% *P*=0.073).

CSF $A\beta_{1-42}$ concentrations were significantly higher in samples kept at RT for 48h (*P*=0.009) and borderline non-significant for 24h at RT (*P*=0.065) in comparison to samples frozen at -80°C. CSF P-tau_{181P} values were significantly lower in samples kept at 2-8°C for 24h before freezing at -80°C in comparison to samples frozen at -80°C (*P*=0.016). This significant difference did not hold when excluding the out-of-range samples (*P*=0.244). 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 **Figure3**.

3.3. Freeze-thaw cycles (Protocol4)

Median protein and glucose concentrations were 35mg/dL (range: 16-105 mg/dL) and 57mg/dL (range: 41-80mg/dL), respectively. Samples were frozen within four hours after sampling (median: 115min; range: 75-230min). Lower $A\beta_{1-42}$ concentrations were found in samples that underwent four freeze-thaw cycles in comparison to all other samples (1/4: *P*=0.022; 2/4: *P*<0.001; 3/4: *P*=0.002) (Figure4). The T-tau concentrations were also lower after four freeze-thaw cycles in comparison to samples that underwent two freeze-thaw cycles (*P*=0.016), but was not significantly different from samples with one or three freeze-thaw cycles. Freezing and thawing of CSF had no effect on P-tau_{181P} concentrations.

4. Discussion

Poor standardization of the pre-analytical sample handling procedures has hampered the comparison of CSF $A\beta_{1-42}$, T-tau and P-tau_{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. Different freezing temperatures and delays in the freezing process introduced differences as compared to freezing at -80°C, which is considered to be the standard procedure. The consecutive freezing and thawing of CSF samples for up to three times demonstrated little effect on biomarker concentrations.

4.1. 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 one large polypropylene tube before aliquoting. Brain-derived proteins usually show a rostro-caudal concentration gradient with higher concentrations in ventricular CSF compared to lumbar CSF (5, 8), implicating that different volumes or fractionated sampling could generate differences in CSF biomarker concentrations. However, differences in $A\beta_{1\text{-}42}$ 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-tau_{181P}, although only a difference of 7.5 mL CSF was investigated (between two fractions) and the estimated lumbosacral CSF volume of healthy subjects, though highly variable, is much more than 7.5 mL (19).

4.2. Centrifugation

In 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 about 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 an overnight incubation of the sample, did not affect $A\beta_{1-42}$ concentration, 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 $A\beta_{1-42}$, two studies that did not freeze-thaw their CSF samples before analysis yielded contradictory results; one showed increasing $A\beta_{1-42}$ concentrations in centrifuged samples independent of the centrifugation temperature (4°C or RT) (6), whereas the other showed stable $A\beta_{1-42}$ concentrations independent of the time lapse (1h, 4h, 48h or 72h) between collection and centrifugation (11). Another study on $A\beta_{1-42}$ that did freeze the centrifuged and non-centrifuged samples before analysis, showed no changes in $A\beta_{1-42}$, independent of the technology platform (12). All studies investigating the effect of centrifugation on T-tau and P-tau_{181P} showed no differences (10-12). Moreover, no significant changes in $A\beta_{1-42}$ arose from the addition of 5000 lysed erythrocytes/mm³ (6). The concentrations of $A\beta_{1-42}$ and T-tau were also determined in one severely blood-contaminated sample (28.800 erythrocytes/mm³), that had been aliquoted with one aliquot being centrifuged before freezing and another one not, which did not lead to a considerable difference (11). The present data, combined with the majority of data from 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 $A\beta_{1-42}$ can also be found in plasma, originating from different pools such as platelets, muscle, 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 inter-assay CV for the assays used (10, 17). In addition, centrifugation of the non-blood-contaminated samples did not lead to a significantly different intra-assay CV for $A\beta_{1-42}$ (4.6% versus 3.8%), T-tau (4.1% versus 4.8%), or P-tau_{181P} (2.2% versus 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.

4.3. Freezing temperature

Freezing of samples can be done at different temperatures, of which -20°C, -80°C and liquid N_2 (-196°C) are most commonly used. From long-term stability studies, it is already known that $A\beta_{1-42}$ concentration in samples frozen at -80°C are stable for at least 2 years (6, 18) and T-tau and P-$tau_{181P,}$ 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 N_2, or direct freezing of a sample in liquid N_2 as compared to other freezing conditions has never been investigated extensively. Freezing in -80°C in comparison to (initial freezing in) liquid N_2 resulted in borderline significantly lower $A\beta_{1-42}$ concentrations, while the comparison of -80°C with -20°C was not found significant. We can speculate that freezing in liquid N_2 quickly reduces degradation of proteins by protease activity and stabilizes all proteins in the CSF, thereby preventing loss of

full-length $A\beta_{1-42}$. However, freezing in liquid N_2 immediately after CSF collection should then have led to higher $A\beta_{1-42}$ concentrations in comparison to freezing in liquid N_2 after 2h, 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-tau_{181P} concentrations differed significantly between -80°C and -20°C frozen samples, but not $A\beta_{1-42}$. Although a significant difference was found for $A\beta_{1-42}$ after freezing in liquid N_2 in comparison to freezing at -80°C, these results should be interpreted with caution, because of the relatively large CI for $A\beta_{1-42}$ in comparison to T-tau and P-tau_{181P}, indicative of a larger variation during measurement 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 three markers but is not the most practical solution for routine and freezing of CSF samples at -20°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 three biomarkers means that differences between liquid N_2, -80°C and -20°C are most likely attributed to the initial freezing conditions. This is also seen for tubes that immediately adsorb $A\beta_{1-42}$ (25).

4.4. Freezing delay

CSF samples are frequently shipped to reference laboratories, either at RT, cooled, or after freezing (**Figure1**). Shipment of samples by means of regular mail takes at least 24h. Therefore, $A\beta_{1-42}$, T-tau and P-tau_{181P} should preferably be stable at RT for several days, also because shipment of CSF samples on dry ice is very costly. In comparison to immediate (within 4h) freezing of samples at -80°C, the $A\beta_{1-42}$ concentration gradually increased by almost 15% during 48h of storage at RT.

Bjerke et al. (6) stated that storage at RT for more than 24h did not significantly affect the $A\beta_{1-42}$ concentration in comparison to both fresh samples and after storage at -80°C until analysis. Another study even found relatively stable CSF biomarker concentrations after four days, although unsystematic variation increased over the course of days (18). In contrast to this, $A\beta_{1-42}$ has been found to decrease during the first 48h (11) or, more in line with the present study, even to increase within 24h (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 Aβ in plasma and CSF is bound to proteins (26, 27). A higher ratio of free/bound Aβ could substantially alter the $A\beta_{1-42}$ 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 also seem to have the same effect on $A\beta_{1-42}$ (24h at 2-8°C or 24h at RT: 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 which was seen for samples from AD patients, not controls. T-tau was found to be stable at RT (14, 16, 28), for up to 22 days in one 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 RT on P-tau_{181P} were found in previous studies (10, 14, 16). One study examined possible differences between storage at 4°C for 4h, 24h and 72h and immediate freezing at -80°C and 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.

4.5. 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 freeze-thaw cycle (-15%) (11, 12, 17), whereas no differences were ever found between fresh CSF samples and samples frozen and thawed once (11, 13, 17). T-tau showed a (non-significant) increase after two freeze-thaw cycles whereupon a decreasing trend is imposed, resulting in a significant difference between freeze-thaw cycle 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 up to six 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 CI point to a low variability in evolution of P-tau_{181P} among the different subjects, 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 three 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.

4.6. 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 pre-analytical variables under study) and a limitation, as the limited physiological variability might not necessarily reflect daily clinical practice.

Moreover, the impact of the pre-analytical variables might depend on the brain pathology (AD versus non-AD dementias versus 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 manuscript.

4.7. Conclusions

Following recommendations can be proposed as a result of the present study:

* **Fractionated sampling**

  It is possible to collect CSF in one 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 N_2 is not recommended.

* **Freezing delay**

  The delay in freezing should be minimized.

* **Freeze-thaw cycles**:

  One freeze-thaw cycle just prior to analysis is standard procedure, but one or two additional freeze-thaw cycles are allowed.

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Table 1. Median concentrations, range (min-max) and relative median differences of CSF biomarker concentrations for protocol 1 (fractionated sampling) and 2 (centrifugation).

	C3 (n=20)	C7.1 (n=20)	C7.1* (n=20)	Protocol 1 C3-C7.1 (fractions)		Protocol 2 C7.1-C7.1* (centrifugation)	
				median % difference (95%CI)	p-value	median % difference (95%CI)	p-value
$A\beta_{1-42}$	421 (269-855)	421 (223-907)	426 (246-875)	-1.10 (-5.46 - 3.47)	0.615	2.29 (-2.30 - 7.10)	0.315
T-tau	411 (71-2183)	368 (63-1224)	386 (63-1466)	4.32 (-4.91 - 14.45) 0.53 (-7.33 – 9.06)	0.351 0.892	0.91 (-5.01 - 7.19)	0.758
P-tau_{181P}	61 (13-239)	59 (13-299)	58 (13-318)	-0.18 (-3.94 - 3.72)	0.921	-0.03 (-3.63 - 3.71)	0.988

Concentrations are expressed in pg/mL. None of the comparisons were found statistically significant. Abbreviations: 95%CI = 95% confidence interval; $A\beta_{1-42}$= β-$amyloid_{1-42}$ protein; C3 = 3rd consecutive CSF fraction; C7.1 = 7th consecutive CSF fraction, not centrifuged; C7.1* = 7th consecutive CSF fraction, centrifuged; T-tau = total tau protein; $P\text{-}tau_{181P}$ = tau phosphorylated at threonine 181.

Table 2. Median concentrations and ranges (min-max) of CSF biomarker concentrations for protocol 3 (freezing temperature and delay) and 4 (freeze-thaw cycles). See also **Figure 2**.

		$A\beta_{1-42}$ (pg/mL)	***P*-value in reference to standard procedure***	**T-tau (pg/mL)**	***P*-value in reference to standard procedure***	**$P\text{-tau}_{181P}$ (pg/mL)**	***P*-value in reference to standard procedure***
Protocol 3 freezing temperature and delay (n=22)	**-80°C**	300 (165-579)		359 (46-1875)		53 (14-172)	
	-20°C	313 (166-636)	0.135	348 (52-1550)	**0.012**	52 (10-165)	**0.005**
	Liquid N_2	335 (172-594)	**0.048**	396 (63-1604)	0.405	55 (10-173)	0.068
	24h 2-8°C	307 (145-576)	0.624	386 (52-1890)	0.296	50 (7-169)	**0.016**
	24h RT	324 (178-612)	0.065	403 (48-1871)	0.732	55 (10-171)	0.625

	48h RT	320 (195-558)	**0.009**	384 (50-1919)	0.804	54 (8-169)	0.180
Protocol 4 freeze-thaw cycles (n=20)	**f/t 1**	428 (190-837)		417 (89-2063)		48 (14-162)	
	f/t 2	443 (229-892)	0.667	422 (90-2228)	0.133	47 (14-155)	0.742
	f/t 3	411 (229-939)	0.783	413 (86-1928)	0.672	49 (14-171)	0.245
	f/t 4	398 (178-833)	**0.022**	406 (80-2070)	0.491	50 (15-162)	0.749

Table legend: * *P*-values are given for comparisons in reference to the standard procedure: for protocol 3 freezing at -80°C and for protocol 4 one freeze-thaw cycle. Standard procedures are indicated with a blue background and significant differences are indicated in bold. Abbreviations: $A\beta_{1-42}$ = β-amyloid$_{1-42}$ protein; f/t = freeze-thaw cycles; N_2 = nitrogen; P-tau$_{181P}$ = tau phosphorylated at threonine 181; RT = room temperature; T-tau = total tau protein.

Figure 1. Schematic presentation of sampling procedures in different settings (research and clinical routine).

Figure 2. Schematic presentation of the study protocol (four protocols).

[$]In protocol 1, concentrations in the C3 and C7.1 fraction were compared. Because C7.1 was pipetted into a different vial, 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. Abbreviations: C = cerebrospinal fluid fraction; RT = room temperature; liq N_2 = liquid nitrogen; f/t = freeze/thaw cycle. The boxes explain the goal of the different protocols.

Figure 3. Visualization of the relative median differences (%) between the different freezing conditions and introductions of a delay before freezing, as compared to freezing at -80°C.

Results are presented as the relative median difference with 95% confidence interval. For example, the relative median difference for $A\beta_{1-42}$ comparing samples that remained at room temperature for 48h and samples that were frozen immediately at -80°C is about 8%, indicating that the $A\beta_{1-42}$ concentrations are 8% higher in samples that were kept at room temperature for 48h.

Figure 4. Visualization of the relative median differences (%) between the different freeze-thaw cycles.

Results are presented as the relative median difference with its 95% confidence interval. For example, the relative median difference for $A\beta_{1-42}$ comparing samples that underwent one and four freeze-thaw cycles is about 8%, indicating that the $A\beta_{1-42}$ concentrations were 8% lower in the samples that underwent four freeze-thaw cycles in comparison to samples that underwent only one freeze-thaw cycle.

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