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Comparison of Different Matrices as Potential Quality Control Samples for Neurochemical Dementia Diagnostics

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Abstract.

Background: Assay-vendor independent quality control (QC) samples for neurochemical dementia diagnostics (NDD) biomarkers are so far commercially unavailable. This requires that NDD laboratories prepare their own QC samples, for example by pooling leftover cerebrospinal fluid (CSF) samples.

Objective: To prepare and test alternative matrices for QC samples that could facilitate intra- and inter-laboratory QC of the NDD biomarkers.

Methods: Three matrices were validated in this study: (A) human pooled CSF, (B) Aβ peptides spiked into human prediluted plasma, and (C) Aβ peptides spiked into solution of bovine serum albumin in phosphate-buffered saline. All matrices were tested also after supplementation with an antibacterial agent (sodium azide). We analyzed short- and long-term stability of the biomarkers with ELISA and chemiluminescence (Fujirebio Europe, MSD, IBL International), and performed an inter-laboratory variability study.

Results: NDD biomarkers turned out to be stable in almost all samples stored at the tested conditions for up to 14 days as well as in samples stored deep-frozen (at –80°C) for up to one year. Sodium azide did not influence biomarker stability. Inter-center variability of the samples sent at room temperature (pooled CSF, freeze-dried CSF, and four artificial matrices) was comparable to the results obtained on deep-frozen samples in other large-scale projects.

Conclusion: Our results suggest that it is possible to replace self-made, CSF-based QC samples with large-scale volumes of QC materials prepared with artificial peptides and matrices. This would greatly facilitate intra- and inter-laboratory QC schedules for NDD measurements.

Keywords: Alzheimer's disease, amyloid-β, biomarkers, cerebrospinal fluid, laboratory diagnostics, quality control, tau

INTRODUCTION

Neurochemical dementia diagnostics (NDD), along with the amyloid-β (Aβ) PET imaging, has become the most important modality for the early diagnosis of Alzheimer's disease (AD) [1, 2]. Cerebrospinal fluid (CSF) biomarkers, particularly Aβ peptides, show alterations early in the preclinical stage of the disorder, probably decades before the onset of the first clinical symptoms [3, 4]. Therefore it is not surprising that an increasing number of laboratories is establishing NDD as their routine tool either for an early AD diagnosis or to increase the probability to enroll patients with underlying AD pathology in clinical trials [5, 6]. On the other hand, partially due to the physicochemical properties of Aβ peptides and tau proteins, quality control (QC) of the NDD analyses is very difficult; large-scale international studies performed in the last years [7, 8] have shown that especially the inter-laboratory precision of the NDD measurements requires further optimization. Insufficient precision and reproducibility of the NDD measurements are considered most important factors preventing CSF biomarkers from general acceptance as a routine AD diagnostic tool [9] and certainly consensus protocols on collection and storage of the samples for NDD biomarkers are crucial. Nevertheless, we believe that standardized and assay vendor-independent QC samples, which could be universally used, would very logically complement reference methods and standards currently tested [10] and hopefully would improve our knowledge on possible sources of the intra- and inter-center imprecision of measurements.

So far, an assay-independent QC sample for any of the NDD biomarkers has not been commercially available, which forces laboratories to develop their own procedures to prepare QC materials, most commonly based on pooling of available, otherwise discarded, leftover CSF samples. Such an approach makes intra-laboratory QC difficult and inter-laboratory QC even impossible, for at least three reasons: (a) the concentrations of the biomarkers in samples prepared by different centers will be different; (b) the quality of leftover CSF samples used for the preparation of the QC material is not always optimal, and (c) operating procedures to prepare the QC material differ among centers. Moreover, longitudinal QC, taken together with the growing frequency of the analyses, forces preparation of the QC material in very large amounts, which is difficult in laboratories with limited access to large numbers and volumes of CSF samples. Equally important is that it is easier to obtain different biomarkers concentrations (for example "normal" and "pathologic" levels) in synthetic matrices than in pooled CSF leftovers. In our previous study [11], we addressed the feasibility of generating the NDD QC material based on large-scale CSF pools. In the current study, we tested also other, partially artificial, matrices and sample preparation procedures with the rationale to test if application of such matrices for QC of AD biomarkers is possible and plausible.

MATERIALS AND METHODS

Preparation of the samples; conditions for the short- and long-term stability testing; influence of the antibacterial factor (sodium azide); homogeneity testing

The Ethical Committee of the University of Erlangen-Nuremberg approved use of human CSF samples for this study. Block-flows of the preparation of the samples for short-term stability (STS) are presented in Supplementary Figure 1 and Supplementary Table 1. Short- and long-term stability was evaluated in the following matrices:

(A) Human pooled CSF without (A0) and with (*A*+) addition of sodium azide (NaN_3, final concentration 0.1%). Briefly, freshly collected human CSF samples were immediately pooled (to assure anonymity and non-traceability), centrifuged (1,600 g, 10 min.), frozen and stored at –80°C. For this project, the samples were thawed and pooled again to obtain appropriate volumes of homogenous samples; aliquots were then prepared and stored at the conditions described below.

(B) Synthetic Aβ peptides ($A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$; AnaSpec Inc., Fremont, USA) spiked into human EDTA-plasma prediluted 1:200 with 0.97% phosphate-buffered saline (PBS, pH 7.4; Amresco, Solon, USA) and Tween 20 (Roth, Karlsruhe, Germany; final concentration 0.05%), without (B0) and with (*B*+) addition of NaN_3 (final concentration 0.1%). Briefly, 5 mL of human plasma was diluted with 995 mL of PBS + 500 µL of Tween 20, and divided into two equal volumes. To one portion (500 mL), 5 mL of 10% NaN_3 stock was added. Synthetic Aβ peptides (0.5 mg) were reconstituted in 1 mL of dimethyl

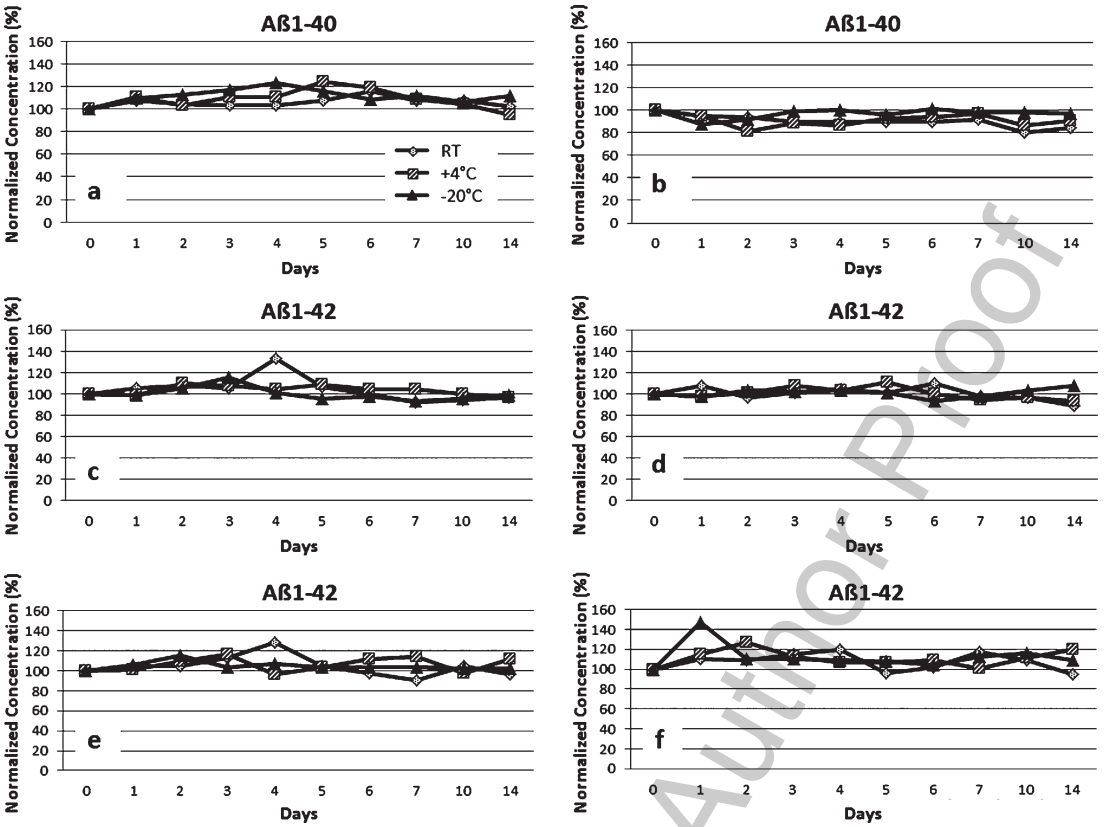

Fig. 1. Results of the short-term stability testing of the samples based on prediluted plasma (Material B), when single peptides were spiked, presented as normalized concentration (in percent) of the reference samples; horizontal axes show storage time (days) at the corresponding storage conditions: RT, room temperature; +4°C, refrigerator; –20°C, frozen at –20°C. a) Non-stabilized $A\beta_{1\text{-}40}$; b) Stabilized $A\beta_{1\text{-}40}$; c) Non-stabilized $A\beta_{1\text{-}42}$ (Innotest); d) Stabilized $A\beta_{1\text{-}42}$ (Innotest); e) Non-stabilized $A\beta_{1\text{-}42}$ (MSD); f) Stabilized $A\beta_{1\text{-}42}$ (MSD).

Table 1
Normalized concentrations of the biomarkers tested for short-term stability in human pooled CSF samples (A0 and A+)

Biomarker	Conditions					
	RT	RT, NaN_3	+4°C	+4°C, NaN_3	–20°C	–20°C, NaN_3
$A\beta_{1\text{-}40}$	99.7 (5.4; NS)	94.2 (4.3; 0.8%/d)	101.9 (4.3; NS)	97.7 (2.5; NS)	104.7 (4.1; NS)	103.4 (2.8; NS)
$A\beta_{1\text{-}42}$ (Innotest)	91.5 (12.5; NS)	100.4 (7.0; 1.5%/d)	118.7 (2.5; NS)	108.4 (2.8; 0.5%/d)	125.3 (5.2; 1.3%/d)	110.7 (3.9; NS)
$A\beta_{1\text{-}42}$ (MSD)	78.2 (27.2; 4.5%/d)	88.7 (7.5; 1.5%/d)	102.0 (10.8; NS)	94.8 (4.1; NS)	111.6 (6.7; NS)	101.7 (8.2; NS)
Tau (Innotest)	99.8 (8.9; NS)	101.2 (5.8; NS)	113.7 (6.4; NS)	116.8 (4.3; NS)	123.5 (7.0; NS)	111.5 (8.7; 2%/d)
Tau (MSD)	83.8 (13.5; 2%/d)	76.5 (11.3; 1.8%/d)	92.6 (8.0; NS)	83.6 (5.9; NS)	91.1 (9.0; NS)	75.3 (6.7; NS)
pTau181 (Innotest)	99.6 (7.6; NS)	104.0 (5.9; NS)	105.6 (4.3; NS)	107.5 (5.4; NS)	98.9 (6.1; NS)	102.8 (5.7; NS)

In brackets, coefficients of variation and average daily decrease (%/d, percent per day) of the concentrations are presented. The averages and the CVs were calculated from the results of the days 1–7, 10, and 14 (i. e. omitting the results of the reference samples of the "Day 0", defined as 100%). NaN_3, a sample containing NaN_3 as an antibacterial stabilizer; NS, non significant.

sulfoxide (DMSO; Sigma, St. Louis, USA) followed by further dilution of 1:1000 (in two steps) in DMSO to the stock-concentration of 500 ng/mL. These stock solutions were then aliquoted into portions of 400 µL in polypropylene Eppendorf test tubes, and frozen at –80°C. Immediately before the preparation of the aliquots, 250 µL of a peptide/DMSO solution was added to 9.750 mL of prediluted plasma (with and without NaN_3).

Furthermore, material B was also tested as a mixture of $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ in one sample. Briefly, artificial peptides were added to the prediluted EDTA-plasma to achieve the same final concentrations as in the separate $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ samples.

(C) Synthetic Aβ peptides spiked into 0.04% bovine serum albumin (BSA; Roth, Karlsruhe, Germany) prediluted in PBS/Tween 20, without (C0) and with (C+) addition of NaN_3 (final concentration 0.1%). Briefly, the material C was prepared analogously to the material B with the exception that instead of prediluted plasma, 0.04% BSA/PBS+Tween was used as a diluent.

All six types of the samples (three matrices, each with- and without NaN_3) were aliquoted immediately after the preparation and stored: (a) at room temperature (RT), (b) in a refrigerator (+4°C), or (c) frozen at –20°C. One aliquot from each of these six sets was deep frozen (–80°C) immediately after the preparation, and served as a reference sample. The remaining aliquots were kept for: 1–7, 10, and 14 days following transfer into –80°C. After the completion of the storage time (i.e., 14 days), all aliquots of a given sample type were thawed and immediately tested in one analytical run (one ELISA or electrochemiluminescence plate) per biomarker. All measurements were performed in duplicates.

The influence of NaN_3 (as an antibacterial agent) on the concentrations of the biomarkers was tested by comparing their concentrations following storage for five days at room temperature with and without NaN_3.

To test for homogeneity, 31 aliquots of Material B were prepared, with both $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ spiked into the same stock sample. One aliquot was immediately transferred into –80°C and served as a reference sample. Remaining 30 aliquots were divided into three groups of 10 samples and stored for five days at: (a) room temperature (b) +4°C or (c) –20°C, following transfer into a deep freezer. All aliquots, including the reference sample, were then assayed in duplicates on one ELISA plate per biomarker, whereas different aliquots were treated as they were separate samples.

To test for homogeneity after freeze-dry procedure (see below), three CSF pooled samples, divided into ten aliquots each, were tested for $A\beta_{1\text{-}42}$ and pTau181 concentrations.

To test long-term stability (LTS), samples were prepared analogously to those for the STS experiment, deep-frozen, and stored at –80°C for 6, 9, and 12 months pending analyses.

Assays

STS and LTS tests were performed with the following assays: $A\beta_{1\text{-}40}$ (IBL International GmbH, Hamburg, Germany), $A\beta_{1\text{-}42}$ (IBL International GmbH, Hamburg, Germany, Fujirebio Europe, formerly Innogenetics, Ghent, Belgium) and Meso Scale Discovery, Rockville, USA), Tau (Fujirebio Europe, and Meso Scale Discovery), pTau181 (Fujirebio Europe), all according to the instruction of the manufacturers.

Preparation of the freeze-dried samples

Aliquots containing 1 mL of the CSF pool were filled into 2-mL SCHOTT TopLyo® glass vials (SCHOTT AG, Germany), and partially stoppered with 13 mm lyophilization stoppers. Freeze-drying of CSF was performed using a commercial Martin Christ Epsilon 2–4 LSC freeze-dryer (CHRIST, Germany). Samples were placed on a heating shelf of the freeze-dryer at a temperature of +4°C and slowly pre-frozen (1 degree/min) down to –80°C with additional frozen hold step for 3 h. Primary and secondary drying was achieved at a vacuum of 30 mTorr and 15 mTorr, respectively. The temperature of the samples was monitored during the freeze-drying process using sample vials with thermoprobes. Cycle time was 26 h. In the end of the cycle, freeze-dried samples were closed, removed from the freeze-dryer, sealed to inhibit the humidification, and kept in the dark at –80 °C until shipment. Shipment has been done at ambient temperatures. Freeze-dried samples were rehydrated with deionized water.

Inter-laboratory variability testing (external quality control, EQC)

For the inter-laboratory variability testing, the participating 25 laboratories obtained the following samples, all of them shipped by regular mail at ambient conditions:

EQC-1: Human pooled CSF;

EQC-2: Sample EQC-1 freeze-dried and sent to the participating laboratories as powder to be reconstituted in 1 mL of distilled water;

EQC-3: $A\beta_{1\text{-}42} + A\beta_{1\text{-}40}$ dissolved in prediluted EDTA-plasma. Briefly, 50 µL of a 500 ng/mL $A\beta_{1\text{-}42}$ stock and 320 µL of a 500 ng/mL stock $A\beta_{1\text{-}40}$ was diluted in 17 mL of human plasma prediluted 1:200 in PBS/Tween.

EQC-4: $A\beta_{1-42} + A\beta_{1-40}$ dissolved in prediluted EDTA-plasma, analogously to the material EQC-3, whereas the volumes of the spiked stocks were 35 µL of $A\beta_{1-42}$ and 160 µL of $A\beta_{1-40}$.

EQC-5: $A\beta_{1-42} + A\beta_{1-40}$ diluted in BSA/PBS +Tween. Briefly, 50 µL of a 500 ng/mL $A\beta_{1-42}$ stock and 320 µL of a 500 ng/mL $A\beta_{1-40}$ stock was diluted in 17 mL of 0.04% BSA/PBS+Tween.

EQC-6: $A\beta_{1-42} + A\beta_{1-40}$ diluted in 0.04% BSA in PBS+Tween, analogously to the material EQC-5, whereas the volumes of the spiked stocks were 35 µL of $A\beta_{1-42}$ and 160 µL of $A\beta_{1-40}$.

The summary of the samples for the EQC is presented in Supplementary Table 2.

Inter-assay imprecision; maximal acceptable instability and variation

For inter-assay imprecision, a set of aliquots of human pooled CSF was prepared and promptly refrozen at –80°C. One aliquot was freshly thawed immediately before the analyses, which were performed by different operators and on different days in the time span of fourteen months. Maximal acceptable instability and variation were defined as ± 20% deviation from the concentration measured in the reference sample (for the STS and LTS studies) or ± 25% from the average of the measurements in the inter-assay imprecision testing.

Statistical analyses

If not stated otherwise, results are presented as averages and standard deviations (or coefficients of variation, CV); results of duplicate measurements are expressed as averages and the absolute difference between the single measurements divided by their average; for determination of total variations and uncertainty, the absolute differences of duplicates were recalculated into standard deviations or relative standard deviations (CVs). Partial uncertainty and partial variation of the measurements are expressed in this study as standard deviations or relative standard deviations (CVs); total variation/uncertainty is calculated as squared root of the sum of the squared contributing variabilities. Stabilities of the biomarkers concentrations over time were analyzed by linear regression with Statistica 12 (StatSoft, Tulsa, USA); a $p < 0.05$ was considered significant.

RESULTS

Short-term stability

If not stated otherwise, the results of the stability studies are presented as normalized concentrations. The normalization was performed by division of the concentration measured in a given sample by the concentration measured in the reference sample, i.e., the sample placed into –80°C freezer immediately after its preparation.

The results of the STS testing of the human pooled CSF samples (A0 and A+) are presented in Supplementary Figure 2, with the concomitant normalized concentrations, CVs and average concentration decrease per day shown in Table 1. $A\beta_{1-40}$ was very stable in the CSF samples stored at the three tested conditions up to 14 days, whereas $A\beta_{1-42}$, expectedly, was apparently less stable. In a sample supplemented with NaN_3 and stored under room temperature, a marginal but significant decrease of $A\beta_{1-40}$ concentration was observed (ca. 0.8%/day). Similar minimal, yet significant, daily decrease of $A\beta_{1-42}$ concentration (ca. 0.5–1.5%/day) was also noticed in this matrix stored under all three conditions. Exception was $A\beta_{1-42}$ measured with MSD method, which showed average concentration decrease of 4.5%/d in a sample stored at room temperature. Interestingly, addition of NaN_3 resulted in an improved stability of $A\beta_{1-42}$ in the CSF samples (compare Supplementary Figure 2c and 2d for $A\beta_{1-42}$ tested with Innotest assays, and Supplementary Figure 2e and 2f for MSD assays). Tau tested with Innotest assays (Supplementary Figure 2g, 2h) showed relatively low discrepancies of concentrations over the whole tested period, with significant daily average decrease only in a sample stored at –20°C. When tested with MSD assays (Supplementary Figure 2i, 2j), its concentration dropped after 2–3 days of storage (ca. 2%/day on average during overall storage time). Phosphorylated tau, expectedly, showed stable concentrations for up to 14 days of storage without significant daily concentration decreases (Supplementary Figure 2k, 2l).

The results of the STS testing of the prediluted plasma-based samples (B0 and B+) are presented in Fig. 1 and Supplementary Figure 3, and the resulting normalized concentrations, their CVs and average concentration decrease per day are presented in Table 2 and Supplementary Table 3. Taken together, both biomarkers turned out stable when stored under all three conditions, with only single cases of the

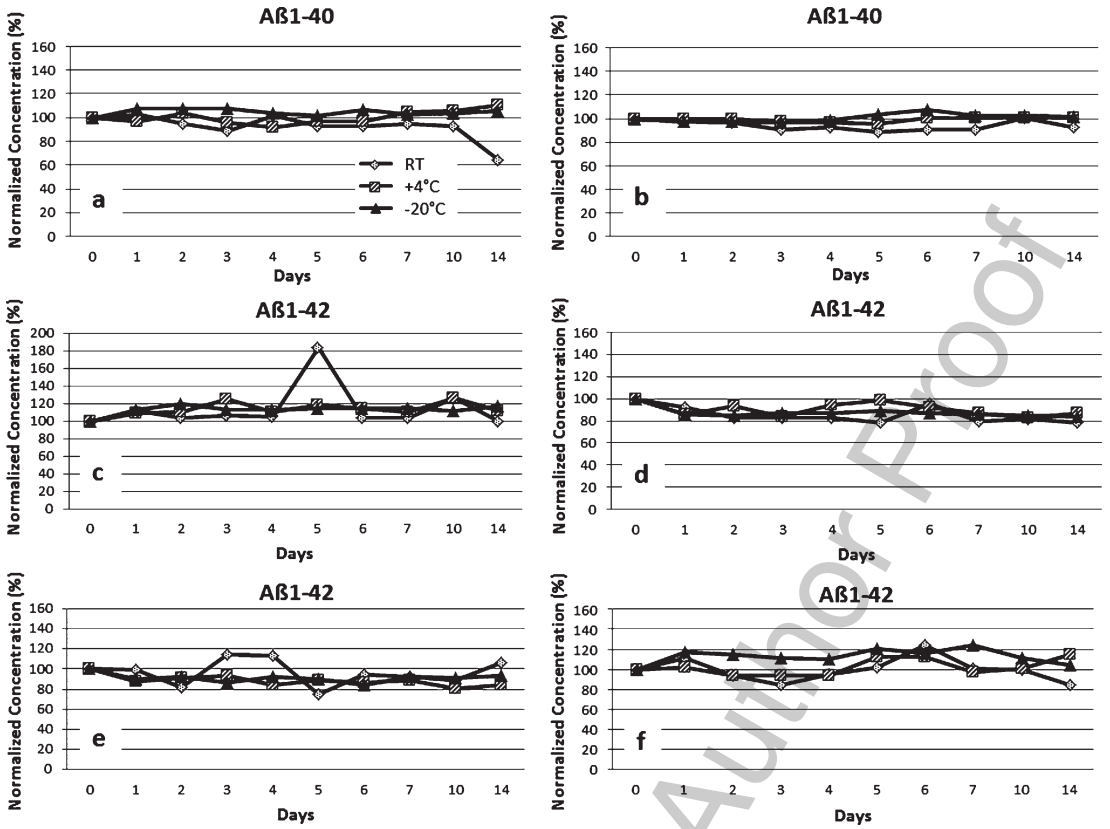

Fig. 2. Results of the short-term stability testing of the biomarkers diluted in BSA/PBS+Tween (Material C), presented as normalized concentration (in percent) of the reference samples; horizontal axes show storage time (days) at the corresponding storage conditions: RT, room temperature; +4°C, refrigerator; –20°C, frozen at –20°C. a) Non-stabilized $A\beta_{1-40}$; b) Stabilized $A\beta_{1-40}$; c) Non-stabilized $A\beta_{1-42}$ (Innotest); d) Stabilized $A\beta_{1-42}$ (Innotest); e) Non-stabilized $A\beta 1_{-42}$ (MSD); f) Stabilized $A\beta_{1-42}$ (MSD).

concentrations exceeding 80%–120% range of the starting concentrations, irrespectively whether the two peptides were tested separately (Fig. 1) or as a combination (Supplementary Figure 3). Expectedly, the samples stored at –20°C were even more stable than those stored at +4°C or room temperature. Correspondingly, only marginal concentration decrease trends were observed in this matrix, not exceeding 1.3%/day.

The results of the STS testing of the peptides diluted in BSA/PBS+Tween (C0 and C+) are presented in Fig. 2, and the corresponding normalized concentrations, their CVs and average concentration decrease per day are shown in Table 3. Also in this matrix, $A\beta_{1-40}$ turned out very stable at all three tested conditions, with an apparent drop of concentration in a sample stored for longer than 10 days at room temperature without NaN_3 (Fig. 2a). Interestingly, also $A\beta_{1-42}$ was relatively stable in this matrix, however, with unexpected and inexplicable rise in its concentration in a RT sample at day 5 when tested with Innotest (Fig. 2c) but not with MSD (Fig. 2e) assay.

Influence of the antibacterial factor

The differences in the concentrations of the biomarkers possibly resulting from addition of sodium azide are presented in Supplementary Table 4. Application of 0.1% NaN_3 neither changed starting concentrations of the two peptides nor influenced their concentrations after 5 days at room temperature.

Homogeneity

Results of the homogeneity testing of the sample based on pre-diluted plasma (Material B) are presented in Supplementary Table 5, together with the analysis of the intra-assay variation and the total variation resulting from the two sources. Inhomogeneity of the samples was below 3% for $A\beta_{1-42}$ and below 4% for $A\beta_{1-40}$.

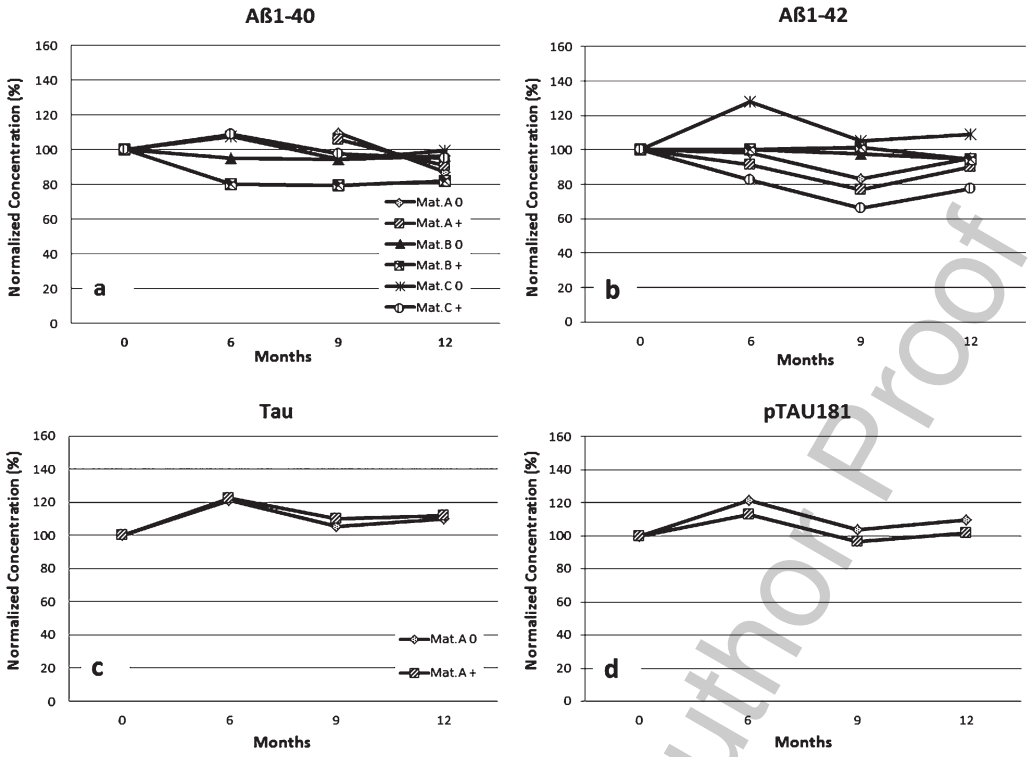

Fig. 3. The results of the long-term stability testing. a) $A\beta_{1\text{-}40}$; b) $A\beta_{1\text{-}42}$; c) Tau; d) pTau181.

Table 2
Normalized concentrations of the biomarkers tested for short-term stability in the prediluted-plasma based samples (B0 and B+), when one peptide isomer (either Aβ1-42 or Aβ1-40) was spiked into the samples

Biomarker	Conditions					
	RT	RT, NaN_3	+4°C	+4°C, NaN_3	–20°C	–20°C, NaN_3
$A\beta_{1\text{-}40}$	106.3 (3.9; NS)	89.4 (5.0; 1%/d)	109.3 (7.9; NS)	90.3 (5.7; NS)	112.5 (4.4; NS)	96.8 (4.5; NS)
$A\beta_{1\text{-}42}$ (Innotest)	104.6 (11.7; NS)	100.3 (6.5; 1%/d)	104.0 (4.5; NS)	100.4 (5.9; NS)	100.4 (6.6; NS)	100.8 (4.2; NS)
$A\beta_{1\text{-}42}$ (MSD)	105.1 (10.3; NS)	108.1 (8.2; NS)	107.2 (6.9; NS)	111.9 (7.0; NS)	105.3 (3.7; NS)	113.9 (11.1; NS)

In brackets, coefficients of variation and average daily decrease (%/d, percent per day) of the concentrations are presented. The averages and the CV's were calculated from the results of the days 1–7, 10, and 14 (i. e. omitting the results of the reference samples of the "Day 0", defined as 100%). NaN_3, a sample containing NaN_3 as an antibacterial stabilizer; NS, non significant.

Table 3
Normalized concentrations of the biomarkers diluted in BSA/PBS+Tween, tested for short-term stability (C0 and C+)

Biomarker	Conditions					
	RT	RT, NaN_3	+4°C	+4°C, NaN_3	–20°C	–20°C, NaN_3
$A\beta_{1\text{-}40}$	91.9 (12.4; 2%/d)	93.3 (4.2; NS)	100.2 (5.9; NS)	98.8 (2.1; NS)	105.1 (2.0; NS)	100.9 (3.4; NS)
$A\beta_{1\text{-}42}$ (Innotest)	115.7 (22.9; NS)	83.7 (7.5; NS)	115.0 (5.6; NS)	89.6 (5.9; NS)	114.3 (2.1; NS)	86.2 (1.8; NS)
$A\beta_{1\text{-}42}$ (MSD)	96.2 (14.1; NS)	99.4 (12.8; NS)	87.2 (4.5; NS)	102.1 (8.7; NS)	90.3 (3.5; NS)	114.3 (5.2; NS)

In brackets, coefficients of variation and average daily decrease (%/d, percent per day) of the concentrations are presented. The averages and the CVs were calculated from the results of the days 1–7, 10, and 14 (i.e., omitting the results of the reference samples of the "Day 0", defined as 100%). NaN_3, a sample containing NaN_3 as an antibacterial stabilizer; NS, non significant.

Average inhomogeneity of $A\beta_{1\text{-}42}$ and pTau181 in three samples after freeze-dry procedure was 3.4% and 3.8%, respectively.

Long-term stability

The results of the LTS experiments are presented in Fig. 3. The concentrations of $A\beta_{1\text{-}40}$ in the samples stored deeply frozen for one year were apparently stable in all samples, with an exception of the sample based on prediluted plasma not supplemented with NaN_3 (Fig. 3a, Mat. B0). Interestingly, $A\beta_{1\text{-}42}$ was very stable in prediluted plasma-based samples (Fig. 3b, Mat. B0 and B+) as well as, to a lesser degree, CSF samples (Fig. 3b, Mat. A0 and A+), but

Table 4
Average concentrations (in pg/mL) and the coefficients of variation in the inter-laboratory variability study

Biomarker (number of the centers)	Samples					
	EQC-1	EQC-2	EQC-3	EQC-4	EQC-5	EQC-6
$A\beta_{1-40}$ (8)	8,591.5 (20.5%)	6,229.1 (45.4%)	8,748.5 (19.0%)	4,460.9 (22.8%)	8,276.2 (19.0%)	4,150.3 (22.0%)
$A\beta_{1-42}$ (23)	783.9 (25.1%)	643.4 (29.1%)	667.4 (27.8%)	389.1 (23.1%)	581.5 (25.3%)	397.3 (21.4%)
Tau (24)	337.3 (16.5%)	530.3 (16.2%)	ND	ND	ND	ND
pTau181 (25)	55.9 (9.8%)	37.6 (12.9%)	ND	ND	ND	ND

Aβ1-42 and Aβ1-40 were measured with assays from Innogenetics, IBL International or MSD; Tau was measured with assays from Innogenetics or MSD; pTau181 was measured with assays from Innogenetics.

much less stable (in terms of rising and dropping concentrations) in a BSA/PBS samples (Fig. 3b, Mat. C0 and C+). Tau (Fig. 3c) and pTau181 (Fig. 3d) showed stable concentrations in the CSF samples stored for up to one year.

Inter-assay variability

The results of the inter-assay variability of the biomarkers tested in a pooled human CSF sample are presented in Supplementary Figure 4. Inter-assay imprecision (the number of the repetitions are in brackets) of the biomarkers was: 11.7% (25), 10.4% (30), 9.4% (17), and 6.8% (20) for $A\beta_{1-42}$, $A\beta_{1-40}$, tau, and pTau181, respectively.

Inter-laboratory variability

The results of the biomarker measurements reported by the participants of the inter-center study are presented in Fig. 4 and Table 4. The concentrations in the pooled CSF sample (EQC-1 on Fig. 4a, c, e, and f) showed moderate variation, expectedly slightly higher in case of $A\beta_{1-42}$ (∼25%) than in case of other biomarkers, with the lowest variation, as expected, in case of pTau181 (<10%). Comparable variation was obtained in case of freeze-dried CSF (EQC-2 on Fig. 4a, c, e, and f), with the exception that one participant reported unexpectedly low $A\beta_{1-40}$ concentration in EQC-2 (particularly when compared to the EQC-1 result), which increased overall variation of $A\beta_{1-40}$ in the freeze-dried material to 45%. Interestingly to note is that the concentrations of $A\beta_{1-40}$, $A\beta_{1-42}$, and pTau181 reported in the freeze-dried material (EQC-2) paralleled very well, with two or three exceptions, the concentrations in the native CSF (EQC-1) but were consistently 20–30% lower. In the case of tau, however, the reverse was true: The concentrations in freeze-dried material were consistently 50–60% higher than in the native CSF.

$A\beta_{1-40}$ in the artificial samples showed variation of about 20% (Fig. 4b). $A\beta_{1-40}$ concentrations reported by the participants were almost identical irrespectively of the material tested (compare EQC-3 versus EQC-5 and EQC-4 versus EQC-6 on Fig. 4b); furthermore, the concentrations in EQC-4 and EQC-6 were, as expected (considering the amount of the spiked synthetic peptides), almost ideally halves of the concentrations in EQC-3 and EQC-5, respectively.

Similar results were obtained for $A\beta_{1-42}$ (Fig. 4d); with overall inter-center variation around 25%, the concentrations in EQC-3 and ECQ-5 were very well comparable to each other (with the exception of the participants #2 and 5), and the same was observed regarding the samples EQC-4 and EQC-6. Also in case of $A\beta_{1-42}$, the concentrations in EQC-4 and EQC-6 turned out, as expected (considering the amount of the spiked peptides), almost ideally 70% of the concentrations in EQC-3 and EQC-5, respectively.

DISCUSSION

We present in this study the results of the validation of three matrices (one based on human CSF, and two artificial) as potential QC samples for NDD biomarkers [12].

At the beginning of this study (Summer 2012), no commercial material was available for any kind of control of the quality of AD biomarkers assays; meanwhile the situation has improved to some extent, as the majority of assay vendors include some form of control samples in their kits for the validation of a particular analytical run; however, such samples are specific-assay-tailored, and cannot be reliably used to control the quality of other manufacturers' assays. These run-validation samples do not necessarily have the same performance as biological material, and as such they are not representative to document the most important variables in the assays. It is also worth stressing that in case of the NDD biomarkers, which

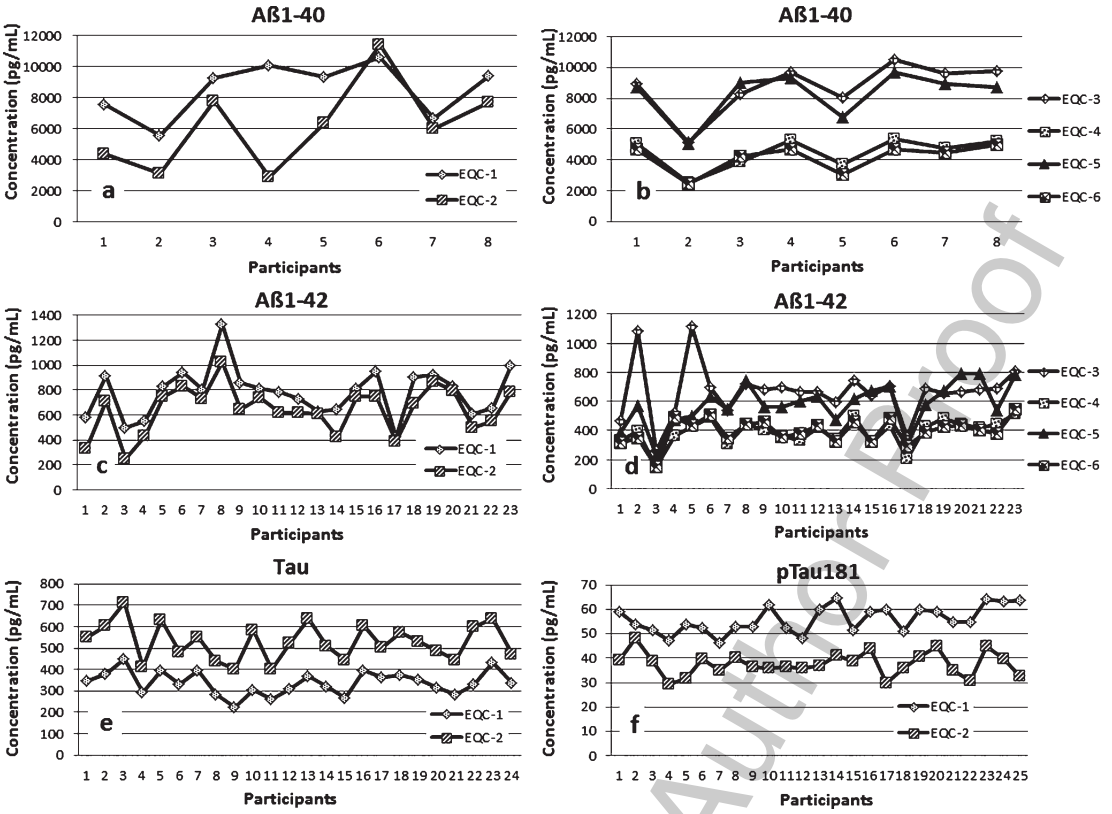

Fig. 4. Results of the inter-center variability testing. Horizontal axes represent the participants (in a random order); vertical axes present concentrations of the biomarkers (pg/mL). a) $A\beta_{1\text{-}40}$ in liquid CSF (EQC-1) and in freeze-dried CSF (EQC-2); b) $A\beta_{1\text{-}40}$ in prediluted plasma (EQC-3 and EQC-4), and in BSA/PBS+Tween (EQC-5 and EQC-6); c) $A\beta_{1\text{-}42}$ in liquid CSF (EQC-1) and in freeze-dried CSF (EQC-2); d) $A\beta_{1\text{-}42}$ in prediluted plasma (EQC-3 and EQC-4), and in BSA/PBS+Tween (EQC-5 and EQC-6); e) Tau in liquid CSF (EQC-1) and in freeze-dried CSF (EQC-2); f) pTau181 in liquid CSF (EQC-1) and in freeze-dried CSF (EQC-2).

are not listed in the Annex II of the 98/79/EC IVDD Directive [13], a vendor's self-declaration of conformity is enough to CE-mark the product, which does not necessarily correspond to its high quality; for example, Production Quality Assurance is not requested in such cases.

In our study, three matrices were tested: (A) human pooled CSF, (B) Aβ peptides spiked into prediluted human plasma, and (C) Aβ peptides spiked into BSA/PBS solution. Currently most, if not all, of the centers use self-collected pooled CSF QC samples for their purposes. This approach requires collection of large-scale otherwise discarded CSF leftovers, which should meet at least minimal quality criteria. For example, such samples should be collected in a relatively short time, to avoid obsolescing of the CSF samples before required volume has been collected. Moreover, it cannot be excluded that, similarly to misfolded prion proteins triggering misfolding of normal prion proteins ongoing degradation of Aβ peptides in one sample may hypothetically influence or perhaps trigger degradation in other samples after pooling them together. Human-born material should also be tested for potentially harmful infectious diseases. Further limitation is that it is difficult to control the target concentrations of the biomarkers, and, unless a given center has an access to large-scale number and volumes of pathological (in the sense of neurodegenerative diseases) samples, it is difficult to prepare samples with different levels of the biomarkers concentrations. To avoid all these limitations, an artificial sample could be prepared by spiking defined amounts of artificial peptides/proteins into a matrix mimicking CSF. Human plasma, prediluted 1:200, to achieve the CSF-level of the albumin concentration, i.e., the most abundant CSF protein [14], or the solution of bovine serum albumin at the concentration of 0.4 g/L, seemed the most obvious candidates. On the other hand such artificial matrices have limitations too; none of them would correspond ideally to

the human ("real") CSF. In prediluted plasma, other blood-derived proteins (for example immunoglobulins) are overconcentrated compared to the CSF, whereas they are absent in BSA/PBS solution; in both solutions, brain-derived proteins (other than those spiked) are absent or very low. Unpredictable aggregation of synthetic Aβ peptides cannot be excluded also in artificial matrices.

Following the recommendations by Linsinger et al. [15], we tested stability of the biomarkers in two settings: Short-term, which included three temperature conditions, and long-term, which was performed on the samples kept at –80°C (usual long-term storage condition).

To test short-term stability, we applied the isochronous method [16, 17], which means that the aliquots were stored at different conditions (room temperature, refrigerator, and –20°C) for a defined time following transfer to the reference condition (deep freezer) for the time remaining to the end of the study, and then simultaneously analyzed, together with a reference sample stored at the reference conditions (–80°C) from the beginning. For our study, –80°C was chosen as the reference condition, since convincing data are available that NDD biomarkers are sufficiently stable at this temperature [18, 19] and deeper freezing (in liquid nitrogen, for example) is probably not necessary. The greatest advantage of the isochronous method is that all aliquots can be analyzed in the same analytical run (on the same ELISA plate) irrespectively of the time they had been stored at a tested condition, which eliminates the influence of the inter-assay measurement imprecision. Stability of the samples is expressed in this study as CV of the average normalized concentrations, and as average daily decrease of concentration in percent per day. In most cases coefficient of variation of the concentrations stored up to 14 days was lower than 10%, with only a few exceeding 15%. Daily decrease trends, when significant at all, were rarely higher than 1.5% per day. We believe that for the proper interpretation of the data, combination of the two statistical approaches must be considered: Where as time trends in concentration decrease, when significant, show systematic degradation of the samples (or actually lack of it, in most cases), variation of the concentrations tells more about nonsystematic changes, partially explainable by marginal inhomogeneity of aliquots and imprecision of the methods. Taking together both approaches, we think that up to one working week (5–7 days) all matrices are stable when kept at the routine storage working conditions usually applied in medical laboratories (room temperature, refrigerator, –20°C freezer). This conclusion corresponds well to the results of our previous results [11].

In this study, we also tested whether the stability of the biomarkers could be improved by addition of an antibacterial agent, sodium azide (NaN_3), which was brought about by the suggestion of decreased Aβ concentrations in CSF samples due to bacterial growth [20]. We did not observe any additional benefit (nor any negative effect) of the addition of this antibacterial agent on the stability of the NDD biomarkers, with the exception of an improved stability of $A\beta_{1\text{-}42}$ in the human CSF stored at room temperature (Supplementary Figure 2c versus 2d, and Supplementary Figure 2e versus 2f). Correspondingly, we do not recommend supplementation of QC samples with NaN_3.

For the LTS testing, samples were stored deeply frozen and periodically analyzed on ELISA plates of different production batches and, in one case, even with vendor-introduced modifications of the assay format. Interpretation of these results must therefore take into consideration that the obtained variability is the superposition of the variability of the biomarkers concentrations and the inter-assay imprecision of the measurement methods. Long-term variation of measurements, usually large compared to the degree of degradation of biomolecules, is one the major problems in the determination of long-term stability and shelf-life [21]; on the other hand, we believe that such approach is more reliable, compared to the extrapolations of the results of accelerated ageing studies with application of mathematical equations, as proposed by other investigators [18]. Similarly, Linsinger et al. do not recommend attempts to estimate LTS by extrapolating STS data via the Arrhenius-equation [15]. To our opinion, the observed maximal variability in the range of $\pm 20\%$ should be considered an acceptable result. As a matter of fact, the concentrations of $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ in prediluted plasma without NaN_3 supplementation (B0) deviated not more than $\pm 10\%$ in all measurement points, i.e., actually within expected inter-assay variability.

For the inter-center study, six samples were prepared and sent to the participants under room temperature. This approach differs from the protocols of other large-scale inter-center projects, where samples were sent frozen [7, 8, 22]. Observed inter-center variability of the biomarkers obtained in this study in pooled CSF was compared to the results of the studies coordinated by the group at the University of Gothenburg, with the variation of tau and pTau

expectedly lower that this of $A\beta_{1-42}$ [8]. Interestingly, comparable variation was obtained with freeze-dried sample, with the exception of $A\beta_{1-40}$, which had higher variability, which can be explained by one outlier center (#4) and by the relatively low number of the participants. To our best knowledge, no data has been published so far on the inter-center variability of Aβ peptides in artificial matrices. The results of this study show moderate variation obtained in these matrices (∼20–25%) and plausible distribution of the results across the centers: In majority of cases, the participants reported either higher or lower concentrations of a given biomarker in all samples, which might denote that the performance of a center, and not the samples, require some further optimization. Furthermore, the distribution of measurements from the different laboratories was uniform above and below the average values, which indicates there is no systematic matrix effect; it is also important to consider that the total variabilities of the inter-center study include also variabilities resulting from intra-assay imprecision and inhomogeneity of the aliquots. Noteworthy to mention is also that spiking lower amounts of the Aβ peptides into artificial matrices resulted in their concentrations almost ideally correspondingly lower (70% and 50% of $A\beta_{1-42}$ and $A\beta_{1-40}$, respectively).

We are aware that use of synthetic QC samples by itself will not solve the problem of inter-center variability, but we are convinced that use of one sample type by many laboratories and on long-term basis would improve our knowledge on possible sources of the variability problem. To our best knowledge, this is the first study addressing the question if synthetic matrices can be used for QC of the AD biomarkers. Certainly further studies are warranted to address more detailed questions, like robustness of the samples or molecules absorption.

Our study has at least one limitation: Due to dynamical processes of phosphorylation/dephosphorylation of the tau molecule, and resulting problems of its molecular instability, it was currently impossible to spike tau and phosphorylated tau (pTau) into the artificial matrices tested in this study. Certainly further work is warranted to facilitate the inclusion of these important biomarkers in artificial QC samples.

In conclusion, we believe that it is possible to replace self-made CSF-based QC samples for the NDD with large-scale volumes of the samples prepared with artificial peptides and matrices and at different concentrations, which would greatly facilitate intra- and inter-center QC of the NDD measurements. Furthermore, most probably a calibration reference material, currently being tested, will be based on human CSF [10], and hence it makes sense to offer QC material based on a different matrix. It will be also very interesting to see if application of the same calibrators and the same QC material will reduce intra- and inter-laboratory variability. Also longitudinal statistical evaluation of assays performance will be easier if the same QC sample (generated in large volume and obviously stable for longer time) is used for many analytical runs, all of them performed according to one set of standardized reference calibrators. We believe that our study has implication also for other biomarkers, e.g., CSF α-synuclein in Parkinson Syndromes. Such artificial CSF QC samples can also be used in biospecimen Proficiency Testing (PT) programs, like the ISBER-endorsed PT program for biorepositories and other laboratories [23].

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SUPPLEMENTARY MATERIAL

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