CSF Aβ1-42 - an excellent but complicated Alzheimer biomarker - the IFCC WG-CSF 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 (AD). Several immunoassays for $A\beta_{1-42}$ are commercially available, but can suffer from between laboratory and batch-to-batch variability and 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 standardization of $A\beta_{1-42}$ measurement results. The efforts of the IFCC WG-CSF include development of certified reference materials (CRMs) and reference measurements procedures (RMPs) for key biomarkers.

Two candidate RMPs for quantification of $A\beta_{1-42}$ in CSF based on liquid chromatography tandem mass spectrometry (LC-MS/MS) have been developed and tested in two ring trials. Furthermore, two commutability studies including native CSF pools, artificial CSF and spiked material 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.

Keywords: Alzheimer's disease; $A\beta_{1-42}$; cerebrospinal fluid; reference measurement procedure; certified reference material

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 total tau (T-tau) and phosphorylated tau (P-tau) protein (1). The decrease of $A\beta_{1-42}$ levels in CSF reflects its deposition into amyloid plaques in the brain (2), that is one of the hallmarks of the disease. The CSF levels of $A\beta_{1-42}$ have been shown to exhibit high diagnostic accuracy for AD dementia (3) and prodromal AD (4-7). Furthermore, CSF $A\beta_{1-42}$ levels show high concordance with amyloid positron emission tomography (PET) scans (8). What makes this biomarker particularly useful for early diagnosis is the fact that it is the first one that shows changes, many years before onset of clinical symptoms. Current clinical routine measurement procedures are based on enzyme-linked immunosorbent assays (ELISA) or immunoassays on other technology platforms. Marked variability in measurement values was observed between analytical procedures and between 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 lack of standardisation. This variability is partly due to differences in laboratory procedures for sample collection, storage and analysis, as well as variability linked to the manufacturing process for the assays resulting in batch-to-batch variations. However the lack of standardisation is the main reason for which different immunoassays give different concentrations when measuring the same sample (10). The availability of a commutable certified reference material (CRM) could dramatically decrease the

variability of measurement results, specifically batch-to-batch variability and the bias between assays.

Since the AD research field 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 standardization of CSF $A\beta_{1-42}$ measurements, this analyte was chosen first as a timely development of RMPs seemed more feasible (11). These developments happened at an opportune moment as the first promising results of clinical trials have been reported (12, 13) and there was an increased need to select individuals at early stages to enrol them in clinical trials and adapt current diagnosis criteria and clinical guidance. The Institute for Reference Materials and Measurements (JRC-IRMM), which is one of the seven European Commission's Joint Research Centres, 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 International Organisation for Standardisation (ISO) Guide 34:2009 (14).

The aim of standardisation is to create measurement results for the same sample produced over time, by different laboratories or with different routine measurement procedures that are equivalent within their corresponding uncertainties (15, 16). This requires the setup of a calibration hierarchy that allows traceability of measurement results to a higher order measurement standard (Figure 1A). The first concern in the standardisation of $A\beta_{1-42}$ is the evaluation of the degree of correlation between

routine measurement procedures. Comparability of measurement results can only be achieved if results from different routine measurement procedures correlate. Next, the upper part of the calibration hierarchy needs to be built up (Figure 1B). This includes the development of a RMP that provides results that correlate with 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 $A\beta_{1-42}$.

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 biomarkers and several LC-MS/MS methods for the quantification of CSF $A\beta_{1-42}$ have been reported in the literature (17-19). This indicated that the development and validation of a LC-MS/MS-based RMP should be feasible. One of the methods (19) has been used in the two commutability studies reported in section 3 of this report. Results from this method showed good correlation with those from routine measurement procedures (Figure 2). The bias (slope ≠ 1) that can be observed is likely due to differences in calibration, which could easily be removed by calibration with a common calibrant. Since then, two RMPs for the quantification of CSF $A\beta_{1-42}$ based on LC-MS/MS have been developed and submitted to the JCTLM on behalf of the WG-CSF. Both methods are based on a procedure published by Lame *et al*. (18)

that uses guanidine hydrochloride treatment followed by a solid phase extraction step as sample preparation and multiple reaction monitoring for quantification. The two RMPs use the same sample preparation, but they differ in instrumentation and more importantly in the matrix used for preparing the calibration solutions. The method developed by Leinenbach *et al.* (20) (RMP1) uses human CSF as calibrator matrix spiked with ^{15}N-labelled Aβ_{1-42} peptide, as a surrogate analyte, whereas the second method described by Korecka *et al.* (21) (RMP2) uses calibrators prepared from artificial CSF (aCSF) containing 4 mg/mL BSA, as a surrogate matrix, spiked with recombinant Aβ_{1-42} peptide (Table 1). Despite the differences, the results of the two RMPs correlate very well (R^2 = 0.98) as shown in Figure 3.

2.1 LC-MS/MS ring trial 1

A ring trial was organized in collaboration with the GBSC to evaluate the correlation between results from different LC-MS/MS methods for the quantification of Aβ_{1-42} and to estimate the inter-laboratory variability for those methods. The following four laboratories that are involved in the GBSC participated: University of Gothenburg, University of Pennsylvania, Waters Corporation (Milford, MA, USA) and PPD (Richmond, VA, USA). Each laboratory received 12 human CSF pools (Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden) and was asked to use their validated in-house method to quantify Aβ_{1-42}. In addition to that, the INNOTEST β-AMYLOID (1-42) assay (Fujirebio-Europe, Inc., Ghent, Belgium) was used to analyse the correlation of the LC-MS/MS methods to a routine immunoassay measurement procedure. The details of this study have been reported elsewhere (22). In short, all laboratories used the same sample preparation scheme, and while all applied selected reaction monitoring (SRM) for LC-MS/MS

quantification, different instrumentation and calibration methods were used. The results of all methods 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 %.

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 used in a ring trial and by how much its use could reduce the inter-laboratory variability. This was an important step, 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) that contained recombinant $A\beta_{1-42}$ peptide (rPeptide, Bogart, GA, USA) in 20 % acetonitrile and 1 % ammonium hydroxide in water. The calibrator was provided to the participating laboratories (University of Gothenburg, University of Pennsylvania, Waters Corporation (Milford, MA, USA), PPD (Richmond, VA, USA) and Roche Diagnostics GmbH (Penzberg, Germany)) along with one aliquot of 20 individual CSF samples (Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden). Each lab was instructed to use the common calibrator with their validated in-house method as well as with 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 methods 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 same way as representative clinical samples. This material property is required for a material to be used for calibration and trueness control to assure accurate clinical results. Laboratory medicine applications like this usually require that a matrix reference material is developed, rather than a pure substance material. Matrix CRMs are often more demanding and expensive to produce, but much more likely to be commutable in clinical routine assays than a pure protein solution. In addition to that, spiking of native sample pools with the analyte is often performed to create the desired concentration level(s). Two commutability studies were conducted to select the most suitable starting material for the production of a commutable CRM for $A\beta_{1-42}$. 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 the development of a reference material for $A\beta_{1-42}$. The following five immunoassay-based routine measurement procedures were used: 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), 4) INNOTEST® β-AMYLOID (1-42) and 5) INNO-BIA AlzBio3 (both Fujirebio-Europe, Inc., Ghent, Belgium). In addition to that, a LC-MS/MS method was applied to compare the results to those obtained with the routine measurement procedures. A total of 48 individual CSF clinical samples (Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden) and 16 candidate CRM formats were tested. The candidate CRM formats included native CSF pools with low and high intrinsic $A\beta_{1-42}$ concentrations as well as aCSF and PBS containing recombinant $A\beta_{1-42}$ (rPeptide, Bogart, GA, USA). 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 selected produced highly correlated results (Figure 4), but also showed that different routine measurement procedures gave results that varied up to a factor 2.6. This is consistent with previously published data (26) and a problem that can be solved with the use of a commutable CRM for calibration. The neat CSF pools behaved like the individual CSF clinical samples for most method combinations, but none of the artificial matrices (aCSF and PBS) tested in the study were commutable for all method combinations tested (Figure 5A). The addition of detergent did not improve the results and even caused the native CSF pools to be non-commutable for some method combinations. The conclusion of this study was that 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 the native CSF pool with recombinant $A\beta_{1-42}$ was an option to create the desired concentration levels of commutable CRM and construct calibration curves. For this study eight routine measurement procedures and one LC-MS/MS method were used to measure 32 individual CSF clinical samples (Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden), which covered the clinical spectrum of $A\beta_{1-42}$ 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) (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). To create the CRM candidates, a total of 24 individual CSF clinical samples (Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden) foreseen for the production of the CRM were combined to produce a test pool with a final $A\beta_{1-42}$ concentration of around 760 ng/L (value determined 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 $A\beta_{1-42}$ spiking 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 method combinations (Figure 5B). However, the spiked levels were only commutable for some method combinations, with the lower spike level being commutable for more method combinations than the highest spike level. The results of the study clearly showed 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 multi-level calibrators for the calibration of current routine measurement procedures.

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

Since the first 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. The production of the CRM is done by the 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 the native CSF pools with recombinant $A\beta_{1-42}$ resulted in non-commutable candidate CRM formats, this approach could not be used to construct multi-level calibration curves to calibrate routine measurement procedures. Therefore, the decision was taken to produce three candidate CRMs with different $A\beta_{1-42}$ concentrations 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, the JRC-IRMM performed several feasibility studies to evaluate different manual and automated processing conditions as well as freezing procedures. For these studies, aCSF spiked with recombinant $A\beta_{1-42}$ and human CSF were used. Samples of the filling were analysed by Roche Diagnostics with the Elecsys β-Amyloid (1-42) immunoassay (27). Several issues concerning the homogeneity of the filled materials with regard to the concentration of $A\beta_{1-42}$ were encountered. Once the appropriate processing and freezing conditions were determined, the three CSF pools were processed. The vials were frozen at -70 ˚C and a required 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 a between unit heterogeneity (u_{bb}) below 1.5 % for all three levels. This value is calculated with an ANOVA on results from triplicate measurements on a set of samples, and does not include the contribution from method repeatability. The next step is the value assignment of the candidate CRMs, which is currently being organized.

5. Next steps

The next steps in the characterisation of the candidate CRMs include the value assignment. The proposed calibration hierarchy has been defined early in the development of the project and was already mentioned in paragraph 1. In principle, a CRM can be value assigned using either immunoassays or the RMPs based on LC-MS/MS (28). In the case of $A\beta_{1-42}$ the use of immunoassays for value assignment is not possible, since a calibrator that is commutable for all methods is very hard to produce. Therefore, the RMPs will be used for the value assignment.

Some challenges remain related to the characterisation of the $A\beta_{1-42}$ calibrator, which was used in the second LC-MS/MS ring trial mentioned in paragraph 2.2. A combination of purity assessment and amino acid analysis (AAA) is used to assign a value and an uncertainty 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 effort is needed to investigate the source of the variability.

The second LC-MS/MS ring trial using the common calibrator, showed that the candidate RMPs can be used 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 uncertainties.

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

6. Conclusion

The WG-CSF has accomplished several important milestones essential for the development and release of a reference system for CSF $A\beta_{1-42}$. 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 methods. The correlation of different LC-MS/MS methods used for the quantification of CSF $A\beta_{1-42}$ has been investigated in a ring trial, which showed that results of these methods have high analytical precision and are highly correlated. A second LC-MS/MS ring trial has almost been completed to scrutinise the use of a common $A\beta_{1-42}$ calibrator for the envisioned value transfer to the candidate CRMs. In addition to that, several potential candidate CRM formats were tested in two commutability studies, which helped determine the most suitable format for the CRM. Since then, the raw material for the production of the candidate CMRs has been collected and several feasibility studies performed which helped to determine the most appropriate processing condition for the production of the final candidate CRMs. The three candidate CRMs have been produced by the JRC-IRMM and the homogeneity assessment showed low between unit heterogeneity. With these steps accomplished, the release of three CRMs for CSF $A\beta_{1-42}$ is well underway, which will enable standardisation of this important measurand.

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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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Tables

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	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 x 250 mm, 50 °C	Waters BEH 300 2.1 x 150 mm, 60 °C
MS	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.

Figures

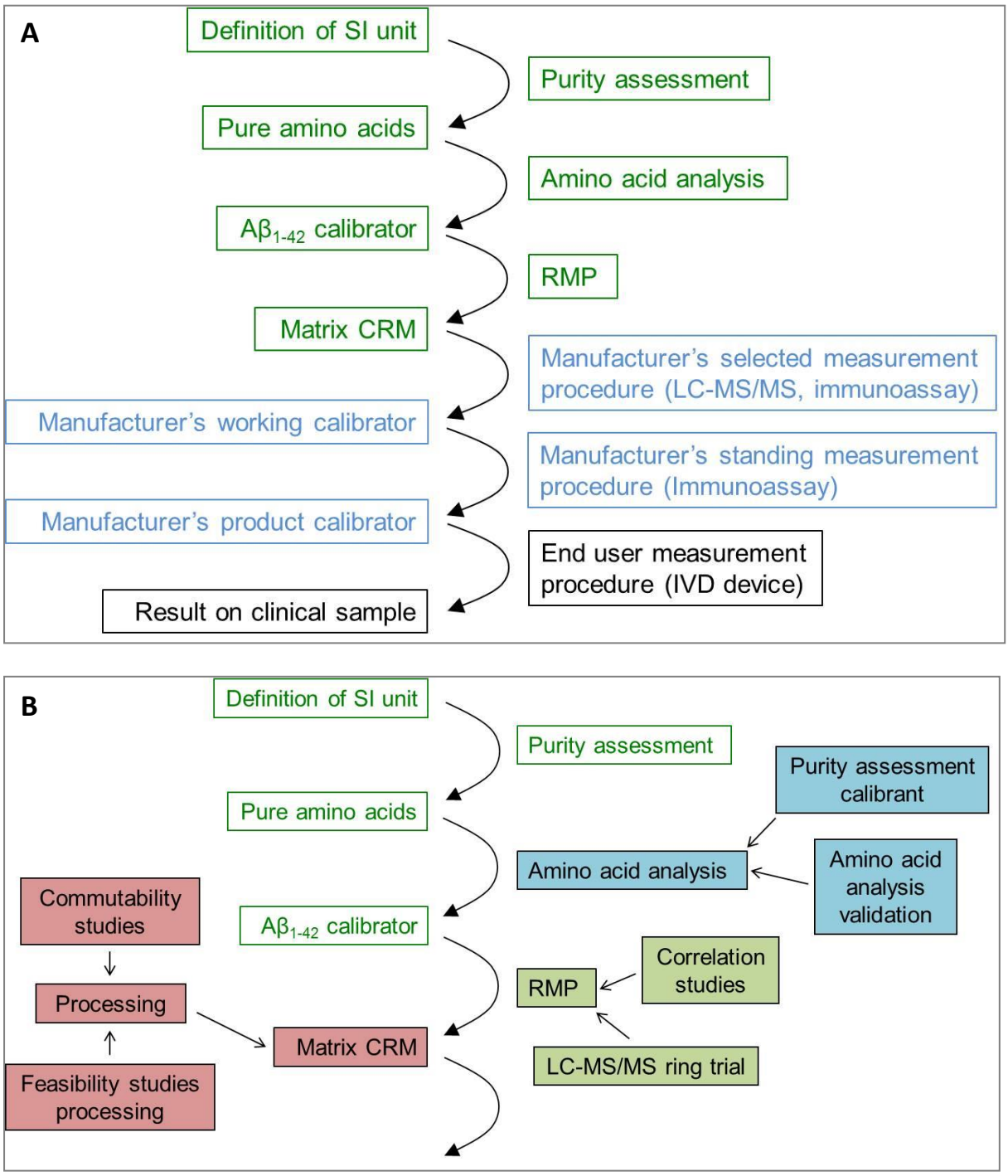

Figure 1. (A) Overview of the traceability chain for $A\beta_{1-42}$, linking results of routine samples to the international system of units (SI) as a common reference. (B) Steps necessary to establish the upper part of the traceability chain. Abbreviations: RMP, reference measurement procedure; CRM, certified reference material; SI,

International System of Units (Système International d'Unités); IVD, in vitro diagnostic.

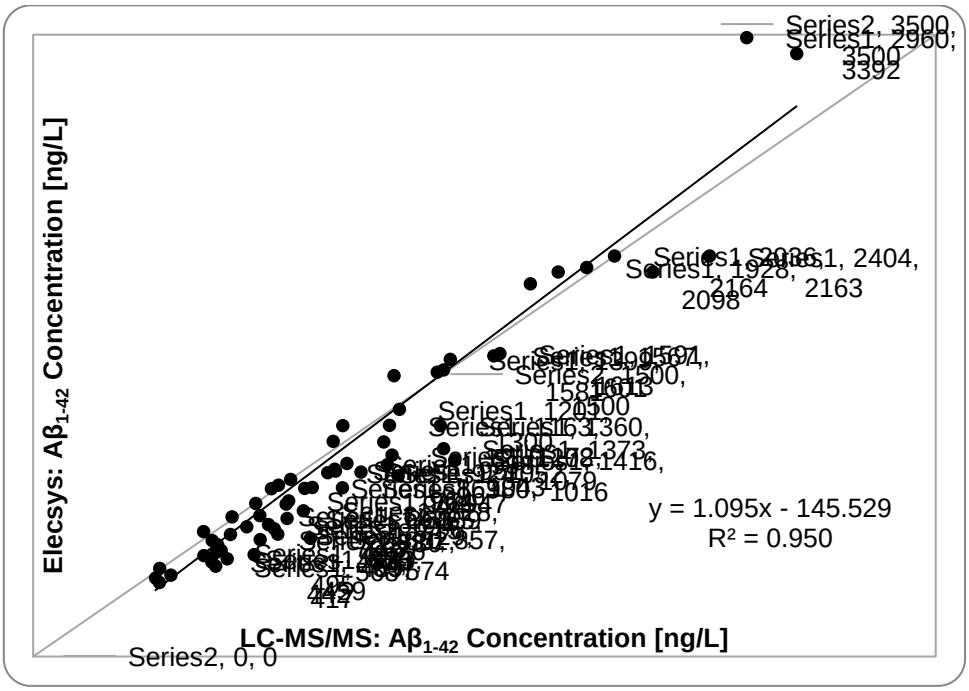

Figure 2. Correlation of the average results of CSF samples measured in duplicate with a LC-MS/MS method and a representative routine measurement procedure.

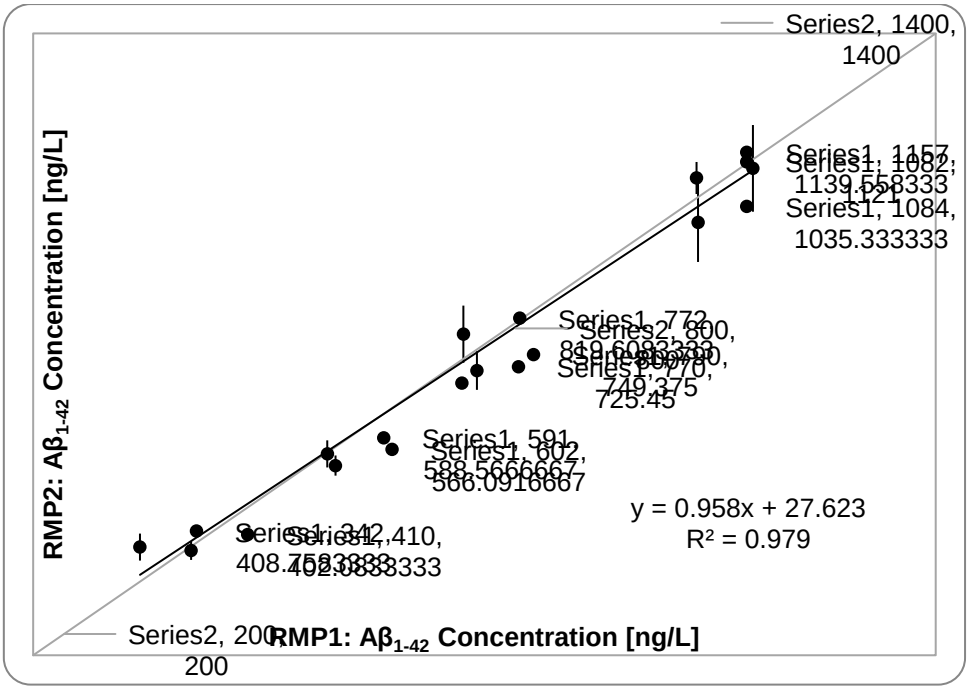

Figure 3. Correlation of the results of 10 CSF pools measured with the 2 reference measurement procedures (RMPs) for $A\beta_{1-42}$ quantification by LC-MS/MS. Over the course of 3 days, 2 aliquots per CSF pool were measured in duplicate. Both methods were calibrated with a common $A\beta_{1-42}$ calibrator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

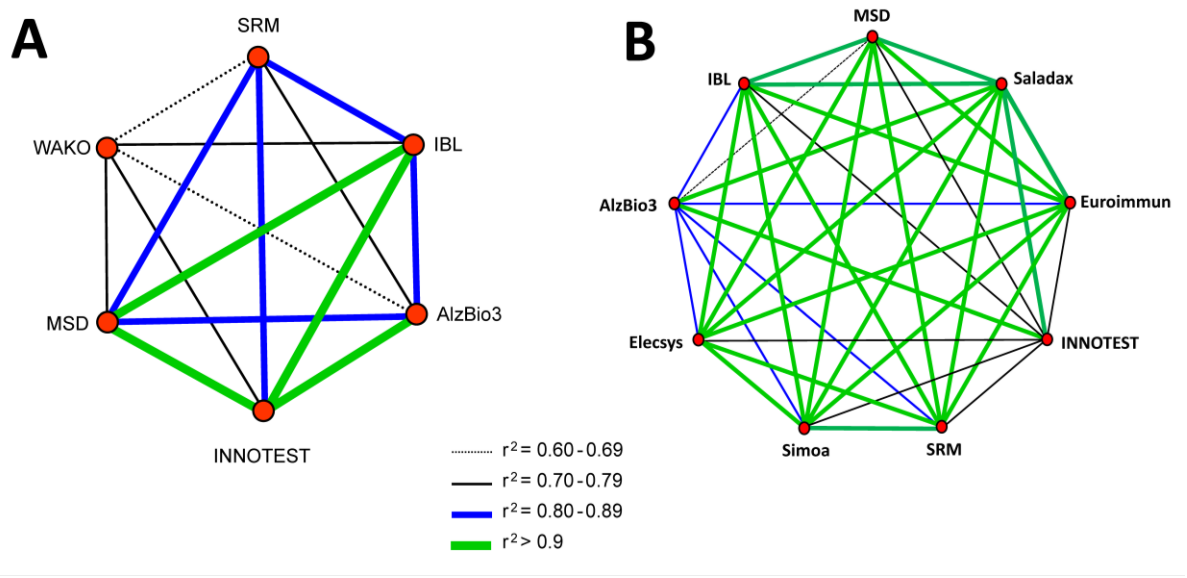

Figure 4. Correlation of routine measurement procedures used in the (A) first and (B) second commutability study.

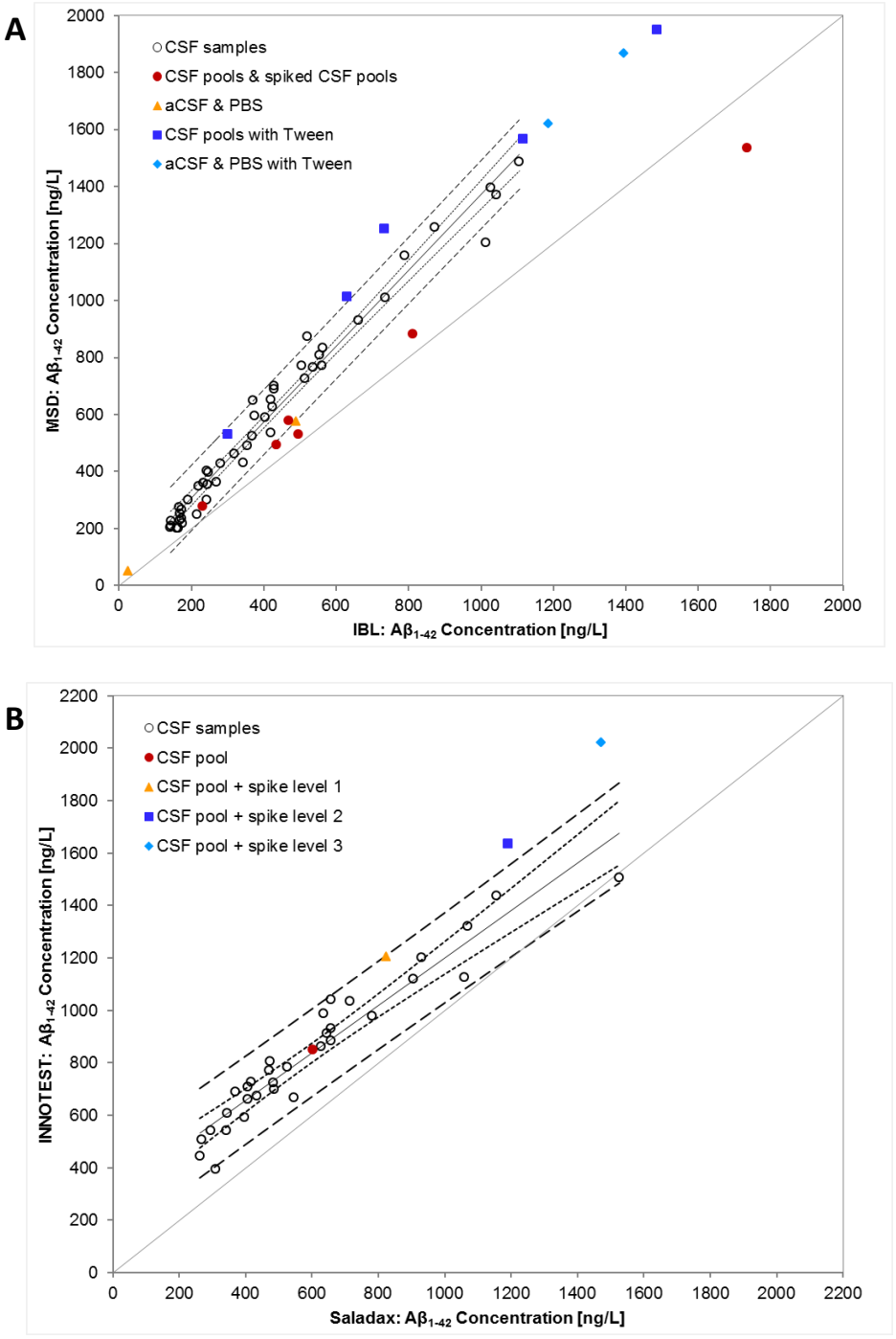

Figure 5. Linear regression with 95 % confidence band and 95 % prediction interval of data from (A) the first and (B) the second commutability study. The results of the first study showed that only native pools are commutable for all method combinations, but not artificial solutions. The second study showed that while CSF pools spiked with recombinant $A\beta_{1-42}$ peptide can be commutable for a few method

combinations, they are not commutable for the majority of method combinations tested.