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Improved protocol for measurement of plasma β -amyloid in longitudinal evaluation of Alzheimer's Disease Neuroimaging Initiative study patients

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Abstract

Background—The interassay variability and inconsistency of plasma β -amyloid ($A\beta$) measurements among centers are major factors precluding the interpretation of results and a substantial obstacle in the meta-analysis across studies of this biomarker. The goal of this investigation was to address these problems by improving the performance of the bioanalytical method.

Methods—We used the Luminex immunoassay platform with a multiplex microsphere-based reagent kit from Innogenetics. A robotic pipetting system was used to perform crucial steps of the procedure. The performance of this method was evaluated using two kit control samples and two quality control plasma samples from volunteer donors, and by retesting previously assayed patient samples in each run. This setup was applied to process 2454 patient plasma samples from the Alzheimer's Disease Neuroimaging Initiative study biofluid repository. We have additionally evaluated the correlations between our results and cerebrospinal fluid (CSF) biomarker data using mixed-effects modeling.

Results—The average precision values of the kit controls were 8.3% for $A\beta_{1-40}$ and 4.0% for $A\beta_{1-42}$, whereas the values for the plasma quality controls were 6.4% for $A\beta_{1-40}$ and 4.8% for $A\beta_{1-42}$. From the test–retest evaluation, the average precision was 7.2% for $A\beta_{1-40}$ and 4.5% for $A\beta_{1-42}$. The range of final plasma results for Alzheimer's Disease Neuroimaging Initiative patients was 13 to 372 pg/mL (median: 164 pg/mL) for $A\beta_{1-40}$ and 3.5 to 103 pg/mL (median: 39.3 pg/mL) for $A\beta_{1-42}$. We found that sample collection parameters (blood volume and time to freeze) have a small, but significant, influence on the result. No significant difference was found between plasma $A\beta$ levels for patients with Alzheimer's disease and healthy control subjects. We have determined multiple significant correlations of plasma $A\beta_{1-42}$ levels with CSF biomarkers. The relatively strongest, although modest, correlation was found between plasma $A\beta_{1-42}$ levels and CSF p-tau₁₈₁/ $A\beta_{1-42}$ ratio in patients with mild cognitive impairment. Plasma $A\beta_{1-40}$ correlations

with CSF biomarkers were weaker and diminished completely when we used longitudinal data. No significant correlations were found for the plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio.

Conclusions—The precision of our robotized method represents a substantial improvement over results reported in the literature. Multiple significant correlations between plasma and CSF biomarkers were found. Although these correlations are not strong enough to support the use of plasma $A\beta$ measurement as a diagnostic screening test, plasma $A\beta_{1-42}$ levels are well suited for use as a pharmacodynamic marker.

Keywords

β -amyloid; Longitudinal; Plasma; xMAP immunoassay; ADNI study subjects; Robotic pipetting

1. Introduction

The formation and aggregation of β -amyloid ($A\beta$) into plaques in the brain is one of the major pathologic events associated with the development of Alzheimer's disease (AD). Hence, the research community has great interest in evaluating $A\beta$, particularly $A\beta_{1-42}$ (the least soluble member of the family of $A\beta$ peptides), as a biomarker of the disease.

Measurements of $A\beta_{1-42}$ levels in cerebrospinal fluid (CSF), along with the tau peptide and its phosphorylated counterpart p-tau₁₈₁ (products of neuronal degeneration and the main components of tangles also found in autopsied brains of AD patients), already have a prominent role in the early detection of AD pathology. The clinical performance for the detection of AD using these three CSF biomarkers is relatively well characterized. Threshold levels were established for them using CSF samples collected premortem from AD patients who had autopsy-based diagnoses [1].

Nevertheless, owing to the invasiveness of CSF sampling, there is an increasing interest in the development and validation of biomarkers measurable in blood or plasma, and many researchers are investigating whether plasma $A\beta$ could be one of them. Unfortunately, the reports are contradictory in many instances, where some researchers report higher and others report lower plasma $A\beta$ levels in AD patients compared with control subjects. Moreover, the correlations reported for plasma $A\beta$ levels are also conflicting; some authors report a higher plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio in patients with less education [2], whereas others report a higher ratio in patients with the highest education level [3].

A substantial contributor to this lack of consistency, as many investigators agree, is the poor within- and between-assay precision and the low accuracy of the immunoassay test methods used in the analysis of $A\beta$. Additionally, there is a general difficulty of comparing the results from different studies, as the reports differ substantially from one another in the reported ranges and threshold values. These additional confounding factors hinder the interpretation of the data [4] and make the meta-analysis of $A\beta$ results challenging.

The procedure presented in this study is a step toward further standardization and improved performance by implementing automation in critical steps of the procedure. The goal of this work was to improve the performance of the immunoassay method for measuring plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in terms of run-to-run reproducibility, and to apply this improved methodology to measure plasma $A\beta$ levels collected at baseline and subsequent annual visits in the Alzheimer's Disease Neuroimaging Initiative (ADNI) study patients and compare them with CSF results.

2. Methods

2.1. ADNI study cohort

The ADNI study (<http://adni.loni.ucla.edu>) was launched in 2004 by the National Institute of Aging, the Foundation for the National Institutes of Health, and by a group of private–public partners as a precompetitive AD biomarker consortium that now is in its seventh year and will continue for approximately another 6 years. Enrollment target in the first phase of ADNI (ADNI-1) was 800 participants: 200 healthy control subjects, 400 subjects with amnesic mild cognitive impairment (MCI), and 200 subjects with mild AD (although this cohort has been enlarged in the second phase of ADNI [ADNI-2]). The ADNI protocol was approved by the human studies committee at the 58 institutions participating in the ADNI study in the United States and Canada. Written and verbal informed consent was obtained from participants at screening and enrollment. At sample collection, participants were 60 years of age and in good general health, with no other neurological, psychiatric, or major medical diagnoses that could contribute importantly to dementia. Further details regarding the ADNI study, including participant selection procedures and complete study protocol, have been presented elsewhere and can be found online at <http://www.alzheimers.org/clinicaltrials/fullrec.asp?PrimaryKey=208>. A schematic depiction of ADNI biomarker specimen collection, analytical procedures, and data flow is shown in Fig. 1.

2.2. Samples

Plasma samples used in this study were obtained from the ADNI biofluid repository at the University of Pennsylvania. All ADNI study patients having a baseline and at least one scheduled follow-up plasma samples were included in the study ($n = 722$; 422 men [58.4%] and 300 women [41.6%], with 2454 samples). The mean age of all patients at baseline was 75.3 ± 6.79 years. Patients were age-matched across genders (75.7 ± 6.73 years in male and 74.8 ± 6.87 years in female subjects, $P = .12$) and across diagnosis groups (75.9 ± 5.03 years in AD, 75.0 ± 7.3 years in MCI, and 75.3 ± 7.57 years in cognitively normal (NL) patients, $P = .78$).

The plasma samples were collected at the participating ADNI centers. After overnight fasting, plasma was collected in the morning by venipuncture into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing potassium K3 ethylene tetraacetate as an anticoagulant. After centrifugation, samples were placed in transfer tubes (13 mL polypropylene, Sarstedt Inc., Newton, NC, catalog number 60.541), frozen, and shipped on dry ice to the UPenn Biomarker Core Laboratory, where they were stored temporarily at -80°C . The average time from blood collection to freezing of plasma for shipment was 67 ± 41 minutes (95% confidence interval [CI]: 21–180 minutes). Within several weeks of receipt, the samples were thawed, aliquoted by 500 μL into aliquot tubes (1.5 mL polypropylene, Thermo Fisher Scientific, Waltham, MA, catalog number 05-408-129), and stored at -80°C pending biochemical analyses.

2.3. Analytical method

The levels of $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ peptides were quantified in plasma using “INNO-BIA plasma $\text{A}\beta$ forms” (module A kit; Innogenetics NV, Ghent, Belgium), a multiplex microsphere-based xMAP technology research use-only reagent kit, on a Luminex 100 with IS version 2.3 software (Luminex, Austin, TX). The kit uses monoclonal antibodies (MAbs) covalently coupled to spectrally specific fluorescent beads to detect $\text{A}\beta_{1-42}$ (MAb 21F12) and $\text{A}\beta_{1-40}$ (MAb 2G3). Full details of the implementation of this kit on the Luminex platform are described elsewhere [5].

The same lot number of the kit reagents as well as of calibrators and controls was used throughout the study to avoid possible lot-to-lot variability. Reagents were prepared according to the manufacturer's instructions, except for the coated beads' dilution, which was decreased by a factor of two (to $\times 50$, a twofold increase in the number of beads per well) to ensure a consistently sufficient bead count number (100). Samples and calibration standards were run in duplicate, and every set of samples from a single subject was analyzed within the same run.

2.4. Sample preparation procedure

Sample preparation was performed using an automated pipetting system epMotion 5075 (Eppendorf, Hamburg, Germany), except for the filter plate draining step, which was performed using a stand-alone vacuum manifold (Milli-pore, Brussels, Belgium). The schematic diagram of the setup and automation is displayed in Fig. 2. Further details, including epMotion bench layout and program, can be found in Fig. S1 and Tables S1 and S2.

Briefly, thawed plasma samples were centrifuged at room temperature (3000 rpm for 10 minutes) to remove solid particles that could cause problems during further steps in the analyses. Subsequently, a 1:3 dilution of samples was prepared by adding 160 μL of diluent to 80 μL of plasma. Then, a 96-well filter plate was washed with 225 μL of washing solution per well and drained using the vacuum manifold, followed by the addition of a mixture of the coated beads (100 μL /well) to all wells. The wells were drained, and “conjugate 1” (25 μL /well) was added, followed by 75 μL /well of blanks, standards, quality controls (“ConA” and “ConB”), diluted quality control plasma samples (collected from individual volunteer donors), and diluted patient plasma samples. After an overnight incubation at 2°C to 8°C in the dark (plate covered with aluminum foil, continuous shaking), the filter plate was drained and washed three times. Subsequently, phycoerythrin-labeled streptavidin was added (100 μL /well), and the plate was incubated on a shaker for another 1 hour in the dark at room temperature. After the subsequent washing step, reading solution (phosphate-buffered saline, 100 μL /well) was added and again incubated at room temperature for 3 minutes in the dark. The assay was read on a Luminex 100 IS instrument. For each set of micro-spheres, 100 beads were analyzed, and the median fluorescence intensity (MFI) was used for quantification.

2.5. Evaluation of method performance

To closely evaluate the performance of this procedure, two additional quality control samples (“QC1” and “QC2”) were run on each plate apart from the kit quality controls (ConA and ConB). These samples were plasma aliquots from volunteer donors. Additionally, on every plate except the first one, we included 2 to 3 never-before-thawed aliquots of samples that were run previously within this project, thus implementing a continuous test–retest evaluation.

2.6. Assay data processing

For efficient processing of large amounts of data from the multiple runs performed using the aforementioned procedure, we developed a set of scripts for the “R” statistical analysis language (R version 2.12.2 [6]). The scripts were collected in a library named “immunoassay,” currently available from R-Forge (version 0.3, <http://immunoassay.r-forge.r-project.org>) [7]. An important functionality of this library from our perspective was the ability to batch process the data from multiple Luminex runs and create a cumulative table of results.

In brief, the functions in the immunoassay library were used to load run data into R. Only the tables of bead counts and MFI were loaded, along with basic run information. Kit information (concentrations of analytes in standards and kit quality controls) was loaded from a separate table. Subsequently, the fitting of sigmoidal models to the calibrators was performed using five-parametric logistic model with either “1/y” or “sqrt(1/y)” weighting, and the fitting function was allowed to automatically select the best fit based on the criteria of residual sum of squares [8]. Final results for plasma samples, including QC1 and QC2, were multiplied by the factor of 3 because of the initial step of dilution of the plasma samples.

Subsequently, the final results were evaluated for acceptability based on three predefined criteria: minimum bead counts of 20, minimum MFI greater than or equal to the MFI of the lowest calibrator, and maximum percent coefficient of variation (%CV) between replicates of 20%. Results not meeting these criteria were removed from the data set.

2.7. Statistical analysis of results

For statistical analysis of results, the R software package version 2.12.2 was used [6]. The results from the “test–retest” experiment were analyzed with the Passing–Bablok [9] and Deming method [10] using the “MethComp” library version 1.3 [11]. By-patient means of A β results across all visits were used in the analysis of time-independent variables [12]. Analysis of time-dependent variables in the longitudinal data set was performed with mixed-effects models using the library “nlme” version 3.1–98 [13]. Determination of statistical significance of model terms was done by comparing the “maximum likelihood” estimated models with and without those terms using analysis of variance [14]. Summary results are presented as mean $\pm$ standard deviation, and statistical tests for significance were performed using nonparametric Kruskal–Wallis rank sum test, unless noted otherwise. Correlations were evaluated using Spearman test. A *P* value of .05 was assumed to be the threshold for statistical significance.

3. Results

3.1. Analytical performance

A total of 2454 plasma samples from the ADNI study patients were run in duplicate using 76 plates. The first 24 plates were run with calibrators St7 and St8 prepared by sequential 1:3 dilution of the St6 calibrator to obtain concentrations of, respectively, 5 and 1.6 pg/mL for A β _{1–40} and of 1.6 and 0.53 pg/mL for A β _{1–42}. However, the results for St8 of A β _{1–42} were not satisfactory: the average imprecision amounted to 31.8 %CV, and the average inaccuracy amounted to –18.5%. Therefore, according to the US Food and Drug Administration guidance [15], this calibrator was removed from consideration for those 24 plates, and St7 was considered the lowest calibrator. For further runs, we replaced St7 and St8 with sequential 1:2 dilutions of St6, resulting in the concentrations of, respectively, 7.5 and 3.75 pg/mL for A β _{1–40} and 2.5 and 1.25 pg/mL for A β _{1–42}. Performance of those calibrators (back-calculated values) is summarized in Table 1. Summary of the sigmoidal fits to those calibrators is also displayed on log concentration versus MFI plots in Fig. 3, along with box-and-whisker plots of measured patient results. The fitting function selected “1/y” weighting in 38 runs and “sqrt(1/y)” weighting in 40 runs for A β _{1–40}, and “1/y” weighting in 49 runs and “sqrt(1/y)” weighting in 29 runs for A β ₄₂.

A summary of the kit controls and the human plasma quality control samples is shown in Table 2 and in the form of stability plots in Fig. 4. In the first 42 runs, we used sample “a” as the human plasma QC2 sample; subsequently, we replaced the QC2 with sample “b” from

another individual because sample “a” was similar in concentration to QC1 and did not offer enough coverage of the measured concentration range.

A total of 195 samples were retested in the continuous test–retest performance evaluation. The average magnitudes of differences were 8.4 pg/mL for $A\beta_{1-40}$ (5.2% of the average concentration of 160.7 pg/mL) and 1.1 pg/mL for $A\beta_{1-42}$ (2.8% of the average concentration of 39.0 pg/mL). The slope of the Passing–Bablok regression line for test versus retest results was 0.971 (95% CI: 0.9057–1.0335) for $A\beta_{1-40}$ and 0.936 (95% CI: 0.8939–0.9789) for $A\beta_{1-42}$. The parameters used were as follows: R^2 of 0.746, root-mean-square error of 7.73, average bias of –4.14 pg/mL, and 7.24% average coefficient of variation of residuals for the $A\beta_{1-40}$ fit; R^2 of 0.908, root-mean-square error of 1.18, average bias of –2.67 pg/mL, and 4.51% average coefficient of variation of residuals for $A\beta_{1-42}$ fit. The results of this experiment are graphically summarized in Fig. 5.

3.2. Patient results

For the 2454 samples from the ADNI study patients, 33 results did not meet the acceptability criteria for $A\beta_{1-40}$ (2 owing to bead count criteria, 8 owing to %CV criteria, and 23 owing to MFI criteria), and 24 results did not meet the criteria for $A\beta_{1-42}$ (5 owing to %CV criteria and 19 owing to MFI criteria). The range of the final results was from 12.6 to 371.7 pg/mL (95% CI: 54.8–256.2 pg/mL, median: 163.6 pg/mL) for $A\beta_{1-40}$ and from 3.47 to 102.8 pg/mL (95% CI: 17.9–62.1 pg/mL, median: 39.3 pg/mL) for $A\beta_{1-42}$.

When evaluating these data, we noticed small, but significant, effects of certain sample collection parameters on the results: collected blood volume ($A\beta_{1-40}$: Spearman $\rho = 0.098$, coefficient = 2.33, $P < .0001$; $A\beta_{1-42}$: $\rho = 0.063$, coefficient = 0.311, $P = .0033$) and time to freeze ($A\beta_{1-40}$: $\rho = -0.068$, coefficient = –0.13, $P = .0015$; $A\beta_{1-42}$: $\rho = -0.056$, coefficient = –0.034, $P = .0006$). Therefore, all further analyses were adjusted for these variables.

The $A\beta_{1-40}$ and $A\beta_{1-42}$ results were found to be moderately correlated ($\rho = 0.599$, $P < .0001$), and the equation estimated using Deming method to predict $A\beta_{1-42}$ levels from those of $A\beta_{1-40}$ was as follows: $A\beta_{1-42} = 0.152 \times A\beta_{1-40} + 4.9$ pg/mL.

No statistically significant differences were found in plasma levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ and the plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio between the three ADNI diagnosis groups at any of the time points. The average concentrations of $A\beta$ peptides in the diagnosis groups stratified by visit are displayed in Table 3, along with P values for statistical significance. However, both the $A\beta_{1-40}$ and the $A\beta_{1-42}$ results (but not the $A\beta_{1-42}/A\beta_{1-40}$ ratio) were found to be significantly correlated with time, as shown in Table 3. Spearman correlation coefficients were found to be 0.182 ($P < .0001$) for time and $A\beta_{1-40}$ levels, 0.226 ($P < .0001$) for time and $A\beta_{42}$ levels, and –0.039 ($P = .053$) for time and the $A\beta_{1-42}/A\beta_{1-40}$ ratio.

3.3. Correlations with CSF data

We have extended and modified the evaluation of plasma $A\beta$ correlations with CSF biomarkers performed by Toledo et al by incorporating additional CSF biomarkers (p-tau₁₈₁ and p-tau₁₈₁/ $A\beta_{1-42}$ ratio), by using longitudinal CSF data in addition to the baseline data, and by using additional adjustment factors.

When using the “baseline” visit data with 359 results (each for different patient), the results were adjusted for patient's gender, age, and the APOE ϵ allele count, which were previously found to be significantly associated with plasma $A\beta$ levels [16], and additionally adjusted for collected blood volume and time to freeze, which retained statistical significance in this setting. We have found multiple significant correlations: plasma $A\beta_{1-42}$ was correlated to CSF p-tau₁₈₁ ($\rho = -0.180$, $P = .0006$), CSF p-tau₁₈₁/ $A\beta_{1-42}$ ratio ($\rho = -0.171$, $P = .0011$),

CSF tau/A β_{1-42} ratio ($\rho = -0.137$, $P = .009$), CSF tau ($\rho = -0.116$, $P = .028$), and CSF A β_{1-42} ($\rho = 0.110$, $P = .036$). Plasma A β_{1-40} was significantly correlated to the CSF p-tau₁₈₁ ($\rho = -0.149$, $P = .0046$) and CSF p-tau₁₈₁/A β_{1-42} ratio ($\rho = -0.130$, $P = .013$). The A β_{1-42} /A β_{1-40} ratio was significantly correlated to CSF tau/A β_{1-42} ratio ($\rho = -0.127$, $P = .016$), CSF tau ($\rho = -0.121$, $P = .021$), and CSF p-tau₁₈₁/A β_{1-42} ratio ($\rho = -0.112$, $P = .034$).

Using the mixed-effects model of the longitudinal data, we have evaluated these correlations in a multivariate way. There were a total of 140 patients having multiple results for both plasma and CSF studies at different points in time (404 total data points). Plasma A β_{1-42} was found to be significantly correlated to the CSF p-tau₁₈₁/CSF A β_{1-42} ratio ($P = .0011$) and the CSF p-tau₁₈₁ ($P = .0234$), and borderline significantly correlated to the CSF A β_{1-42} ($P = .0432$). Interestingly, A β_{1-40} levels and A β_{1-42} /A β_{1-40} ratio were not found to be significantly correlated to any of the CSF biomarkers using this approach.

Applying the autopsy-derived threshold values, we additionally used the CSF biomarker data from baseline visit to divide the plasma results into two groups [1]. We have used the following thresholds for CSF biomarkers: 192 pg/mL for A β_{1-42} , 93 pg/mL for tau, 23 pg/mL p-tau₁₈₁, 0.39 for tau/A β_{1-42} ratio, and 0.1 for p-tau₁₈₁/A β_{1-42} ratio [1]. For the 359 available data points, there were no significant differences in plasma A β_{1-40} levels for any of the CSF biomarkers. For plasma A β_{1-42} levels, the most significant differences were found between groups stratified by CSF A β_{1-42} levels (38.6 ± 12.06 pg/mL in the “above” group and 35.1 ± 10.46 pg/mL in the “below” group, $P = .0053$) and CSF p-tau₁₈₁/A β_{1-42} ratio (35.2 ± 10.45 pg/mL in the “above” group and 39.1 ± 12.34 pg/mL in the “below” group, $P = .0029$). Also significant differences were found between groups divided by CSF tau levels (34.4 ± 10.47 pg/mL in the “above” group and 37.5 ± 11.40 pg/mL in the “below” group, $P = .0168$), CSF tau/A β_{1-42} ratio (35.3 ± 10.36 pg/mL in the “above” group and 37.9 ± 12.15 pg/mL in the “below” group, $P = .0202$), and CSF p-tau₁₈₁ levels (35.3 ± 10.49 pg/mL in the “above” group and 38.0 ± 12.04 pg/mL in the “below” group, $P = .0210$). Significant differences were also found for the plasma A β_{1-42} /A β_{1-40} ratio when the groups were stratified by CSF tau/A β_{1-42} ratio (0.251 ± 0.082 in the “above” group vs 0.271 ± 0.082 in the “below” group, $P = .003$), CSF A β_{1-42} levels (0.271 ± 0.083 in the “above” group versus 0.253 ± 0.082 in the “below” group, $P = .008$), and CSF p-tau₁₈₁ levels (0.253 ± 0.083 in the “above” group versus 0.268 ± 0.080 in the “below” group, $P = .012$).

Drilling down these correlations, we have evaluated the by-CSF-threshold division separately in each diagnosis group. Again, there were no statistically significant differences in plasma A β_{1-40} levels in any of the groups when stratified by any of the CSF biomarker thresholds. Interestingly, however, significant differences for A β_{1-42} levels according to CSF biomarker thresholds were found exclusively in the MCI group, and not in the AD or NL groups. The plasma A β_{1-42} levels stratified by CSF biomarker thresholds in the MCI group ($n = 164$) are displayed in Table 4, along with corresponding P values from comparisons of the “above-” and “below-threshold” groups. Additionally, Fig. 6 displays the plasma A β_{1-42} levels in the MCI group, stratified by the thresholds of CSF A β_{1-42} levels and the CSF p-tau₁₈₁/A β_{1-42} ratio.

For the plasma A β_{1-42} /A β_{1-40} ratio, the by-CSF-threshold evaluation separately for diagnostic groups revealed several significant differences between the AD and MCI diagnosis groups. In the AD group, the A β_{1-42} /A β_{1-40} ratio was different when stratified by the CSF tau/A β_{1-42} ratio (0.249 ± 0.068 in the “above-threshold” group versus 0.291 ± 0.046 in the “below-threshold” group, $P = .013$) and CSF A β_{1-42} levels (0.286 ± 0.039 in the “above-threshold” group vs 0.251 ± 0.068 in the “below-threshold” group, $P = .024$). In the MCI group, significant difference in the A β_{1-42} /A β_{1-40} ratio was found when stratified

by CSF p-tau₁₈₁ levels (0.247 ± 0.088 in the “above-threshold” group versus 0.262 ± 0.078 in the “below-threshold” group, $P = .030$).

4. Discussion

In this study, we developed an improved protocol for analysis of A β _{1–40} and A β _{1–42} levels in human plasma and applied it to evaluate a cohort of 722 longitudinally followed patients from the ADNI study. We have automated the major steps of the procedure, as schematically depicted in Fig. 2, which enabled us to achieve substantial improvements in the precision and repeatability of the method.

As shown in Table 1, the precision of the calibrators for A β _{1–42} levels was highest for the nominal concentrations in the range from 5 to 530 pg/mL and lower for the 1.25- to 2.5-pg/mL calibrators, whereas for A β _{1–40}, it was best at the low limit of concentrations and more than 20% at the high end. However, the majority of measured results (95% CI: 18.3–85.4 pg/mL for A β _{1–40} and 5.97–20.7 pg/mL for A β _{1–42}) were between the concentrations of St6 and St3, in the area of rising slope on the log concentration plot (Fig. 3). Therefore, in both cases, the measured results were within a well-defined part of the calibration curve, although the calibration ranges were exceedingly broad at the upper end.

The described procedure enabled us to achieve excellent kit control sample precision for A β _{1–42} levels (3.4%–4.6%); the precision for A β _{1–40} levels was also well within the acceptable range (5.2%–11.3%) [15], especially considering the fact that the “high” kit control sample ConB for A β _{1–40} had an average concentration between calibrators “St1” and “St2”—the least precise calibrators for this biomarker. Other authors using the same assay have reported the average between-run precision of 9.3% for A β _{1–42} and 9.9% for A β _{1–40} results [2]. Between-run precision for another type of assay (enzyme-linked immunosorbent assay) was reported to be 17.3% on average [17]. Thus, the procedure described here provides a considerable improvement in measurement precision.

However, kit control samples are spiked samples prepared using an artificial aqueous matrix, and their performance does not always reflect the actual performance of the assay on patient samples. A better performance measure for the method was, therefore, reflected by the QC1 and QC2 samples that were plasma samples obtained from volunteers. Both of these samples had excellent precision ranging from 4.1% to 7%—on par with many mass spectrometry methods for much simpler analytes. With the exception of one run, as can be seen in Fig. 4, the results were stable across many runs, spanning approximately 7 weeks of time.

The results of the continuing test-retest evaluation also indicated good performance of the method, with an overall precision of 7.2 %CV for A β _{1–40} and 4.5 %CV for A β _{1–42}—well within the acceptable range of 15 %CV [15]. There was considerably more variability in A β _{1–40} results, as shown in Fig. 5, compared with A β _{1–42}, and A β _{1–40} results additionally showed more bias (on average, –4.1% compared with –22.7% for A β _{1–42}). However, the bias, although statistically significant in cases of both peptides, was rather small in scale and lower than the general between-aliquot variability; therefore, we did not consider it a problem.

Many authors performing meta-analysis of existing plasma A β results have attributed the conflicting reports about the clinical value of plasma A β to general lack of consistency across analytical methods. Therefore, the method presented here is a substantial step toward eliminating this confounding factor. This improved protocol has provided results for 2454 samples from patients participating in the ADNI study. The in-depth analysis of this data set

in the clinical context can be found in the article by Toledo et al [16]. Here we present only a brief summary of the data.

As expected, no significant differences in the $A\beta_{1-40}$ levels, the $A\beta_{1-42}$ levels, and the $A\beta_{1-42}/A\beta_{1-40}$ ratio were found between the clinical diagnostic groups, at any of the time points, as shown in Table 3. We have also studied the rates of the change of $A\beta$ peptides and their ratio across diagnostic groups and found no significant differences (data not shown). Toledo et al have additionally evaluated the progression of the disease and found that plasma $A\beta$ measures have limited value as prognostic factors [16]. However, to rigorously assess the potential utility of plasma $A\beta$ measurements for prediction of progression, it is important to emphasize the need to apply this improved analytical methodology to earlier stages of MCI, which this study did not include.

However, there is a general steady increase of the $A\beta_{1-40}$ and $A\beta_{1-42}$ levels with time [16], a fact reported previously by others although with less detail and statistical power [17,18]. The mixed-effects modeling of our data revealed that the age has both a “between-patient” and the “within-patient” effect. At the individual patient level, although peptide concentrations decreased in some patients, with some having too much variability to determine the trend, the concentrations increased in a majority of patients with time. In general, older patients in our group had significantly higher $A\beta$ levels than younger patients, and the effect was strong enough to be detected in our generally well age-matched population. Substantial effect can also be attributed to patient's gender and *APOE* $\epsilon 4$ status, as described elsewhere [16]. Other allele types (2 and 3) did not seem to influence the plasma $A\beta$ levels. In contrast, patients with two *APOE* $\epsilon 4$ alleles seem to have much increased variability of $A\beta_{1-42}/A\beta_{1-40}$ ratio, which is more stable in other patients.

We were able to determine significant correlations between the plasma $A\beta_{1-42}$ levels and the CSF biomarkers: $A\beta_{1-42}$, tau, and p-tau₁₈₁ levels as well as their ratios. Interestingly, these correlations were found exclusively in the MCI group of patients, and not in the AD or NL groups. There currently are very few reports in the literature on this topic, and the reported correlations between plasma and CSF biomarkers are rather weak [5,19]. However, such observations may highly depend on the choice of population, as also indicated by the conflicting reports relating plasma $A\beta$ results to the diagnosis group.

Another interesting fact is that the correlation of baseline plasma $A\beta_{1-42}$ with CSF p-tau₁₈₁ results is much stronger than that with CSF $A\beta_{1-42}$ results. Using common sense, the correlation of the same species in two compartments of the body should be stronger than that of different species. This indicates that the dynamic balance between plasma and CSF biomarkers is very complex, and much more data are needed to characterize this, preferably covering a substantially broader period and spanning across the AD continuum.

Using the CSF biomarker thresholds [1], we found that the $A\beta_{1-42}$ levels in the MCI group were significantly different in the “above” and “below” subgroups when stratified by those thresholds. Nevertheless, as shown in Fig. 6, even for the strongest stratification by CSF p-tau₁₈₁/ $A\beta_{1-42}$ ratio, the difference in plasma $A\beta_{1-42}$ levels was small between the “below”- and “above”-threshold groups. Using receiver operating characteristic analysis of the plasma $A\beta_{1-42}$ levels, we were able to predict to which CSF p-tau₁₈₁/ $A\beta_{1-42}$ group a patient belongs only with 63% specificity and 55% selectivity at best. Therefore, plasma $A\beta$ measurements are not yet suitable as a diagnostic test. Moreover, as in the case of univariate correlations, the association between CSF and plasma $A\beta_{1-42}$ levels was weaker than that of other CSF biomarkers when evaluated by CSF threshold stratification.

The plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio, in contrast to many reports in the literature, did not turn out to be a good candidate for a biomarker in our study [20,21]. According to our data, the

association of this ratio with various patient characteristics and CSF data was relatively weak.

Perhaps a diagnostic modifier could help extract more value from plasma A β measurements. In a recent animal model study, Takeda et al [4] reported that plasma A β levels respond differently to an oral glucose loading dose in mice with and without AD. Other authors have suggested other modifiers that could potentially be used to dynamically alter plasma A β levels. If these methods prove successful, plasma A β_{1-42} level could potentially emerge as a valuable screening biomarker to be used before considering CSF testing, as it is a simpler and less invasive procedure.

Nevertheless, despite the current moderate diagnostic utility of this protocol, it also has practical utility in the determination of drug effect of anti-A β therapies. One report showed the effect of an investigational drug on plasma A β levels in a range from -75% to +150% throughout 24 hours [22]. Our protocol is perfectly suited for this type of study because of its high level of automation and high throughput, and it will allow researchers to determine A β concentrations with the greatest possible precision.

5. Conclusions

Using our robotic pipetting platform, we were able to substantially improve the analytical performance of the plasma A β_{1-40} and A β_{1-42} assay, eliminating a substantial amount of interassay variability. The excellent performance of our method was demonstrated using plasma quality control samples obtained from volunteers and run repeatedly across many plates throughout the study, as well as through retesting of the study patients' samples in every run. This improved protocol significantly reduces the effect of an important confounding factor that contributes to the lack of consistency between reports on plasma A β levels.

Using the described method, we processed 2454 samples from the ADNI study patients. We have determined significant correlations of the obtained results with sample collection parameters: time of storage at room temperature (time to freeze) and the volume of collected blood. We have confirmed significant correlations of plasma A β levels with age and gender and also with the *APOE* $\epsilon 4$ allele. Additionally, we have determined significant correlations between plasma A β levels and CSF biomarker levels and thresholds. However, these correlations are not strong enough to support research use as a diagnostic screening test at this point. Nevertheless, this biomarker test is well suited for use as a pharmacodynamic marker for therapeutic agents that alter the biosynthesis of A β or its clearance.

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Fig. 1.
Schematic diagram of the ADNI biomarker specimen collection, bioanalytical procedures, and data flow.

Fig. 2. Schematic diagram of the analytical procedure. The steps inside the box are performed by the epMotion 5075 robotic pipettor.

Fig. 3. Summary log concentration versus MFI plots of the calibration curves for the 76 runs and box-and-whisker plots of the results. Solid thick line on the top of the individual run lines represents median.

Fig. 4. Stability plots for kit control samples (ConA and ConB) and for two human plasma controls (QC1 and QC2b). Thick solid line represents the average concentration, and dark and light shaded areas around the mean represent ± 1 standard deviation and ± 2 standard deviations, respectively.

Fig. 5.

Passing–Bablok regression and Bland–Altman plots for the test–retest experiment. On the Passing–Bablok regression plots, the thick solid line represents the regression line, the dashed line is the line of identity, and the shaded area is the 95% confidence interval for the fit. In the Bland–Altman plots, the thick solid line represents the average difference between test and retest results, whereas dashed lines are drawn at ± 1.96 standard deviation (95% confidence interval). Results are expressed as picograms per milliliter.

Fig. 6. Box-and-whisker plots with superimposed aligned dot plots of plasma Aβ₁₋₄₂ levels stratified by cerebrospinal fluid thresholds.

Table 1

Summary of calibration information

Standard	<u>Aβ₁₋₄₀</u>				<u>Aβ₁₋₄₂</u>			
	Nominal (pg/mL)	Mean $\pm$ SD (pg/mL)	Precision (%)	Percent inaccuracy	Nominal (pg/mL)	Mean $\pm$ SD (pg/mL)	Precision (%)	Percent inaccuracy
St1	1256	1046 $\pm$ 224	21.4	16.75	530	521 $\pm$ 26.3	5.06	1.78
St2	438	556 $\pm$ 93.0	16.7	-27.01	180	187 $\pm$ 9.66	5.15	-4.08
St3	198	176 $\pm$ 18.5	10.5	11.04	73	70.8 $\pm$ 3.34	4.72	3.05
St4	58	59.2 $\pm$ 3.61	6.10	-2.14	30	29.6 $\pm$ 0.98	3.30	1.44
St5	32	32.4 $\pm$ 1.94	5.98	-1.25	12	12.9 $\pm$ 0.47	3.67	-7.24
St6	15	14.5 $\pm$ 0.79	5.43	3.13	5	4.65 $\pm$ 0.26	5.53	7.10
St7	7.5	7.66 $\pm$ 0.41	5.33	-2.14	2.5	2.65 $\pm$ 0.21	7.99	-5.87
St8	3.75	3.72 $\pm$ 0.15	3.95	0.69	1.25	1.31 $\pm$ 0.20	15.0	-4.45

Abbreviations: A β , β -amyloid; SD, standard deviation.

Summary for 54 runs (of 78) performed with St7 and St8 as subsequent 1:2 dilutions of St6.

Table 2

Quality control sample summary

Sample	Aβ ₁₋₄₀			Aβ ₁₋₄₂		
	n	Mean ± SD (pg/mL)	CV (%)	n	Mean ± SD (pg/mL)	CV (%)
ConA	78	52.5 ± 2.72	5.19	78	179 ± 8.28	4.62
ConB	78	506 ± 57.3	11.3	78	33.4 ± 1.14	3.42
QC1	75	63.8 ± 4.49	7.04	75	14.7 ± 0.60	4.09
QC2a	42	57.1 ± 3.15	5.52	42	15.9 ± 0.82	5.15
QC2b	39	38.7 ± 2.59	6.70	39	7.73 ± 0.39	5.08

Abbreviation: CV, coefficient of variation.

“ConA” and “ConB” are kit controls prepared in artificial matrix by the kit manufacturer. “QC1,” “QC2a,” and “QC2b” are human plasma samples obtained from volunteer donors.

Table 3

Average biomarker values for each diagnosis group stratified by visit

Visit	Group	n	$A\beta_{1-40}$		$A\beta_{1-42}$		$A\beta_{1-42}/A\beta_{1-40}$	
			Mean $\pm$ SD (pg/mL)	P value	Mean $\pm$ SD (pg/mL)	P value	Mean $\pm$ SD (–)	P value
BL	NL	206	151 $\pm$ 48.6	.998	37.7 $\pm$ 12.1	.290	0.266 $\pm$ 0.080	.165
	MCI	350	151 $\pm$ 53.9		36.3 $\pm$ 11.9		0.261 $\pm$ 0.102	
	AD	162	152 $\pm$ 46.7		36.4 $\pm$ 10.4		0.249 $\pm$ 0.059	
M12	NL	201	164 $\pm$ 45.2	.830	39.5 $\pm$ 11.2	.863	0.249 $\pm$ 0.058	.373
	MCI	348	166 $\pm$ 46.0		39.7 $\pm$ 11.1		0.252 $\pm$ 0.090	
	AD	155	162 $\pm$ 42.4		39.3 $\pm$ 9.89		0.250 $\pm$ 0.051	
M24	NL	183	164 $\pm$ 43.8	.117	40.8 $\pm$ 10.4	.774	0.258 $\pm$ 0.067	.193
	MCI	286	173 $\pm$ 43.6		41.5 $\pm$ 10.5		0.252 $\pm$ 0.092	
	AD	120	167 $\pm$ 35.5		40.1 $\pm$ 8.98		0.246 $\pm$ 0.046	
M36	NL	167	173 $\pm$ 39.3	.644	41.8 $\pm$ 9.72	.602	0.252 $\pm$ 0.062	.204
	MCI	213	177 $\pm$ 44.8		41.8 $\pm$ 11.1		0.247 $\pm$ 0.080	
	AD	9	190 $\pm$ 52.6		39.8 $\pm$ 8.76		0.219 $\pm$ 0.051	
M48	NL	3	207 $\pm$ 43.1	.448	42.0 $\pm$ 10.6	.507	0.204 $\pm$ 0.035	.555
	MCI	2	185 $\pm$ 30.1		44.1 $\pm$ 2.75		0.241 $\pm$ 0.024	
	AD	2	177 $\pm$ 23.3		41.4 $\pm$ 0.25		0.235 $\pm$ 0.032	

Abbreviations: NL, cognitively normal; MCI, mild cognitive impairment; AD, Alzheimer's disease.

Table 4Plasma A β_{1-42} values stratified by CSF biomarker thresholds in the MCI diagnosis group

CSF biomarker (cutoff)	Above			Below			P value
	n	Mean $\pm$ SD (pg/mL)	95% confidence interval (pg/mL)	n	Mean $\pm$ SD (pg/mL)	95% confidence interval (pg/mL)	
A β_{1-42} (192)	45	40.1 $\pm$ 11.9	20.8–59.4	124	34.5 $\pm$ 10.3	11.3–55.5	.0064
Tau (93)	71	32.9 $\pm$ 10.8	10.1–56.6	98	38.3 $\pm$ 10.7	20.3–58.9	.0026
p-tau ₁₈₁ (23)	118	34.2 $\pm$ 10.5	10.8–55.8	51	40.3 $\pm$ 11.1	21.3–59.1	.0008
Tau/A β_{1-42} (0.39)	115	34.4 $\pm$ 10.4	10.8–56.1	54	39.4 $\pm$ 11.6	20.2–59.0	.0069
p-tau ₁₈₁ /A β_{1-42} (0.1)	130	34.3 $\pm$ 10.2	12.1–55.4	39	41.7 $\pm$ 11.7	21.4–59.7	.0004

Abbreviation: CSF, cerebrospinal fluid.