
Pre-Analytical and Analytical Critical Factors Influencing the High Variability of the Concentrations Levels of Alzheimer Disease Biomarkers in Cerebral Spinal Fluid

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1. Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative disorder characterized by a progressive neuronal death and loss of cognitive functions. AD is the most common type of dementia and its incidence rise to 10% in people aged over 90 [1]. Due to Increased longevity, it has been estimated that the number of people suffering from this neurodegenerative disorder will rise from 26.6 million cases in 2006 to 106,8 million worldwide in 2050 [2].

Although clinical intervention to halt the disease is inefficient, the clinical and psychological cares are likely known to significantly improve the quality of life of the patient but also those of the family. At the prodromal stage of the disease (Mild cognitive Impairment linked to AD), there are no sufficient evidences that treating the patient improves the patient outcome. This lack of evidence poses in some cases an ethical problem that is to announce the diagnosis of AD at an autonomous patient who will shift irreversibly in the coming years to the dementia stages. However, as reported in new criteria established by the National Institute on Aging (NIA) and the Alzheimer's Association, core clinical criteria could be used by healthcare providers without access to advanced imaging techniques or cerebrospinal fluid analysis. Criteria including these last advanced tools still remain in the research field [3]. On the contrary, the diagnosis is highly aimed to be accurate at least at the clinical stage of mild dementia, to detect the AD pathology. Core clinical criteria seems to be enough to ensure the AD diagnosis and the use of biomarkers (imaging or CSF biomarkers) can only increase the certainty that the basis of the clinical dementia syndrome is the AD pathophysiological process

in a patient presenting the core clinical diagnosis [4]. The CSF biomarker panel of AD is a picture of the neurodegeneration, the neuronal loss, the tangle formation and $A\beta$ -amyloid₄₂ ($A\beta_{42}$) peptide accumulation in the brain. Indeed, the core CSF biomarkers for AD diagnosis are a decrease of $A\beta_{42}$ levels and more recently a decrease of the ratio of $A\beta$ -amyloid₄₂ / $A\beta$ -amyloid₄₀ ($A\beta_{42}/A\beta_{40}$) which reflect senile plaques pathology as well as an increase of total tau (T-tau) and phosphorylated tau (P-tau) which reflect axonal degeneration [5,6]. The use of AD biomarker tests for routine diagnostic purposes at the present time, is only proposed as optional for use in patients with dementia when deemed appropriate by the clinician. From the several reasons for this limitation, the workgroup with the task of revising the 1984 criteria for Alzheimer's disease (AD) dementia, highlighted the limited standardization of biomarkers from one locale to another [4]. Despite a decrease in the number of side effects associated with the puncture, lumbar puncture remains an invasive procedure that is clearly the main factor preventing the wide dissemination of these biomarkers in the routine. However, we cannot ignore that the significant variability in measured biomarkers levels found in various studies, resulting in a high variability of both the diagnostic accuracy [7] and of the clinical cut-off for the diagnostic of AD [8], is a hindrance to the spread of these markers and their integration in the diagnostic criteria [3]. The cut-offs obtained in Europe for CSF total tau and beta-amyloid measured by the ELISA assays from the same manufacturer, were reported highly diverse, with two to three fold differences between the highest and lowest reported values [8]. Three major explanations are proposed in this report: first, the inter-laboratory comparisons are very difficult, as some laboratories have adopted the cut-off values from the research literature whereas others have established their own controls, these last controls being likely different in neuropsychology evaluation, neuroimaging and the follow-up. Secondly, the lack of standardized material between the different assays but also the lack of standardized protocols, seem to be a major source of this variation. Finally, pre-analytical factors are those factors that contribute to the variation of the laboratory results before the analysis of the sample. One consensus report has already established the main pre-analytical factors that should be standardized for CSF AD biomarkers analysis [9]. However, the importance of some pre-analytical confounding factors highlighted in this report remained to be elucidated. The aim of this report is to discuss and focus on main critical points in the different preanalytical steps likely to be responsible of data variability. For analytical steps, the introduction since 2009 of an external quality control at a large scale gave an overview of the «desaster», in the same line that prior results. We will discuss rapidly the prior results reported in 2011 and we will underline the urgent need for standardization.

2. Influence of confounding factors in pre-analytical phases on the analysis of AD biomarkers

The confounding factors in pre-analytical phases have a great importance to biochemical analysis and can affect the reliability of the results. Specially in the context of biomarkers of AD in CSF, there are some experimental studies that support this proposition [10,11,12]. Those factors are classically dichotomized in two different groups, «in vivo» and «in vitro». The «in

vivo» factors are those biological factors that are linked directly to the patient, the «in vitro» factors are linked to the procedure of sample handling and processing.

2.1. *In vivo* factors

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

Answering this issue needs to know if a nycthemeral cycle exists that could modify the concentrations levels of AD CSF biomarkers during the day. Although a lack of standardization in the diagnostic strategy of the patient still exists, in most cases, after a first examination including a clinical and a neuropsychological evaluation, if needed, the lumbar puncture is generally scheduled in a second visit with morphological brain imaging in the same time, with the aim to minimize the duration of the hospitalization. As the time of the lumbar puncture is highly dependent of the coordinated organization of the clinical memory centre, of the biological laboratory and of the imaging department associated with it (waiting homeostasis results, scheduling imaging...), this question is highly relevant.

Previous results have suggested the existence of a large diurnal variability in A β levels during a time period of 36 hours, but without significant differences between the hours all along the day period [13]. Following these amazing and unexplained data, recent studies were unable to demonstrate the existence of a temporal fluctuation in CSF biomarker levels, not only for A β , but also for T-tau and P-tau [10, 14, 15]. Therefore, there is no need to standardize a specific time interval during the day for CSF collection dedicated to the AD biomarkers assays.

2.1.2. Is fasting able to modify the concentrations levels of AD biomarkers ?

At our knowledge, there are no study that has analyzed the influence of fasting on AD CSF biomarkers. The comparison of patients with and without fasting would give a set of indirect and biased data without clear conclusion. Moreover, for ethical reasons, it seems to be impossible to start a research study focused on this topic, as this study would imply a protocol with the realization of successive lumbar punctures in a short delay. Therefore, it is not possible to answer scientifically this issue. Nevertheless, it has been shown that, independently of the patient food intake, A β levels in plasma are very stable [10]. As there is a lack of data concerning this topic, as those kind of data could probably never be obtained, and taking account of the large diversity in the locale organization, it is not logical to recommend fasting for the analysis of AD biomarkers in CSF.

2.2. *In vitro* factors

2.2.1. Localization of the puncture

Due to the possible decreased rostro-caudal concentration gradient, the site of CSF withdrawal must be also standardized. At our knowledge, there is no study reporting any difference between AD biomarkers concentrations obtained by a ventricular puncture and those obtained by lumbar puncture. Therefore, it is not recommended to analyse these markers in the

ventricular punctures obtained during neurosurgical interventions. Nowadays, diagnostic CSF is usually obtained by LP between the L3/L4 and L4/L5 intervertebral space.

2.2.2. Does a CSF gradient of AD biomarkers exist ?

Most brain-derived proteins have a decreased rostral-caudal concentration gradient [16]. Therefore, the volume of CSF taken can influence protein concentration. Using unpublished data from Le Bastard et al., Vanderstichele et al reported the absence of a gradient effect in AD CSF biomarkers concentrations during [9]. It was confirmed by another experimental study analyzing the gradient effect in the spinal cord on $A\beta_{42}$ [10]. Therefore, there is no reason to recommend any specific fraction of CSF volume for the assay of AD biomarkers.

2.2.3. What kind of needle for the puncture ?

The type of needle is likely known to influence the percentage of side effects in patients and to be a factor leading to the presence of red cells [17, 18]. Therefore, the needle could influence the biomarkers concentrations. It has been shown that post-lumbar puncture headache (PLPH) severity was significantly decreased when a 22G needle was used instead of a 20G needle [18]. Moreover, using a 22G atraumatic needle it was also observed a remarkably decrease of PLPH in comparison with 22G traumatic needles [19]. Finally, as lumbar puncture is sometimes difficult with 25G needle in elderly people, a Korean group has compared the prevalence of PLPH using 23G and 25G needles. They concluded that the choice of a 23 or 25 gauge Quincke needle has no significant influence on post-dural puncture headache for Korean patients greater than 60 years old. Therefore, the 23 gauge Quincke needle is an option for lumbar punctures in this patient population [20].

2.2.4. Types of sampling tubes

It was established that polypropylene (PP) tubes should be preferred to glass or polystyrene tubes for collection of the CSF since $A\beta$ peptides, but also T-tau and P-tau, bind in a non-specific manner to the polystyrene tubes and to the glass tubes [10, 21]. However, two independent studies reported significant differences on $A\beta_{42}$ levels (up to 50 % compared to basal values !) when CSF was collected in PP tubes from different suppliers [11, 22]. For $A\beta_{42}$, we found that adsorption was effective in a contact time less than 15 minutes, the loss of $A\beta_{42}$ levels being highly significant [11]. Moreover the adsorption intensity was highly dependent on the levels of total proteinorachia, since we abolished this phenomenon when we spiked the CSF with solutions of bovine serum albumin. Amazingly, we also shown that, whereas all the tubes that we studied were commercialized by the providers as tubes in PP, a calorimetry and a spectroscopy analysis revealed that just one out of 11 tubes was pure PP while the others were copolymers made of PP and polyethylene (PE) [11]. Moreover, we also shown that the pure PP causes more adsorption of amyloid peptides than tubes in copolymers of PE and PP, with or without treatment surface, and that some tubes in copolymers could be worse than classical polystyrene: these highly striking results were reproducible in the independent laboratories which have collaborated in this study [11]. Moreover, it was also observed that the tubes that performed better for $A\beta_{42}$ were the worst for P-tau suggesting that hydrophilic-

hydrophobic balance is a important point in protein adsorption [11, 23]. The variability of adsorption intensity of proteins onto the plastic of the tube is the result of the incredible jungle of the manufacturing of different tubes called PP: difference in the nature and in the percentage of the copolymers in the plastic, presence of additives, surface treatments, modification of the surface by the sterilization process... The possibility of modifying the protein adsorption by additives or surface treatments was underlined by different reports. First, when Tween-20 was added in the tube containing the CSF, the adsorption of amyloid peptides was significantly reduced [22]. Secondly we recently reported similar results using various plasma treatments of the tube surface, able to modify the adsorption of different proteins like prion protein, Tau and alpha synuclein [23]. These data highlight the need to standardize also the type of test tube used since the great variability found could even lead to a possible AD misdiagnosis. In our laboratory, we shifted to the best tube that we found in this study. This shift has introduced an averaged increase of 25 % of $A\beta_{42}$ levels leading to a modification of our cut off diagnostic value from 500 ng/L to 700 ng/L (data submitted). Currently the members of the Joint Programming Neurodegenerative Disease research (JPND) are performing a study which includes the analysis of the most suitable type of tube for AD CSF biomarkers research. Therefore, it is not reasonable to follow the actual guidelines recommending the use of generic PP tubes. Since the data of the JPND collaboration will probably not be available before 2 or 3 years, the best compromise would be that each laboratory concerned by these markers, compares its local tube with the best tubes identified in our study, which are easily available in the commercial market.

2.2.5. Time delay between CSF collection and storage before assay

This is an issue difficult to standardize due the high variety of existing procedures and its probable dependance of confounding factors (hemorrhagic puncture, hemolyzed samples, high levels of total protein, one sampling tube for AD biomarkers and various markers of others pathologies...) which could modify the stability of the biomarkers during this critical period.

For that, we will discuss first the need to centrifuge and the protocol of centrifugation. This step is able to avoid the presumed influence of the blood cells introduced by the hemorrhagic puncture. These hemorrhagic punctures occur in 14-20% cases of lumbar puncture. Bjerke et al. were unable to detect any difference in $A\beta_{42}$ levels when up to 5000 erythrocytes/ μ l were spiked to the CSF. This value was found ten fold higher than those recommended in the regulation's document included in the Innogenetics kits. 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 proteins [10]. We cannot also neglect the presence of plasmatic proteases able to digest the peptides since it has been shown that blood contamination of CSF can also lead to protein degradation [25]. The guidelines of Vanderstichele et al. pointed out the absence of difference on the levels of $A\beta_{42}$, T-tau and P-tau between centrifuged and non-centrifuged samples (N. Le Bastard, unpublished data) [9] which could be explained by the fact that they used clear CSF samples. In these guidelines, it was pointed out that spinning speed did not modify significantly the concentration levels of the biomarkers. More recently, it was reported that the

sample temperature was always similar to the temperature set up in the centrifuge showing that temperature is not increased by spinning itself [26]. We can then recommend, that centrifugation should be performed at 2,000 g during 10 minutes at room temperature (RT) following the standardized protocol [26].

If several publications and recommendations are related to the delay between sampling and storage [27], it seems that there is a lack of conclusive data about the influence of the delay between sampling and centrifugation for AD biomarkers, mainly for hemorrhagic puncture. Nevertheless, it was reported significant changes of various metabolites, various amino acids and proteins in presence of white blood cells in the CSF, using a proteomics approach when the CSF were left at RT in the first 30 minutes [28]. These data could explain the apparent discrepancy between the study of Kaiser et al, describing a significant increase of the levels of $A\beta_{42}$ after 24 hours [29] and those of Bjerke, describing that $A\beta_{42}$ concentrations remained stable up to 24 hours after the sampling (storage at RT) [10]. The lack of centrifugation prior incubation is likely the reason of the increase in $A\beta_{42}$ previously observed. Taken all together, all these data highlight the importance of centrifugation to be realized, as soon as possible after sampling, for CSF biomarker analysis.

Although the aspect of the CSF was not always indicated, we can imagine that the different studies which have reported a stability of the CSF levels of $A\beta_{42}$, $A\beta_{40}$, T-tau and P-tau over a period of 24 h at least, were done with clear CSF. Thus, the concentrations of $A\beta_{42}$ were found stable 24 h [10], 72 h when the sample was stored at 4°C [12] and up to 7 days after LP at RT [30]. It was the same for the concentrations of T-tau [10, 12, 29, 30]. 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 [9, 10].

2.2.6. Freezing process

This process is complex since different factors could influence the biomarkers concentrations: although it seems clear that heterogeneity also exists for storage tubes, the temperature of freezing, the volume of the aliquots, the length of the storage and the possible effect of freezing / thawing cycles are potential factors to evaluate. Moreover, these factors can be synergistic: the adsorption of proteins onto the tube walls could be increased by the lower volume of the aliquot and mainly by the ratio volume / surface, or by the temperature of freezing (-20 versus -80°C).

The first step is to choose a storage tube. In parallel to the test realized with 11 sampling tubes [11], we selected 9 different commercially available polypropylene storage tubes (Table 1, tubes 13 to 21), some of them being used by different clinical teams in the AD field. The volume capacity was ranged from 0,5 to 1,5 mL. We performed an analysis of the surface polymer composition using differential scanning calorimetry and Fourier Transformed Infrared spectroscopy. This revealed the same surprising results than obtained with the sampling tubes [11]: only one tube was constituted by pure polypropylene, the others being copolymers with at least polyethylene, with or without surface treatment. Using the same protocol as described for the sampling tubes [11], biomarkers concentrations showed variations that were significantly different for $A\beta_{42}$ peptide. Median values for $A\beta_{42}$ peptide varied from 94 % to 127 %.

These data confirmed those obtained for sampling tubes, although the variability was lower than those found with these last tubes. The effect was present after 15 min, but increasing the incubation time to 24h at 2-8°C, the values did not significantly change compared to 15 minutes incubation.

The next step consists to standardize if needed the temperature, i.e. the speed, of freezing.

Freezing temperatures may affect CSF proteins concentrations as it has previously been reported for cystatin C, which undergoes a proteolysis at -20°C but not at -80°C [31]. Recently, the levels of T-tau and P-tau were reported significantly lower when CSF samples were immediately frozen at -20°C instead of -80°C (N. Le Bastard, unpublished data) [19]. However, this group did not find any difference for the A β_{42} levels when the CSF were frozen at -20°C or -80°C, confirming previous results [10]. Therefore, freezing and storage at -80°C the CSF samples, seem to be logical.

Aliquoting the supernatant of CSF is absolutely necessary since it avoids different Freeze/thaw cycles (see below). Although we did not realize a study designed to evaluate the possible synergy between the ratio volume/surface and the speed of freezing onto the absorption phenomenon in these storage tubes (total volume less than 1.5 ml), some procedures issued from previous reported guidelines can be logically applied [27]. They pointed out the need to use small volumes (never more than 0.5 ml), which would allow: a/ to realize at least the assay of the 3 classical AD biomarkers and if needed the assay of A β_{40} , b/ to prevent freeze/thaw cycles and c/ fill the tube up to 75% to minimize the adsorption and the evaporation effect, this last effect being negligible when the sample is stored frozen at -80°C [26].

As mentioned before, the guidelines recommend separating the supernatant in several fractions, that which will reduce the numbers of freeze/thaw cycles since freezing was shown able to affect protein stability [32]. Some studies have already analyzed the influence of freeze/thaw cycles on AD CSF biomarkers. Most studies using an ELISA format no have found any change on A β_{42} and Tau CSF levels after one freeze/thaw cycle [10, 12, 30, 33], whereas a significant loss of A β_{42} was found after one single cycle in one study using a semi-quantitative method [34]. Increasing the number of cycles was reported able to modify the stability of A β_{42} CSF levels. However, about the exact numbers of cycles able to impact the levels, no real consensus was found between the different studies. If the Tau CSF levels seem to be unaffected by 3 or 6 freeze/thaw cycles [30, 12], the A β_{42} CSF levels were found either stable after 3 cycles [30], either were significantly decreased after the third cycle [12]. In case of immunoassay analysis, it is logically recommended to limit the number of freeze/thaw cycles up to two as maximum [9].

Finally, the length of storage at -80°C does not seem to present a major influence on stability of CSF AD biomarkers, at least for 2 years [30] according to unpublished data from Blennow K. et al., referenced in the guideline published by Vanderstichele et al. [19]. Moreover, the levels of A β_{42} and T-tau but not A β_{40} remained stable up to 6 years [35]. In summary, we can conclude that CSF can be stored up to 2 years at -80°C as previously reported [19].

3. Variability introduced by the analytical step

There are several available assays for the determination of CSF $A\beta_{42}$, T-Tau and P-Tau, commercialized by different companies (Covance, Cusabio, IBL international, Innogenetics, Invitrogen, Millipore, Meso Scale Discovery, Wako... list not exhaustive). Large variation, in assay performance and outcomes of CSF $A\beta_{42}$, T-Tau and P-Tau levels was observed between laboratories also when the same assay format was used, reaching in some cases an inter-assay and inter-laboratory coefficient variations of 20 to 35% [7, 36]. As shown in conclusions of the first report of the external quality control (EQC) program started by the Alzheimer's association [37], ELISA techniques dominate the market while multiplex techniques are used less. In this program, for $A\beta_{42}$, T-Tau and P-Tau, most of laboratories [26 laboratories] used the INNOTEST enzyme-linked immunosorbent assays (ELISAs) (Innogenetics, Ghent, Belgium, www.innogenetics.com), whereas 14 laboratories used the bead-based Luminex xMAP platform with the INNO-BIA AlzBio3 (Innogenetics, Ghent, Belgium, www.innogenetics.com). Moreover, for $A\beta_{42}$ and T-Tau, 5 laboratories used Meso Scale Discovery (MSD, Gaithersburg, MD, www.mesoscale.com) technology [37].

3.1. Principles of assays

INNOTEST enzyme-linked immunosorbent assays (ELISAs) (Innogenetics) are classical ELISAs with colorimetric detection.

INNO-BIA AlzBio3 allows the simultaneous quantification of $A\beta_{42}$, T-Tau and P-Tau in CSF using xMAP® technology (xMAP is a registered trademark of Luminex Corp). The microsphere-based Luminex xMAP technology involves covalent coupling of a capture antibody to spectrally specific fluorescent microspheres [38]. Each microsphere number has a unique spectral identity. The classification of each bead is made by excitation at 635 nm. Each bead number is linked with only one antibody and the signals from analytes in the mixture are identified unequivocally. The quantification of the molecular reaction that has occurred at the microsphere surface, is done using a fluorochrome, the phycoerythrin coupled to streptavidin. The intensity of the fluorescence, derived after excitation of PE at 532 nm, is reported.

MSD offers the possibility to measure in simplex or multiplex format, depending on the biomarker analysed. Whereas t-Tau is measured in simplex format by the participants of the external control program, $A\beta_{42}$ can be measured in simplex or multiplex format in combination with $A\beta_{38}$ and $A\beta_{40}$. Multi-array plate formats include 96- and 384-well plates. The multi-spot plates are available with up to 100 spots per well. MSD uses electrochemiluminescence to detect binding events on patterned arrays. Electrochemiluminescence detection uses labels that emit light at ~620 nm when electrochemically stimulated, the stimulation mechanism (electricity) being decoupled from the signal (light). The signals are treated by the SECTOR Imager Instrument, which is medium throughput imaging detection systems (charge-coupled device camera), capable of multiplexing in all spot formats and reads 96- and 384-well plates.

3.2. Extent of the variability highlighted by this EQC program [37]

3.2.1. Total variability

In this report, results were grouped according to analytical techniques and samples [37]. The total CVs among centers were 16% to 28% for ELISA, 13% to 36% for xMAP, and 16% to 36% for MSD. CVs for MSD must be interpreted with caution, because they included 2 different Monoclonal antibodies (Mab) for $A\beta_{42}$ assays, binding to different epitopes on the amyloid peptide. These data were totally conformed to those reported earlier [7, 36]. There was no major modification of the CV in the longitudinal evaluation, except a decrease in variation for T-tau measured by ELISA. This was expected, since there was no active intervention between the 2 rounds [37].

3.2.2. Within-laboratory precision

Within-laboratory CVs were examined at the reference laboratories for ELISA and xMAP in two consecutive rounds. CVs were 3.2% to 24% for ELISA and 2.3% to 26% for xMAP, but differed between analytes within individual laboratories, indicating assay-dependent variations [37].

3.2.3. Differences in absolute values

The analytical techniques reported different absolute values for the biomarkers. ELISA values for $A\beta_{42}$ were about 2 fold higher than xMAP values. MSD values for $A\beta_{42}$ were dependent of the Mab used. ELISA values for T-Tau were about 3 fold higher than xMAP values. Finally for P-Tau, the differences inter techniques were clearly decreased in comparison to $A\beta_{42}$ and T-Tau. Considerable variability exists among the same manufacturer between mono and multiplex technology. For example, the decision threshold of clinical disease was reported to be at 86 pg / mL and 350 pg / mL for T-TAU measured by xMAP technology of Innogenetics on the platform Luminex and the conventional ELISA, respectively [39]. Factors of correction between values obtained by xMAP and ELISA, were used for global comparison of groups of patients, i.e. controls, Mild Cognitive Impairment and AD patients to predict incipient AD by CSF biomarkers [40]. In an other side, it was clearly shown that the use of factors of correction did not resolve the discrepancy in values observed between xMAP and ELISAs [41]. 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 [39] or for detecting early AD [41, 42].

3.3. Possible sources of variability

In this study analysing the variability of results from only two rounds of an EQC program and from many different assay lots used, the authors limited their interpretation of the relative contributions from between-laboratory, within-laboratory, and between-lot components to the total variability [37]. Differences in within-laboratory CVs among the biomarkers within individual reference laboratories suggest that assay-related factors are important. Moreover,

the high variability of the results of biomarkers measured by different commercial kits can be explained, by the use of different antibodies, the nature of the calibrator, the calibration method and many others factors as for example the nature of standard. Increasing data during years and by incorporation of new centers (since this first report concerning 40 laboratories, in summer of 2012, 64 laboratories were participating at this program) will permit to better identify the major sources of variability in analytical steps. Thus, we can just list the different points to be further investigated.

In the laboratory, the biologist will take care for:

a. Pipetting

The pipetting mode (inverse pipetting...) is not specified by the manufacturer. Using a single tip can influence the standard curve accuracy. However, the magnitude of this effect, if any, should be tested, to provide a better basis for recommendation [43].

b. Calibration

For lyophilised standard, accurate solubilization and accurate pipetting is critical. Moreover, since for INNOTEST Ab42, the first point of the curve calibration must be adjusted depending of the set value, accurate pipeting is absolutely needed. The type of curve fitting used and the software for data calculation were shown as possible factors of variability [43].

c. Reagent handling and adhesion of biologists to the manufacturer standard operating procedure (SOP)

The adhesion of routine laboratories to the manufacturer SOP is absolutely needed to reduce the part of the variability found in CSF biomarkers analysis. For that, a great effort must be done by the different manufacturers to limit individual interpretation of the technical instructions. The best example consists in the definition of the «room temperature» which can mainly vary from the north to the south of Europe. The maintenance of laboratory equipment is a crucial point to ensure the accuracy of pipeting volumes, the accuracy of temperatures, the accuracy of detection signals and the quality and reproducibility of washing steps.

d. Familiarization with the method and Competency Train

Implementing these techniques in the laboratory needs a training program ensured by the manufacturer. Moreover, habilitation and qualification of the laboratory staff must be done.

e. Validation criteria of runs for rejecting data

Different means are used to ensure validation of results. The definition of the criteria of acceptance of results must be strict. They include the calibration curve parameters, the CV of the duplicate samples and the use of an internal quality control program. For the CV criteria acceptance, in our experience, it seems that they are to be adequately defined since, the recommendation of $CV < 20\%$ done in the INNOTEST documentation, is not acceptable all along the dynamical range of the assay, in particular when the concentration level is near the clinical cut-off. Moreover, in the absence of QC samples in the kit, the biologist needs to implement its own QC program with different crucial points to resolve: the nature of the

sample (native CSF pools, spiked CSF with standards, peptides...) how many QC samples, range of concentrations to cover, absence of reference material. This point is crucial for laboratories concerned by accreditation scheme based on the application of ISO15189 standard.

3.3.1. Issues to be solved by manufacturers

Many crucial points need to be solved as the poor quality of the test procedure instructions to decrease variability induced by misunderstanding of the protocols. This lacking information is often an indicator of minimal method optimization of the protocol (for example incubation steps, handling the reagents...). The reagents must be proposed in a manner that permits to decrease variability, for instance the «ready to use» calibrators. The absence of quality control included in the kit is a major problem. In fact, part of the discrepancy observed in the concentrations levels between the analytical techniques ELISA, xMAP, and MSD is caused by the lack of certified reference materials (CRMs). This could mainly impact the interlot variability and is at least, a brake to standardization. Antibody purification, coating of plates and beads are also factors of lot-lot variability.

4. Conclusion

The present chapter highlights two main issues responsible for the lack of harmonization of CSF AD biomarkers cut-offs values: the lack of standardization of the pre-analytical steps and the high variability of results linked to the analytical step. This latter issue can be explained by the absence of transferability of results between the different platforms but also by the high inter laboratory dispersion within the same assays. Previous consensus guidelines for pre-analytical factor standardization gave the way to resolve this issue, evidencing the need to standardize sampling and storage tubes, the type of the needle for the CSF puncture and the long term storage. Establishing SOPs for sample processing would allow to compare diagnostic conclusions between different laboratories. The implementation of those SOPs in the clinical community may reduce part of the variability found in the analysis of AD CSF biomarkers. Antibody purification, coating surfaces, preparation of standards, manufacturers instructions are also sources of variation, which need to be decreased and requires increased efforts by kit manufacturers. The optimal approach is a collaborative effort between commercial kit and instrument platform manufacturers, laboratories concerned by those methods, and reference standardization programs.

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