



HAL
open science

Pre-analytical and analytical factors influencing Alzheimer's disease cerebrospinal fluid biomarker variability

Anthony Fourier, Erik Portelius, Henrik Zetterberg, Kaj Blennow, Isabelle Quadrio, Armand Perret-Liaudet

► To cite this version:

Anthony Fourier, Erik Portelius, Henrik Zetterberg, Kaj Blennow, Isabelle Quadrio, et al.. Pre-analytical and analytical factors influencing Alzheimer's disease cerebrospinal fluid biomarker variability. *Clinica Chimica Acta*, 2015, 449, pp.9-15. 10.1016/j.cca.2015.05.024 . hal-04796026

HAL Id: hal-04796026

<https://hal.science/hal-04796026v1>

Submitted on 23 Jan 2025

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

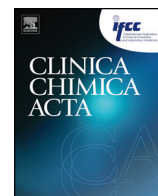


Distributed under a Creative Commons Attribution 4.0 International License



Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Invited critical review

Pre-analytical and analytical factors influencing Alzheimer's disease cerebrospinal fluid biomarker variability

Anthony Fourier^{a,b}, Erik Portelius^d, Henrik Zetterberg^{d,e}, Kaj Blennow^d,
Isabelle Quadrio^{a,b}, Armand Perret-Liaudet^{a,b,c,*}

^a Neurobiology Laboratory, Biochemistry and Molecular Biology Department, Hôpitaux de Lyon, Lyon, France

^b University of Lyon 1, CNRS UMR5292, INSERM U1028, BioRan, Lyon, France

^c Société Française de Biologie Clinique (SFBC), Alzheimer Biomarkers group co-coordination, France

^d Clinical, Neurochemistry Laboratory, Institute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, the Sahlgrenska Academy at the University of Gothenburg, Sweden

^e Department of Molecular Neuroscience, UCL Institute of Neurology, London, UK

ARTICLE INFO

Article history:

Received 29 May 2015

Accepted 30 May 2015

Available online xxxx

Keywords:

Alzheimer diagnosis

Dementia

CSF

Biomarkers

Pre-analytical confounders

Analytical confounders

ABSTRACT

A panel of cerebrospinal fluid (CSF) biomarkers including total Tau (t-Tau), phosphorylated Tau protein at residue 181 (p-Tau) and β -amyloid peptides ($A\beta_{42}$ and $A\beta_{40}$), is frequently used as an aid in Alzheimer's disease (AD) diagnosis for young patients with cognitive impairment, for predicting prodromal AD in mild cognitive impairment (MCI) subjects, for AD discrimination in atypical clinical phenotypes and for inclusion/exclusion and stratification of patients in clinical trials. Due to variability in absolute levels between laboratories, there is no consensus on medical cut-off value for the CSF AD signature. Thus, for full implementation of this core AD biomarker panel in clinical routine, this issue has to be solved. Variability can be explained both by pre-analytical and analytical factors. For example, the plastic tubes used for CSF collection and storage, the lack of reference material and the variability of the analytical protocols were identified as important sources of variability. The aim of this review is to highlight these pre-analytical and analytical factors and describe efforts done to counteract them in order to establish cut-off values for core CSF AD biomarkers. This review will give the current state of recommendations.

© 2015 Published by Elsevier B.V.

Contents

1. Introduction	0
2. Influence of confounding factors in the pre-analytical phase	0
2.1. Confounding factors with major effects	0
2.1.1. The kind of needle used for CSF collection	0
2.1.2. The nature of sampling tubes	0
2.1.3. Centrifugation and time delay between CSF collection and storage before assay	0
2.1.4. The nature of storage tubes	0
2.1.5. Storage conditions	0
2.2. Other factors with minor or without effect: no need of specific recommendation	0
2.2.1. Is there a specific time of day needed to collect the CSF?	0
2.2.2. Is fasting able to modify levels of AD biomarker levels?	0
2.2.3. Is there a gradient of CSF AD biomarker concentrations?	0
2.2.4. Temperature management between CSF centrifugation and storage before assay	0
3. Variability introduced by the analytical phase	0
3.1. An inventory of CSF biomarker determination's variability	0
3.2. Variability linked to kit providers	0

* Corresponding author at: Neurobiology Laboratory, Biochemistry and Molecular Biology Department, Hôpitaux de Lyon, Lyon, France.
E-mail address: armand.perret-liaudet@chu-lyon.fr (A. Perret-Liaudet).

3.3.	Variability linked to kit users	0
3.3.1.	Compliance to standard operating procedures (SOPs)	0
3.3.2.	The maintenance of laboratory equipment	0
3.3.3.	Familiarization with the method, competency training and experience	0
4.	Conclusion	0
	Acknowledgments	0
	References	0

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia and is characterized by progressive neuronal degeneration, aggregation of β -amyloid and hyper phosphorylated Tau proteins into plaques and tangles, leading to progressive loss of cognitive functions [1]. A diagnosis of AD made on pure clinical criteria is uncertain even in the clinical stage of mild dementia; this uncertain diagnosis has caused problems in clinical trials, where 10–30% of enrolled patients did not have AD pathology [2]. In the prodromal stage of the disease (mild cognitive impairment due to AD), the diagnostic criteria, including cerebrospinal fluid (CSF) biomarkers, still remain in the research field [3,4]. It is well accepted that the use of biomarkers (imaging or CSF biomarkers) in specialized centers can improve the diagnostic certainty for AD [5]. The core CSF biomarker panel for AD diagnosis includes a decrease in the concentration of the 42 amino acid long amyloid- β peptide ($A\beta_{42}$) reflecting plaque pathology, together with an increase of total Tau (t-Tau) and phosphorylated Tau 181 (p-Tau) proteins, which reflect axonal degeneration and Tau pathology [6,7]. More recently, a decrease of the $A\beta_{42}/A\beta_{40}$ ratio has also been implemented in several specialized centers [8–10], [Dorey et al. submitted]. The use of AD biomarkers for routine diagnostic purposes is at the present time only proposed to be optional for demented patients when deemed appropriate by the clinician, especially in patients with early-onset dementia, or with atypical AD [11].

The significant variability in measured biomarker levels found in various studies, resulting in a high variability of both the diagnostic accuracy [12] and the clinical cut-off for the diagnostic of AD, with two to threefold differences between the highest and the lowest reported cut-off values in Europe [13], is a hindrance to the general implementation of these markers and their integration in the diagnostic criteria [3]. Recently, a consensus report established the main pre-analytical factors contributing to the variation of the laboratory results before the analysis of the sample and concluded that pre-analytical phase should be standardized for CSF AD biomarker analysis [14]. However, the importance of some pre-analytical confounding factors highlighted in that report remains to be elucidated. Concerning the analytical phase, the introduction of an external quality control program revealed a great dispersion of results among participants [15]. This variability could be partly explained by the lack of reference material and relatively unstandardized operating procedures. The aim of this report is to discuss and focus on main critical points in the pre-analytical and analytical steps likely to be responsible for the variability of data.

2. Influence of confounding factors in the pre-analytical phase

The confounding factors in pre-analytical phases of biochemical analysis may have a great impact on the reliability of the results. Several experimental studies support this assessment for the core CSF AD biomarkers [16–18]. Confounding factors are classically listed in a “catalog” dichotomized in two different groups: “in vivo” or biological factors directly linked to the patient and “in vitro” factors linked to the procedure of sample handling and processing. However, we chose to present them based on the effect size of their potential influence: main factors requiring standardization and minor factors for which no specific recommendation is needed.

2.1. Confounding factors with major effects

Here we present factors causing major modifications of CSF biomarkers concentrations in a logical order, from sampling to freezing/thawing of samples before analysis.

2.1.1. The kind of needle used for CSF collection

The type and the internal diameter of needle may be a factor contributing both to the side effects observed in some patients and to the presence of blood contamination. Comparative studies gave a consensus that decreasing the inner diameter of the needle and using preferentially atraumatic than traumatic needles could decrease the percentage of hemorrhagic CSF samples and the percentage of post-lumbar puncture headaches [19–22]. However, the exact inner diameter to be used remains debating and seems to depend partially on the age of patients [23].

2.1.2. The nature of sampling tubes

Several reports have shown that polypropylene (PP) tubes should be preferred to glass or polystyrene (PS) tubes for collection of CSF since $A\beta$ peptides, but also t-Tau and p-Tau, may bind in a non-specific manner to the two last ones [16,18,24]. Yet, these studies generalized the results to generic PP tubes whereas they did not test a large panel of different PP tubes leading to the conclusion about the apparent superiority of PP tubes against PS or glass tubes. It should be noted that the guidelines used today are based on these reports. Within the PP family, there is a high heterogeneity of plastic polymer composition as we have reported by calorimetry and spectroscopy analysis [17]. Moreover, surface treatments (as plasma gas treatment of tetra fluorine carbon, of anionic or cationic detergents...) at the late stages of their manufacturing are also a source of variability, modifying the hydrophobic/hydrophilic properties of their surface. For example, two independent studies reported significant differences on $A\beta_{42}$ levels when CSF was collected in PP tubes from different suppliers [17,25]. This adsorption occurs quickly (15 min) and is highly dependent on the total amount of proteins present in CSF [17]. The main message learnt from these studies is that pure untreated PP tubes were the worst, probably due to their hydrophobic nature which enables hydrophobic interactions with $A\beta$ peptides. Finally, the best tubes regarding $A\beta$ recovery were found to have been treated onto the walls, independently of the nature of plastic. The exact nature of this treatment is unfortunately not available, the information being protected by companies [17,26]. It has been shown that the adsorption of $A\beta$ peptides was significantly reduced when Tween-20 was mixed with CSF in the tube for example [25,27]. In line with this, we reported similar results using various plasma treatments of the tube surface, which modified the adsorption of different proteins such as prion protein, Tau and alpha synuclein [26]. Indeed, tubes that performed better for $A\beta_{42}$ gave on the other side a slight decrease of p-Tau levels (only a trend with a mean decrease of 10%, in the analytical coefficient of variation of the assay) while t-Tau levels remained unmodified, suggesting once again that hydrophobic/hydrophilic balance is an important aspect in protein adsorption [17,26]. In addition to the previous observation, it is worth noting that adsorption was most pronounced if the sample volume was low, i.e., if there was a low volume to surface ratio [28].

These data highlight the need to standardize the nature of sampling tubes used since the high variability observed could lead to possible AD misdiagnosis in worst cases. Based on the data presented in our study [17], we shifted to the tube giving the best results for AD biomarker determination (tube Sarstedt® catalog no. 62.610.201). This change resulted in an average increase of 25% of $A\beta_{42}$ levels, whereas no significant difference was seen for t-Tau and p-Tau [29]. However, this modification of method introduced the need to revalidate our previously established cut-off values for all three CSF biomarkers. In the frame of the “AD biomarkers working group” of the French Society of Clinical Biology (Société Française de Biologie Clinique), three centers using three different PP sampling tubes participated in a study with the aim of comparing data before and after standardization. Before standardization, one center presented a significant increase of the CSF $A\beta_{42}$ levels for AD patients against the other centers whereas no difference was seen among centers for t-Tau and p-Tau. Adopting the same collection tube in the three centers abolished the significant difference of $A\beta_{42}$ in AD patients among centers. Even if differences in optimal cut-offs still existed (linked to the cohorts and other confounding factors), the range of these optimal cut-offs decreased from 20% around the mean to 10% (382–570 ng/L to 669–831 ng/L with the common tube) [29]. In conclusion, all these data clearly suggest that the nature of sampling tubes is a major critical point for standardization of AD biomarkers.

2.1.3. Centrifugation and time delay between CSF collection and storage before assay

This is a complex issue involving different confounding factors including hemorrhagic puncture, hemolysis CSF samples, and high levels of total proteins, which all could affect the stability of biomarkers. Even if it seems logical trying to reduce these effects by decreasing optimally the time delay between sampling and storage, by the introduction of a centrifugation, there are some different conclusions about the need of a centrifugation. In the guidelines of Vanderstichele et al., the absence of difference on the levels of $A\beta_{42}$, t-Tau and p-Tau between centrifuged and non-centrifuged samples was pointed out [14], confirming the study of Bjerke et al., showing that $A\beta_{42}$ concentrations remained stable up to 24 h after sampling (storage at RT) [16]. However, the study of Kaiser et al. described a significant increase of the levels of $A\beta_{42}$ after 24 h [30]. Using a proteomic approach on CSF samples which had been left at room temperature (RT) for 30 min, significant changes of various metabolites, amino acids and proteins were reported in the presence of white blood cells in CSF [31]. These discrepancies could be explained by the selection of non-hemorrhagic CSF samples. Unfortunately, hemorrhagic punctures occur in 14–20% of cases of lumbar puncture. Bjerke et al. were unable to detect any difference in the $A\beta_{42}$ levels after spiking CSF with up to 5000 erythrocytes/ μ L, although they found significantly decreased CSF $A\beta_{42}$ levels when plasma was added which was attributed to the binding of $A\beta_{42}$ to different plasma proteins [16]. Another possibility is that $A\beta_{42}$ is degraded by plasmatic proteases [32]. In a recent study done in the frame of JPND initiative, we found a slight increase of $A\beta_{42}$ levels after spiking CSF with up to 5000 erythrocytes/ μ L, but even if this increase was significant, it remained in the analytical inter-CV of the assay (Leitao MJ et al. submitted). Moreover, we detected that centrifugation temperature conditions (4 °C versus RT) could slightly modify $A\beta_{42}$ levels when the amount of total protein was superior to 1 g/L confirming previous data of Zimmerman et al. [33]. To confirm these trends, more work is needed, for example by increasing the amount of cells in CSF. In summary a centrifugation step, which is already done in most specialized clinical biochemistry laboratories, seems to be relevant at least for hemorrhagic samples. For the moment, centrifugation could be performed within 2 h after sampling at 2000 g during 10 min at controlled room temperature (RT) following a standardized protocol [14]. Finally, centrifuged samples with an erythrocyte count below 5000/ μ L can be analyzed without clear interference, modifying the previous recommendations reporting a cell count of 500/ μ L [14,22].

2.1.4. The nature of storage tubes

Selecting nine different commercial PP storage tubes with a volume capacity ranging from 0.5 to 1.5 mL, we found a significant difference in CSF $A\beta_{42}$ concentrations, ranging from 94% to 127% around the overall mean. The effect was present after 15 min at RT, and an additional 24 h incubation time at 2–8 °C did not significantly change these values [17].

Within the different PP tubes tested, we found the same heterogeneity of plastic polymer composition as described for sampling tubes [17]. These data have been confirmed in an independent study including five other tubes containing CSF from 8 patients (Fig. 1). In this experiment, the variation of $A\beta_{42}$ concentrations ranged from 73% to 128% for the different tubes compared to the overall mean.

Finally, it was recently reported that $A\beta_{42}$ peptides in CSF are able to significantly interact to the tube surface each time they are exposed to a new plastic surface [27]. This effect was less pronounced for Tau proteins. In conclusion, we hypothesize that the amyloid content of a CSF sample can be depleted just by transferring the fluid from tube to tube. Thus, it is not reasonable to follow the actual guidelines recommending the use of generic PP tubes. The best compromise would be that each laboratory analyzing these markers compares the tubes used in their laboratory with the best tubes identified in our study, which are easily available in the market.

2.1.5. Storage conditions

Freezing process is a complex issue since different factors could influence the apparent concentrations of the biomarkers: the temperature of freezing, the possible effect of freezing/thawing cycles, the volume of CSF aliquots and length of storage. Moreover, these factors could be synergistic: the absorption of proteins onto the tube walls could be increased by a low volume to surface ratio or by the temperature of freezing (–20 versus –80 °C).

Freezing and storing the CSF at –80 °C seem to be logical, as it was reported that freezing at –80 °C could prevent modification of CSF AD biomarker levels after storage up to 2 years [16]. In line with this, CSF t-Tau and p-Tau levels were reported to be significantly lower when CSF samples were frozen at –20 °C instead of –80 °C [16,34].

Most studies using enzyme-linked immunosorbent assays (ELISA) have not found any difference in CSF $A\beta_{42}$ and Tau levels after one freeze/thaw cycle [16,18,33,35,36], [Leitao submitted]. In contrast, one

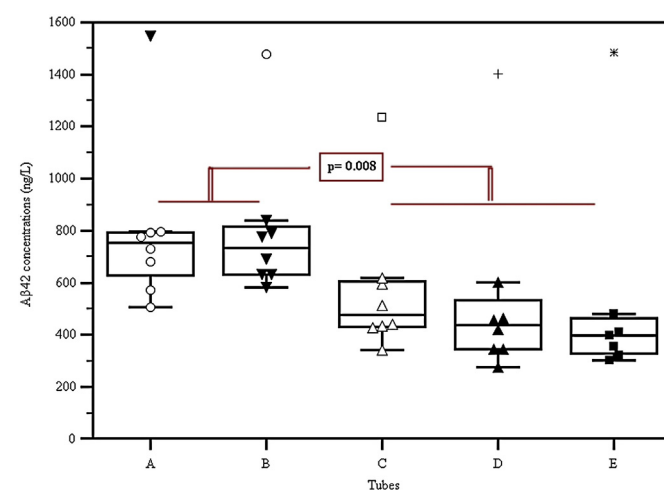


Fig. 1. Variability of CSF $A\beta_{42}$ levels linked to 5 different storage tubes. CSF from eight Alzheimer's disease patients was used. CSF characteristics were: total protein <0.4 g/L, t-Tau ranged between 431 and 791 ng/L, p-Tau ranged between 69 and 110 ng/L and $A\beta_{42}$ ranged between 624 and 1472 ng/L. After incubation 1 h at 22 °C +/- 1 °C in each storage tube (from A to E), CSF was frozen before further comparative analysis for $A\beta_{42}$ (Fujirebio Innostest). In tubes A and B, the mean of the 8 CSF biomarkers was significantly higher than for the three other tubes, C, D and E (Friedman global test $p < 0.001$ followed by Wilcoxon tests, $p = 0.008$).

study found a significant loss of A β ₄₂ after one single freeze/thaw cycle using a semi-quantitative method [37]. CSF Tau levels seem to be stable if the sample undergoes three [18], five [Leitao submitted] or even six freeze/thaw cycles [33]. Concerning CSF p-Tau levels, Leitao et al. found stable concentrations after five freeze/thaw cycles. Besides, CSF A β ₄₂ levels were found to be either significantly decreased [18] or stable after three freeze/thaw cycles [33,35], [Leitao submitted]. We have now enough evidence to propose a modification of the current recommendation [14], by increasing the maximum number of freeze/thaw cycles impacting CSF AD biomarker levels from two to three.

Aliquoting CSF is a common practice since it avoids different freeze/thaw cycles. It was reported that CSF A β ₄₂ levels were significantly decreased when storage tubes were filled with low CSF volumes [28], [Leitao submitted]. This phenomenon is potentially linked to absorption of A β ₄₂ onto the walls of the tube as Tween 20 was able to prevent the decrease of CSF A β ₄₂ levels [28]. The procedures issued from previous reported guidelines can be applied for the moment pointing out the need to use small volume aliquots in adequate volume tubes [22,28]. Besides the time of storage at -80°C doesn't seem to influence stability of CSF AD biomarkers, since levels of A β ₄₂, t-Tau and p-Tau remained stable up to at least 2 years [38] and at least 6 years in one study [39]. In summary, we can conclude that CSF can be stored up to 2 years at -80°C as previously reported [13].

2.2. Other factors with minor or without effect: no need of specific recommendation

2.2.1. Is there a specific time of day needed to collect the CSF?

As the time of the lumbar puncture is highly dependent on the organization of both the clinical memory center and those of the biological laboratory/imaging department (waiting hemostasis results, scheduling imaging...), this question is highly relevant. A diurnal variability was observed for A β peptides according to one study [40]. However, more recent studies were unable to replicate the finding that there is a temporal fluctuation in the CSF biomarker levels (A β , t-Tau and p-Tau) [16,41,42]. Therefore, there is no need to standardize a specific time interval during the day for CSF collection dedicated to AD biomarker assays.

2.2.2. Is fasting able to modify levels of AD biomarker levels?

To our knowledge, there is a lack of data concerning this topic. However, our recommendation is that it is not reasonable to recommend fasting for the analysis of CSF AD biomarkers.

2.2.3. Is there a gradient of CSF AD biomarker concentrations?

CSF is usually obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space and there is no scientific reason to proceed differently for AD biomarkers. Most brain-derived proteins have an increased rostro-caudal concentration gradient [43]. Therefore, it is not recommended to run these markers in the ventricular punctures obtained during neurosurgical interventions. No gradient effect in the spinal cord was observed for the CSF AD biomarkers [16]. Therefore, there is no reason to recommend any specific fraction of CSF, except for hemorrhagic samples.

2.2.4. Temperature management between CSF centrifugation and storage before assay

Regarding the temperature during the time delay, no significant difference was found between the storage of the CSF samples at RT, 4°C or frozen in any of the studies performed [13]. Therefore, there is no reason to recommend any specific temperature of CSF which should be at least controlled.

3. Variability introduced by the analytical phase

Considerable variability exists between the different assays produced from different kit providers, between different technologies

from the same manufacturer (for example ELISA and multiplex), but also between clinical laboratories using the same kits. An important step towards identification of the problems and standardization was the introduction in 2009 of an external quality assessment (EQA) program (Cerebrospinal Fluid Quality Control Program supported by the Alzheimer's Association) [15].

3.1. An inventory of CSF biomarker determination's variability

There are several available assays for the determination of CSF A β ₄₂, t-Tau and p-Tau, commercialized by different companies. As shown in the conclusions in the two reports of the EQA supported by the Alzheimer's Association [15,38], among the 70 participants, INNOTEST® ELISA (Fujirebio, Gent, Belgium) dominates the market while multiplex techniques are less frequently used. Multiplex techniques include bead-based xMAP® platforms with INNO-BIA AlzBio3 reagents (Fujirebio®) to quantify A β ₄₂, t-Tau and p-Tau and electrochemiluminescence assays (Meso Scale Discovery Gaithersburg, MD) to quantify the A β triplex constituted by A β ₄₂, A β ₄₀, and A β ₃₈ peptides [38]. Large variations in assay performance of CSF A β ₄₂, t-Tau and p-Tau levels were reported between laboratories with coefficients of variation (CVs) ranging from 20% to 35% [12,44]. Depending on the biomarker tested, mean CVs were 18% to 23% for ELISA, 20% to 28% for xMAP and 24% to 27% for electrochemiluminescence [38], confirming previously published data [12,15,44]. Overall variability was mainly impacted by important between-laboratory variability (19–28%) which may be explained by a few laboratories having high bias and high imprecision [38]. Moreover, within-laboratory longitudinal variability impacted also overall variability (5–19%), contrary to within-run variability (5–10%) which was in agreement with the criteria given by kit providers.

Finally, biomarkers' concentrations vary according to analytical techniques [15]. For example, CSF A β ₄₂ values obtained by ELISA were about twofold higher than xMAP values and CSF t-Tau were about threefold higher than xMAP respectively [45]. This last point has a major impact on the decision threshold used to diagnose patients correctly. Although correction factors were applied to harmonize the values obtained by xMAP and ELISA techniques for global comparison of groups of patients to predict incipient AD thanks to CSF biomarkers [46], it was clearly shown by the authors that the use of these factors did not totally solve the discrepancy in values obtained by both techniques for all the patients. Thus, the data could not be used at an individual level for diagnosis. Although the observed biomarker concentrations may vary significantly between platforms, including MSD, xMAP and ELISA, these techniques seem to have similar diagnostic accuracy for patients with AD versus controls [46] or for detecting early AD [47,48].

3.2. Variability linked to kit providers

The bias observed between kit providers may partially be explained by the use of different antibodies having different epitopes and affinities for the target antigen. The nature of the calibrators and calibration models could be other factors explaining this variability. This bias will not disappear without standardization efforts including the implementation of certified reference materials (CRMs) and reference measurement procedures (RMPs). Production of candidate CRMs for A β ₄₂ is an ongoing work with the efforts from the IFCC working group on CSF proteins [49] and from the BIOMARKAPAD consortium. Thereby, two candidate RMPs for quantification of A β ₄₂ [50,51] and one for quantification of t-Tau [52] were recently reported.

The between-lot variability was also shown to be a contributor for the overall variability, mainly for A β ₄₂ INNOTEST ELISA® [38]. The global production of the kits may contribute to this problem: antibody manufacturing, coating of plates and beads, calibrators and many other steps constituting a large list of variation. The availability of CRMs and RMPs would be parts of the solution, but efforts must also

be done by the manufacturers to include stricter quality criteria for the release of their kits.

Others crucial points which need to be solved include the actual poor quality of the instructions of use introducing variability by misunderstanding of the protocols. This lack of information is often an indicator of minimal method optimization of the protocol from the suppliers (for example influence of the incubation steps, handling the reagents...). Moreover, reagents should be delivered in a manner that permits to decrease variability. For instance, ready to use calibrators should be privileged to lyophilized calibrators. Nowadays, some kits do not contain specific calibrators and controls unfortunately.

3.3. Variability linked to kit users

We learnt from the data of Cerebrospinal Fluid Quality Control Program supported by the Alzheimer's Association and from the International Workshop of standardization organized by Amsterdam's group [53], that many analytical steps could be rapidly optimized in users' laboratories, given that many crucial issues were studied and yet reported in a guideline [53]. Thus, we can just point out the different issues and possible solutions.

3.3.1. Compliance to standard operating procedures (SOPs)

The compliance of laboratories to SOPs given by the manufacturers is absolutely needed to reduce the part of variability found in CSF biomarker analysis. For that, a great effort must be done by the different kit suppliers to limit individual interpretation of their technical instructions.

For example, the terminology Room Temperature (RT) given by the kit suppliers may introduce between-laboratory variation in incubation steps since RT most likely differs across Europe and also introduces within-laboratory variation (winter/summer variation). In our experience, we have found variability in $A\beta_{42}$ concentrations when we shifted from a manual procedure (RT at 20 °C \pm 1 °C) to a semi-automatized procedure using the DSII instrument (Dyner Technologies®) in which temperature was 25 °C \pm 1 °C inside the instrument. In addition to that, we also observed that levels increased when temperature increased from 20 to 25 °C, this increase being significantly different for concentrations above 900 ng/L. After discussion with the manufacturer and a complete re-validation of the assay, we adopted a temperature of 25 °C for incubation steps anticipating that this modification should be included in the new instructions from the kit supplier.

Another typical example involves the use of a pre-analysis polypropylene (PP) 96-well plate, which was recommended so all CSF samples could have the same incubation time (1 h each) with the capture antibody in the INNOTEST® $A\beta_{42}$ ELISA plate. As the pre-analysis 96-well plate is in PP, we have tested the adsorption of $A\beta_{42}$ onto the pre-analysis plate. We observed that CSF $A\beta_{42}$ levels were significantly reduced ($p < 0.05$) when the samples were incubated in the pre-analysis PP 96-well plate during 5 min (reduction of 14.3%) and 15 min (reduction of 24.8%). This phenomenon was confirmed in an independent study [22]. In the absence of commercially available pre-analysis 96-well plate with minimal adsorption of amyloids to plastic, we investigated if there were significant within-plate variability, from the first to the last rows of wells. In order to elucidate this question, we used the same sample which was distributed into 84 wells following calibrators and QC samples, without using a pre-analysis 96-well plate. We manually distributed the sample with an overall delay of 15 min between the start and the end of distribution, which corresponds to the classical delay for an experienced technician to manage a full 96-well plate, consequently increasing the incubation time by 15 min for the first rows. An $A\beta_{42}$ assay was then run as recommended by Fujirebio®. The overall CV was $< 10\%$ and fulfilled the criteria of acceptance provided by the manufacturer. When we separated the rows in three equal parts of 28 wells each (first part = A, second part = B and third part = C), the mean of $A\beta_{42}$ levels was significantly higher in part A than in part

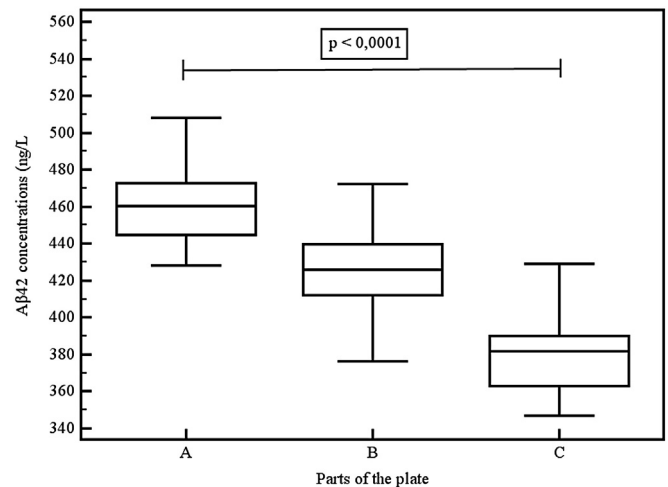


Fig. 2. Comparison of $A\beta_{42}$ concentrations of the same sample distributed in each well of a plate. A QC sample was distributed in 84 wells of an ELISA plate (the other wells were occupied by standards in duplicate). This plate was decomposed in three equal parts of 28 wells each (first part = A, second part = B and third part = C). The mean of $A\beta_{42}$ levels was significantly higher in part A than in part C (463 versus 380 ng/L respectively, $p < 0.0001$).

C (463 versus 380 ng/L respectively, $p < 0.0001$) (Fig. 2). This result highlighted the need to avoid running a full plate. Consequently, the recommendation done by the “AD biomarkers working group” of SFBC is to run no more than a half-plate for $A\beta_{42}$ at the same time.

How to handle the calibrators used to construct the calibration curve is of great importance. Concerning lyophilized standards, accurate solubilization is critical. When dilutions are needed from the first calibrator, variability is known to increase. In the worst cases, standards need to be prepared from a stock solution whose concentration varies across batches of the same assay, so accurate pipetting is absolutely needed. The type of curve fitting used was shown as possible factors of variability [53], therefore it is recommended to strictly use the curve fitting recommended in the kit instruction which must be written unambiguously.

Finally, the mode of pipetting, such as using reverse pipetting or not, is generally not specified by the manufacturer. Moreover, the use of a single pipette tip may influence accuracy for duplicates of the standard curve. However, the magnitude of this effect, if any, should be tested, to provide a better basis for recommendation [53].

The definition of the criteria of acceptance of results must be well defined. They have to include the calibration curve parameters (which should be included as a certificate for each new lot) and an acceptance criteria based on the CVs for samples run in duplicate. For the CV criteria acceptance, it seems that the classical recommendation in different instructions is a CV below 20%. However, this criterion may introduce difficulties when the measured concentration is near the clinical cut-off.

QC samples are now included in most of the kits to validate the calibration curve. However, depending on the kit supplier, they cannot be used as an internal longitudinal QC sample because they are often the same peptide or recombinant protein as calibrators. For the moment, laboratories should implement their own QC samples, in addition to those from the kit, to check reproducibility of assays. Some crucial issues have yet to be solved: the nature of samples (native CSF pools, spiked CSF with standards, peptides...), their number and the range of concentrations.

3.3.2. The maintenance of laboratory equipment

It is necessary to ensure the accuracy of pipettes, the control of temperatures for incubation steps, the accuracy and reproducibility of absorbance of microplate readers, and the quality and reproducibility of washing steps.

3.3.3. Familiarization with the method, competency training and experience

It is of great importance to have a training program given by the manufacturer followed by qualification and habilitation of the laboratory. Moreover, experience is absolutely needed to define the cut-offs of the center. Then, confrontation of biological data obtained must be done in routine in comparison to imaging, clinic and neuropsychological findings with clinicians thanks to multidisciplinary meetings.

4. Conclusion

From the lumbar puncture to CSF biomarker analytical measurement, multiple errors can introduce differences in the measured concentration impacting the clinical cut-off of AD biomarkers. The present review highlights two main issues responsible for the lack of harmonization of CSF AD biomarker cut-off values: the lack of standardization of the pre-analytical steps and the high variability of results linked to the analytical phase. This last issue is explained by the absence of transferability of results between the different platforms but also by the high inter-laboratory dispersion within the same assay. Previous consensus guidelines for standardization of pre-analytical factors pinpointed the variation obtained according to the type of the needle used for CSF puncture and the need to standardize components and volumes of sampling/storage tubes. Based upon our experience and previous published data, we conclude that the variability linked to the nature of tubes is a major critical point.

Otherwise, establishing standard operating procedures (SOPs) for sample processing and handling would allow different laboratories to compare diagnostic conclusions. The implementation of SOPs in laboratories may partly reduce the variability found in the analysis of AD CSF biomarkers. Nature of antibodies, preparation of standards and manufacturer instructions are also sources of variation, requiring increased efforts by kit providers. The optimal approach to manage this issue is a collaborative effort between kit and instrument platform manufacturers and laboratories, thanks to reference standardization programs. The future availability of certified reference materials and reference measurement procedures opens the gate for this collaboration. Without this standardization, clinicians could fall into the suspicion of the real added-value of these biomarkers for AD diagnosis. However, standardization implies checking a potential modification of cut-off values in each laboratory. Then, clinical chemists would be the central actors to convince clinicians about the importance of harmonization to improve diagnostic accuracy of AD biomarkers.

Acknowledgments

We wish to thank all our collaborators of the JPND BIOMARKPD program, those of the French Society of Clinical Biology (SFBC) and those of the NEUROSCREEN European project for their valuable assistance.

References

- [1] K. Blennow, M.J. de Leon, H. Zetterberg, Alzheimer's disease, *Lancet* 368 (2006) 387–403.
- [2] S. Salloway, R. Sperling, N.C. Fox, et al., Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease, *N. Engl. J. Med.* 370 (2014) 322–333.
- [3] M.S. Albert, S.T. DeKosky, D. Dickson, et al., The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease, *Alzheimers Dement.* 7 (2011) 270–279.
- [4] B. Dubois, H.H. Feldman, C. Jacova, et al., Advancing research diagnostic criteria for Alzheimer's disease: the IWG-2 criteria, *Lancet Neurol.* 13 (2014) 614–629.
- [5] G.M. McKhann, D.S. Knopman, H. Chertkow, et al., The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease, *Alzheimers Dement.* 7 (2011) 263–269.
- [6] C.E. Teunissen, J. de Vente, H.W. Steinbusch, C. De Bruijn, Biochemical markers related to Alzheimer's dementia in serum and cerebrospinal fluid, *Neurobiol. Aging* 23 (2002) 485–508.
- [7] K. Blennow, H. Hampel, M. Weiner, H. Zetterberg, Cerebrospinal fluid and plasma biomarkers in Alzheimer disease, *Nat. Rev. Neurol.* 6 (2010) 131–144.
- [8] P. Lewczuk, N. Lelental, P. Spitzer, J.M. Maler, J. Kornhuber, Amyloid-beta 42/40 cerebrospinal fluid concentration ratio in the diagnostics of Alzheimer's disease: validation of two novel assays, *J. Alzheimers Dis.* 43 (2015) 183–191.
- [9] S. Slaets, N. Le Bastard, J.J. Martin, et al., Cerebrospinal fluid Abeta1–40 improves differential dementia diagnosis in patients with intermediate P-tau181P levels, *J. Alzheimers Dis.* 36 (2013) 759–767.
- [10] M. Sauvee, G. Didierlaurent, C. Latache, M.C. Escanye, J.L. Olivier, C. Malaplate-Armand, Additional use of Abeta42/Abeta40 ratio with cerebrospinal fluid biomarkers P-Tau and Abeta42 increases the level of evidence of Alzheimer's disease pathophysiological process in routine practice, *J. Alzheimers Dis.* 41 (2014) 377–386.
- [11] J.L. Molinuevo, K. Blennow, B. Dubois, et al., The clinical use of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: a consensus paper from the Alzheimer's Biomarkers Standardization Initiative, *Alzheimers Dement.* 10 (2014) 808–817.
- [12] N.A. Verwey, W.M. van der Flier, K. Blennow, et al., A worldwide multicentre comparison of assays for cerebrospinal fluid biomarkers in Alzheimer's disease, *Ann. Clin. Biochem.* 46 (2009) 235–240.
- [13] J. Hort, A. Bartos, T. Pirttila, P. Scheltens, Use of cerebrospinal fluid biomarkers in diagnosis of dementia across Europe, *Eur. J. Neurol.* 17 (2010) 90–96.
- [14] H. Vanderstichele, M. Bibl, S. Engelborghs, et al., Standardization of preanalytical aspects of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: a consensus paper from the Alzheimer's Biomarkers Standardization Initiative, *Alzheimers Dement.* 8 (2012) 65–73.
- [15] N. Mattsson, U. Andreasson, S. Persson, et al., The Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers, *Alzheimers Dement.* 7 (2011) 386–395.
- [16] M. Bjerke, E. Portelius, L. Minthon, et al., Confounding factors influencing amyloid Beta concentration in cerebrospinal fluid, *Int. J. Alzheimers Dis.* 2010 (2010). <http://dx.doi.org/10.4061/2010/986310>.
- [17] A. Perret-Liaudet, M. Pelpel, Y. Tholance, et al., Risk of Alzheimer's disease biological misdiagnosis linked to cerebrospinal collection tubes, *J. Alzheimers Dis.* 31 (2012) 13–20.
- [18] N.S. Schoonenboom, C. Mulder, H. Vanderstichele, et al., Effects of processing and storage conditions on amyloid beta (1–42) and tau concentrations in cerebrospinal fluid: implications for use in clinical practice, *Clin. Chem.* 51 (2005) 189–195.
- [19] S. Chevallier, M. Monti, P. Michel, P. Vollenweider, Lumbar puncture, *Rev. Med. Suisse* 4 (2008) 2312–2314.
- [20] M. Dieterich, Post-lumbar puncture headache syndrome, in: E.A. Press (Ed.), *Neurological disorders: course and treatment*, 59, 1996.
- [21] R. Lavi, D. Yarnitsky, J.M. Rowe, A. Weissman, D. Segal, I. Avivi, Standard vs atraumatic Whitacre needle for diagnostic lumbar puncture: a randomized trial, *Neurology* 67 (2006) 1492–1494.
- [22] M. del Campo, B. Mollenhauer, A. Bertolotto, et al., Recommendations to standardize preanalytical confounding factors in Alzheimer's and Parkinson's disease cerebrospinal fluid biomarkers: an update, *Biomark. Med* 6 (2012) 419–430.
- [23] M. Kim, H. Yoon, Comparison of post-dural puncture headache and low back pain between 23 and 25 gauge Quincke spinal needles in patients over 60 years: randomized, double-blind controlled trial, *Int. J. Nurs. Stud.* 48 (2011) 1315–1322.
- [24] P. Lewczuk, G. Beck, H. Esselmann, et al., Effect of sample collection tubes on cerebrospinal fluid concentrations of tau proteins and amyloid beta peptides, *Clin. Chem.* 52 (2006) 332–334.
- [25] A.M. Pica-Mendez, M. Tanen, A. Dallob, W. Tanaka, O.F. Laterza, Nonspecific binding of Abeta42 to polypropylene tubes and the effect of Tween-20, *Clin. Chim. Acta* 411 (2010) 1833.
- [26] F. Poncin-Epaillard, C. Mille, D. Debarnot, et al., Study of the adhesion of neurodegenerative proteins on plasma-modified and coated polypropylene surfaces, *J. Biomater. Sci. Polym. Ed.* 23 (2011) 1879–1893.
- [27] J. Toombs, R.W. Paterson, J.M. Schott, H. Zetterberg, Amyloid-beta 42 adsorption following serial tube transfer, *Alzheimers Res. Ther.* 6 (2014) 5.
- [28] J. Toombs, R.W. Paterson, M.P. Lunn, et al., Identification of an important potential confound in CSF AD studies: aliquot volume, *Clinical chemistry and laboratory medicine: CCLM/FESCC* 51 (2013) 2311–2317.
- [29] S. Lehmann, S. Schraen, I. Quadrio, et al., Impact of harmonization of collection tubes on Alzheimer's disease diagnosis, *Alzheimers Dement.* 10 (2014) S390–S394.
- [30] E. Kaiser, P. Schonknecht, P.A. Thomann, A. Hunt, J. Schroder, Influence of delayed CSF storage on concentrations of phospho-tau protein (181), total tau protein and beta-amyloid (1–42), *Neurosci. Lett.* 417 (2007) 193–195.
- [31] T. Rosenling, C.L. Slim, C. Christin, et al., The effect of preanalytical factors on stability of the proteome and selected metabolites in cerebrospinal fluid (CSF), *J. Proteome Res.* 8 (2009) 5511–5522.
- [32] J.S. You, V. Gelfanova, M.D. Knierman, F.A. Witzmann, M. Wang, J.E. Hale, The impact of blood contamination on the proteome of cerebrospinal fluid, *Proteomics* 5 (2005) 290–296.
- [33] R. Zimmermann, N. Lelental, O. Ganslandt, J.M. Maler, J. Kornhuber, P. Lewczuk, Preanalytical sample handling and sample stability testing for the neurochemical dementia diagnostics, *J. Alzheimers Dis.* 25 (2011) 739–745.
- [34] N. Le Bastard, P.P. De Deyn, S. Engelborghs, Importance and impact of preanalytical variables on Alzheimer disease biomarker concentrations in cerebrospinal fluid, *Clin. Chem.* 61 (2015) 734–743.
- [35] A.H. Simonsen, J.M. Bahl, P.B. Danborg, et al., Pre-analytical factors influencing the stability of cerebrospinal fluid proteins, *J. Neurosci. Methods* 215 (2013) 234–240.
- [36] M. Sjogren, H. Vanderstichele, H. Agren, et al., Tau and Abeta42 in cerebrospinal fluid from healthy adults 21–93 years of age: establishment of reference values, *Clin. Chem.* 47 (2001) 1776–1781.

- [37] M. Bibl, H. Esselmann, M. Otto, et al., Cerebrospinal fluid amyloid beta peptide patterns in Alzheimer's disease patients and nondemented controls depend on sample pretreatment: indication of carrier-mediated epitope masking of amyloid beta peptides, *Electrophoresis* 25 (2004) 2912–2918.
- [38] N. Mattsson, U. Andreasson, S. Persson, et al., CSF biomarker variability in the Alzheimer's Association quality control program, *Alzheimers Dement.* 9 (2013) 251–261.
- [39] C.G. Schipke, F. Jessen, S. Teipel, et al., Long-term stability of Alzheimer's disease biomarker proteins in cerebrospinal fluid, *J. Alzheimers Dis.* 26 (2011) 255–262.
- [40] R.J. Bateman, G. Wen, J.C. Morris, D.M. Holtzman, Fluctuations of CSF amyloid-beta levels: implications for a diagnostic and therapeutic biomarker, *Neurology* 68 (2007) 666–669.
- [41] A. Moghekar, J. Goh, M. Li, M. Albert, R.J. O'Brien, Cerebrospinal fluid Abeta and tau level fluctuation in an older clinical cohort, *Arch. Neurol.* 69 (2012) 246–250.
- [42] D. Slats, J.A. Claassen, P.E. Spies, et al., Hourly variability of cerebrospinal fluid biomarkers in Alzheimer's disease subjects and healthy older volunteers, *Neurobiol. Aging* 33 (831) (2011) e831–e839.
- [43] H. Reiber, Dynamics of brain-derived proteins in cerebrospinal fluid, *Clin. Chim. Acta* 310 (2001) 173–186.
- [44] P. Lewczuk, G. Beck, O. Ganslandt, et al., International quality control survey of neurochemical dementia diagnostics, *Neurosci. Lett.* 409 (2006) 1–4.
- [45] A. Olsson, H. Vanderstichele, N. Andreasen, et al., Simultaneous measurement of beta-amyloid(1–42), total tau, and phosphorylated tau (Thr181) in cerebrospinal fluid by the xMAP technology, *Clin. Chem.* 51 (2005) 336–345.
- [46] N. Mattsson, H. Zetterberg, O. Hansson, et al., CSF biomarkers and incipient Alzheimer disease in patients with mild cognitive impairment, *JAMA* 302 (2009) 385–393.
- [47] W. Jongbloed, M.I. Kester, W.M. van der Flier, et al., Discriminatory and predictive capabilities of enzyme-linked immunosorbent assay and multiplex platforms in a longitudinal Alzheimer's disease study, *Alzheimers Dement.* 9 (2013) 276–283.
- [48] C.G. Schipke, S. Prokop, F.L. Heppner, I. Heuser, O. Peters, Comparison of immunosorbent assays for the quantification of biomarkers for Alzheimer's disease in human cerebrospinal fluid, *Dement. Geriatr. Cogn. Disord.* 31 (2011) 139–145.
- [49] M.C. Carrillo, K. Blennow, H. Soares, et al., Global standardization measurement of cerebral spinal fluid for Alzheimer's disease: an update from the Alzheimer's Association Global Biomarkers Consortium, *Alzheimers Dement.* 9 (2013) 137–140.
- [50] M. Korecka, T. Waligorska, M. Figurski, et al., Qualification of a surrogate matrix-based absolute quantification method for amyloid-beta(4)(2) in human cerebrospinal fluid using 2D UPLC-tandem mass spectrometry, *J. Alzheimers Dis.* 41 (2014) 441–451.
- [51] A. Leinenbach, J. Pannee, T. Dulffer, et al., Mass spectrometry-based candidate reference measurement procedure for quantification of amyloid-beta in cerebrospinal fluid, *Clin. Chem.* 60 (2014) 987–994.
- [52] T. McAvoy, M.E. Lassman, D.S. Spellman, et al., Quantification of tau in cerebrospinal fluid by immunoaffinity enrichment and tandem mass spectrometry, *Clin. Chem.* 60 (2014) 683–689.
- [53] C.E. Teunissen, N.A. Verwey, M.I. Kester, K. van Uffelen, M.A. Blankenstein, Standardization of assay procedures for analysis of the CSF biomarkers amyloid beta((1–42)), Tau, and phosphorylated Tau in Alzheimer's disease: report of an international workshop, *Int. J. Alzheimers Dis.* 2010 (2010).