Reference measurement procedure for CSF $A\beta_{1-42}$ and the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio – a cross-validation study against Amyloid PET

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Keywords

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Abstract

A clinical diagnosis of Alzheimer's disease can currently be made on the basis of results from cognitive tests in combination with medical history and general clinical evaluation, but the peptide amyloid-beta (Aβ) in cerebrospinal fluid (CSF) is increasingly used as a biomarker for amyloid pathology in clinical trials and in recently proposed revised clinical criteria for AD. Recent analytical developments have resulted in mass spectrometry (MS) reference measurement procedures for absolute quantification of $A\beta_{1-42}$ in CSF. Our aim was to cross-validate the $A\beta_{1-42}$ concentrations, the $A\beta_{1-42}/A\beta_{1-40}$ and the $A\beta_{1-42}/A\beta_{1-38}$ ratios in CSF measured by MS by comparing with plaque load as measured by amyloid positron emission tomography (PET). Validation results of the MS method showed that the method was linear (R^2 >0.99) throughout the measurement range for all calibrator curves with relative errors below 15%. Repeatability and the intermediate precision were below 10% and 15 % respectively. Samples can go through up to five

freeze/thaw cycles and be stored in -20° C with no significant effect on the measured concentration of the analyte. We included 100 non-demented patients with cognitive symptoms from the Swedish BioFINDER study, all of whom had undergone both lumbar puncture and ^{18}F-flutemetamol PET. When using the CSF $A\beta_{1-42}$ concentration for comparison with ^{18}F-flutemetamol PET, 66% of all patients were classified identically with an area under the receiver operating characteristic (ROC) curve of 0.85 (95% CI, 0.78-0.93) and a sensitivity and specificity of 82% and 81% respectively. Using the ratio of $A\beta_{1-42}/A\beta_{1-40}$ significantly improved the concordance, classifying 89% of the patients identically, with an area under the ROC curve of 0.95 (95% CI, 0.90-1.00) and a sensitivity and specificity of 96% and 91% respectively. Similar results were obtained when using the $A\beta_{1-42}/A\beta_{1-38}$ ratio.

These results show that the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio using the described MS method is strongly associated with the amount of cortical Aβ deposition measured by ^{18}F-flutemetamol PET.

Introduction

Alzheimer's disease (AD) affects about 35 million people worldwide, making it the most common form of dementia[1]. Neuropathologically, the disease is characterized by intracellular neurofibrillary tangles of accumulated hyperphosphorylated tau protein[2], and extracellular plaques consisting of aggregated amyloid-β (Aβ) peptides, which are widely believed to lie at the core of AD pathogenesis[3, 4]. In line with this, the plaque pathology *in vivo*, assessed by biomarkers, has recently been included in the research criteria for AD[5].

There are currently two established methods to identify Aβ accumulation in the brain *in vivo*: by positron emission tomography (PET) imaging of the brain using different Aβ-binding tracers and by measuring the concentration of $A\beta_{1-42}$ in the cerebrospinal fluid (CSF). Several amyloid tracers are available to assess the amount of deposited Aβ in the cortex, with ^{18}F-flutemetamol [6, 7], Pittsburg compound B [8, 9] and ^{18}F-florbetapir[10] being the most widely studied. These tracers have been validated against Aβ plaque load

determined using histopathology[11-14], and a high degree of concordance has also been demonstrated between the tracers[12, 15].

For CSF measurements of $A\beta_{1-42}$, several immunoassays are available and used in many clinical laboratories [16]. The concentration of $A\beta_{1-42}$ in CSF is approximately 50% lower in AD patients than in cognitively normal elderly, reflecting the deposition of the peptide in senile plaques in the brain[17]. The combination of CSF $A\beta_{1-42}$ with total tau (T-tau) and phosphorylated tau (P-tau) can predict progression from mild cognitive impairment (MCI) to AD as early as ten years before clinical dementia [18-21]. Further, emerging evidence suggest that a ratio of CSF $A\beta_{1-42}$ to $A\beta_{1-40}$ is superior to CSF $A\beta_{1-42}$ alone when detecting cortical Aβ pathology [22-26].

There is a very high concordance between CSF Aβ and amyloid PET measurements [27], but both PET and CSF methodologies suffer from similar standardization issues, especially regarding uniform cut-off levels but also in reproducibility [27-30]. Efforts to standardize procedures and harmonize levels between assay formats have been initiated to address these issues and to harmonize results across laboratories together within The Alzheimer's Association Quality Control program for CSF biomarkers which currently involves 84 laboratories globally [29] as well as the International Federation of Clinical Chemistry Working Group for CSF proteins (IFCC WG-CSF). The production of a certified reference material (CRM), with exact levels of $A\beta_{1-42}$ determined using a mass spectrometry (MS)-based reference measurement procedure (RMP), will enable the introduction of global cut-off levels and a more general use of $A\beta_{1-42}$ in CSF as a diagnostic tool. Two RMPs using antibody independent MS for quantification of CSF $A\beta_{1-42}$ [31, 32] have recently been accepted and listed by the Joint Committee for Traceability in Laboratory medicine (JCTLM database identification number C11RMP9).

In the present study we compared our RMP for $A\beta_{1-42}$ [31] with ^{18}F-flutemetanol PET imaging to study the concordance between the methods. Further, since the CSF sample preparation allows quantification of multiple Aβ species within a single MS analysis, we also measured $A\beta_{1-38}$ and $A\beta_{1-40}$ using the same

antibody-independent MS technique to analyze whether the ratios of $A\beta_{1-42}/A\beta_{1-40}$ or $A\beta_{1-42}/A\beta_{1-38}$ in CSF improve the diagnostic accuracy of cortical amyloid accumulation as compared with $A\beta_{1-42}$ alone.

Materials and methods

Study population

The study was approved by the Regional Ethics Committee in Lund, Sweden, and the Swedish Medical Products Agency, Sweden. All the patients provided their written informed consent to participate in the study.

The study population was derived from the prospective and longitudinal Swedish BioFINDER study, which consecutively enrolled patients at three memory outpatient clinics in Sweden (further information available at: www.biofinder.se). The present study included 100 non-demented patients with mild cognitive complaints, who all had undergone lumbar puncture and ^{18}F-flutemetamol PET. The patients were referred for assessment of their cognitive complaints and were included between 2010 and 2014. They were thoroughly assessed for their cognitive complaints by physicians with special interest in dementia disorders. The inclusion criteria were: 1) cognitive symptoms; 2) not fulfilling the criteria for dementia; 3) a Mini-Mental State Examination (MMSE) score of 24–30 points; 4) age 60–80 years; and 5) fluent in Swedish. The exclusion criteria were: 1) cognitive impairment that without doubt could be explained by another condition (other than prodromal dementias); 2) severe somatic disease; and 3) refusing lumbar puncture or neuropsychological investigation.

CSF sampling and analysis

The procedure and analysis of the CSF followed the Alzheimer's Association Flow Chart for CSF biomarkers [17]. Lumbar CSF samples were collected at the three centers and analyzed according to a standardized protocol [17, 33].

^{18}F-flutemetamol PET

The deposition of cerebral Aβ was visualized with the PET tracer ^{18}F-flutemetamol (approved by the European Medical Agency and the Food and Drug Administration). PET/CT scanning of the brain was conducted using a Philips Gemini TF PET scanner. Sum images, obtained 90-110 min post injection, were analyzed using the software NeuroMarQ (GE Healthcare). A volume of interest (VOI) template was applied for the following 9 bilateral regions: prefrontal, parietal, lateral temporal, medial temporal, sensorimotor, occipital, anterior cingulate, posterior cingulate/precuneus and a global neocortical composite region [34]. The standardized uptake value ratio (SUVR) was defined as the uptake in a VOI normalized for the cerebellar cortex uptake. Recently, we have established that the composite SUVR of ^{18}F-flutemetamol data show a bimodal distribution and can be separated in two populations (normal vs abnormal) using a cutoff of 1.42 SUVR [33].

Mass spectrometry

Calibration and sample preparation

Native (unlabeled) and ^{15}N uniformly labeled $A\beta_{1-38}$, $A\beta_{1-40}$, $A\beta_{1-42}$ and ^{13}C uniformly labeled $A\beta_{1-42}$ (rPeptide, Bogart, GA, USA) were dissolved in 20% acetonitrile (ACN) and 1% ammonium hydroxide (NH_4OH) to a concentration of 50 µg/mL. Aliquots were stored in -80° C. Artificial CSF (aCSF) was prepared as described elsewhere [35].

Calibration samples for $A\beta_{1-42}$ were prepared in human CSF as previously described [31]. For $A\beta_{1-38}$ and $A\beta_{1-40}$, aCSF was spiked to a final concentration of 150, 500, 1000, 2000, 3000 and 4000pg/mL native $A\beta_{1-38}$ and 1500, 5000, 10000, 20000, 30000 and 40000 pg/mL native $A\beta_{1-40}$ and a constant concentration of ^{15}N-$A\beta_{1-38}$ and ^{15}N-$A\beta_{1-40}$ at 1600 pg/mL as internal standard (IS).

CSF samples (180 µL) were spiked with 20 µL IS to a final concentration of 1600 pg/mL ^{13}C-$A\beta_{1-42}$, ^{15}N-$A\beta_{1-38}$ and ^{15}N-$A\beta_{1-40}$.

Solid phase extraction

Solid phase extraction (SPE) using a mixed-mode cation exchange SPE 96-well plate (Oasis MCX µElution, Waters) was conducted as previously described [31]. In brief, 200 µL of 5 M guanidine hydrochloride was added to 200 µL spiked CSF (180 µL CSF with 20 µL IS) and mixed on a vortex mixer at 1000 rpm for 20 min before adding 200 µL of 4% aqueous phosphoric acid (total volume 600 µL). The SPE wells were washed with 200 µL methanol and equilibrated with 200 µL 4% aqueous phosphoric acid prior to loading the prepared samples (600 µL). The wells were washed twice with 200 µL 4% phosphoric acid before the analytes were eluted with 2 × 50 µL 75% ACN and 2.5% NH_4OH. The eluates were collected in 0.75 mL tubes (Micronic, Lelystad, The Netherlands) and dried using vacuum centrifugation. Prior to analysis the samples were re-dissolved in 50 µL aqueous 20% ACN and 1% NH_4OH and gently mixed on shaker for 20 min at room temperature. The vials were briefly centrifuged to collect the volume at the bottom of the vial and placed in the autosampler.

Chromatography

Chromatography was performed using an UltiMate 3000 binary pump, column oven and autosampler (Thermo Scientific, Amsterdam, Netherlands). Separation was performed using a reversed-phase monolithic ProSwift RP-4H 1.0 mm x 250 mm column (Thermo Scientific, Sunnyvale, CA, USA) maintained at 50°C. Mobile phase A consisted of aqueous 5% ACN with 0.075% NH_4OH and mobile phase B of aqueous 95% ACN with 0.025% NH_4OH. Gradient elution was performed after injection of 25 µL processed sample at a flow rate of 0.3 mL/min with the following linear gradient steps: 0 min, 5% B; 1 min, 5% B; 6 min, 20% B; 7 min, 90% B; 9 min, 90% B; 10 min, 5% B; 15 min, 5% B. Eluent flow before 2 min and after 10 min was discarded using a divert valve to reduce contamination of the mass spectrometer. The total run time per sample was 15 minutes (including column re-equilibration). CSF samples were injected between calibrators (six calibrators low to high), blank, response factor and QC samples. Blanks were injected after each calibrator set, each response factor sample and each set of unknowns. The autosampler injector needle and tubing were washed after each sample injection with a solution consisting of aqueous 50% ACN with 1% NH_4OH.

Mass spectrometric analysis

Quantification was performed using a quadrupole-Orbitrap hybrid mass spectrometer (Q Exactive) equipped with a heated electrospray ionization source (HESI-II) (Thermo Scientific, Bremen, Germany). The following parameters were obtained by tuning the instrument on native $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ infused directly: sheath gas 50, auxillary gas 6, spray voltage 4.40 kV, S-lens RF 61, heater +190 °C, and capillary temperature +350 °C. Parallel reaction monitoring [36] was performed by fragmenting the 4+ charge states precursor ions for native and labeled $A\beta_{1-38}$, $A\beta_{1-40}$ & $A\beta_{1-42}$ and using the sum the fragment peak areas of the product ions post-acquisition (Supplemental Data Table 1). The precursors were isolated with an isolation width of 2.5 *m/z* followed by fragmentation using a normalized collision energy of 17.0 (Targeted-MS^2). Fragment spectra were recorded at a resolution of 17500 using an automatic gain control target of 2×10^5 charges and a maximum injection time of 250 ms. Spectra were internally mass calibrated using lock masses recorded in full scan mode during retention time 2.0 – 2.5 min. Before starting an analysis, a system suitability test was performed with an aqueous solution containing native $A\beta_{1-42}$, ^{13}C-$A\beta_{1-42}$ and ^{15}N-$A\beta_{1-42}$ (250 pg/mL each). Resulting signals were required to have a signal-to-noise ratio (S/N) ≥ 10.

Data processing

Xcalibur 2.2 Quanbrowser (Thermo Scientific, Waltham, MA, USA) was used for raw file processing (peak integration and S/N were determined using the built in ICIS peak detection algorithm and generation of calibration curve for the ^{15}N-calibrators). Quantification was performed using summed transitions. Value assignment for endogenous Aβ of unknown samples was calculated in Excel using the calibration function constructed in Quan browser software.

Validation of the mass spectrometry method

Measurement range

Linearity, measurement range, and limits of quantification were evaluated by measuring calibration samples (150-4000 pg/mL and 1500-40000 pg/mL for $A\beta_{1-38}$ and $A\beta_{1-40}$ respectively). The linear calibration curve had to have an R^2 >0.99 (of the Pearson correlation coefficient). The coefficient of variation (CV) for the back calculated concentrations of the data from the calibrator curves had to be <20% at the lower level of quantification (LLOQ) and upper level of quantification (ULOQ), while <15% for all other levels. Calibration curve data from five runs were used and the relative error of the back-calculated concentrations for the calibrators was plotted as a function of concentration.

Precision

Two human CSF pools with high and low concentrations, respectively, of $A\beta_{1-42}$ were constructed and aliquoted in 25 vials each. The vials were stored at -80 °C pending analysis. Five replicates for each concentration were measured in five different days. The experiments were performed by two technicians on a single SPE batch of the assay. For the precision samples the repeatability (CVr) had to be < 10% and the intermediate precision (CV_{RW}) < 15%. One-way ANOVA was used to calculate CV_r and CV_{RW} in accordance with ISO 5725-2 employing a published excel tool [37].

Sample stability

We investigated the effect on measured concentrations as a function of freeze-thaw cycles and different storage conditions. Five different fresh CSF samples were each divided into nine aliquots. One aliquot from each sample was immediately placed in the -80 °C freezer (aliquot 1) and the rest were treated as follows. Aliquot 2-5, two to five freeze/thaw (F/T) cycles, respectively (aliquot 1 undergoes one cycle). Aliquot 6, twenty-four hours (24h) at 5-8 °C; aliquot 7, one week (1w) at 5-8 °C; aliquot 8, twenty-four hours at room temperature (RT); and aliquot 9, one month (1M) at -20 °C.

At the end of the incubations or freeze/thaw cycles the aliquots were placed at -80 °C pending analysis. All nine aliquot for a given sample were run in duplicates. Aliquot 1 serves as a reference sample to which the other were compared, calculating recoveries relative to the reference sample. The evaluation is

qualitative and there are no quantitative demands for sample stability but useful information can be obtained on how to store the samples with minimal effect on the results.

Statistical Analysis

An unbiased cutoff value for an abnormal ^{18}F-flutemetamol scan finding has previously been established [33] using mixture modeling [38]. Cutoff values were established for $A\beta_{1-42}$ concentration, the $A\beta_{1-42}/A\beta_{1-40}$ and the $A\beta_{1-42}/A\beta_{1-38}$ ratios using the same method.

Results

Validation of the mass spectrometry method

Measurement range

The relative errors for the back-calculated calibrators were below 15% in the whole range defined by the calibrator curve (150-4000 pg/mL and 1500-40000 pg/mL for $A\beta_{1-38}$ and $A\beta_{1-40}$ respectively, Supplemental Data Figure 1). The calibration curves for the different analytes all had a goodness-of-fit of R^2 >0.99.

Precision

CV_r and CV_{RW} for both high and low levels were well below demanded limits for the different analytes (Supplemental Table 2).

Sample stability

A sample can go through up to five freeze/thaw cycles with no significant effect on the measured concentration of the analyte (Supplemental Figure 2). Storage in -80° C is preferred, but the other storage conditions tested were also acceptable.

Association between CSF Aβ and ^{18}F-flutemetamol PET

The unbiased cutoff for CSF $A\beta_{1-42}$ to distinguish two populations within the sample set was 1059 pg/mL. Comparing the ^{18}F-flutemetamol PET and CSF $A\beta_{1-42}$ concentrations using this cutoff showed 66% concordance (Figure 1A). We further studied the association between ^{18}F-flutemetamol PET and the ratios of CSF $A\beta_{1-42}/A\beta_{1-40}$ and $A\beta_{1-42}/A\beta_{1-38}$. The unbiased cutoff values to distinguish two populations within the sample set were 0.085 for the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio and 0.40 for the CSF $A\beta_{1-42}/A\beta_{1-38}$ ratio. Comparing the ^{18}F-flutemetamol PET and CSF Aβ ratios using these cutoff values showed 89% concordance for both $A\beta_{1-42}/A\beta_{1-40}$ (Figure 1B) and $A\beta_{1-42}/A\beta_{1-38}$ (Figure 1C).

When dichotomized for positive or negative amyloid PET, using cutoff values derived from receiver operating characteristic (ROC) analysis and Youden's index, the CSF $A\beta_{1-42}$ concentration could distinguish individuals with an abnormal amyloid PET scan finding with a sensitivity of 82% and a specificity of 81%, area under the ROC curve (AUC) 0.85 (95% CI, 0.78-0.93). For the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio the sensitivity and specificity was 96% of 91% respectively with an AUC of 0.95 (95% CI, 0.90-1.00), and for the CSF $A\beta_{1-42}/A\beta_{1-38}$ the sensitivity and specificity was 88% and 96% respectively with an AUC of 0.94 (95% CI, 0.88-0.99) (Figure 1D).

Discussion

In this study we demonstrate the accuracy of Aβ measurements in CSF using a MS-based RMP to determine cortical Aβ deposition. The CSF Aβ measurements were in good agreement with plaque load measured using ^{18}F-flutemetamol PET where the level of CSF $A\beta_{1-42}$ identified an abnormal amyloid PET scan with sensitivities and specificities of more than 80%, while using the CSF $A\beta_{1-42}/ A\beta_{1-40}$ ratio increased the sensitivities and specificities to more than 90%.

The cutoff for pathological CSF $A\beta_{1-42}$ using MS was 1059 pg/mL or less, which is significantly higher compared to the cutoff values for many immunoassays used in clinical routine. While this difference in concentration may be explained by differences in calibration and calibrator characterization, it may also reflect the fundamental difference in quantitation between MS and immunoassays: MS does not rely on antibodies and is therefore unaffected by matrix interferences such as epitope masking. The use of denaturing agents in the sample preparation for MS may make a protein-bound fraction of Aβ accessible to the analysis. This difference highlights the need for a CRM for CSF $A\beta_{1-42}$.

Both CSF and plasma contains a variety of Aβ peptides, where $A\beta_{1-40}$ is around ten times more abundant than $A\beta_{1-42}$ [39], and there is a variation between individuals regarding the amount of all (total) Aβ peptides produced [26]. Consequently, when using only $A\beta_{1-42}$, low total Aβ producers might be false positive for AD while the opposite might be true for high producers. While the levels of $A\beta_{1-40}$ in CSF are unchanged in AD, the ratio of $A\beta_{1-42}/A\beta_{1-40}$ has been shown to improve the diagnostic accuracy of AD [22-26, 40]. For this study we expanded the previously published MS method to quantify $A\beta_{1-42}$ in human CSF [31] to also include $A\beta_{1-40}$ and $A\beta_{1-38}$ with results from the validation showing good precision and sample stability for the analytes (see Supplemental data). The present results are in agreement with a recent study, which showed that the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio is better than CSF $A\beta_{1-42}$ to reliably determine abnormal cortical Aβ deposition, even when using different types of immunoassays [22]. These results strongly suggest that the $A\beta_{1-42}/A\beta_{1-40}$ ratio can accurately detect cortical amyloid pathology and can be used to improve the diagnostic work-up of AD in the clinic and when recruiting early AD patients to clinical trials evaluating new disease-modifying therapies.

Since cognitively normal individuals can exhibit cortical Aβ accumulation, and patients showing cognitive symptoms might be clinically misdiagnosed for AD without Aβ pathology, an important aspect of this study was to use an objective method such as amyloid PET as a “standard of truth” instead of clinical diagnosis to only include true AD cases.

In conclusion, the presented results show that the $A\beta_{1-42}/A\beta_{1-40}$ ratio measured in CSF is strongly associated with the amount of cortical Aβ deposition assessed by PET. Further, the data suggest that a ratio of $A\beta_{1-42}/A\beta_{1-40}$ (or $A\beta_{1-42}/A\beta_{1-38}$) is superior to $A\beta_{1-42}$ alone.

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Figure captions

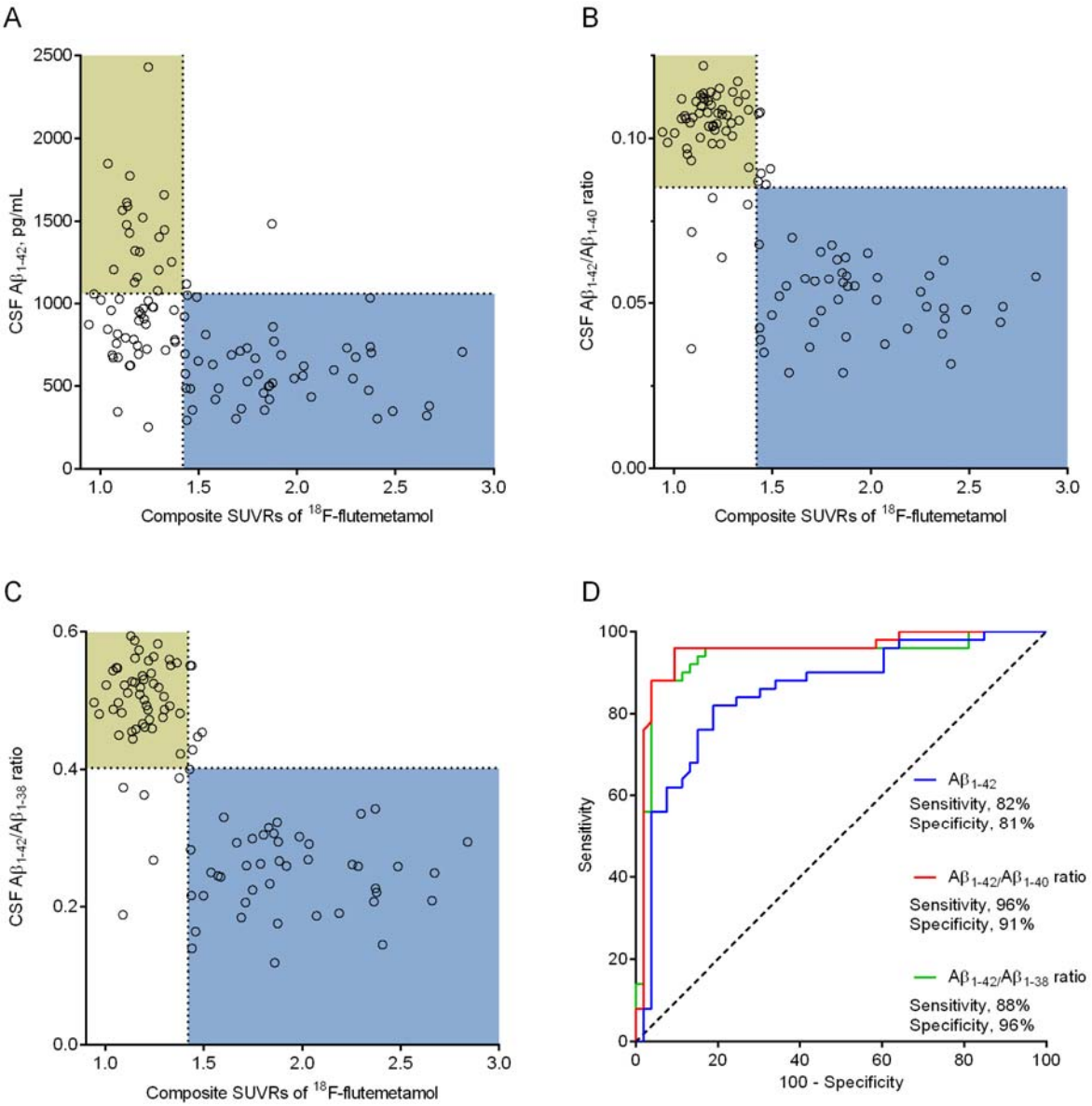
Figure 1.

Scatterplot showing (A) CSF $A\beta_{1-42}$ concentrations with an unbiased cutoff determined to 1059 pg/mL (horizontal dashed line), (B) the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratios with a cutoff determined at 0.0852 (horizontal dashed line) and (C) the CSF $A\beta_{1-42}/A\beta_{1-38}$ ratios with a cutoff determined at 0.402 and the SUVR values of ^{18}F-flutemetanol and SUVR at 1.42 (vertical dashed line). The blue quadrants indicate abnormal amyloid PET and CSF Aβ while tan quadrants indicate normal amyloid PET and CSF Aβ. White quadrants indicate discordant results.

(D) The area under the receiver operating characteristic (ROC) curve (AUC) for the accuracy of CSF $A\beta_{1-42}$ (blue line, AUC 0.85), CSF $A\beta_{1-42}/A\beta_{1-40}$ ratios (red line, AUC 0.95) and CSF $A\beta_{1-42}/A\beta_{1-38}$ ratios (green line, AUC 0.94) to predict an abnormal ^{18}F-flutemetanol scan (standardized uptake value ratio [SUVR] > 1.42).

Figures

Figure 1



Supplemental Data

Supplemental Table 1. Retention times, precursor and product ions mass-to-charge ratios for Aβ peptides.

Peptide	RT (min)	Precursor (m/z)	Products (m/z)
$A\beta_{1-38}$	5.98	1033.90	915.00; 943.29; 976.09; 1000.87; 1015.13; 1125.21; 1162.93; 1200.64; 1219.66; 1257.38; 1301.11
^{15}N-$A\beta_{1-38}$	5.98	1046.57	926.42; 954.96; 988.01; 1013.04; 1027.55; 1139.44; 1177.50; 1215.55; 1234.89; 1272.94; 1317.01
$A\beta_{1-40}$	6.5	1083.47	915.00; 943.29; 953.69; 976.09; 1000.87; 1015.13; 1029.40; 1054.18; 1125.21; 1162.93; 1200.64; 1219.66; 1257.38; 1301.11; 1334.16; 1353.17; 1372.19
^{15}N-$A\beta_{1-40}$	6.5	1096.63	926.42; 954.96; 965.28; 988.01; 1013.04; 1027.55; 1042.06; 1067.09; 1139.44; 1177.50; 1215.55; 1234.89; 1272.94; 1317.01; 1350.38; 1369.73; 1389.08
$A\beta_{1-42}$	6.9	1129.58	915.19; 943.21; 975.98; 1000.74; 1029.51; 1054.03; 1078.79; 1107.06; 1163.23; 1200.25; 1257.29; 1300.96; 1333.66; 1372.00; 1405.02
^{15}N-$A\beta_{1-42}$	6.9	1143.00	926.41; 954.68; 987.95; 1012.71; 1041.22; 1066.99; 1091.75; 1120.28; 1177.18; 1215.55; 1272.58; 1316.92; 1349.94; 1388.63; 1422.31
^{13}C-$A\beta_{1-42}$	6.9	1179.50	955.33; 985.11; 1019.37; 1045.14; 1074.65; 1100.67; 1126.69; 1156.40; 1253.43; 1313.14; 1358.50; 1393.19; 1432.21; 1466.90

Abbreviations: RT, Retention time (minutes). m/z, mass-to-charge ratio.

Supplemental Table 2. Precision, $A\beta_{1-38}$. n=5 per level and day over 5 days.

Sample	Average concentration (pg/mL)	s_r (pg/mL)	CV_r (%)	s_{RW} (pg/mL)	CV_{Rw} (%)
HIGH	2937.9	136.5	4.6	202.6	6.9
LOW	2071.9	71.9	3.5	145.7	7.0

Abbreviations: CV, coefficient of variation. s, standard deviation. r, repeatability. Rw, intermediate precision.

Supplemental Table 3. Precision, $A\beta_{1-40}$. n=5 per level and day over 5 days.

Sample	Average concentration (pg/mL)	s_r (pg/mL)	CV_r (%)	s_{RW} (pg/mL)	CV_{Rw} (%)
HIGH	4197.4	171.8	4.1	252.5	6.0
LOW	2737.5	93.5	3.4	166.5	6.1

Abbreviations: CV, coefficient of variation. s, standard deviation. r, repeatability. Rw, intermediate precision.

Supplemental Table 4. Precision, $A\beta_{1-42}$. n=5 per level and day over 5 days.

Sample	Average concentration (pg/mL)	s_r (pg/mL)	CV_r (%)	s_{RW} (pg/mL)	CV_{Rw} (%)

HIGH	1066	61.5	5.8	111.5	10.5
LOW	301	21.6	7.1	32.3	10.7

Supplemental Table 5. Precision, $A\beta_{1-42}/A\beta_{1-40}$. n=5 per level and day over 5 days.

Sample	Average ratio	s_r	CV_r (%)	s_{RW}	CV_{Rw} (%)
HIGH	0.254	0.011	4.4	0.017	6.6
LOW	0.110	0.006	5.5	0.011	10.2

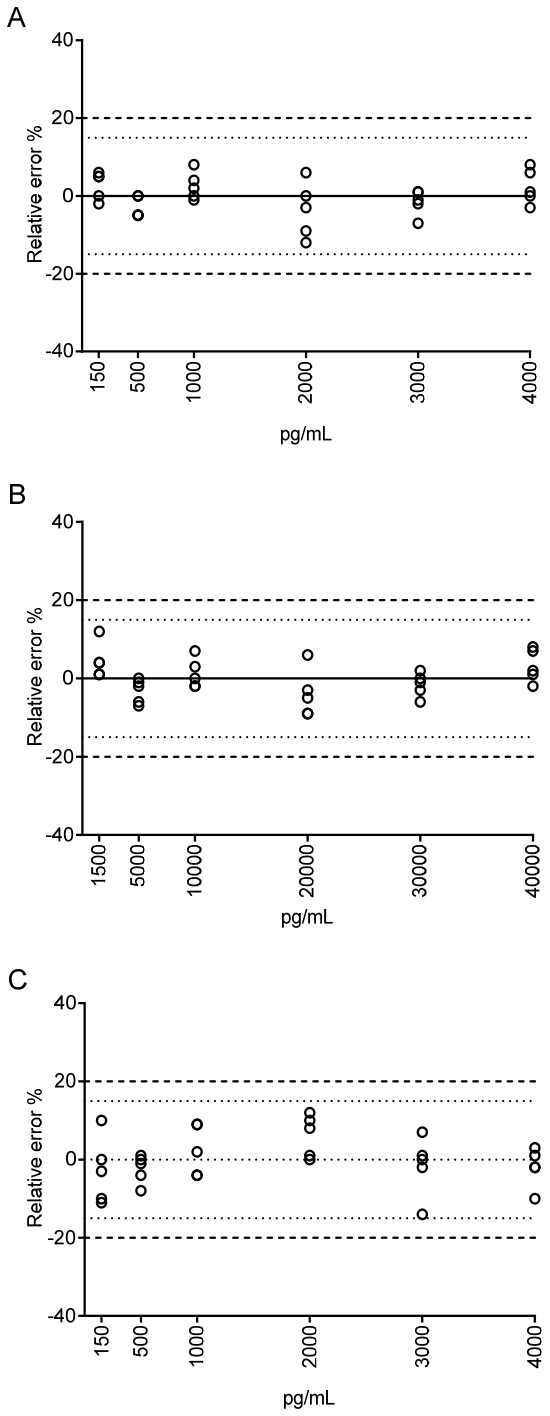
Abbreviations: CV, coefficient of variation. s, standard deviation. r, repeatability. Rw, intermediate precision.

Supplemental Table 5. Precision, $A\beta_{1-42}/A\beta_{1-38}$. n=5 per level and day over 5 days.

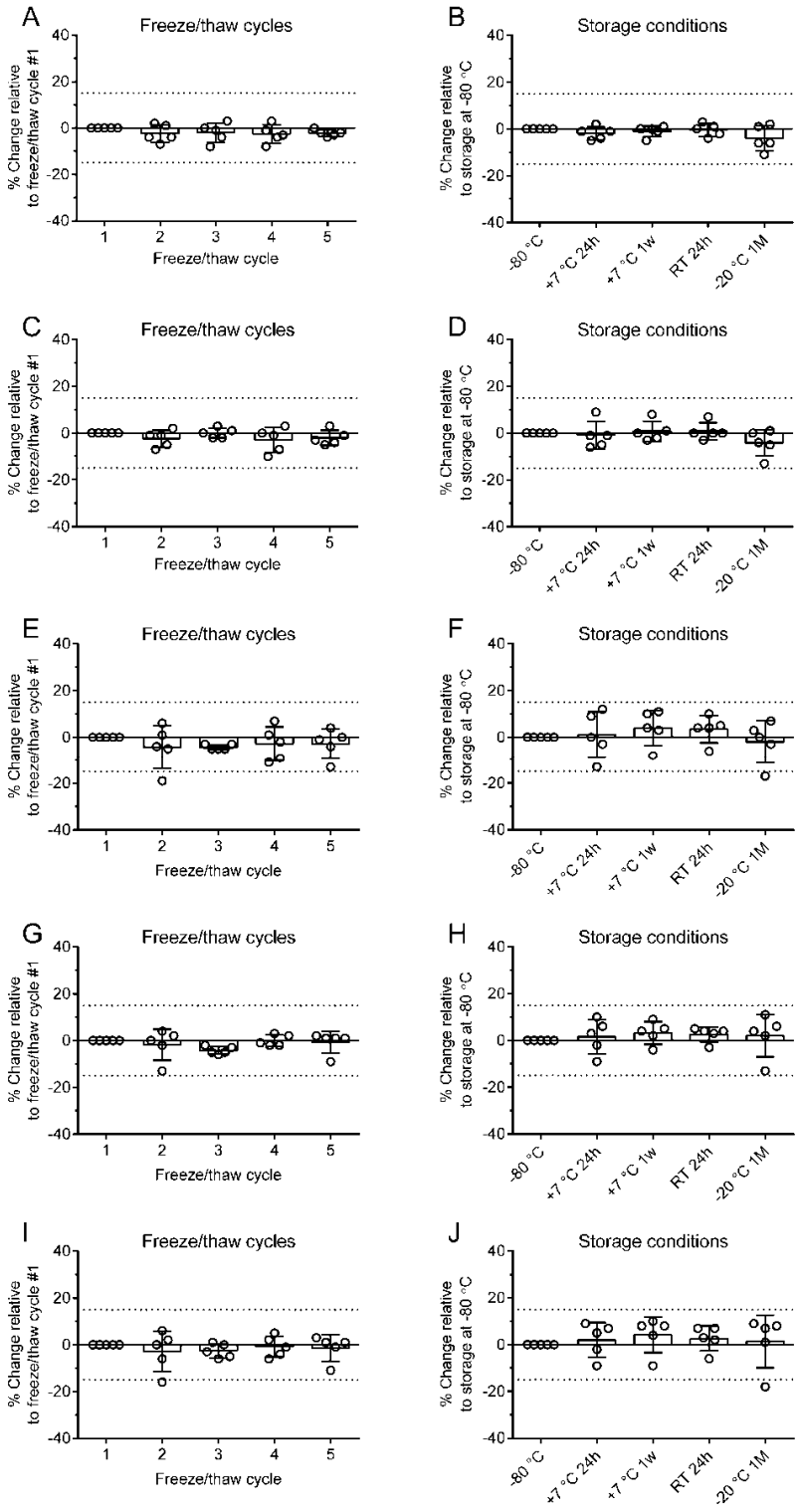
Sample	Average ratio	s_r	CV_r (%)	s_{RW}	CV_{Rw} (%)
HIGH	0.363	0.019	5.2	0.026	7.1
LOW	0.146	0.009	6.2	0.014	9.9

Abbreviations: CV, coefficient of variation. s, standard deviation. r, repeatability. Rw, intermediate precision.

Supplemental Figure 1. Measurement range for (A) $A\beta_{1-38}$, (B) $A\beta_{1-40}$ and (C) $A\beta_{1-42}$.



Supplemental Figure 2. Sample stability for (A-B) $A\beta_{1-38}$, (C-D) $A\beta_{1-40}$, (E-F) $A\beta_{1-42}$, (G-H) $A\beta_{1-42}/A\beta_{1-40}$ ratio and (I-J) $A\beta_{1-42}/A\beta_{1-38}$ ratio.


A
Freeze/thaw cycles
% Change relative to freeze/thaw cycle #1
Freeze/thaw cycle
B
Storage conditions
% Change relative to storage at -80 °C
-80 °C
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+7 °C 1w
RT 24h
-20 °C 1M
C
D
E
F
G
H
I
J