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Qualification of the analytical and clinical performance of CSF biomarker analyses in ADNI

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Conflict of interest

Leslie Shaw has served on technical advisory boards for Bristol Myers Squibb and Innogenetics; Piotr Lewczuk is a consultant to Innogenetics. Kaj Blennow has served on a technical advisory board for Innogenetics. No conflicts of interest declared by the other authors of this manuscript.

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Alzheimer's Disease Neuroimaging Initiative

Abstract

The close correlation between abnormally low pre-mortem cerebrospinal fluid (CSF) concentrations of amyloid-β1-42 ($A\beta_{1–42}$) and plaque burden measured by amyloid imaging as well as between pathologically increased levels of CSF tau and the extent of neurode-generation measured by MRI has led to growing interest in using these biomarkers to predict the presence of AD plaque and tangle pathology. A challenge for the wide-spread use of these CSF biomarkers is the high variability in the assays used to measure these analytes which has been ascribed to multiple pre-analytical and analytical test performance factors. To address this challenge, we conducted a seven-center inter-laboratory standardization study for CSF total tau (t-tau), phospho-tau (p-tau_{181}) and $A\beta_{1–42}$ as part of the Alzheimer's Disease Neuroimaging Initiative (ADNI). Aliquots prepared from five CSF pools assembled from multiple elderly controls (n = 3) and AD patients (n = 2) were the primary test samples analyzed in each of three analytical runs by the participating laboratories using a common batch of research use only immunoassay reagents (INNO-BIA AlzBio3, xMAP technology, from Innogenetics) on the Luminex analytical platform. To account for the combined effects on overall precision of CSF samples (fixed effect), different laboratories and analytical runs (random effects), these data were analyzed by mixed-effects modeling with the following results: within center %CV 95% CI values (mean) of 4.0–6.0% (5.3%) for CSF $A\beta_{1–42}$; 6.4–6.8% (6.7%) for t-tau and 5.5–18.0% (10.8%) for p-tau_{181} and inter-center %CV 95% CI range of 15.9–19.8% (17.9%) for $A\beta_{1–42}$, 9.6–15.2% (13.1%) for t-tau and 11.3–18.2% (14.6%) for p-tau_{181}. Long-term experience by the ADNI biomarker core laboratory replicated this degree of within-center precision. Diagnostic threshold CSF concentrations for $A\beta_{1–42}$ and for the ratio t-tau/$A\beta_{1–42}$ were determined in an ADNI independent, autopsy-confirmed AD cohort from whom ante-mortem CSF was obtained, and a clinically defined group of cognitively normal controls (NCs) provides statistically significant separation of those who progressed from MCI to AD in the ADNI study. These data suggest that interrogation of ante-mortem CSF in cognitively impaired individuals to determine levels of t-tau, p-tau_{181} and $A\beta_{1–42}$, together with MRI and amyloid imaging biomarkers, could replace autopsy confirmation of AD plaque and tangle pathology as the "gold standard" for the diagnosis of definite AD in the near future.

Keywords

Alzheimer's Disease Neuroimaging Initiative; Cerebrospinal fluid; Amyloid-β1-42; Total tau; p-tau_{181}; Interlaboratory study; Mixed-effects modeling

Introduction

Measurement of the CSF concentrations of $A\beta_{1–42}$, t-tau and p-tau_{181} [using xMAP® (multi-analyte) or ELISA (single analyte)-based methods] has been shown to provide at least

85% sensitivity and 80% specificity for diagnosing AD, predicting conversion from MCI to a diagnosis of probable AD, and identifying elderly clinical dementia rating (CDR) scale 0 individuals likely to progress to CDR > 0 [7, 10, 12, 17, 18, 25, 36, 37, 42]. Changes in CSF $A\beta_{1–42}$, t-tau and p-tau_{181} concentrations reflect on-going amyloidopathy ($A\beta_{1–42}$) and tauopathy as well as damage to neurons (t-tau and p-tau_{181}) in the brain [4, 15, 16, 22, 38, 40]. It is widely recognized that excessive analytical variability across laboratories and across time limits the potential use of these measures to assess the effects of candidate therapies on AD pathology in longitudinal, large world-wide clinical trials [8, 23, 24, 44]. ADNI is specifically designed to define longitudinal changes in imaging, biological markers, clinical and neuropsychological measurements in NCs as well as subjects who have late MCI or early AD to accelerate efforts to develop new treatments and monitor their effectiveness as well as lessen the time and cost of clinical trials. In order to accomplish this challenging goal the ADNI study adopted standardized biochemical and imaging biomarker methods in hopes of improving the ability to optimally detect the natural progression of neurodegenerative processes inherent in AD. Besides development of assays for novel analytes, the University of Pennsylvania (UPenn) ADNI Biomarker Core laboratory has thoroughly studied measurement of $A\beta_{1–42}$, t-tau and p-tau_{181} in highly clinically annotated ADNI CSF samples to identify and attempt to control key sources of analytical variability.

This paper describes the analytical qualification of the INNO-BIA AlzBio3 immunoassay for use by the UPenn Biomarker Core for analyses of CSF samples collected from ADNI subjects. The multiplex xMAP Luminex platform (Luminex Corp, Austin, TX, USA) with Innogenetics (INNO-BIA AlzBio3, Ghent, Belgium) immunoassay reagents are for research use only. At first, different aspects of the analytical precision of the assays were evaluated including implementation of an inter-center qualification study (7 centers, including academic centers and industrial partners) to document the reproducibility and repeatability of the immunoassay using pooled CSF samples ($n = 5$) and aqueous validation samples ($n = 3$), evaluation of the long-term stability of CSF pools, and test/re-test analytical performance over time during the analyses of ADNI CSF samples. Second, the predictive performance of these CSF biomarkers was assessed for risk of progression from MCI to AD dementia. Finally, we discuss recognized pre-analytical factors that affect the results of these biomarker measurements.

Materials and methods

Data used in the preparation of this manuscript were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://www.loni.ucla.edu/ADNI). The ADNI was launched in 2004 by the National Institute on Aging (NIA), the National Institute of Biomedical Imaging and Bioengineering (NIBIB), the Food and Drug Administration (FDA), private pharmaceutical companies and non-profit organizations, as a $60 million, 5-year public–private partnership. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early Alzheimer's disease (AD). Determination of sensitive and specific markers of very early AD progression is intended to aid researchers and clinicians to develop new treatments and monitor their effectiveness, as well as lessen the time and cost of clinical trials. The principal investigator of this initiative is Michael W. Weiner, MD, VA Medical Center and University of California-San Francisco. ADNI is the result of efforts of many co-investigators from a broad range of academic institutions and private corporations, and subjects have been recruited from over 50 sites across the U.S. and Canada. The initial goal of ADNI was to recruit 800 adults, aged 55–90, to participate in the research—approximately 200 cognitively normal older individuals to be followed for 3

years, 400 people with MCI to be followed for 3 years and 200 people with early AD to be followed for 2 years. For up-to-date information, see http://www.adni-info.org.

xMAP platform

Each participating center in this inter-laboratory study used either the Luminex 100 IS (Luminex Corp, Austin, TX, USA) (4 centers) or BioPlex 100 immunoassay platform (Bio Rad, Hercules, CA, USA) (3 centers) with the same Luminex analytical platform but different customized software packages for instrument operation. The selection of the system was at the discretion of each participant and was not considered a variable in the study protocol. The xMAP Luminex technology is a flow cytometric method that allows simultaneous detection of several analytes on different sets of microspheres in a single well. Each set of microspheres has embedded a precise concentration ratio of red- and infrared fluorochromes, resulting in unique spectral identities. This allows flow cytometric discrimination of mixed microsphere sets [14, 28].

The INNO-BIA AlzBio3 immunoassay kits

The full details of the implementation of the INNO-BIA Alz Bio3 immunoassay reagents on the Luminex analytical platform are described elsewhere [29, 42]. Monoclonal antibodies (mAbs) are used in this assay, and the production process for the immunoassay kits includes in current production processes assurance of lot-to-lot consistency. These tests are relative quantitative assays for CSF $A\beta_{1–42}$, t-tau and p-tau_{181} since no international reference standards for the analytes prepared in CSF are available. Each participating center used the same INNO-BIA AlzBio3 immunoassay kit (assay lot # 157353 and calibrator lot # 157379), provided for the study by Innogenetics, Ghent, Belgium. The kit reagents include a mixture of three xMAP color-coded carboxylated microspheres, each containing a bead set coupled with well-characterized capture mAbs specific for $A\beta_{1–42}$ (4D7A3; bead region 56), t-tau (AT120; bead region 2) or p-tau_{181} (AT270; bead region 69), and a vial with analyte-specific biotinylated detector mAbs (3D6 for $A\beta_{1–42}$ and HT7 for t-tau or p-tau_{181}). Ready-to-use vials containing pre-determined calibrator concentrations for the three analytes were provided. Calibration curves were produced for each biomarker using aqueous buffered solutions that contained the combination of three bio-markers at concentrations ranging from 56 to 1,948 pg/mL for recombinant t-tau, 27–1,574 pg/mL for synthetic $A\beta_{1–42}$ and 8–230 pg/mL for a synthetic tau peptide phosphorylated at the threonine 181 position (the p-tau_{181} standard; numbering according to the longest tau isoforms [13]). In addition to the calibrators, the immunoassay kit includes two quality control samples, produced in aqueous diluent, with pre-defined acceptable concentration ranges for the three biomarkers.

The assay procedure

A mixture of the microspheres were added to individual wells of 96-well plates followed by addition of 75 μL of sample (pooled CSF, calibrators, quality control samples) together with 25 μL of a mixture of the biotinylated detector mAbs (Conjugate 1). After an overnight incubation (protected from light; continuous shaking), at room temperature, the antigen–antibody complex was detected by a phycoerythrin-labeled streptavidin conjugate (Detection conjugate). Following a wash step, the signal associated with each analyte was measured by analyzing the resulting bead-capture antibody-analyte-detection antibody-detection conjugate complexes on the Luminex or BioPlex systems. The analyte-specific capture antibodies covalently coupled to spectrally unique microspheric beads provide analytical specificity. The analyte concentration is related to the fluorescence intensity derived from complexed detection conjugates.

Inter-lab study design

Aqueous calibrators and controls are included in the immunoassay kit. Analyses were also performed on separate aqueous validation and human subject CSF pool samples (generated by using residual CSF samples obtained from individuals who were not part of the ADNI cohort). All calibrators, controls and additional samples were analyzed in duplicate in each run. A result (a reported value) was defined as the arithmetic mean of duplicate results. For each of the five CSF pool aliquots, there were two sets of duplicates, i.e., there were four replicates for each of these samples. Each participating laboratory performed three analytical runs using separate frozen, not previously thawed aliquots of each of the three aqueous validation and five CSF pools in each run. The aqueous samples, X-1, X-2 and X-3, were prepared by Innogenetics and contained different concentrations of reference materials [(synthetic peptides, $A\beta_{1–42}$ and p-tau_{181}) or recombinant protein (total tau)] spiked into diluent. Five CSF pools were prepared at the UPenn ADNI Biomarker Core laboratory. Three of the CSF pools, X-41, X-42 and X-43, were prepared from excess routine clinic patient samples, and they also were included in the pre-qualification study. Two other CSF pools, X-40 and X-44, were prepared from aliquots collected from AD patients who were not in the ADNI cohort. The homogeneity of aliquots prepared from the CSF pools was verified prior to the start of the study. The aqueous run validation and the CSF pool aliquots were stored in labeled polypropylene tubes at −80°C. Three each of these aliquots were shipped on dry ice to each participating center with a temperature sensor attached to each shipment container to provide assurance of maintenance of temperature during transit.

For each of the three analytical runs, a test protocol was followed and checklist information provided to assure compliance with test procedure steps and conditions by the seven participating laboratories. The full details of the test protocol are available as an appendix to the report on this seven-center interlaboratory study on the ADNI website at:http://adni-info.org/Scientists/ADNIScientistsHome.aspx.

Statistical analyses

The databanks for this study were prepared in the UPenn ADNI Biomarker Core laboratory and at Innogenetics. Following comparison of the databanks to assure accuracy of transcription of the raw data from each center, the databanks were locked, and statistical analyses performed at both the UPenn ADNI Biomarker Core laboratory and at Innogenetics. Statistical analysis and modeling were performed using ‘R’ software version 2.9.1 [32]. The between-Center and within-Center sources of variation of the results were evaluated using Mixed-Effects Modeling methodology with the ‘lme4’ package for ‘R’ [2]. The general Mixed-Effects Model was defined as: “Concentration ~ Sample + (Sample| Center\Run)”, where ‘Concentration’ is a function of ‘Sample’ (fixed effect), with random effects of ‘Center’ and ‘Run’, estimated for each sample separately.

Biomarker result acceptance criteria

Result acceptance criteria defined prior to study performance included: (1) microsphere (“bead”) counts >50 for a single sample and (2) %CV < 25% for duplicates. Thus, if one of four replicates of a CSF pool had a bead count <50, only that single result was removed, and the remaining three were retained. When the %CV of the biomarker concentration for a pair of results was >25%, that pair was removed, but the remaining pair of quadruplicate results was retained. A checklist was provided to each participant in order to identify possible root causes for the observed differences.

Results

Calibration curves

In order to limit variability related to software and/or curve fit algorithms, curve fitting was performed in an excel format using a four parametric logistic (4-PL) regression model. During the development phase of the immunoassay kit, accuracy plots revealed no improvements when five-parameter curve fit models were applied to the data. The resulting mean ± SD for r^2 for the curve fit to the calibration data, using the four-parameter curve fitting program, across the seven participating laboratories (n = 21 analytical runs) were, respectively, for each bio-marker: 0.998 ± 0.00243 for $A\beta_{1–42}$; 0.999 ± 0.00116 for t-tau; and 0.998 ± 0.00189 for p-tau_{181}. The mean ± SD pg/mL values for back-calculated calibrator concentrations for the 21 analytical runs across the seven centers were as follows: for $A\beta_{1–42}$ 49 (48 ± 4.4), 97 (95 ± 4.5), 209 (213 ± 6.4), 465 (461 ± 9.2), 980 (984 ± 10.4) and 1,960 (1,957 ± 8.0); for t-tau 28 (24 ± 7.8), 65 (66 ± 2.3), 136 (137 ± 4.7), 334 (333 ± 3.3), 739 (740 ± 2.7) and 1,699 (1,698 ± 2.9); for p-tau_{181}: 15 (16 ± 1.2), 26 (26 ± 0.6), 47 (46 ± 1.2), 82 (83 ± 0.8), 147 (147 ± 0.2), 265 (265 ± 0.026).

Biomarker concentration outliers in CSF pool samples

For the acceptance criterion of bead counts >50, three single replicate results did not meet this criterion for CSF pool samples, one $A\beta_{1–42}$, one t-tau and one p-tau_{181} for pools X-40, X-42 and X-43. Thus, a total of nine replicate sample results did not meet this criterion. Two centers experienced this problem: six of these nine out-lier results occurred in one run at one center, and three of these nine outliers occurred in one run at another center. All of these nine outlier results were bead counts of 15 (n = 1), or lower [12 (1), 10 (1), 7 (3), 6 (1), 5 (1) or 2 (1)] and likely due to “leaky” plate wells. For the %CV of paired replicate results, three $A\beta_{1–42}$, two p-tau_{181} and eight t-tau pairs failed this criterion at three centers, one of which also had bead count failures. Thus, there were a total of 13 %CV result failures that involved three centers. Three centers had no result failures for either the bead counts or the %CV criteria. Since these acceptance criteria were established prior to conduct of the inter-laboratory study, the bead count (n = 9) and %CV (n = 13) results that did not meet the respective criteria for inclusion in this study were removed from the precision analyses summarized below.

Within- and between-center precision

Figure 1a–c summarizes the within-center precision (%CV) data for $A\beta_{1–42}$, t-tau and p-tau_{181} by center and by sample type (2 aqueous kit controls, 3 aqueous validation samples, 5 CSF pools).

CSF pools—Table 1 summarizes the within-center precision data for the five CSF pools. Analysis of this data set using mixed-effects modeling resulted in the following within center 95% CI values: 4.0–6.0% (5.3 and 5.4% mean and median values, respectively) for CSF $A\beta_{1–42}$; 6.4–6.8% (6.7% mean and median) for t-tau and 5.5–18.0% (10.8 and 11% mean and median, respectively) for p-tau_{181}. The corresponding inter-laboratory reproducibility for the CSF pools was in the range of 15.9–19.8% (mean and median values of 17.9 and 18.8%) for $A\beta_{1–42}$, 9.6–15.2% (13.1 and 13.6%) for t-tau and 11.3–18.2% (14.6 and 13.9%) for p-tau_{181}.

Aqueous control samples—The within center precision values for the aqueous kit controls and aqueous validation samples were 2.4–22.8% (mean and median values of 8.4% and 7.4%) for $A\beta_{1–42}$, from 0.3 to 13.9% (mean and median values of 5.0% and 4.4%) for t-tau and from 0.1 to 9.1% (mean and median values of 3.4 and 2.8%) for p-tau_{181}.

The inter-laboratory reproducibility values for the aqueous samples X-1, X-2 and X-3 were 0.01–15.7% (mean and median values 6.0 and 7.2%) for $A\beta_{1–42}$; 0.02–8.0% (mean and median 4.5 and 3.8%) for t-tau; and 0.17–2.9% for p-tau_{181} (mean and median 1.3 and 1.1%).

These results reveal better within- and between-center reproducibility for t-tau and p-tau_{181} obtained using aqueous controls compared to performance using CSF pools, but somewhat poorer reproducibility for $A\beta_{1–42}$ in aqueous samples probably reflecting greater difficulties in preparation of homogeneous solutions of $A\beta_{1–42}$ relative to tau in aqueous solutions.

Measured biomarker bias for CSF pools at each center

Assessment of the bias from the overall mean values obtained for $A\beta_{1–42}$, t-tau and p-tau_{181} for the five CSF pools is summarized in Fig. 2a–c. These are graphical summaries of the center-to-center variations for CSF pools in the form of percent deviation from the grand mean (mean value across the centers) of each pool at each center. The average center-to-center percent deviation at centers I through VII, respectively, was: for $A\beta_{1–42}$ = 10.7, −14.7, −6.6, 34.3, −16.0, −8.9 and 1.2%; for t-tau = −7.2, −3.0, −3.6, 25.5, −15.4, −7.5 and 11.2%; and for p-tau_{181} = 6.6, −0.9, −8.8, 5.4, −23.6, 29.3 and −7.8%. The average center-to-center bias for the five aqueous control samples was noticeably lower for each biomarker as compared to CSF: the average center-to-center percent deviation from the overall mean value for the aqueous control samples at centers I through VII, respectively, was: for $A\beta_{1–42}$ = 0.07, −6.68, −1.39, 9.54, −2.79, −2.74 and 3.98%; for t-tau = 0.99, −1.04, −0.57, 7.08, −4.38, −3.14 and 1.07%; for p-tau_{181} = −0.57, 0.15, 0.17, −0.05, −0.04, 0.26 and 0.08%. These data show closer agreement for each center with the overall mean values for each of the three biomarkers measured in the aqueous control samples than achieved with CSF pool samples.

Statistical model for within, between and total precision

Figure 3a–c displays the within-, between-center and total %CV derived from the Mixed Effects statistical model for the data set. It is clear from the figures that between-center effects are the greatest contributor to total variability in the results for the CSF pools for each of the biomarkers with this being greatest for $A\beta_{1–42}$ and least for p-tau_{181}.

Analytical performance in ADNI CSF studies

Between-lot variability of CSF biomarker measurements using three lots of reagents—Following performance of the inter-laboratory study, the UPenn ADNI Biomarker Core laboratory analyzed the ADNI subject BASELINE visit CSF samples [36] and about a year later, simultaneously analyzed never-thawed BASELINE and year 1 visit CSF sample aliquots obtained from the same study subjects (Shaw et al., in preparation, 2011). Both sets of analyses were based on AlzBio3 immunoassay reagents and Luminex platform. During an approximately 2-year period of time, repeated analysis of the ADNI study samples, test samples from other analytical runs unrelated to ADNI and two pools of CSF provided an opportunity to track reproducibility of the method and result stability for the CSF biomarkers using three different lots of AlzBio3 reagents. The resulting quality control data are summarized in Fig. 4a, b. Separate, never-thawed aliquots of CSF pools X-52 and X-45 were included in a total of 51 and 36 analytical runs, respectively. Briefly, these data characterize the stability and precision of this CSF tau and Aβ assay system using the Luminex platform and three different lots of the AlzBio3 immunoassay reagents over a 2-year period. For $A\beta_{1–42}$, the mean concentration ranged from 96 to 100 pg/mL (pool X-45) and 190–218 pg/mL (pool X-52); t-tau, 179–202 pg/mL and 46–52 pg/mL, respectively and p-tau_{181}, 54–64 pg/mL and 11–14 pg/mL, respectively. A comparison of overall mean values for the kit lots used here to the mean values obtained for the single kit

lot with the largest number of analytical runs is summarized in Table 2. These data were further analyzed using a random effects model to assess the relative contributions of within-lot and between-lot variability. Between-lot estimated %CV for pools X-45 and X-52, respectively, were 0 and 6.5% for $A\beta_{1–42}$; 5.4 and 5.4% for t-tau; 6.7 and 10.2% for p-tau_{181}. The within-lot %CV values for pools 45 and 52 were 9.1 and 10.0% for $A\beta_{1–42}$; 7.4 and 11.4% for t-tau; 16.8 and 13.3% for p-tau_{181}. Thus, for each combination of CSF pool and biomarker analyte, the within-lot variance was greater than between lot variance.

Test/re-test performance—Assessment of test/re-test precision for the CSF biomarkers, measured in 118 ADNI CSF sample aliquots, was determined using 2–3 subjects' aliquots, randomly selected from each of 38 analytical runs. For each randomly selected subject, a second never previously thawed aliquot was analyzed in the subsequent run for comparison to the result obtained from the initial aliquot analysis. Linear regression and Bland–Altman bias plot analyses of these data are shown (Fig. 5a–f). For $A\beta_{1–42}$ and t-tau, the test/re-test pairs were highly correlated with respective r^2 values of 0.915 and 0.977 (Fig. 5a, b). Bland–Altman bias analysis showed no evidence of a systematic bias across the range of concentration values obtained for these two bio-markers, and %CV values for the test–retest pairs were 5.7 and 5.6%, respectively (Fig. 5d, e). For p-tau_{181}, the results were more variable: r^2 and %CV values of 0.744 and 11.5% (Fig. 5c, f).

Clinical performance in ADNI CSF studies

Using the analytically qualified Alz Bio3 reagents and the Luminex platform, we developed an AD CSF biomarker profile or signature based on a comparison of results obtained on ante-mortem CSF samples in a cohort of non-ADNI patients with an autopsy-confirmed diagnosis of AD and a clinical cohort of age-matched NCs [36]. In this study, we confirmed the presence of increased t-tau and p-tau_{181} concentrations (2.4 and 2.2-fold increases, respectively) and decreased $A\beta_{1–42}$ concentrations (47% decrease) in the AD compared to NCs. We determined diagnostic cutpoints for CSF $A\beta_{1–42}$, t-tau and p-tau_{181} for these data using receiver-operating-characteristic curve analyses. Using the qualified analytical system including Alz Bio3 reagents and the Luminex xMAP platform, we confirmed the prevalence of increased average concentrations of t-tau and decreased concentrations of $A\beta_{1–42}$ in AD versus NCs in the ADNI study [36]. A question of increasing interest is the assessment of risk in MCI patients for progressing to AD dementia utilizing CSF biomarkers [18, 26, 36]. Figure 6a, b summarizes assessment of the utility of these diagnostic threshold cutpoints for $A\beta_{1–42}$ and for the t-tau/$A\beta_{1–42}$ ratio for prediction of progression from MCI to AD by Kaplan–Meier survival curve analyses. This analysis illustrates that these threshold cutpoints for $A\beta_{1–42}$ and for the ratio t-tau/$A\beta_{1–42}$ provide statistically significant separation of progressors from non-progressors starting at 6 months for t-tau/$A\beta_{1–42}$ and 12 months for $A\beta_{1–42}$, thereby confirming the clinical performance of this test system.

Discussion

There is increasing interest in the measurement of CSF biomarkers, particularly $A\beta_{1–42}$, t-tau and p-tau_{181}, as indices of AD pathology in patients who are at predementia and pre-symptomatic stages of the disease [1, 6, 7, 9, 10, 12, 18-20, 25-27, 30, 33, 36, 37, 39]. These AD biomarkers emerged from a number of earlier studies as prime candidate analytes for inclusion or exclusion criteria of demented or MCI patients in clinical trials of investigational drugs for treatment of AD. These markers are also of potential interest for use in stratification criteria for the assessment of treatment effects of investigational, disease modifying therapies that target Aβ and tau AD neurodegeneration (for recent reviews see [16, 17, 21, 35, 41, 43]). However, experience with a number of CSF tau and Aβ immunoassays reported in the literature reveals significant measurement variability linked to

the following: lack of strict consistent adherence to the assay method protocol; inconsistent manufacture of reagents; differences in analytical test procedures; matrix effects confounding spike recovery and linearity with sample dilutions. A host of pre-analytical issues, including but not limited to patient preparation and CSF sample acquisition, processing and storage also contribute to the variability of reported results [5, 8, 23, 24, 26, 34, 44]. Additionally, there is an overall measurement bias in all commercially available kits because currently available calibrators are prepared in buffer matrixes, rather than authentic CSF or artificial equivalents thereof that are free of matrix interactions.

To address these issues here, we evaluated the inter-laboratory performance achieved with the Innogenetics AlzBio3 immunoassay on the xMAP Luminex platform in seven participating laboratories. The samples tested included aliquots prepared from five pools of human CSF in addition to aqueous controls. Each laboratory performed three analytical runs using not previously thawed aliquots for each of the five CSF pools. Using mixed-effects modeling to analyze precision across the seven participating laboratories for these five CSF pools the mean within-laboratory %CV for these five CSF pools were 5.3% for $A\beta_{1–42}$, 6.7% for t-tau and 10.8% for p-tau_{181}, thereby demonstrating the level of reproducibility a laboratory can expect, on average, for CSF analyses based on the AlzBio3 reagents and the Luminex analytical platform. The UPenn ADNI Biomarker Core laboratory measurement of CSF $A\beta_{1–42}$, t-tau and p-tau_{181} in ADNI CSF sample aliquots was performed over a series of analytical runs (n = 36 for CSF pool X-45 and n = 51 for CSF pool X-52) using three different lots of manufactured reagent kits. These additional data demonstrated very good within-laboratory inter-day precision, thereby confirming and expanding the within-laboratory reproducibility initially demonstrated in the inter-laboratory study.

The average inter-laboratory precision for the five CSF pools was 17.9% for $A\beta_{1–42}$, 13.1% for t-tau and 14.6% for p-tau_{181}. Further studies are required to fully explain the increased variability between centers as compared to within-center reproducibility. Since this is a multistep procedure, there are several manipulations in the procedure that could be a source for variability in the biomarker test results either individually or in specific combinations. Examples include sample handling, e.g., the time and temperature used for thawing CSF aliquots and the mixing procedure intended to assure uniform sample composition; composition of pipetting materials and pipetting technique; incubation conditions including the time and temperature of the overnight incubation and the vacuum pressure utilized in plate washing steps. We believe that certain factors, e.g., silanized pipet tip versus standard polypropylene tip, are not likely a major factor for the $A\beta_{1–42}$ measurement variability since using three CSF pools we obtained a mean difference in results for $A\beta_{1–42}$ of only 1% using the silanized sample pipet tips versus polypropylene, thus precluding adsorption of $A\beta_{1–42}$ to non-silanized pipet tips as a likely factor, and use of silanized sample tips did not give rise to higher concentrations of either t-tau or p-tau_{181}. The vacuum pressure for plate washing steps varied among the seven participating laboratories from 2.2 to 9.0 *in Hg*. We tested for the effect on results of varying plate washing vacuum pressure on aliquots from six CSF pools in a series of experiments testing each of four plate washing vacuum pressures (2, 5, 6 and 9 *in Hg*) and found modest average differences that were within the run to run variability of the method (unpublished results). The temperatures used for the overnight incubation step by participating laboratories (19–24°C) were well within the acceptable range specified by the manufacturer. Another potential source of variability is the type of material used in the manufacture of aliquot tubes. Glass and polystyrene surfaces are prone to adsorb $A\beta_{1–42}$ and to a lesser degree t-tau in comparison to polypropylene [1, 24], but recent studies have detected significant differences between polypropylene tubes made by different manufacturers [3, 31]. However, since the polypropylene aliquot tubes used in this study were the same lot number and manufacturer, the CSF aliquot tube type was not a source of variability here, but other possible factors (e.g., pipetting technique, pipet tip

manufacturer and materials) require further investigation to assess their possible effects on these bio-marker measurements and were beyond the scope of this study.

Notwithstanding the multiple factors potentially impacting analytical performance of the AlzBio3 on the Luminex platform, the results reported herein represent a significant improvement in inter-laboratory reproducibility relative to previously reported multiple center assessments of this commercially available assay kit and analytical platform combination [23, 44]. The clinical implications of this are important. Diagnostic cutpoints were developed using this analytically qualified immunoassay system and ante-mortem CSF samples from non-ADNI patients with an autopsy confirmed diagnosis of AD and age-matched NCs from a non-ADNI clinical cohort, e.g., $A\beta_{1–42}$ was abnormal in 90% of ADNI subjects with probable AD [36], and $A\beta_{1–42}$ and t-tau/$A\beta_{1–42}$ were abnormal in 90.2 or 92.7%, respectively, in ADNI MCI subjects who progressed to AD (unpublished observation on ADNI data as of 6/30/2010) and differentiated progressors from non-progressors in the ADNI MCI cohort as demonstrated in this investigation. The current study is significant because it defines parameters that set the stage for further refinements of CSF tau and Aβ assay performance, thereby offering the prospect of wider use of these assays including the analysis of specimens from transgenic mouse models of AD-like tau and Aβ pathologies and the analysis of specimens from AD patients, MCI patients and cognitively normal subjects participating in clinical research studies to evaluate the diagnostic utility of the methods themselves and the therapeutic impact of investigational therapies for the treatment and prevention of AD. These promising findings together with advances in standardizing AD imaging methods prompt us to speculate that ante-mortem measures of CSF t-tau, p-tau_{181} and $A\beta_{1–42}$, together with MRI and amyloid imaging biomarkers in probable AD patients, could replace autopsy confirmation of AD plaque and tangle pathology as the “gold standard” for the diagnosis of definite AD in the near future.

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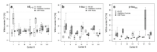

Fig. 1.

Box plots of the within-center %CV for each of seven participating centers for **a** $A\beta_{1–42}$, **b** t-tau and **c** p-tau_{181} measured in two aqueous kit quality control samples, three aqueous run validation samples and five CSF pools in three separate analytical runs using INNO-BIA AlzBio3 reagents and the Luminex platform

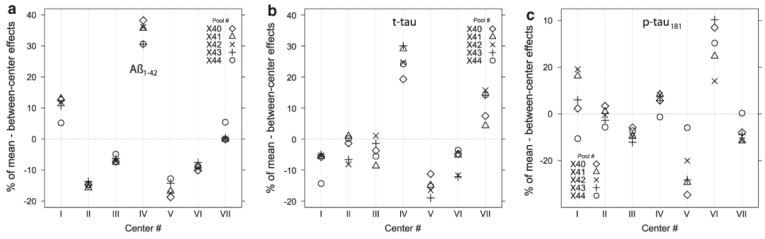

Fig. 2.

Plots of average % of grand mean values for each center and for each of five CSF pools for **a** $A\beta_{1–42}$, **b** t-tau and **c** p-tau_{181}. The concentrations of $A\beta_{1–42}$, t-tau and p-tau_{181} were determined as described using pristine aliquots of each of five CSF pools in three analytical runs, and the grand mean values for each biomarker were determined. For each of the seven participating centers the % of the grand mean value was determined for each of the five CSF pools by dividing the average value over the three analytical runs by the grand average value and plotting the % of the grand mean value for each pool and each center

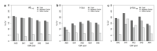

Fig. 3.
Bar chart plots for the total, between-center and within-center %CV values derived for each CSF pool from the Mixed Effects statistical model for the CSF pools data set for **a** $A\beta_{1–42}$, **b** t-tau and **c** p-tau_{181}

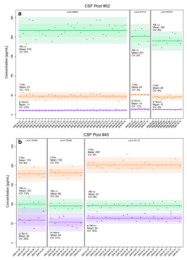

Fig. 4.

Longitudinal plots of $A\beta_{1–42}$, t-tau and p-tau_{181} concentrations measured in never previously thawed aliquots of **a** CSF pool #52 (*n* = 51) and **b** CSF pool #45 (*n* = 36)

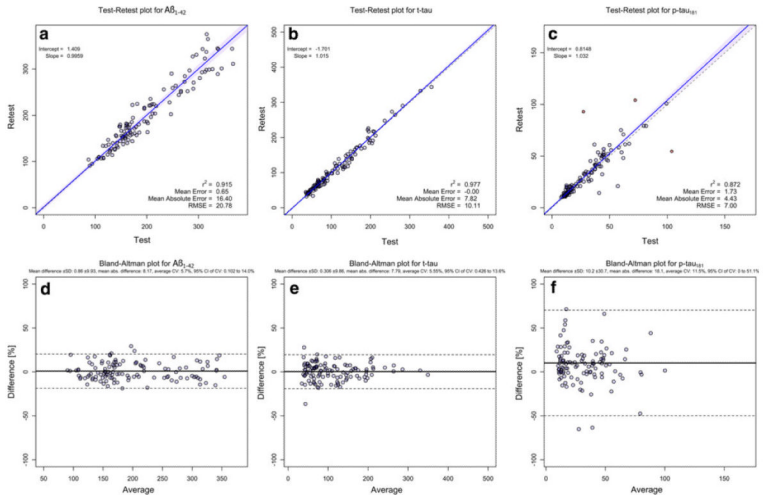

Fig. 5.

Linear regression plots of concentrations, measured in never previously thawed CSF aliquots from 118 ADNI subjects, utilizing 2–3 subjects randomly selected from each of 38 analytical runs. For each randomly selected subject, a second never previously thawed aliquot was included in the run following analysis of the first never previously thawed aliquot. In plots **a–c**, the $A\beta_{1–42}$, t-tau and p-tau_{181} concentration values obtained in the second aliquot (retest) are plotted against the biomarker concentration value obtained in the original analytical run (test) and linear regression analyses performed. In plots **d–f**, the % difference between the test and retest values are plotted versus the average value for each test/retest pair of concentrations. The *shaded area* around each linear regression line is the 95% confidence interval for the regression line. In plots **d–f**, the *dotted lines* are the 95% confidence intervals for the mean difference lines (*solid lines*)

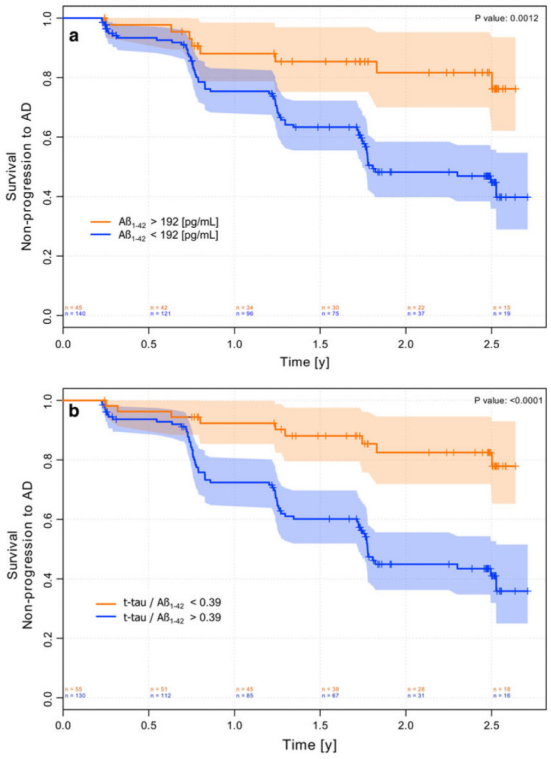

Fig. 6.

Kaplan–Meier time to conversion to AD survival curves for ADNI subjects who had a diagnosis of mild cognitive impairment at their baseline visit. The *small vertical lines* are censored data, and the number of subjects at risk is noted at the *bottom* of the plot. In **a**, the survival curves are shown for MCI subjects with CSF $A\beta_{1–42}$ concentrations above or below the threshold value of 192 pg/mL at their baseline. In **b**, the survival curves are shown for MCI subjects with CSF t-tau/$A\beta_{1–42}$ ratio values above or below the threshold value of 0.39

Table 1

Within-center precision for CSF Aβ1-42, t-tau and p-tau$_{181}$

Biomarker	Center	CSF X40		CSF X41		CSF X42		CSF X43		CSF X44	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
$A\beta_{1–42}$	I	335 ± 20	6.0	388 ± 24	6.2	558 ± 39	7.0	610 ± 72	11.7	239 ± 7	2.9
$\beta_{1–42}$	II	252 ± 12	4.8	282 ± 9	3.4	429 ± 38	8.8	470 ± 36	7.8	195 ± 9	4.7
$A\beta_{1–42}$	III	271 ± 18	6.5	314 ± 23	7.3	455 ± 21	4.6	497 ± 41	8.3	218 ± 13	5.9
$A\beta_{1–42}$	IV	402 ± 22	5.4	458 ± 16	3.6	678 ± 39	5.7	708 ± 34	4.8	297 ± 16	5.3
$A\beta_{1–42}$	V	228 ± 17	7.5	273 ± 19	7.0	405 ± 21	5.2	454 ± 26	5.7	190 ± 20	10.7
$A\beta_{1–42}$	VI	257 ± 19	7.6	311 ± 19	6.1	445 ± 31	6.9	511 ± 59	11.5	207 ± 21	10.1
$A\beta_{1–42}$	VII	298 ± 32	10.7	338 ± 23	6.8	497 ± 29	5.8	552 ± 22	4.0	244 ± 21	8.7
t-tau	I	175 ± 18	10.2	90 ± 5	5.2	50 ± 6	11.8	49 ± 9	19.2	87 ± 7	7.5
t-tau	II	187 ± 18	9.4	97 ± 10	10.1	47 ± 2	4.3	50 ± 1	2.6	102 ± 11	10.8
t-tau	III	181 ± 6	3.2	85 ± 6	6.5	51 ± 2	4.8	52 ± 4	6.9	95 ± 4	4.3
t-tau	IV	229 ± 10	4.2	129 ± 11	8.8	65 ± 8	12.8	70 ± 6	8.0	130 ± 12	9.6
t-tau	V	168 ± 2	1.4	84 ± 3	3.2	42 ± 2	5.0	43 ± 2	5.5	85 ± 3	3.3
t-tau	VII	202 ± 15	7.6	100 ± 4	4.3	61 ± 3	4.6	61 ± 2	3.6	116 ± 6	5.2
p-tau$_{181}$	I	60 ± 8	12.8	35 ± 2	7.0	28 ± 2	7.3	42 ± 2	3.6	111 ± 4	3.4
p-tau$_{181}$	II	55 ± 6	11.5	27 ± 3	11.3	24 ± 2	8.2	44 ± 3	7.1	115 ± 6	5.5
p-tau$_{181}$	III	44 ± 1	2.5	25 ± 0	1.5	22 ± 1	2.7	42 ± 1	1.4	98 ± 3	2.8
p-tau$_{181}$	IV	54 ± 1	2.3	33 ± 1	2.4	27 ± 82	3.0	47 ± 1	2.5	116 ± 10	8.2
p-tau$_{181}$	V	38 ± 3	8.7	22 ± 1	4.1	18 ± 1	3.9	44 ± 1	3.1	70 ± 2	3.1
p-tau$_{181}$	VI	68 ± 22	32.5	32 ± 10	30.3	35 ± 8	21.7	60 ± 6	10.6	147 ± 7	5.0
p-tau$_{181}$	VII	41 ± 2	5.0	25 ± 2	6.4	22 ± 1	5.0	46 ± 4	9.1	97 ± 8	8.2

Table 2

Reproducibilities for two CSF pools using one kit lot of reagents and across three kit lots of reagents

	3-kit lots[b]		1-kit lot[a]	
	CSF pool 45	**CSF pool 52**	**CSF pool 45**	**CSF pool 52**
$A\beta_{1-42}$				
Mean ± SD	97.0 ± 8.3	210 ± 19	96.6 ± 7.3	218 ± 17
%CV	8.6	9.3	7.5	7.8
95% CI	81–112	179–244	81–108	188–244
t-tau				
Mean ± SD	193.3 ± 14.3	47.5 ± 5.0	201.5 ± 10.1	47.4 ± 5.3
%CV	7.4	10.5	5	11.2
95% CI	169–220	37–54	188–224	36–55
$p\text{-}tau_{181}$				
Mean ± SD	60.9 ± 10.1	12.2 ± 1.4	64.0 ± 6.7	11.5 ± 1.1
%CV	16.6	11.8	10.4	9.5
95% CI	39–77	10–14	55–75	10–13

The number of analytical runs was: $n = 22$, pool 45 1-kit lot; $n = 34$, pool 52 1-kit lot; $n = 36$, pool 45 3-kit lots; $n = 51$, pool 52 3-kit lots

[a] For CSF pool 45, 1-kit lot, lot# 191113, and for pool 52, 1-kit lot, lot# 190841 was used

[b] For CSF pool 45, 3-kit lots used were: lot #'s 191113, 176456 and 176496; for CSF pool 52, 3-kit lots used were: lot #'s 190841, 191113 and 197741