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Batch prepared protein standards for cerebrospinal fluid (CSF) biomarkers for neurodegeneration

A. Petzold[a,*], N.A. Verwey[a,b,c], K. van Uffelen[c], M.A. Blankenstein[c], C. Teunissen[c]

[a] Department of Neurology, VU Medical Center, Amsterdam, The Netherlands
[b] Alzheimer Center, VU University Medical Center, Amsterdam, The Netherlands
[c] Department of Clinical Chemistry, VU Medical Center, Amsterdam, The Netherlands

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ABSTRACT

Immuno-assays are increasingly used for quantification of protein biomarkers for neurodegeneration. It has been proposed to use such cerebrospinal fluid (CSF) protein biomarkers as diagnostic tests for Alzheimer's disease. In two recent world-wide validation studies we found the analytical accuracy to be poor (inter-laboratory coefficient of variation, CV > 10%) for CSF tau protein, CSF phospho-tau protein, CSF amyloid beta protein and the CSF neurofilament light chain protein. Retrospectively we suspected that the lack of preparation of accurate and consistent protein standards may have been one reason for the poor inter-laboratory CV. Here we confirm this hypothesis prospectively under standardised and optimised conditions. The CVs for CSF tau, CSF phospho-tau and CSF amyloid beta of individually prepared standards are 8%, 12% and 12% compared to significantly lower CVs for batch prepared standards (5%, 8%, 7%, respectively, $p < 0.05$). This issue will need to be solved in order to ensure that the attempts to include these CSF protein biomarkers either as a diagnostic tool or a secondary outcome measure for treatment trials will be successful.

1. Introduction

Protein biomarkers are important for diagnosis, prognosis, monitoring of disease activity and research on human disease (Teunissen et al., 2005; Blennow et al., 2010). To meet the high expectations a rigorous methodological approach is paramount. In a recent world-wide validation study we *retrospectively* identified the preparation of protein standards as a potentially important source of error (Petzold et al., 2010). To test this finding *prospectively* we set up an experiment in the course of an international hands-on workshop. Participants from laboratories experienced in the analysis of protein biomarkers who had already participated in previous validation studies (Verwey et al., 2009; Petzold et al., 2010) were asked for synchronised preparation of protein standards and analysis of coded samples under controlled conditions in the same laboratory. The hypothesis was that the inter-technician accuracy for quantification of protein biomarkers from coded samples would be better using batch prepared compared to individually prepared standards.

2. Materials and methods

All selected laboratories had already participated in earlier validation studies (Verwey et al., 2009; Petzold et al., 2010). The laboratories were involved both in research and diagnostic testing and as such accredited according to national guidelines.

2.1. Kits and equipment

Commercial kits were purchased by the NeuroUnit Biomarkers for Inflammation and Neurodegeneration (NUBIN, www.vumc.nl/afdelingen/NUBIN) and Department of Clinical Chemistry at the Free University Medical Center (VUmc) Amsterdam. Importantly, for this study all kits, plates, protein standards and antibodies were from the same production lot. Total tau, phospho tau (pTau, phosphorylated at threonine 181) and the Aβ_{1-42} proteins were quantified using the Innotest® hTau and pTau ELISA kits (Innogenetics NV, Ghent, Belgium). According to the data provided by the manufacturer the analytical accuracy (inter-assay coefficient of variation, CV) should be better than 10% for Tau, pTau and Aβ_{1-42}.

2.2. Samples

Pooled cerebrospinal fluid (CSF) samples were aliquoted by one NUBIN technician (KvU), coded and stored at −80 °C as per con-

* Corresponding author at: Free University Medical Center, Amsterdam, The Netherlands.
E-mail addresses: a.petzold@vumc.nl, a.petzold@ion.ucl.ac.uk (A. Petzold).

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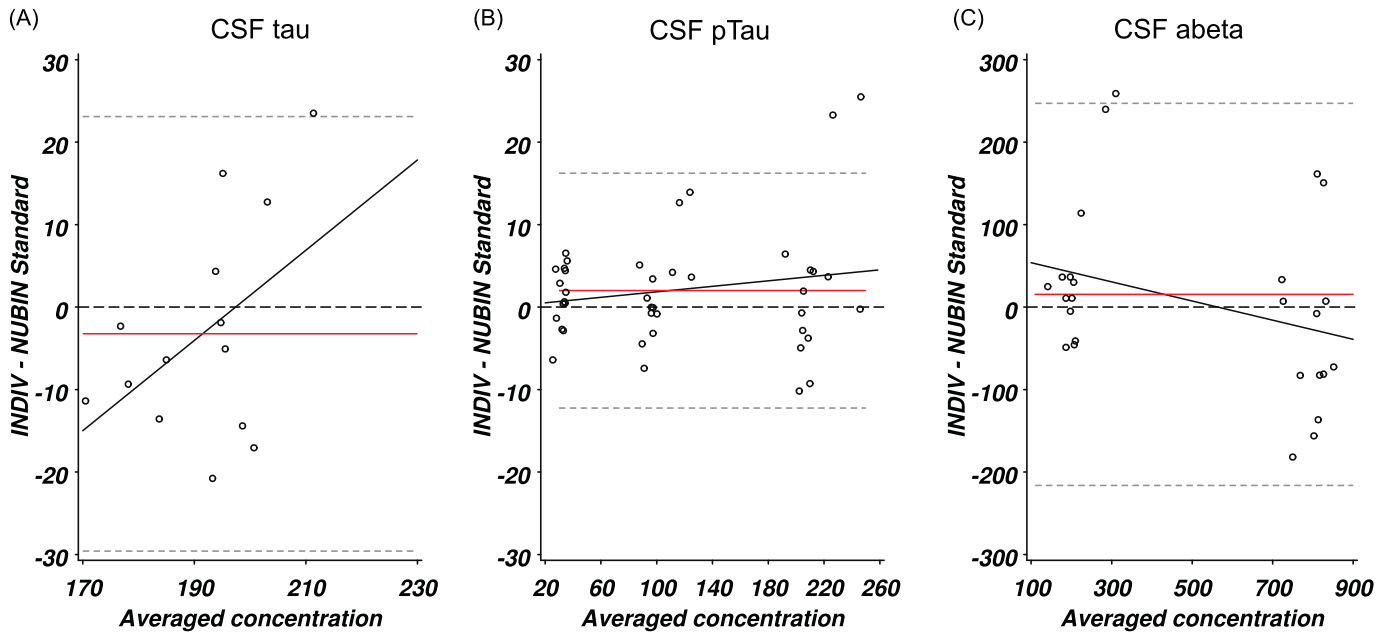

Fig. 1. Bland–Altman plots comparing protein concentrations in coded CSF samples based on a batch prepared standard curve (NUBIN) or individually made up standard curve (INDIV) for (A) CSF tau protein, (B) CSF pTau protein and (C) CSF Aβ_{1-42} protein. The black dashed horizontal zero-line shows where all sample data points should coincide if the agreement between the two standard curves was perfect. The closed horizontal line shows the averaged level of agreement with the 95% CI interval indicated by the grey dashed lines. Regression of the differences between the two standard curves on the averaged values is shown by the oblique black line (Bland and Altman, 1999).

sensus guidelines (Teunissen et al., 2009). There were three CSF samples for pTau, two CSF samples for Aβ_{1-42} and one CSF sample for tau.

2.3. Standards

The top protein standard from the same production lot were batch prepared by one NUBIN technician (KvU) as per manufactures instructions. The top standard was gently mixed by hand (not vortexed) and left on the bench at room temperature for 30 min to allow for complete solubilization of the protein. Next, the top standards were pooled for each assay. The pooled top standards were used to for batch preparation of the standard curves. Finally 14 aliquots were prepared from each pooled, batch prepared standard, coded and stored at −80 °C in 1.5 mL polypropylene Eppendorf tubes three days prior to the NUBIN workshop. For clarity we refer to these standards as in this manuscript as "NUBIN standards".

2.4. Analytical procedures

All participating experts (26 experts from 17 international centers divided into 14 groups, a full list of all participants is given in Acknowledgment section) were advised to follow the manufacturers instructions for performing the ELISAs. The experts used their own, calibrated pipettes for pipetting. All experts made up their own standard curve and also analysed the coded samples. The experts did not know that they were analysing a batch prepared standard curve (NUBIN standards).

2.5. Statistics

The agreement between the NUBIN protein standards and individually prepared standards (using calibrated pipettes) was analysed using Bland–Altman plots, which show the Tukey mean-difference between the outcome of the CSF pools if calculated based on the two different standard curves and. In this paper the two set of protein standards should give the same result if used to calculate the CSF concentration from an unknown sample. The Bland–Altman plot permits to visualise the level of agreement between the two set of standard curves with the Cartesian coordinates of a given sample (S) being calculated as: $S(x, y) = ((S_1 + S_2/2), (S_1 - S_2))$. The inter-technician CV for pooled CSF samples was calculated twice, first based on the NUBIN standard curve and second based on individually prepared standard curves as described (Petzold et al., 2010; Verwey et al., 2009). The Kruskal–Wallis test was used for comparison of two variables. For categorical data analysis Fisher's exact test was used. A p-value of <0.5 was accepted as significant.

3. Results

3.1. Protein standard curve comparison

The Bland–Altman blots for the CSF tau protein concentration (averaging at 193 pg/mL) are shown in Fig. 1A. The CSF tau concentration measured based on the NUBIN standards was 3.2 ± 13.2 pg/mL higher compared to the CSF tau concentration based on individually made up standards (horizontal closed line in Fig. 1A below zero-line of complete agreement).

For CSF ptau three CSF samples were measured with a respective average concentration of 32 pg/mL, 101 pg/mL and 213 pg/mL. Fig. 1B shows that the CSF pTau concentration was about 2.0 ± 7.1 pg/mL lower if calculations were based on the NUBIN standards compared to individually made up standards (horizontal closed line in Fig. 1B above zero-line of complete agreement).

For CSF Aβ_{1-42} two CSF samples were measured with respective average concentrations of 818 pg/mL and 253 pg/mL. Fig. 1C shows that that the CSF Aβ_{1-42} concentration was about 15.4 ± 115.4 pg/mL lower if calculations were based on the NUBIN standards compared to individually made up standards (horizontal closed line in Fig. 1C above zero-line of complete agreement).

Importantly, there was not one single expert responsible for the wide scatter of dots in Fig. 1A–C. Therefore, the analytical accuracy was calculated including the data from all 14 participating experts.