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CSF $A\beta_{1-42}$ – an excellent but complicated Alzheimer's biomarker – a route to standardisation

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ABSTRACT

The 42 amino acid form of amyloid β ($A\beta_{1-42}$) in cerebrospinal fluid (CSF) has been widely accepted as a central biomarker for Alzheimer's disease. Several immunoassays for CSF $A\beta_{1-42}$ are commercially available, but can suffer from between laboratory and batch-to-batch variability as well as lack of standardisation across assays. As a consequence, no general cut-off values have been established for a specific context of use (e.g., clinical diagnostics) and selection of individuals for enrolment in clinical trials (patient stratification) remains challenging.
The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has initiated a working group for CSF proteins (WG-CSF) to facilitate standardisation of CSF $A\beta_{1-42}$ measurement results. The efforts of the IFCC WG-CSF include the development of certified reference materials (CRMs) and reference measurement procedures (RMPs) for key biomarkers. Two candidate RMPs for quantification of $A\beta_{1-42}$ in CSF based on liquid chromatography tandem mass spectrometry have been developed and tested in two ring trials. Furthermore, two commutability studies including native CSF pools, artificial CSF and spiked materials have been completed. On the basis of these studies, human CSF pools containing only endogenous $A\beta_{1-42}$ at three concentrations were selected as the format for future CRMs that are now being processed.

1. Introduction

The 42 amino acid form of amyloid β peptide ($A\beta_{1-42}$) in cerebrospinal fluid (CSF) is widely accepted as a key biomarker for Alzheimer's disease (AD) together with the total tau (T-tau) and phosphorylated tau (P-tau) protein [1]. The decrease of $A\beta_{1-42}$ concentrations in CSF reflects its deposition in amyloid plaques in the brain [2], that is one of the hallmarks of the disease. The CSF concentrations of $A\beta_{1-42}$ have demonstrated a high diagnostic accuracy for AD dementia [3] and prodromal AD [4–7]. Furthermore, CSF $A\beta_{1-42}$ concentrations show high concordance with results of amyloid positron emission tomography (PET) scans of the brain [8]. What makes this biomarker particularly suitable for early diagnosis is the fact that its concentration changes many years before an onset of clinical symptoms. Current clinical routine measurement procedures are based on enzyme-linked immunosorbent assays (ELISAs) or immunoassays on other technology platforms. A significant variability in measured values was observed among analytical procedures and among laboratories [7]. For this reason, the Alzheimer's Association Quality Control (QC) program was initiated among members of the Alzheimer's Association Global Biomarker Standardization Consortium (GBSC) [9]. Although the QC program has been active for several years, variability between routine measurement procedures is still a problem due to a lack of standardisation. This variability arises partly from differences in laboratory procedures for

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sample collection, storage and analysis, as well as from variability linked to the manufacturing process for the assays resulting in batch-to-batch variations. However the lack of standardisation is the main reason that different immunoassays give different concentrations when measuring the same sample [10]. The availability of a commutable certified reference material (CRM) for calibration could dramatically decrease the variability of measurement results, specifically batch-to-batch variability and the bias between assay results.

Since the field of AD research has gone through a thorough validation process to ascertain biologic and diagnostic relevance of the CSF biomarkers, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) approved the setup of a working group on CSF proteins (WG-CSF) with the goal of developing reference systems for CSF biomarker measurements. The immediate tasks of the IFCC WG-CSF included the collection of human CSF for the production of a commutable CRM and the establishment of reference measurement procedures (RMPs). Although the activities are not limited to the standardisation of CSF $A\beta_{1-42}$ measurements, this analyte was chosen for a first development of RMPs [11]. At the same time first promising results of clinical trials have been reported [12,13]. There was also an increased need to select individuals at early stages for clinical trials and to adapt current diagnostic criteria and clinical guidance. The Institute for Reference Materials and Measurements (JRC-IRMM), which is one of the seven institutes of the European Commission's Joint Research Centre, assists the WG-CSF in these efforts with advice on standardisation and by producing the first CRM for CSF $A\beta_{1-42}$ according to the CRM principle laid down in ISO Guide 34:2009 [14].

The aim of standardisation is to ensure that measurement results for the same sample are equivalent over time, among different laboratories or by using different routine measurement procedures [15,16]. This requires the setup of a proper calibration hierarchy that allows traceability of measurement results to a higher order measurement standard. The first concern in the standardisation of CSF $A\beta_{1-42}$ measurements is the evaluation of the degree of correlation between results of routine measurement procedures. Next, the upper part of the calibration hierarchy needs to be built up (Fig. 1). This includes, among other, the development of a RMP that provides results correlating with appropriate routine measurement procedures and a matrix CRM that is commutable for the intended routine measurement procedures. This paper describes the progress achieved and the difficulties encountered in setting up a reference system for CSF $A\beta_{1-42}$ measurements.

2. Reference measurement procedures for CSF $A\beta_{1-42}$

The development and validation of one or more RMPs is a crucial step towards the development of a CRM and the standardisation of biomarker measurements. The RMPs are not only useful to assess the performance of other measurement procedures and assign values to routine calibrators, but are also necessary for value assignment of candidate CRMs. In recent years liquid chromatography tandem mass spectrometry (LC-MS/MS) has been increasingly used for the quantification of protein biomarkers and several LC-MS/MS procedures for the quantification of CSF $A\beta_{1-42}$ have been reported in the literature [17–19]. This indicated that the establishment of a LC-MS/MS-based RMP should be feasible. A selected reaction monitoring method [19] has been applied in the two commutability studies described below (Section 3). Results from this procedure showed good correlation with the Cobas Elecys (Fig. 2). The observed bias (slope ≠ 1) is likely due to differences in calibration, which could be removed by using a common calibrator. Since then, two RMPs for the quantification of CSF $A\beta_{1-42}$ based on LC-MS/MS have been developed and both are accepted and listed by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) as RMPs (no. C11RMP9 and C12RMP1). Both RMPs are based on a procedure published by Lame et al. [18] that includes guanidine hydrochloride treatment followed by a solid phase extraction step as sample preparation and multiple reaction monitoring for quantification. The same sample preparation procedure is applied in both RMPs, but they differ in instrumentation and more importantly in the matrix selected for preparing the calibrator solutions. In the procedure developed by Leinenbach et al. [20] (RMP1) the calibrator matrix is human CSF spiked with ^{15}N-labelled $A\beta_{1-42}$ peptide as a surrogate analyte, whereas the second procedure described by Korecka et al. [21] (RMP2) has calibrators prepared from artificial CSF (aCSF) containing 4 mg/mL BSA as a surrogate matrix, spiked with recombinant $A\beta_{1-42}$ peptide (Table 1). Furthermore, RMP1 uses a quadrupole Orbitrap hybrid instrument operated in the parallel reaction monitoring (PRM) mode where

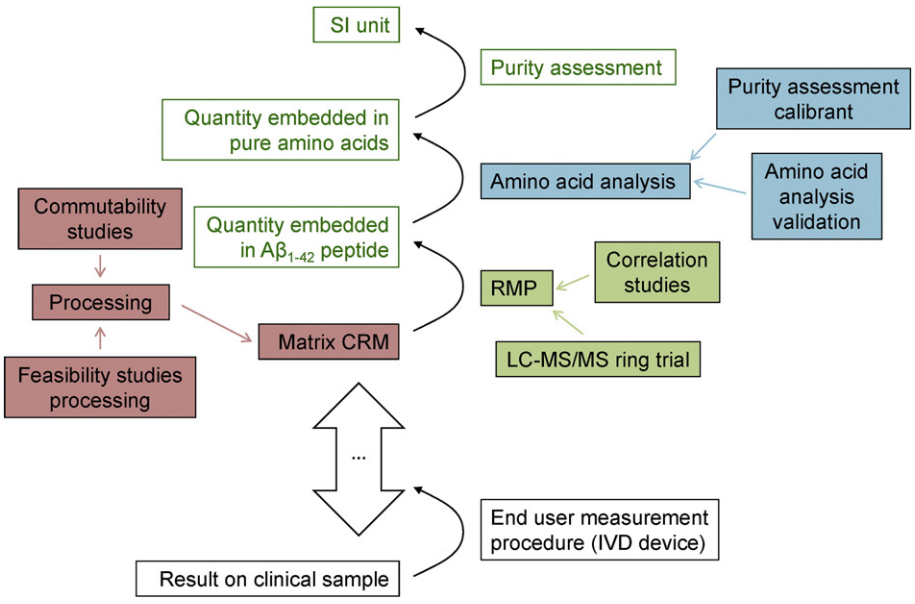

Fig. 1. Overview of the traceability chain for $A\beta_{1-42}$, linking results of routine samples to the international system of units (SI) as a common reference. Steps necessary to establish the upper part of the traceability chain are colour coded. Abbreviations: RMP, reference measurement procedure; CRM, certified reference material; SI, International System of units (Système International d'Unités); IVD, in vitro diagnostic.

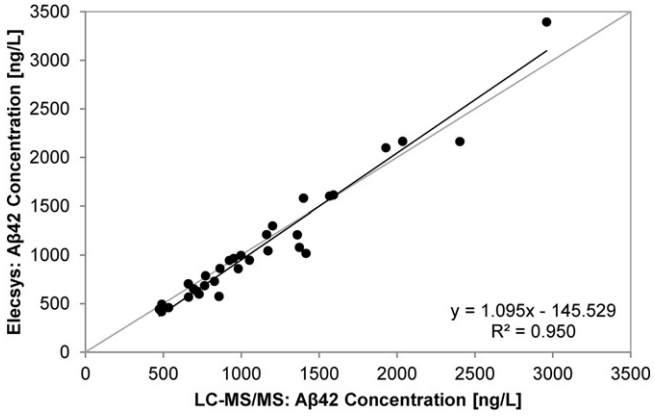

Fig. 2. Correlation of the average results on CSF samples measured in duplicate with a LC-MS/MS procedure and the Cobas Elecsys method for CSF $A\beta_{1-42}$ (ref. 27).

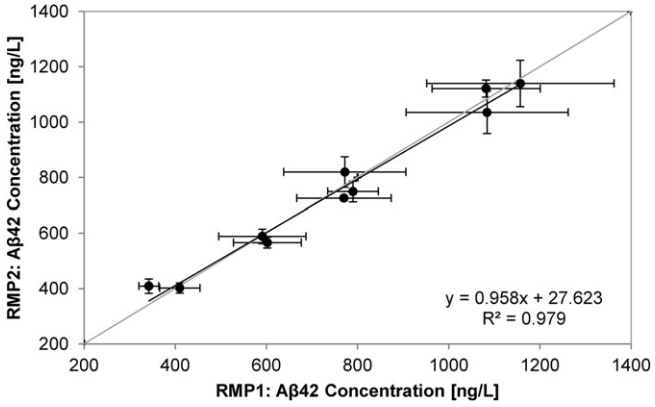

Fig. 3. Correlation of the results on 10 CSF pools measured with the 2 reference measurement procedures (RMPs) for CSF $A\beta_{1-42}$ quantification by LC-MS/MS. Over the course of 3 days, 2 aliquots per CSF pool were measured in duplicate. Both procedures were calibrated with a common $A\beta_{1-42}$calibrator provided by JRC-IRMM. Error bars indicate standard deviations of the daily averages measured with RMP2.

quantifications is performed by using the summed-up peak areas of 15 fragment ions of $A\beta_{1-42}$, while the RMP2 method uses a triple quadrupole instrument using one specific fragment for quantification of $A\beta_{1-42}$. Despite the differences, the results of the two RMPs correlate very well ($R^2 = 0.98$) as shown in Fig. 3.

2.1. LC-MS/MS ring trial 1

A ring trial was organized in collaboration with the GBSC to study the correlation between results from different LC-MS/MS procedures for the quantification of CSF $A\beta_{1-42}$ and to estimate the inter-laboratory variability for those procedures. Laboratories from the University of Gothenburg (Gothenburg, Sweden), University of Pennsylvania (Philadelphia, PA, USA), Waters Corporation (Milford, MA, USA) and PPD (Richmond, VA, USA) have participated. Each laboratory received aliquots of 12 human CSF pools (from the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden) and was asked to apply their validated in-house procedure to quantify $A\beta_{1-42}$. In addition, the INNOTEST β-AMYLOID (1–42) assay (Fujirebio-Europe, Inc., Ghent, Belgium) was employed by the laboratory at the University of Gothenburg to analyse the correlation of the LC-MS/MS procedures to a routine immunoassay measurement procedure. Details of this study have been reported elsewhere [22]. In short, all laboratories applied the same sample preparation scheme, and while all used selected reaction monitoring for LC-MS/MS quantification, different instrumentation and calibration procedures were employed. The results of all procedures correlated well ($R^2 = 0.98$) with high analytical precision and an average intra-laboratory coefficient of variation (CV) of 4.7%. Furthermore, they showed good correlation with the selected routine measurement procedure. However, the average inter-laboratory CV was 12.2%, which was not surprising as no common calibrator was available at the time of the study. Therefore, one CSF sample was selected as reference sample, a correction factor was calculated and when applied the inter-laboratory variability was reduced by 32% to a CV of 8.3%.

Table 1
Comparison of reference measurement procedures for $A\beta_{1-42}$.

	RMP1 [20]	RMP2 [21]
Calibrator	$[^{15}N]A\beta_{1-42}$: 150–4000 ng/L	$A\beta_{1-42}$: 100–3000 ng/L
Internal standard	$[^{13}C]A\beta_{1-42}$: 1600 ng/L	$[^{15}N]A\beta_{1-42}$: 1 ng/mL
CSF volume	180 μL	100 μL
Calibrator matrix	Human CSF	aCSF + 4 mg/mL BSA
LC instrument	Thermo UltiMate 3000	Waters ACQUITY; 2D trapping/eluting
Dilution injection	N/A	50 μL + 50 μL H_2O (25 μL)
LC eluents	A: 5% ACN, 0.075% NH_4OH B: 95% ACN, 0.025% NH_4OH	A: 0.1% NH_4OH B: 70% ACN, 25% MeOH, 5% TFE
Column	Thermo ProSwift RP-4H 1.0 × 250 mm, 50 °C	Waters BEH 300 2.1 × 150 mm, 60 °C
MS instrument	Thermo Scientific Q-Exactive	Waters Xevo TQ-S
$A\beta_{1-42}$ range	150–4000 ng/L	100–3000 ng/L

Abbreviations: CSF, cerebrospinal fluid; aCSF, artificial cerebrospinal fluid; BSA, bovine serum albumin; LC, liquid chromatography; ACN, acetonitrile; MeOH, methanol; TFE, trifluoroethanol; MS, mass spectrometer. Concentrations are given as *v*/v.

2.2. LC-MS/MS ring trial 2

A second LC-MS/MS ring trial was initiated to investigate how a common $A\beta_{1-42}$ calibrator could be implemented in a ring trial and if that could reduce the inter-laboratory variability. This was an important study, since the value assignment of the candidate CRMs is foreseen to be done by LC-MS/MS using a common $A\beta_{1-42}$ calibrator. The JRC-IRMM produced a calibrator based on a procedure adapted from Broersen et al. [23]. It contained a recombinant $A\beta_{1-42}$ peptide (rPeptide, Bogart, GA, USA) in 20% acetonitrile and 1% ammonium hydroxide (*v*/v) in water with an indicative $A\beta_{1-42}$ concentration value of 74 ng/L as determined by amino acid analysis (AAA). The calibrator was provided to the participating laboratories (University of Gothenburg (Gothenburg, Sweden), University of Pennsylvania (Philadelphia, PA, USA), Waters Corporation (Milford, MA, USA), PPD (Richmond, VA, USA) and Roche Diagnostics GmbH (Penzberg, Germany)) along with aliquots of 20 individual CSF samples (from the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden). Each lab was instructed to use the common calibrator in their validated in-house procedure and according to a common protocol for the preparation of calibrators provided by JRC-IRMM. An initial analysis of the data received from the participating laboratories showed good correlation of the data and an inter-laboratory CV of 9% (manuscript in preparation).

3. Commutability studies

One crucial step in the development of a CRM is the assessment of its commutability, which has been defined by the Clinical and Laboratory Standards Institute (CLSI) as "the equivalence of the mathematical relationships among the results of different measurement procedures for a reference material and for representative samples of the type intended to be measured" [24]. In other words, a reference material is commutable if it behaves in the measurement process like representative clinical samples. This material property is required for using a CRM for calibration or trueness control to assure accurate clinical results. Such

laboratory medicine applications usually require that a matrix reference material is developed, rather than a pure substance material. Matrix CRMs are often more delicate and expensive to produce, but they are much more likely to be commutable in clinical routine assays than a pure protein solution. Moreover, spiking of native sample pools with the analyte is often performed to create the desired concentrations. Two commutability studies were conducted to select the most suitable starting material for the production of a commutable CRM for CSF $A\beta_{1-42}$ measurements. The details of those were previously reported elsewhere [25].

3.1. Commutability study 1

The first commutability study was organized to evaluate which matrix format would be most suited for a reference material for $A\beta_{1-42}$. The following five immunoassay-based routine measurement procedures were included: 1) MSD® 96-Well MULTI-ARRAY® Human (4G8) Abeta42 Ultra-Sensitive Kit (Meso Scale Discovery, Gaithersburg, MD, USA), 2) Human β Amyloid(1–42) ELISA Kit Wako High-Sensitive (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 3) Human Amyloid β (1–42)(N) assay kit - IBL (Immuno-Biological Laboratories Co., Ltd., Fujioka, Japan, distributed by IBL International GmbH, Germany), 4) INNOTEST® β-AMYLOID (1–42) and 5) INNO-BIA AlzBio3 (both Fujirebio-Europe, Inc., Ghent, Belgium), see Table 2 for details. In addition, a LC-MS/MS procedure was applied to compare the results to those obtained with the routine measurement procedures. A total of 48 individual CSF clinical samples (from the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden) and 16 candidate CRM formats were tested. The latter included native CSF pools with low and high intrinsic $A\beta_{1-42}$ concentrations as well as recombinant $A\beta_{1-42}$ (rPeptide, Bogart, GA, USA) containing aCSF and PBS. Furthermore, all four matrices were spiked with different concentrations of recombinant $A\beta_{1-42}$ peptide and the addition of the detergent Tween® 20 (Sigma-Aldrich, St. Louis, MO, USA) was tested. The study showed that most of the measurement procedures provided highly correlating results (Fig. 4). However, some routine measurement procedures produced results that varied up to a factor of 2.6. This is consistent with previously published data (26). The problem could be solved by calibration with a commutable CRM. The neat CSF pools behaved like the individual CSF clinical samples for most procedure combinations, but none of the artificial matrices (aCSF and PBS) tested in the study was commutable for all procedure combinations tested (Fig. 5A). The addition of detergent did not improve the results and even caused non-commutability for the native CSF pools to in some procedure combinations. As a conclusion a native CSF pool should be used for the production of the CRM.

3.2. Commutability study 2

A second commutability study was organized to investigate 1) if the foreseen CSF pool would be suitable for the production of the CRMs and 2) if spiking of the native CSF pool with recombinant $A\beta_{1-42}$ would be an option to create the desired concentration concentrations for the CRMs. For this study eight routine measurement procedures and one LC-MS/MS procedure were employed to measure 32 individual CSF clinical samples (Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden), which covered the clinical spectrum of CSF $A\beta_{1-42}$ values. The following routine measurement procedures were included in the study: 1) MSD® 96-Well MULTI-SPOT® Human Aβ42 V-PLEX Kit (Meso Scale Discovery, Gaithersburg, MD, USA), 2) Amyloid-beta (1–42) CSF ELISA (IBL International GmbH, Hamburg, Germany), 3) VITROS® Immunodiagnostic Amyloid Beta 42 Assay (AB-42) (Saladax Biomedical, Bethlehem, PA, USA), 4) Elecsys® β-Amyloid (1–42) immunoassay (Roche Diagnostics, Penzberg, Germany)

Table 2
Analytical methods used in the commutability studies.

Assay name	Short name	Company / Institution	Specificity	Antibodies name (specificity)	Assay principle	Study I	Study II
SRM LC MS/MS Aβ42	SRM	Clinical Neurochemistry Laboratory, University of Gothenburg, Sweden	$A\beta_{1-42}$	None	Triple quadrupole MS on a TSQ Vantage instrument (Thermo Scientific, USA)	X	X
MSD® 96-Well MULTI-ARRAY® Human (4G8) Abeta42 Ultra-Sensitive Kit	MSD	Meso Scale Discovery, USA	Aβ X-42	12F4 (Aβ X-42) and 4G8 (Aβ 17–24)	Sandwich immunoassay, electrochemiluminscence	X	
MSD® 96-Well MULTI-SPOT® Human Aβ42 V-PLEX Kit	MSD	Meso Scale Discovery, USA	Aβ X-42	12F4 (Aβ X-42) and 6E10 (Aβ 6–10)	Sandwich immunoassay, electrochemiluminscence		X
Human β Amyloid(1–42) ELISA Kit Wako High-Sensitive	Wako	Wako Pure Chemical Industries, Japan	$A\beta_{1-42}$	BAN-50 (Aβ 1-X) and BC-05 (Aβ X-42)	Sandwich ELISA	X	
Human Amyloid β (1–42)(N) assay kit	IBL	IBL, Japan	Aβ X-42	44 A3 (Aβ X-42) and 12B2 (Aβ 11–28)	Sandwich ELISA	X	
Amyloid-beta (1–42) CSF ELISA	IBL	IBL International, Germany	$A\beta_{1-42}$	44 A3 (Aβ X-42) and 82E1 (Aβ 1-X)	Sandwich ELISA with ready to use calibrators		X
INNOTEST® β-AMYLOID (1–42)	Innotest	Fujirebio-Europe, Belgium	$A\beta_{1-42}$	21F12 (Aβ X-42) and 3D6 (Aβ 1-X)	Sandwich ELISA	X	
INNOTEST® β-AMYLOID (1–42)	Innotest	Fujirebio-Europe, Belgium	$A\beta_{1-42}$	21F12 (Aβ X-42) and 3D6 (Aβ 1-X)	Sandwich ELISA with ready to use calibrators		X
INNO-BIA AlzBio3	AlzBio3	Fujirebio-Europe, Belgium	$A\beta_{1-42}$	4D7A3 (Aβ X-42) and 3D6 (Aβ 1-X)	Bead-based sandwich immunoassay based on the Luminex xMAP® technique	X	X
VITROS® Immunodiagnostic Amyloid Beta 42 Assay (AB-42)	Saladax	Saladax Biomedical, USA	$A\beta_{1-42}$	Aβ1-IgG (Aβ X-42) and Aβ2-IgG (Aβ 1-X)	Chemiluminescence sandwich immunoassay on a fully automated immunoanalyzer instrument		X
Elecsys® β-Amyloid (1–42) immunoassay	Elecsys	Roche Diagnostics, Germany	$A\beta_{1-42}$	21F12 (Aβ X-42) and 3D6 (Aβ 1-X)	Electrochemiluminescence immunoassay on a fully automated cobas e 601 analyzer		X
EUROIMMUN Beta-Amyloid (1–42)	Euroimmune	ADx NeuroSciences, Belgium	$A\beta_{1-42}$	21F12 (Aβ X-42) and 3D6 (Aβ 1-X)	Sandwich ELISA with ready to use calibrators		X
Simoa Human Aβ42	Simoa	Quanterix Corporation, USA	Aβ X-42	6E10 (Aβ 6–10) and H31L21 (Aβ X-42)	Single Molecule Array immunoassay technology on a Simoa HD-1 Analyzer		X

Abbreviations: SRM: selected reaction monitoring; MS: mass spectrometry; TSQ: triple stage quadrupole.

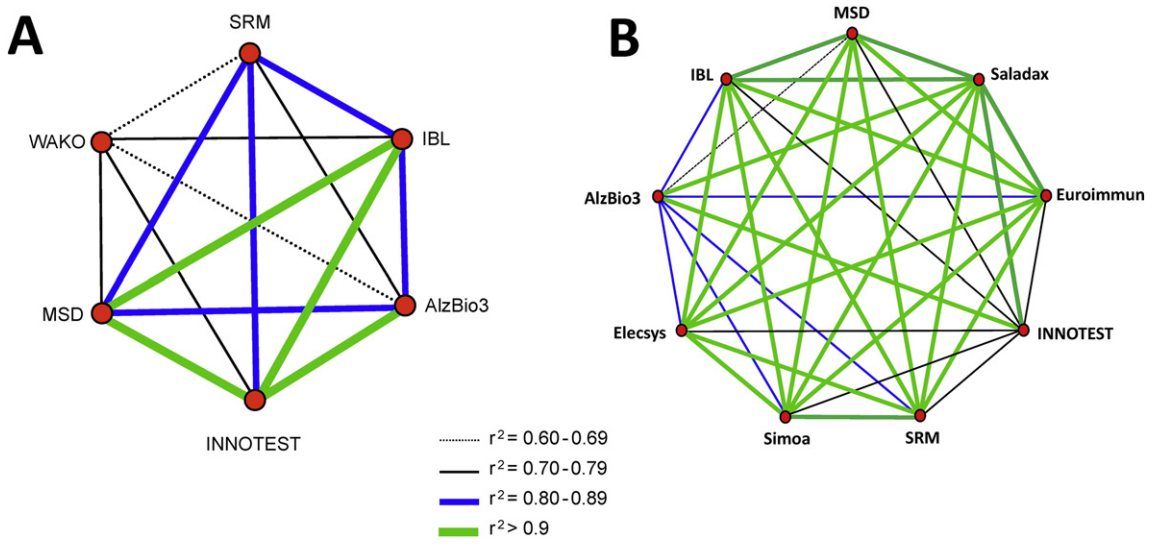

Fig. 4. Ranges of correlations between routine measurement procedures employed in the (A) first and (B) second commutability study (modified from ref. 26).

[27], 5) EUROIMMUN Beta-Amyloid (1–42) (EUROIMMUN, Lübeck, Germany), 6) INNO-BIA AlzBio3 (Fujirebio-Europe, Ghent, Belgium), 7) INNOTEST® β-AMYLOID (1–42) (with ready-to-use calibrators, Fujirebio-Europe), and 8) Simoa Human Aβ42 (Quanterix Corporation, Lexington, MA, USA), see Table 2 for details. To create the candidate CRMs, a total of 24 individual CSF clinical samples (Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden) were combined to prepare a test pool with a final $A\beta_{1-42}$ concentration of about 760 ng/L (value measured with INNOTEST β-AMYLOID (1–42)). Additionally, the neat CSF pool was spiked with the recombinant $A\beta_{1-42}$ calibrator solution prepared by JRC-IRMM to reach $A\beta_{1-42}$ concentrations of 300 ng/L, 800 ng/L, and 1300 ng/L. The results showed that the native CSF pool was again commutable for almost all procedure combinations (Fig. 5B). However, the spiked materials were only commutable for some procedure combinations, with the lower spike concentration being commutable for more procedure combinations than the highest spike concentration. The results of the study demonstrated clearly that only a native CSF pool would be most suitable for the production of a commutable CRM. Furthermore, it indicated that spiking the native CSF pool with recombinant $A\beta_{1-42}$ peptide would not be an option to create calibrators for the calibration of current routine measurement procedures.

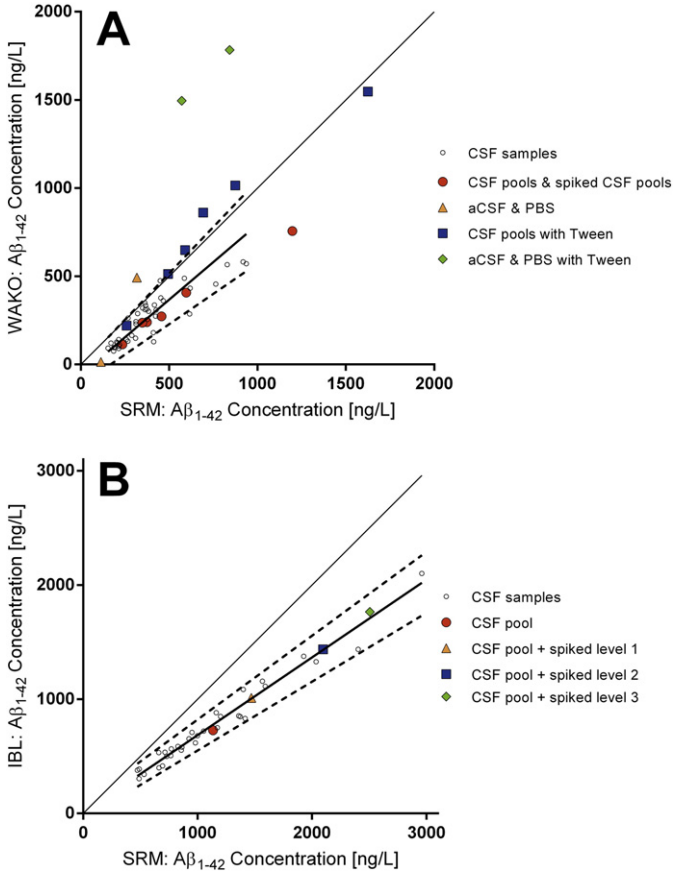

Fig. 5. Examples of linear regressions with 95% confidence interval of data from (A) the first and (B) the second commutability study (modified from ref. 26). Individual CSF samples and different candidate reference materials indicated with colours.

4. Production of candidate CRMs for CSF $A\beta_{1-42}$

Since the commutability study indicated that only a native CSF pool would be suitable as a CRM, the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital in Mölndal, Sweden collected a sufficiently large volume of CSF (de-identified samples according to Swedish Law on Biobanks in Healthcare (2002:297)) from 24 individuals with normal pressure hydrocephalus for the production of a candidate CRM. This production is currently performed by JRC-IRMM according to ISO Guide 34:2009 [14]. Although the initial planning foresaw the production of a single $A\beta_{1-42}$ CRM, the outcome of the second commutability study urged a change in planning. Since spiking of the native CSF pools with recombinant $A\beta_{1-42}$ resulted in non-commutable materials, this approach could not be applied to construct calibration curves for routine measurement procedures. Therefore, three candidate CRMs with different $A\beta_{1-42}$ concentrations will be prepared that could be mixed with each other. The clinical samples initially selected for the production were subsequently divided to create three CSF pools with $A\beta_{1-42}$ concentrations at the low and high end of the clinical range as well as one close to the expected cut-off. In the meantime, JRC-IRMM performed several feasibility studies to evaluate different manual and automated processing steps as well as freezing procedures. For these studies, aCSF spiked with recombinant $A\beta_{1-42}$ and human CSF was used. Samples were analysed by Roche Diagnostics with the Elecsys β-Amyloid (1–42) immunoassay [27]. Several issues concerning the homogeneity of the aliquoted materials with regard to the concentration of $A\beta_{1-42}$ were encountered. Once the appropriate processing and

freezing had been found, the three CSF pools were processed. The vials were frozen at −70 °C and a one-year stability monitoring started. The homogeneity of the candidate CRMs was evaluated by Roche Diagnostics with the Elecsys β-Amyloid (1–42) immunoassay and by ADx NeuroSciences with the EUROIMMUN Beta-Amyloid (1–42) assay. The results showed an uncertainty for between-unit homogeneity (u_{bb}) below 1.5% for all three concentrations. This value is calculated with an ANOVA on results from triplicate measurements on a set of samples, and does not include the contribution from procedure repeatability.

5. Next steps

The next steps in the characterisation of the candidate CRMs include the value assignment. The intended calibration hierarchy has been defined early in the development of the project and was already discussed above. In the case of $A\beta_{1-42}$ value assignment by immunoassays is not possible, since a calibrator that is commutable for all procedures is very difficult to produce. Therefore, the value assignment will be done with the RMPs.

Some challenges remain related to the characterisation of the $A\beta_{1-42}$ calibrator, which was employed in the second LC-MS/MS ring trial mentioned in paragraph 2.2. A combination of purity assessment and AAA was selected to assign a property value to the calibrator. However, the AAA of $A\beta_{1-42}$ is more challenging than expected. While results for individual amino acids showed low CVs, large variability for the results between different amino acids was observed. One explanation could be the presence of peptide contaminations rich in certain amino acids or the presence of free amino acids. However, purity assessment of the calibrator by high-resolution LC-MS/MS did not confirm that suspicion. Another potential explanation could be that the peptide is not fully digested using the conditions selected. Consequently, additional efforts are needed to investigate the source of the variability.

The second LC-MS/MS ring trial using the common calibrator showed that the candidate RMPs are suitable for value assignment of the reference materials. Thus, once the calibrator is fully characterised, value assignment using the RMPs should lead to a certified value with appropriate uncertainty.

Another issue that needs to be addressed is the proper use of the CRMs for calibration of immunoassays. Preferably procedures should be developed for the transfer of property values from the matrix CRMs to (possibly non-commutable) in-house calibrators and kit calibrators in such a manner that results for clinical samples will be equivalent (Fig. 1).

6. Summary

The WG-CSF has accomplished several important milestones essential for the development and release of a reference system for CSF $A\beta_{1-42}$ measurements. Since the initiation of the working group, two RMPs for CSF $A\beta_{1-42}$ based on LC-MS/MS quantification have been developed and submitted to the JCTLM for listing them as ISO 15193:2009 compliant procedures. The correlation of results from different LC-MS/MS procedures used for the quantification of CSF $A\beta_{1-42}$ has been investigated in a ring trial, which showed that results of these procedures have high analytical precision and are highly correlated. A second LC-MS/MS ring trial has almost been completed to scrutinise the implementation of a common $A\beta_{1-42}$ calibrator for the envisioned value transfer to the candidate CRMs. In addition, several potential candidate CRM formats were tested in two commutability studies, which helped determining the most suitable format for the CRM. Since then, the raw material for the preparation of the candidate CRMs has been collected and several feasibility studies have been performed which helped to determine the most appropriate processing conditions for the preparation. The three candidate CRMs have been prepared by JRC-IRMM and the homogeneity assessment demonstrated low between unit heterogeneity.

7. Conclusion

With the important steps accomplished, the certification of three CRMs for CSF $A\beta_{1-42}$ measurements is well underway, which will enable standardisation of this important measurement process.

Conflict of interest

EP, HS, IZ, JK, JP, MB, MK, RJ and UA declare no conflict of interest. ES is an employee of ADx NeuroSciences (Ghent, Belgium). HV is a co-founder of ADx NeuroSciences and a founder of Biomarkable bvba. KB has served at Advisory Boards for IBL International and Roche Diagnostics GmbH. HZ and KB are co-founders of Brain Biomarker Solutions AB (Gothenburg, Sweden), a GU Venture-based platform company at the University of Gothenburg. LMS serves as a consultant to Eli Lilly, Novartis and AbbVie. PL received consultation and/or lecture honoraria from Innogenetics/Fujirebio, AJ Roboscreen, Roche, Virion\Serion GmbH, and IBL International. TB and AL are employees of Roche Diagnostics GmbH (Penzberg, Germany).

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