

Chapter 7

Recommendations to standardize Pre-analytical confounding factors in Alzheimer's and Parkinson's disease CSF biomarkers: an update.

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Marta del Campo
Brit Mollenhauer
Antonio Bertolotto
Sebastiaan Engelborghs
Harald Hampel
Anja Hviid Simonsen
Niels Kruse
Nathalie Le Bastard
Sylvain Lehmann
Armand Perret-Liaudet
Javier Sáez-Valero
Esen Saka
Andrea Urbani
Eugeen Vanmechelen
Charlotte E. Teunissen

Abstract

Early diagnosis of neurodegenerative disorders as Alzheimer's or Parkinson's disease (AD and PD) is needed to slow down or halt the disease at the earliest stage. Cerebrospinal fluid (CSF) biomarkers can be a good tool for early diagnosis. However, their use in clinical practice is challenging due to the high variability found between centers in the concentrations of both AD CSF biomarkers ($A\beta_{42}$, T-tau and P-tau) and PD CSF biomarker (α -synuclein). Such a variability has been partially attributed to different pre-analytical procedures between laboratories, thus highlighting the need to establish standardized operating procedures (SOPs). Here, we merge two previous consensus guidelines for pre-analytical confounding factors in order to have one exhaustive guideline updated with new evidences for $A\beta_{42}$, T-tau and P-tau and α -synuclein. The proposed SOP is applicable not only to novel CSF biomarkers in AD and PD but also to biomarkers for other neurodegenerative disorders.

Key words: Alzheimer's disease, biomarkers, CSF, early diagnosis, guidelines, Parkinson's disease, pre-analytical factors, recommendations, SOPs.

Introduction

Alzheimer's disease (AD), the most common type of dementia, and Parkinson's disease (PD) are age-related irreversible neurodegenerative disorders. Recent investigations indicate that about two-thirds of dementia cases may be undiagnosed in non-specialized centers¹. Importantly, it has been estimated that the initial AD (but also PD) typical molecular alterations may take place even several decades before the appearance of clinical signs. Thus, owing to the long duration of this asymptomatic phase, during which pathophysiological mechanisms, together with structural and functional changes are developing^{2,3}, it is of paramount importance to detect biological markers accurately reproducing features of AD and PD pathophysiology before the expression of clinical symptoms^{4,5}. Biomarkers reflecting amyloid deposition and neuronal changes in early stages of the disease can be an efficient tool for early diagnosis. Cerebrospinal fluid (CSF) is considered one of the main sources for central nervous system biomarker discovery since it directly interacts with the extracellular space of the brain and mirrors biochemical alterations occurring in it⁶. The current AD CSF biomarker model parallels the prevailing hypothesis for its pathogenesis, i.e. the amyloid cascade hypothesis characterized by an early β -amyloid ($A\beta$) peptide accumulation, representing the central event, followed by tangle formation, synaptic dysfunction, neurodegeneration and neuronal loss. Thus, the core CSF biomarkers for AD diagnosis are a decrease of $A\beta_{42}$ levels which reflects senile plaques pathology as well as an increase of total tau (T-tau) and phosphorylated tau (P-tau) which reflect axonal degeneration⁷⁻⁹. Recently, the new diagnostic criteria and guidelines for AD established by the National Institute on Aging and the Alzheimer's Association (NIA-AA) discuss the role of CSF biomarkers in AD diagnosis. The value of these biomarkers for AD diagnosis changes according to the stage of the disease¹⁰. Actually the new NIA-AA guidelines recommend the use of those biomarkers to increase the confidence in establishing that the underlying dementia syndrome of a patient is due to an AD pathophysiological process but not for routine diagnosis.

In parallel, in PD the core CSF biomarker is α -synuclein: its intracellular accumulation is characteristic for PD and other Parkinsonism syndromes like Multiple System Atrophy (MSA) and dementia with Lewy-bodies (DLB). α -synuclein is following a gene doses event, and by genome wide association studies of sporadic PD and MSA a strong association between disease risk and distinct single nucleotide polymorphisms in the α -synuclein coding gene, *SNCA*, has been shown as risk factor for developing PD¹¹. Therefore misprocessing of α -synuclein is a seminal event in synucleinopathies. The quantification of α -synuclein in CSF has been described by several groups in smaller studies which

mostly showed reduced levels of CSF α -synuclein in α -synuclein related diseases (PD, MSA and DLB)¹²⁻¹⁵.

One of the main reasons that renders the use of AD and PD biomarkers challenging in routine diagnosis is the great variability found between the levels of the biomarkers measured in different studies and the diagnostic accuracy of those measurements¹⁶⁻¹⁸ reaching in some cases an inter-assay and inter-laboratory coefficient variations of 20 to 35%¹⁹⁻²¹. The lack of standardized protocols seems to be the major source of this variability²². The sources of variability includes batch-to-batch variation of the immunoassay kit as well as several pre-analytical and analytical factors²³. Some studies have already addressed the involvement of lot-to-lot variation or analytical factors²⁴ and a quality control program for CSF $A\beta_{42}$, T-tau and P-tau measurements initiated by the Alzheimer Association continues running in order to analyze the differences between assay procedures¹⁶. Pre-analytical bias affects the quality of samples and the reliability of the data. Pre-analytical bias is of great importance on biochemical analysis as it was reported that approximately 40-60% of total laboratory errors were due to pre-analytical procedures²⁵. They can act both *in vivo* and *in vitro*. The *in vivo* pre-analytical factors are those biological variables that act in the subject at the time of sample collection as fasting or diurnal and physical exercise. *In vitro* factors act during sample handling and processing including sample collection mode, type of test tubes, tube/plate adsorption, freeze/thaw cycles and length of storage. Several experimental studies in CSF have already demonstrated the importance of pre-analytical confounding factors on biochemical analysis^{23,26,27}. Such results highlight the need to establish standard operating procedures (SOPs) for sample handling and processing which would allow comparison of methods and diagnostic conclusions among different laboratories. The adherence to and implementation of these SOPs in the scientific and clinical community may reduce the great variability found in the analysis of AD and PD CSF biomarkers. A reduced variability would permit not only to establish general cut-off values for CSF biomarkers but it may also improve the predictive value of CSF biomarkers along the disease progression in routine diagnosis. Two consensus reports have already established the main pre-analytical factors that should be standardized for CSF analysis, one was focused on AD biomarkers²⁸, the other more generally on CSF biobanking for biomarker research protocols²⁹. However, the importance of some pre-analytical confounding factors highlighted in these reports remained to be experimentally sustained. The aim of this review is to merge the two current guidelines and to include new evidence in order to establish new pre-analytical SOPs for the analysis of AD CSF biomarkers and the PD CSF biomarker, α -synuclein. Moreover, several of the issues discussed here can be applied to novel biomarkers in AD, PD as well as other neurodegenerative disorders.

Pre-analytical factors in the analysis of AD and PD CSF biomarkers

Issue 1. Diurnal variation.

Diurnal variation can be a critical factor in the analysis of specific biochemical compounds that are influenced by circadian rhythms. In those cases, the time of the day for sample withdrawal is of great importance³⁰. Several studies have already analyzed the diurnal variation of AD CSF biomarkers. A study performed by Bateman et al. showed a large diurnal variability in A β (1-40 and 1-42) levels during a time period of 36 hours³¹. However no significant differences were observed between the hours during the day time period. Several recent studies have shown no temporal fluctuations in CSF biomarker levels not only for A β but also for T-tau and P-tau^{23,32,33}. For CSF α -synuclein only a slight diurnal variation was shown³⁴. Another recent study did not observed sinusoidal fluctuations in α -synuclein CSF concentrations over 33-hours period³⁵. Taking into account that the time of CSF withdrawal is during the day due to clinic time schedule, and that no significant changes have been found in the levels of AD CSF biomarkers and CSF α -synuclein between the different times of the day, there is no need to standardize a specific time interval during the day for CSF collection²⁹. Nevertheless, in case of novel CSF biomarkers it is recommended to record time withdrawal information in order to detect the possible diurnal variation of the new analyte.

Issue 2: CSF gradient

Most brain-derived proteins have a decreased rostro-caudal concentration gradient³⁶. Therefore, the volume of CSF taken can influence protein concentration. A proteomics study observed a gradient effect in protein concentration just in two (Albumin and Apolipoprotein CI) out of the 41 proteins³⁷. Vanderstichele et al. concluded that there is no gradient effect in AD CSF biomarkers concentrations (Le Bastard, unpublished data)²⁸ which has also been confirmed by other independent group (Verbeek, unpublished data). Additionally, another experimental study analyzed the spinal cord gradient effect on A β ₄₂ and they did not find significant differences between the level of A β ₄₂ in four successive 10 ml portions of CSF²³. Nevertheless, other biochemical compounds can be affected^{38,39}. CSF α -synuclein levels show a slight reduction from rostral to caudal (thereby supporting its neuronal origin) in a small set of gradient samples (7 consecutive samples of 5 ml each) from patients with normal pressure hydrocephalus⁴⁰. Therefore a standardized volume of CSF collection is recommended for analysis of a broad range of biomarkers. Although a minimum of 1.5 ml of CSF is needed for routine analysis²⁸, we recommend to standardize the volume of CSF withdrawal to 12 ml which will allow to have enough material to do

additional studies. It must be noted that there is no correlation between the volume of CSF collected and the risk of post-lumbar puncture headache⁴¹.

Issue 3: Location of lumbar puncture (LP) and type of needle.

Due to the decreased rostral-caudal concentration gradient previously mentioned, the site of CSF withdrawal must be also standardized. Diagnostic CSF is usually obtained by LP between the L3/L4 and L4/L5 intervertebral space. Although there is no experimental evidence that the type of needle influences biomarker concentration, this issue should be taken into consideration since it can affect patient side effects. It has been shown that both post-lumbar puncture headache severity and recovery time was remarkably decreased when an atraumatic 25G needle was used instead of a 20G needle. Moreover, it was also shown that the use of a 25G needle decreased blood contamination, defined as $>5/\mu\text{l}$ red blood cells in the first tube of CSF collected, from 39.1% with a 20G needle to 19.7% (Bertolotto, manuscript submitted). Blood contamination of CSF is an important issue in biomarker analysis and will be discussed later (*Issue 7*). In view of these results, CSF should be taken by LP between the L3/L4 and L4/L5 intervertebral space with a 25G atraumatic needle. If not possible, the type of needle used should be documented.

Issue 4: Fasting

To the best of our knowledge, there are no studies that have analyzed the influence of fasting on AD and PD CSF biomarkers. Nevertheless, it has been shown that A β levels in plasma are very stable, independently of the patient food intake²³. Therefore, it is unlikely that CSF biomarkers levels would be affected by fasting if plasma levels are not. Indirect support to this idea comes from the only study that analyzed the effect of fasting on the levels of a CSF biomarker, S100B, a protein involved in neurodegeneration, in rat CSF and serum. It was found that while the levels of S100B in serum were affected by fasting, the levels in CSF remained stable⁴². As there is no specific evidence demonstrating that fasting influences individual CSF biomarkers levels, it can be concluded that fasting is not a requirement for the analysis of CSF biomarkers. However, it is important to remark that especially in case of novel metabolic markers, the effect of fasting should be evaluated.

Issue 5: Matched serum, plasma and DNA/RNA information linked to CSF collection

It is recommended to collect matched serum and/or plasma samples for CSF biomarkers analysis since it helps not only to study the intrathecal origin of a specific biomarker but also its specificity within the central nervous system. Of note, plasma EDTA rather than serum is often preferred to run blood proteomics biomarker discovery programs⁴³. Moreover, DNA

collection will also contribute to the development of biomarkers. With DNA information, novel biomarkers can be compared, or related to specific genotypes and phenotypes within individuals which may unravel new characteristics of the pathology²⁹. Finally, collection of whole blood using specific tubes will allow the realization of transcriptomic profiles using microarrays, qPCR or sequencing. This biomarker approach is now been used in many pathological contexts, including AD^{44,45}.

Issue 6: Types of tubes

It has been well established that polypropylene (PP) tubes should be used for CSF collection since lipophilic proteins, like A β peptides and α -synuclein bind in a non specifically manner to non-PP tubes. Actually, lower values of A β_{42} , T-tau and P-tau were found when using glass or polystyrene tubes^{23,26,46}. However, it has also been shown that some vials of pure PP cause more adsorption than tubes made with copolymers of polyethylene (PE) and PP⁴⁷. Moreover, treatment of the tube surface with Tween-20 reduces amyloid peptide adsorption⁴⁸. The use of siliconized low-binding tubes has been shown to lower adsorption of CSF α -synuclein (Mollenhauer, unpublished data). Strikingly, significant differences on A β_{42} , T-tau and P-tau levels have been recently published when CSF was collected in PP tubes from 11 different suppliers. Although those tubes were labeled as PP tubes, a calorimetry and spectroscopy analysis revealed that just 1 out of 11 tubes was pure PP while the others were copolymers of PP with PE. Moreover, it was also observed that the tubes that performed better for A β_{42} were the worst for P-tau suggesting that hydrophilic-hydrophobic balance is a important point in protein adsorption²⁶. These data highlight the need to standardize the type of test tube used since the great variability between tubes could even lead to a possible AD misdiagnoses. Currently, the members of the Joint Programming Neurodegenerative Disease Biomarkapd (JPND-BIOMAKAPD) are performing a study which includes the analysis of the most suitable type of tube for AD CSF biomarker research. Until then, it is recommended to keep using PP tubes²⁹. Additionally, adsorption may also take place in the 96 well PP plate that is used for biomarker analysis which may affect the final biochemical results. In order to have an accurate incubation time of each CSF sample with the corresponding antibodies in the ELISA assay for A β_{42} measurements, it is recommended by the supplier (Innogenetics, Ghent, Belgium) to load CSF samples in a pre-analysis PP 96-well plate so that all samples are transferred simultaneously with a multichannel pipette to the final analysis plate. However, it has not been reported if plate adsorption in the pre-analysis plate influences the final biochemical results. To test plate adsorption we analyzed A β_{42} levels in 10 individual CSF samples using a pre-analysis 96-well PP plate and the results were compared with those obtained when the CSF samples were directly loaded from the test tube to the final analysis plate

in a timed manner. We found that the use of a pre-analysis plate lead to significantly decreased of $A\beta_{42}$ levels ($p = 0.027$) (Figure 1A). In another independent experiment we have also tested the influence of pre-analytical plate adsorption by measuring the levels of $A\beta_{42}$ when samples were incubated in a pre-analytical plate for 5 and 15 minutes. We observed that the $A\beta_{42}$ levels were reduced 14.3% and 24.8% when samples were incubated in a pre-analytical plate fro 5 and 15 minutes respectively. These reductions were also significant ($p < 0.05$) (Figure 1B). This decrease may contribute to the inter-laboratory variability observed in CSF biomarkers depending on the use of pre-analytical plate. Therefore, its use should be further evaluated for future standardization between all the kit manufacturers. The use of a pre-analytical plate only affects $A\beta_{42}$ measurements and not to T-tau and P-tau measurements, since the incubation time for tau analysis (i.e. overnight sample incubation) does not need to be strictly accurate.

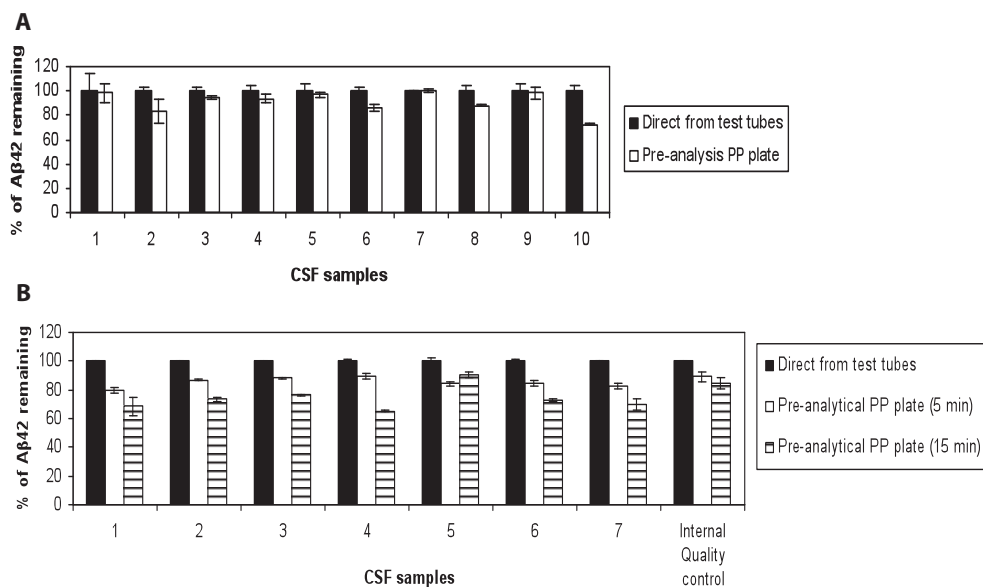


Figure 1. The use of pre-analytical 96 well plate in ELISA significantly reduces $A\beta_{42}$ levels. **A**, $A\beta_{42}$ levels in CSF from 10 different subjects measure either by loading the CSF directly from the tube or using a pre-analytical plate **B**, $A\beta_{42}$ levels in CSF from 7 different subjects measured in another independent study. CSF was measured directly from the tube or after incubation during 5 and 15 minutes in a pre-analytical plate. Results are expressed as the % of remaining $A\beta_{42}$ compared to direct pipeting. Samples were analyzed in duplicates. Errors bars represent CV percentage between sample duplicates which were within the range of intra-assay CVs. Using paired t-test analysis, values were significantly lower when a pre-analytical plate was used in both experiments ($p < 0.05$).

Issue 7: Spinning conditions

Blood contamination of CSF occurs in 14-20% cases due to traumatic LP⁴⁹. Minor blood contamination during LP can influence biomarker analysis since CSF total protein concentration is approximately 0.5% compared to blood⁵⁰. For some CSF markers, such as α -synuclein, the concentrations in blood are much higher than in CSF (10 fold in serum and plasma and up to 10,000 fold in whole blood)¹⁸. Moreover it has been shown that blood contamination of CSF can also lead to protein degradation⁵⁰. The effect of blood contamination on $A\beta_{42}$ was analyzed by Bjerke et al who found no significant difference in $A\beta_{42}$ levels when up to 5000 erythrocytes/ μ l were added to the CSF. However, they found significant decreased $A\beta_{42}$ levels in CSF when plasma was added which was attributed to the binding of $A\beta_{42}$ to different plasma proteins²³. Those results highlight the importance of blood contamination for CSF biomarker analysis. Nevertheless, centrifugation before initial freezing highly reduced the amount of blood proteins due to removal of blood cells^{51,52}. Very few studies have analyzed the effect of centrifugation on CSF biomarker analysis. Bjerke et al. found a significant decrease in $A\beta_{42}$ CSF levels possibly due to cell lysis after centrifugation²³. However, the guidelines of Vanderstichele et al. pointed out no differences on the levels of $A\beta_{42}$, T-tau and P-tau between centrifuged and non-centrifuged samples (Le Bastard, unpublished data)²⁸. Additionally, it is conceivable that sample centrifugation leads to changes in the temperature of the sample which can modify the final biochemical results. We analyzed the temperature of CSF samples after centrifugation at RT, at 4°C and 20°C. Prior centrifugation the samples were maintained at RT or 4°C. The sample temperature was always similar to the temperature set up in the centrifuge (Table 1) demonstrating that temperature is not increased by spinning itself. Since spinning may influence biomarker values, our recommendation is that for CSF biomarker research, centrifugation should be always performed following the standardize protocol of 2.000xg for 10 minutes at RT²⁹. In this way, biochemical results can always be

Table 1. Effect of centrifugation on CSF sample temperature (°C)

Centrifugation temperature (°C)	Samples pre-incubated on ice		Samples pre-incubated at RT	
	Start (°C)	End (°C)	Start (°C)	End (°C)
RT	1	23	25	26
4°C	1	5	26	8
20°C	2	20	26	23

Prior centrifugation samples were pre-incubated on ice or at RT. Centrifugation of samples with both conditions was then performed at different temperatures. Temperatures were measured prior (start) and post (end) centrifugation.

compared despite traumatic LP. For proteins more abundant in peripheral blood, like CSF α -synuclein, samples with > 50 erythrocytes/ μ l should be excluded.

Issue 8: Time delay between CSF collection and storage

It has been reported that different laboratories use different timing and temperatures between sample collection and final storage^{28,29}. Proteomics studies have revealed that this issue is more crucial for serum or plasma proteins than for CSF^{52,53} although significant changes have also been found in a proteomics study when CSF was left at room temperature (RT) in the first 30 minutes after collection⁵⁴. Schoonenboom et al found that $A\beta_{42}$ and tau concentrations measured by ELISA remained stable up to 72 hours after LP (storage at 4°C)²⁷. Kaiser et al. confirmed also the stability of tau concentrations. However in that study a significant increase of the levels of $A\beta_{42}$ after 24 hours at RT was found⁵⁵. The effect of time delay on sample handling on $A\beta_{42}$ levels has also been analyzed by another study and $A\beta_{42}$ concentrations remained stable up to 24 hours after withdrawal (storage at RT)²³. Additionally, the guidelines of Vanderstichele et al reported that there were no significant effects on $A\beta_{42}$, T-tau and P-tau levels at least for the first 5 days after CSF collection (Le Bastard et al, unpublished data)²⁸. Moreover, in a recent study it was also shown that after LP the levels of $A\beta_{42}$, $A\beta_{40}$, T-tau and P-tau remained stable up to 7 days when stored at RT⁵⁶. The lack of centrifugation prior to incubation is likely the reason for the increase in $A\beta_{42}$ observed in one of the previous studies. These data support the importance of centrifugation before CSF biomarker analysis as previously discussed (Issue 7). In case of α -synuclein we observed up to 40% decrease in CSF concentration upon storage at 4°C for 4 days (Figure 2) using a specific α -synuclein assay⁵⁷.

Regarding the temperature during the time delay, no significant differences were found between storage of the CSF samples at RT, 4°C or -80°C in any of the studies performed^{23,28}. According to the experimental data obtained so far, CSF samples can be stored at RT up to 5 days after collection before freezing for the analysis of $A\beta_{42}$, T-tau and P-tau²⁸. However, since RT can greatly diverge between different countries we recommend to store the samples at 4°C since it can be easily done and it does not modify the biochemical results. Nevertheless, in case of novel biomarkers, protein stability in relation to time delay should be analyzed.

Issue 9: Freeze/thaw cycles

As freezing may affect protein stability⁵⁸, it has been recommended to avoid freeze/thaw cycles²⁹. Moreover, it has been shown that protein levels in serum were modified by repeated freeze/thaw cycles⁵⁹. In CSF, the levels of some proteins remains unaltered by freeze/thaw cycles⁶⁰, whereas others are affected⁶¹. Indeed, some studies have already

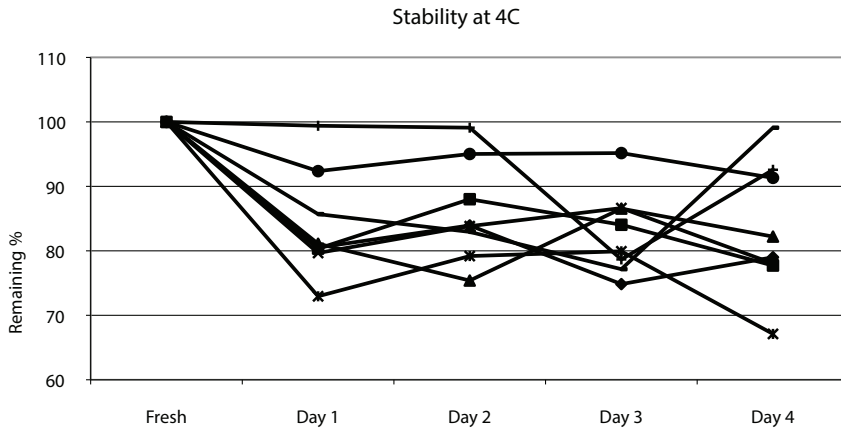


Figure 2. α -synuclein concentrations in CSF are reduced after 4 days of storage at 4°C. α -synuclein levels from 5 different CSF samples were measured immediately after withdrawal (fresh) and during 4 consecutive days (stored at 4°C). Results expressed the remaining percentage of α -synuclein along time delay. In one sample a α -synuclein loss of 35% is observed after 4 days of storage at 4°C. Using paired t-test analysis, values were significantly lower when results were compared to the ones from fresh samples (always $p < 0.01$).

analyzed the influence of freeze/thaw cycles on AD CSF biomarkers. Most studies found a decrease in $A\beta_{42}$ concentration as a result of freeze/thaw cycles. However, different results were found in the number of cycles that led to a decrease in $A\beta_{42}$ concentrations. Two studies have reported no changes on $A\beta_{42}$ and T-tau levels after one freeze/thaw cycle^{23,62}. These results were confirmed by another study which further showed a decrease of approximately 20% in $A\beta_{42}$ levels after the third cycle and a reduction of almost 80% after 6 freeze/thaw cycles. Additionally, no significant changes on T-tau levels were found after up to 6 cycles²⁷. However, in one study, a significant decrease of $A\beta_{42}$ levels was found after one single cycle⁶³. In a more recent study the levels of $A\beta_{42}$, $A\beta_{40}$, T-tau and P-tau remained stable up to 3 freeze/thaw cycles⁵⁶. In case of α -synuclein, we have found a decrease concentration in CSF of up to 50% after 6 freeze/thaw cycles (Figure 3). A proteomics study showed significant differences in a selected peptide profile of CSF samples just after 10 freeze/thaw cycles⁵⁴. In case of immunoassay analysis it is recommended to limit the number of freeze/thaw cycles up to two as maximum²⁸, and in case of novel biomarkers the effect of freeze/thaw cycles should be evaluated. Thus, it is essential to split the pooled CSF in small aliquots and document accurately the amount of freeze/thaw cycles that the sample has gone through.

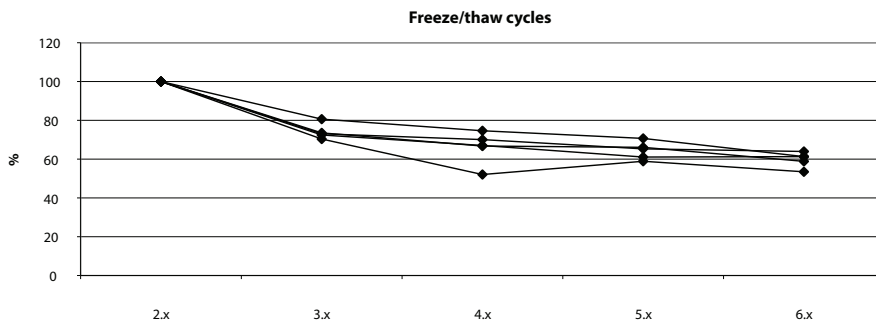


Figure 3. α-synuclein concentrations in CSF are reduced with freeze/thaw cycles. α-synuclein levels from 5 different CSF samples were measured after up to 6 freeze/thaw cycles. Results expressed the remaining percentage of α-synuclein in each cycle. In some samples a loss of 50% is observed after 6 freeze/thaw cycles.

Issue10: Aliquots

As previously mentioned, aliquoting the CSF pool is absolutely necessary. When aliquots are prepared not only freeze/thaw cycles should be taken into account but also tube surface adsorption and evaporation. We have analyzed the influence of volume on evaporation (or sublimation in case of frozen samples) at different temperatures, since it could lead to changes in protein concentration. To this end, we stored different volumes of deionized water (0.05 to 1.5 ml) at RT, 4, -20 and -80°C and we analyzed the weight of the tubes after several time points from 1 day to 2 years. The maximum percentage changes found were 68, 34 and 14% corresponding to the tubes stored at RT and with the lowest initial volumes (0.05, 0.1 and 0.25 ml respectively). This decrease was observed after 1 year. In contrast, no change in volume was observed in any of the tubes stored at -20 or -80°C (Figure 4). It can be concluded that evaporation (and not sublimation) occurs when small volumes are stored at RT. It remains to be studied whether these results can be extrapolated to protein-rich solutions. Thus it is recommended to use small volumes (0.25 or 0.5 ml tube) to prevent freeze/thaw cycles and to fill the tube up to 75% to minimize the adsorption and evaporation effect²⁹. Before aliquoting the centrifuged sample should be gently mixed to remove gradient effects. Regarding the tubes used for aliquoting, most “ependorf” or screw-cap tubes are made of PP and should be used. These microtubes present in general a low adsorption of AD biomarkers and in particular of Aβ₄₂, (<5%, Lehmann and Perret-Liaudet, unpublished data).

Issue 11: Freezing temperature

Freezing temperatures may have an effect on CSF proteins as it has previously been reported for cystatin C or for Reelin, which undergo a proteolysis at -20°C but not at

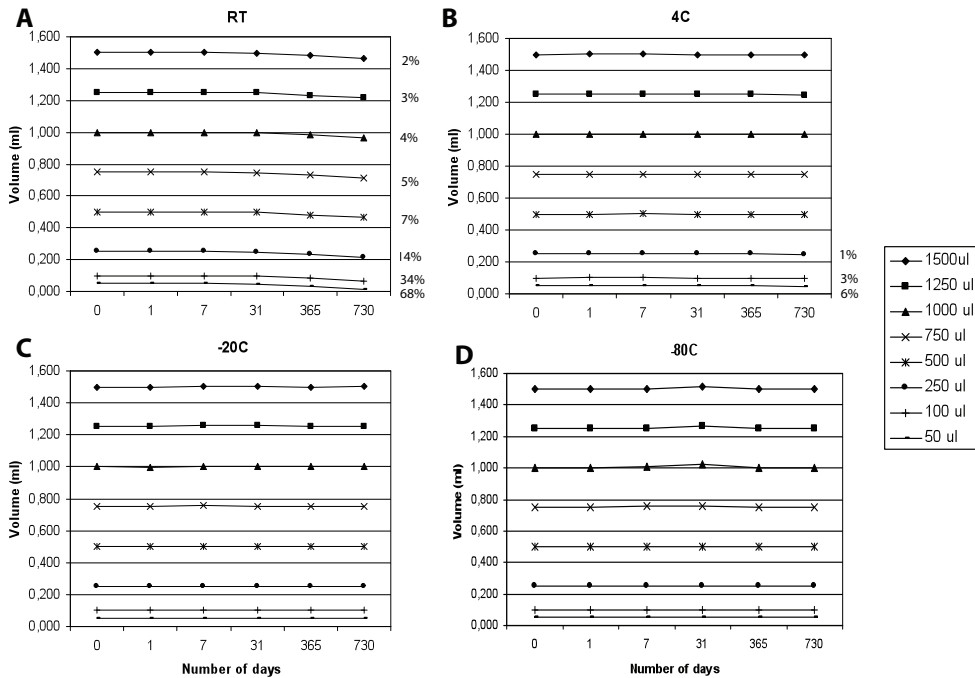


Figure 4. Evaporation effect on stored samples over time at different temperatures. Different volumes of deionized water (0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.25 and 1.5ml) were stored during 2 years at (A) RT, (B) 4, (C) -20 and (D) -80°C. Volumes were analyzed after 1, 7, 31, 365 and 730 days. The percentage of change was calculated and it is shown at the right of the graphs only when values were $\geq 1\%$. The highest percentage of change was found at RT with 0.05, 0.1 and 0.5 ml. No changes were found in tubes stored at -20 or -80°C.

-80°C^{61,64}. Recent studies compared the stability of AD CSF biomarkers at different freezing temperatures. One study showed no difference in $A\beta_{42}$ levels when CSF was frozen at -20°C or -80°C²³. These results have been confirmed by another study which additionally reported that the levels of T-tau and P-tau were significantly lower when CSF samples were immediately frozen at -20°C instead of -80°C (Le Bastard, un published data)²⁸. The effect of freezing temperatures on Tau concentrations must be further analyzed in order to confirm these results. No data so far reported any benefits of storage CSF samples on dry ice. The latter is of importance since many samples are shipped on dry ice. Therefore, CSF samples should be frozen and stored at -80°C as previously reported²⁹.

Issue 12: Length of storage

It has been suggested that CSF protein stability might be affected by the length of storage²⁷ and actually very few studies have been performed with samples stored for many years. It has been shown that the levels of $A\beta_{42}$ and T-tau but not $A\beta_{40}$ remained stable up to

6 years⁶⁵. Another study has also shown no variation on AD CSF biomarkers in samples stored for more than 2 years⁵⁶ which agrees with the comments in the guidelines of Vanderstichele et al which reported stability up to 10 years at -80°C (Blennow, unpublished data)²⁸. Additionally, in our evaporation experiment mentioned above we did not observe any volume changes when samples were stored at -20 or -80°C for up to two years (Figure 2, C-D). In summary, we conclude that CSF can be stored for up to 2 years at -80°C without significant loss as previously reported²⁸. Data about the concentrations of CSF α -synuclein depending on the storage time and temperature still have to be investigated.

Conclusion

The present review merges two previous consensus guidelines for pre-analytical factor standardization on AD biomarkers^{28,29}. Additionally, some new evidences have been reported related to the type of needle, pre-analytical PP adsorption, sample evaporation and effect of spinning in sample temperature. Moreover, we also included new data about the main CSF biomarker for PD, α -synuclein. As a result, Table 2 outlines the main recommendations to standardize the pre-analytical factors that can influence CSF biomarkers concentrations for AD ($A\beta_{42}$, T-tau and P-tau) and PD (α -synuclein). Therefore, Table 2 can be used as a SOP for CSF biomarkers analysis. Moreover, these recommendations may also serve as a guideline for novel CSF biomarkers analysis in AD, PD and other neurodegenerative disorders. However, some issues should be re-considered in case of novel biomarkers (Table 2). Recording of all variables mentioned is essential for standardization upon need. Nevertheless, some critical points remain to be unrevealing such as the type of test tubes that should be used which will be soon defined by the JPND-BIOMAKAPD members. In case of α -synuclein more studies are needed in order to confirm the results obtained. The influence of medication (i.e. dopaminergic medication in PD) on biomarkers levels should also be analyzed. Therefore, it is a continuous process and more experiments and new updates to the current SOP will be expected in the future.

Future Perspectives

The further analysis of pre-analytical confounding factors in CSF biomarkers will permit the establishment a consolidated SOP applicable to the analysis of CSF biomarkers in different neurodegenerative disorders. Nevertheless, the adherence of routine and research laboratories to the SOPs is of paramount importance. The utilization of the SOP may reduce the great variability found in biomarkers analysis between centers. This would allow the

Table 2. New consensus-based recommendations for pre-analytical issues on AD and PD CSF biomarkers analysis

Key issue	Procedure	Recommendation	Check Novel biom.¹
1. Diurnal variation	Time of day withdrawal	Day time	X
2. CSF gradient	CSF volume withdrawal	12 ml	
3 Lumbar puncture	Type of needle	25G atraumatic	
	Location LP	Intervertebral space L3-L5	
4. Fasting	Meal consumption	No criterium	X
5. Matched samples	Serum, Plasma and DNA collection	If possible, yes (same time LP)	
6. Types of tube	Material	PP (further research is needed)	
	Pre-analysis 96 well PP Plate ²	Further evaluation needed for standardisation	
7. Spinning conditions	Centrifugation	Should always be performed 2000 g - 10 minutes RT	
	Erythrocyte count	<50/ul ³	
8. Time delay before storage		< 5 days, 4°C ⁴	X
9. Freezethaw cycles		maximum 2	X
10. Aliquots	Tube volume	0.25-0.5 ml	
	Tube filling	> 75%	
13. Freezing temperature		-80°C	
14. Length of storage	Long-term storage	up to 2 years	X

¹ Items that should be check for novel biomarker analysis

² Only for A β ₄₂

³ only for proteins highly abundant in peripheral blood

⁴ Optimally freeze as soon as possible

establishment of general cutoff values and the comparison of diagnostic conclusions, which will facilitate collaboration between different research centers. Furthermore, since the application of this SOP may greatly increase the predictive, prognostic and/or diagnostic value of a CSF biomarker, we believe that a consolidate SOP will aid clinical trials, effective clinical intervention and it will facilitate the implementation of the current biomarkers in the routine diagnostic guidelines of neurodegenerative disorders.

Executive summary

- New evidences of pre-analytical issues in the analysis of AD CSF biomarkers has been discussed.
 - New data of pre-analytical issues in the analysis of the PD biomarker α -synuclein has been included
 - Table 2 can be used as a SOP for CSF biomarker analysis
 - Critical issues as the type of test tubes remains to be further investigated
 - A consolidate SOP is need for CSF biomarkers analysis.
 - Laboratories should adhere to the consolidated SOP in order to reduce the variability on CSF biomarkers analysis
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